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СТАНДАРТИЗОВАННЫЕ ПРОЦЕДУРЫ ВАЛИДАЦИИ МЕТОДИК КОНТРОЛЯ КАЧЕСТВА ЛЕКАРСТВЕННЫХ СРЕДСТВ

STANDARDIZED VALIDATION SCHEMES FOR DRUG QUALITY CONTROL PROCEDURES

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INTRODUCTION

In accordance with the requirements of the European Pharmacopoeia (Eur.Ph.) [3] and harmonized with it the State Pharmacopoeia of Ukraine (SPU) [1], all analytical procedures of quality control of medicinal products, used for the official analyses, must be validated. The validation process is regulated by the Eur.Ph. Technical Guide [4]. However, this Guide determines only general principles of it. The acceptability criteria and the validation scheme should be designed for every particular procedure, taking into account its specificity. So, generally speaking, we can offer the different criteria and approaches, which formally comply with the requirements of the Eur.Ph.-SPU, but can lead to different conclusions about the procedure correctness, based on the results of validation. The same problems arise in the validation of the methods included in the Pharmacopoeia.

In this connection there is a need to develop standardized validation schemes for medicines quality control procedures and to formulate the principles of this standardization. Such validation schemes were developed [5-10, 69-73, 110] and showed themselves to good advantage for a large number of the real specification procedures. This has made it possible to introduce these schemes as references in the SPU [11] and the Guidelines [12] adopted as formal recommendations in the Russian Federation.

This monograph presents a systematic consideration of the theoretical basis of the standardized validation schemes.

This monograph presents a systematic consideration of the theoretical basis of the standardized schemes for drug quality control procedures, as well as the specific features of their application to all basic quantitative pharmacopoeial tests: assay, related substances, residual solvents, “Dissolution”, “Content uniformity”, *in vitro* bioequivalence study. There are used the main pharmacopoeial analytical methods: UV-VIS spectrophotometry, liquid and gas chromatography, atomic absorption spectrophotometry, titration.

The standardized schemes are developed for all basic options of standardization: reference standard method, calibration graph method, standard addition method, specific absorbance method.

The specificity of validation of quality control procedures of summarized drugs are discussed as well.

All developed standardized procedures are illustrated by examples of their application to validation of quality control procedures of real pharmaceutical objects.

The developed schemes and approaches are intended to validate the procedures of drug quality control, but the main points and conclusions are common enough in quality control of any serial product with regulated tolerances.

Addendum 1 provides a metrological ground scheme for conducting the tests for content uniformity and dissolution by chromatographic methods, which allows reducing significantly the experiment volume. Addendum 2 describes the profiles of release of active ingredients from medicinal substances and different dosage forms. These addendums are closely related to the validation of procedures for these tests.

Compared to the 1st Edition [110], in the 2nd Edition chapters 2-5 are updated and chapters 6-9 and annex 2 are added. This allows us to cover most of the pharmacopoeial procedures with the standardized validation schemes.

Table of contents

3.2.2. Specificity and robustness
3.2.2.1. Specificity
3.2.2.2 Solution stability study
3.2.3. Model solutions, measurements and calculations

3.2.4. Results and discussion

3.2.4.1. System suitability
3.2.4.2. Specificity
3.2.4.3. Stability of the solutions through time
3.2.4.4. Linearity
3.2.4.5. Repeatability and accuracy
3.2.4.6. Limit of quantitation and limit of detection

4. Validation of procedures for the residual solvents control in drug products by gas chromatography

4.1. Theoretical part

4.1.1. Target uncertainty of the procedure
4.1.2. Detection limit
4.1.3. Procedure and range
4.1.3.1. Reference standard method
4.1.3.2. Standard addition method
4.1.4. Specificity: effect of interfering peaks of the substance and the solvent
4.1.4.1. Effect of irremovable rests of RS
4.1.4.2. Effect of impurities in the solvent and the test substance
4.1.4.2.1. Standard addition method
4.1.4.2.2. Reference standard method
4.1.5. Requirements to linearity
4.1.5.1. Reference standard method
4.1.5.1.1. Correlation coefficient
4.1.5.1.2. Requirements to *Y*-intercept
4.1.5.2. Standard addition method
4.1.5.2.1. Correlation coefficient
4.1.5.2.2. Requirements to *Y*-intercept
4.1.6. Accuracy and precision
4.1.7. Robustness, system suitability test
4.1.7.1. Stability of the solutions
4.1.8. System suitability test

4.2. Example. Validation of the control procedure of toluene and isopropanol residuals in fensuccinal medicinal substance

4.2.1. Procedure to be validated
4.2.2. Preparation of model and reference solutions
4.2.3. Chromatographic results
4.2.4. Results and discussion

4.2.4.1. Repeatability of replicate injections

9.4. Linearity

9.4.1. Problem of linearity study for an assay of summarized drugs
9.4.2. Normalized coordinates
9.4.3. Range and number of points for obtaining the calibration line
9.4.4. Model mixtures, their analysis and processing

9.4.4.1. Calculations for the reference standard method

9.5. Validation criteria

9.5.1. Uncertainty of a calibration line
9.5.2. Uncertainty of a test sample in the calibration line method
9.5.3. Uncertainty of a test sample in the reference standard method
9.5.4. Intermediate precision

9.6. Example. Validation of the assay of the carbohydrates content in CDDL

9.6.1. Carbohydrates determination procedure to be validated
9.6.2. Obtaining the calibration line
9.6.3. Analysis of model mixtures

9.6.3.1. Specificity
9.6.3.2. Preparation and analysis of model solutions
9.6.3.3. Intermediate precision

Addendum 1

Performing tests "Uniformity of dosage units" and "Dissolution test for solid dosage forms" by liquid chromatographic procedures under the production quality control of drug products

1. Requirements to the uncertainty of the analytical results obtained in "Content uniformity" and "Dissolution" tests

1.1. Content uniformity

1.2. Dissolution

2. Calculation of a sufficient number of replicate chromatograms when performing the "Content uniformity" and "Dissolution" tests

2.1. General assumptions

2.2. Role of the chromatographic system suitability test

2.3. The experiment scheme for conducting the "Content uniformity" and "Dissolution" tests

2.3.1. Content uniformity
2.3.2. Dissolution
2.3.3. Recommendations for conducting the "Content uniformity" and "Dissolution" tests

3. Example. Content uniformity control of the "Renalgan" tablets with use of liquid chromatography

3.1. Test subject

3.2. Content uniformity procedure

3.3. Results and their discussion

4. Using two reference standard solutions when conducting the “Dissolution” and “Content uniformity” tests by chromatographic methods

4. 1. Experiment scheme
4.2. Standardized *ST1* and *ST2* peak areas
4.3. Requirements to *RSD* values
4.4. Content uniformity
4.5. Dissolution
4.6. Recommendations for conducting the “Content uniformity” and “Dissolution” tests
4.7. Supplementary control of the reference standard solutions

Addendum 2

Description of active substance release profiles for various dosage forms

1. Description of *in vitro* release profiles of biologically active substances for suppositories and ointments

1.1. Description of release profiles at a constant layer thickness

1.1.1. Experiment
1.1.2. Results and discussion

1.2. Dependence of release degree on drug layer thickness

1.2.1. Theory
1.2.2. Experiment
1.2.3. Results and discussion

1.3. Standardized release procedure for ointments and suppositories

1.3.1. Experiment
1.3.2. Results and discussion

2. Dependence of substance solubility on its particle size

2.1. Experiment

2.1.1. Substance purity
2.1.2. X-ray phase analysis of different samples of fensuccinal substance
2.1.3. Production of fractions
2.1.4. Dissolution media
2.1.5. Solubility study
2.1.6. Actual distribution of various fensuccinal substance batches (samples) on particle size

2.2. Theory

2.2.1. Dependence of fensuccinal substance solubility on dissolution time
2.2.2. Problem of uniform precision for ordinate values

1. GENERAL NOTES

1.1. What is the Validation of an Analytical Procedure?

The objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose [1, 3].

Any analytical procedures (including a quality control procedure of a specific medicinal product), which is expected to include into a specification or which is some kind of official tasks (a formal opinion on the quality of any object, any research processes, etc.) must be validated. Otherwise, you cannot be sure of the correctness of the results.

Validation could not be performed for some abstract procedure, for example, an assay of acetylsalicylic acid in tablets by acid-base titration. Only the specific procedure may be validated, for example, an assay of acetylsalicylic acid in 0.500 g tablets of pharmaceutical company "Zdorovie" using acid-base aqueous titration by 0.1 M sodium hydroxide aqueous solution when using the indicator phenolphthalein. The procedure should be clearly described, indicating the requirements for the titrant, burette, indicator solution and acetylsalicylic acid tolerances. Even changing the tablet manufacturer requires revalidation as excipients and their proportions may be different.

Validation of an analytical procedure involves analytical tasks are articulated clearly - for what purpose the analytical procedure is used. From this point of view, a distinction should be made between determining the concentration of a substance in a sample (for example, determination of acetylsalicylic acid in tablets) from the quality control of the same object (tablets of acetylsalicylic acid) in terms of acetylsalicylic acid. Validation criteria for these cases, generally speaking, are different.

If the analytical task is determination of the concentration (for example, tablets of acetylsalicylic acid content), it means that we are interested in is the concentration, to be defined in the specified limits with specified accuracy and precision (with confidence interval). Accordingly, the statement of procedure validation task is formulated. This problem affects, for example, when examining the stability, dissolution profiles in proving *in vitro* bioequivalence or defining the content of the tablets when tested on the content uniformity [1].

If the analytical task is to control the quality of an object, then the problem is changing. Quality control implies that we with some degree of reliability (to be determined) make the conclusion whether the concentration of the substance is in the acceptable range (tolerances) or not. If it is, then the drug quality is acceptable, if it is not, then the drug is of substandard quality. In this case the concentration value, generally speaking, might not be interested for us. An example is the routine quality control of a specific drug product (DP) (for example, Paracetamol tablets 0.5 g) in the manufacturer control laboratory or at the stage of State control.

Quality control involves the definition of what a substandard production is, and this may be different in different cases. Depending on this definition the validation

criteria will differ. Therefore, the task of quality control is more complex and is not limited to determining the concentration alone.

1.2. Types of analytical procedures to be validated

All analytical procedures and tests that are included in the specifications must be validated [4, 11, 12]. In the pharmaceutical analysis of greatest interest is the validation of the following tests:

- Identification tests;
- Quantitative tests for impurity tests;
- Limit test for the control of impurities;
- Quantitative tests of the active moiety in samples of drug substance or drug product or other selected component(s) in the drug product (e.g., preservatives).

These tests are designed to solve the following tasks [4, 11, 12]:

- Identification tests are intended to ensure the identity of an analyte in a sample. This is normally achieved by comparison of a property of the sample (e.g. spectrum, chromatographic behavior, chemical reactivity, etc.) to that of a reference substance.
- Testing for impurities can be either a quantitative test or a limit test for the impurity in a sample. Either test is intended to accurately reflect the purity characteristics of the sample. Different validation characteristics are required for a quantitative test than for a limit test.
- Assay procedures are intended to measure the analyte present in a given sample. For the drug product, similar validation characteristics also apply when assaying for the active or other selected component(s), The same validation characteristics may also apply to assays associated with other analytical procedures (e.g. “Dissolution”).

In general, the validation of an analytical procedure involves the following steps:

1. Statement of the validation problem.
2. Selection of the validation characteristics.
3. Select reasonable acceptability criteria of validation characteristics.
4. Experimental acquisition of validation of characteristics for the validated procedure.
5. Comparison of the received validation characteristics with the acceptability criteria and conclusions of conformity or nonconformity.
6. Prognosis of the procedure uncertainty in other laboratories.

Steps 1-5 are, in general, the obvious. Some explanation requires only Step 6 – the uncertainty prognosis of the procedure in other laboratories. The fact of the matter is that the procedure development is carried out in a single laboratory (usually in the laboratories of drug manufactories), and the official analysis on this procedure (for example, the State quality control of the drug product) in another. The another

laboratory can be much worse equipped than a laboratory- developer and the procedure validated by manufacturer will give incorrect results. To avoid this, make sure that you carry out the uncertainty prognosis in other laboratories, provided that they have standard equipments meeting the specific (usually pharmacopoeia) requirements.

1.3. Qualification of analytical equipment

Qualification is the experimental proof that the analytical equipment is able to ensure the task decision. The analytical equipment is made for a variety of tasks, and its qualification by the manufacturers for a general case, of course, cannot take into account the specificity of the pharmaceutical analysis. Therefore, the supplementary qualification of the equipment for pharmaceutical purposes is needed. As a basis, the Pharmacopoeial requirements outlined in the appropriate general articles on analytical methods are used. These requirements are mandatory when conducting any formal analysis of a drug product.

1.3.1. Chromatographs

The State Pharmacopoeia of Ukraine (SPU) [13] sets requirements to the relative standard deviation (RSD) of replicate injections in the system suitability test for chromatographic assays (see Table 1.1).

Table 1.1

SPU repeatability requirements to the chromatographic assays (the system suitability test). It is assumed that uncertainty of sample preparation is insignificant in comparison with the total assay uncertainty [13, Table 2.2.46-2]

Number of individual injections							
n_o	2	3	4	5	6	7	8
	Maximal permitted standard deviation RSD_{max} (%)						
***B* = upper limit – 100%**	**Drug substances**						
1	0.16	0.42	0.60	0.74	0.86	0.96	1.06
1.5	0.24	0.63	0.90	1.11	1.29	1.44	1.58
2	0.32	0.84	1.20	1.48	1.72	1.93	2.11
3	0.48	1.26	1.80	2.23	2.58	2.89	3.17

Half-sum of upper and lower limits given in the definition of the individual monograph in (percent of the label claim)	**Drug products**						
5	0.25	0.67	0.96	1.19	1.38	1.54	1.69
7.5	0.38	1.01	1.44	1.78	2.06	2.31	2.53
10	0.51	1.34	1.92	2.37	2.75	3.08	3.38
15	0.76	2.01	2.88	3.56	4.13	4.62	5.07
20	1.01	2.68	3.85	4.75	5.50	6.16	6.76

From Table 1.1 should be an important conclusion: it is difficult enough to get total uncertainty below 2% for a routine chromatographic assay. In the case of drug substances, maximal permitted procedure uncertainty is the symmetrical tolerances (deviation from 100%) (see below). So for the chromatographic assays SPU-Eur.Ph. [1, 3] sets tolerances not closer than 98.0%-102.0%.

To use chromatograph must be able to obtain such *RSD* values; otherwise a correct analysis is not possible. If the data are absent in the manufacturer specification, you should design your own chromatograph procedure qualification by the repeated injection *RSD*.

This procedure is described for liquid chromatographs [14]. The initial requirements for it are the following [14]:

1. The procedure of liquid chromatograph qualification must be the same for all types of chromatographs.
2. This procedure must provide as high metrological characteristics as possible with the given equipment.
3. The procedure must be as insensitive as possible to the particular differences in chromatographic properties of similar sorbents (e.g. various types of packing with chemically bonded octadecyl silane).
4. The procedure must be carried out in the mostly often used regime, i.e. inverse-phase HPLC.
5. The procedure must be carried out according to criteria significant for the pharmacopeial analysis: reproducibility of quantities V or t_R, S, H, or their ratios V^ or t_R^, S^, H^.

These requirements are satisfied for the set of 4 phenylalkylketones (PK) C_8 – C_{11}. PK chromatography is close to ideal one, PK are easy separated from one another and cover the wide range of retention volumes. It ensure the correct qualification of a chromatograph.

The brief scheme of the experiment [14]:

- ambient temperature: 20 ± 2^0 C,
- wavelength of the UV detector: 230 nm,
- column: Nucleosil ODS,
- mobile phase: water - acetonitrile (40:60),
- sample introduced: the solution of PK in the mobile phase (0.002% for the solutions of C_8 and C_9; 0.004% for the solutions of C_{10} and C_{11}); sample volume is 20 μl.

The chromatographic measurements for the PK sample is repeated 10 times and for 4 PK peaks the retention times, areas and heights are determined using the integrator. The following quantities are calculated for each peak (except C_{10}) of each chromatogram :

$V^{\wedge}(i) = V(i)/V(i)_{10}$;
$S^{\wedge}(i) = S(i)/S(i)_{10}$;
$H^{\wedge}(i) = H(i)/H(i)_{10}$;

where: $V^{\wedge}(i)$ is the relative retention volume for PK peaks C_8, C_9 and C_{11};
$S^{\wedge}(i)$ is the relative area of PK peaks C_8, C_9 and C_{11};
$H^{\wedge}(i)$ is the relative height of PK peaks C_8, C_9 and C_{11};
$V(i)_{10}$, $S(i)_{10}$ and $H(i)_{10}$ are the retention volumes, the peak areas and the peak heights for PK C_{10} respectively;
i is the chromatogram number.

For each peak and for each value $V(i)$, $(V)^{\wedge}(i)$ $S(i)$, $S^{\wedge}(i)$, $H(i)$, $H^{\wedge}(i)$ calculate the mean and relative standard deviation (*RSD*). *RSD* for each value average out as the root mean square [26], on all PK. The average *RSD* have $v = 4\text{-}(10\text{-}1) = 36$ degrees of freedom for the absolute values (V, S, and H) and $v = 3\text{-}(10\text{-}1) = 27$ degrees of freedom for the relative values ($S^{\wedge}$, $V^{\wedge}$ and $H^{\wedge}$).

Examples of the metrological characteristics calculation for a particular chromatograph are illustrated in Table 1.2. For comparison, the *k'* values are shown as well. Summary on all investigated chromatographs is given in Table 1.3 [14].

Table 1.2

The metrological characteristics for the peaks of PK homologues, obtained with the use of HP 1050 chromatograph

	Homologue				
	C_8	**C_9**	**C_{10}**	**C_{11}**	**Mean**
$S_{x,r}(V)$	0.073	0.12	0.13	0.13	0.11
$S_{x,r}(V^{\wedge})$	0.064	0.087	-	0.027	0.056
$S_{x,r}(S)$	0.18	0.33	0.30	0.41	0.32

$S_{x,r}(S^)$	0.41	0.49	-	0.61	0.44
$S_{x,r}(H)$	0.24	0.44	0.72	1.12	0.82
$S_{x,r}(H^)$	0.84	0.87	-	1.06	0.80
k'^{*}	1.27	1.82	2.65	4.11	

* - the dead volume is measured using KNO_3

An important question is what the requirements must be applied to the average *RSD* values. Different approaches are possible here. In the case of the analytical signal (peak heights and areas) it is reasonable to proceed from the hardest case of Table 1.1 (substances with tolerances of $\pm$1.0%) and the most common number of replicate injections (n = 5). For this case RSD_{max} = 0.74%. We want to the phenylalkylketone *RSD* of 5 replicates do not exceed this value. Fisher's criteria for probability of 0.95 and degrees of freedom 4 and 27, 36, are respectively [26]: *F(0.95; 4, 27)* = 2.73 and *F(0.95; 4, 36)* = 2.63. So the critical *RSD* values are respectively 0.74/$\sqrt{2.73}$ = 0.45 and 0.74/$\sqrt{2.63}$ = 0.46. These critical values are presented in Table 1.3.

In the case of repeatability of retention volumes (*V* and *V^*) it is reasonable to proceed from the generally accepted requirements to retention parameters repeatability of 2% [14] and the criterion of 3*S*. So the critical *RSD* value for the retention parameters would be 2/3 = 0.67%. This value is also presented in Table 1.3.

Table 1.3

The metrological characteristics of some liquid chromatographs (compliances with criteria are italicized)

	***RSD* (%)**					
	$S_{x,r}(V)$	$S_{x,r}(V^)$	$S_{x,r}(S)$	$S_{x,r}(S^)$	$S_{x,r}(H)$	$S_{x,r}(H^)$
Criteria	$\leq$ 0.67	$\leq$ 0.67	$\leq$ 0.46	$\leq$ 0.45	$\leq$ 0.46	$\leq$ 0.45
Milichrom:						
No 657	0.69	*0.48*	1.9	1.2	2.7	1.1
No 710	0.76	*0.36*	1.7	1.1	1.5	1.1
No 801	1.12	*0.39*	4.1	2.4	3.1	1.3
No 294	1.2	*0.60*	4.5	2.2	3.8	1.4
Waters 1	*0.18*	*0.053*	0.81	0.77	1.8	0.82
Waters 2	*0.36*	*0.16*	1.01	0.95	0.78	*0.43*
HP 1050	*0.11*	*0.056*	*0.32*	*0.44*	0.82	0.80

The researches [14] were carried out in 1995, when there was quite another level of the chromatographs. In particular, the current generation of "Milichrom" chromatographs doesn't yield to Western brands by the metrological characteristics. So interest is the application of the above mentioned the modern critical values of metrological characteristics to old chromatographs, as it allows us to check out their differentiative ability. Table 1.3 shows that for chromatograph brands Waters and HP is not a problem the repeatability of retention parameters - both for the absolute values, as for the case of internal standard method: they meet the criteria (≤ 0.67). Chromatograph brand «Milihrom-4» meets the criteria only for the internal standard method. Absolute calibration for them does not provide an acceptable repeatability of the retention parameters.

Table 1.3 shows that only the HP chromatograph meets the *RSD* criterion ($\leq 0.46\%$) to repeatability of the replicate injections - both for the absolute calibration (0.32%) and for the internal standard method ($\leq 0.44\%$). Any other chromatograph does not meet the *RSD* criterion ($\leq 0.46\%$), i.e. their operation can cause problem at the stage of checking system suitability for the chromatographic analysis of drug substances with tolerances of $\pm$ 1.0%. In the case of the peak heights only Waters 2 meets the criterion ($\leq 0.46\%$), and even then, only for the internal standard method. In the case of the absolute calibration no chromatograph meets criterion ($\leq 0.46\%$).

Table 1.3 allows concluding that the modern liquid chromatographs enable to ensure compliance with the requirements of Table 1.1 but any chromatograph needs in qualification, since it may not meet the pharmacopoeial requirements of Table 1.1. The causes may include the ageing of the chromatograph and technical faults.

It should be noted that such a scheme may be offered for the qualification of a gas chromatograph as well (for example, separation of some homologues on a nonpolar phase). However, it needs in a separate qualification for packed columns, capillary columns and vapor-phase analysis, as apparatus equipments for them differ.

1.3.2. Spectrophotometers

To carry out a spectrophotometric assay a spectrophotometer must comply with the requirements of the corresponding general article [15]. These requirements we discussed in detail in the chapter "Application of the spectrophotometry in the quality control of medicines" [63].

The general conclusion: the specific absorbance method usually can't ensure the uncertainty below 3.0% for a pharmacopoeial drug assay. In practice this uncertainty usually much higher because of sample preparation errors [17]. So the specific absorbance method as a rule isn't applied to drug quality control in Ukraine and its validation for the routine laboratory drug control needs additional spectrophotometry check.

1.4. Balances and volumetric laboratory glassware qualification

In accordance with the requirements of SPU-Eur.Ph. [1, 3], only Class A volumetric glassware may be used for the pharmacopoeial analysis. The Class A requirements are equivalent to the 1-rst Class requirements of GOST [18-23]. The requirements to balances, volumetric flasks and pipettes are summarized in SPU [11] and given in the Table.1.4. The requirements to burettes (Table 1.5) are taken from the GOST [18-20]. For comparison the Class B requirements are presented in Table 1.5 as well. As can be seen the Class B uncertainties are much higher than the Class A ones so the Class B glassware use is not allowed in the compendial analysis [1, 3]. Similarly, for quantitative dilutions the pipettes with one mark are usually used.

Table 1.4

The requirements to the maximal permitted uncertainty of weighing, volumetric flasks and pipettes [11]

<table>
<tr><td colspan="6">Balances</td></tr>
<tr><td colspan="3">Weighing uncertainty</td><td colspan="3">0.2 мг</td></tr>
<tr><td colspan="6">Volumetric flasks</td></tr>
<tr><td colspan="3">Capacity, ml</td><td colspan="3">Uncertainty, %</td></tr>
<tr><td colspan="3">10</td><td colspan="3">0.5</td></tr>
<tr><td colspan="3">20</td><td colspan="3">0.28</td></tr>
<tr><td colspan="3">25</td><td colspan="3">0.23</td></tr>
<tr><td colspan="3">50</td><td colspan="3">0.17</td></tr>
<tr><td colspan="3">100</td><td colspan="3">0.12</td></tr>
<tr><td colspan="3">200</td><td colspan="3">0.10</td></tr>
<tr><td colspan="3">250</td><td colspan="3">0.08</td></tr>
<tr><td colspan="3">500</td><td colspan="3">0.07</td></tr>
<tr><td colspan="3">1000</td><td colspan="3">0.05</td></tr>
<tr><td colspan="6">Pipettes</td></tr>
<tr><td colspan="3">Pipettes with one mark</td><td colspan="3">Graduated pipettes</td></tr>
<tr><td rowspan="2">Capacity, ml</td><td colspan="2">Uncertainty</td><td rowspan="2">Capacity, ml</td><td colspan="2">Uncertainty</td></tr>
<tr><td>ml</td><td>% (for the total volume)</td><td>ml</td><td>% (for the total volume)</td></tr>
</table>

1	0.010	0.98	0.5	0.0061	1.23
2	0.012	0.61	1	0.0074	0.74
5	0.018	0.37	2	0.012	0.57
10	0.025	0.25	5	0.037	0.69
20	0.037	0.18	10	0.062	0.57
25	0.037	0.15	25	0.123	0.46
30	0.062	0.12	-	-	-

Table 1.5

Permitted tolerances for burettes [18-20]

Capacity ml	Graduating mark, ml	Tolerances ($max\ \Delta_{bur}$)					
		Class A			Class B		
		± ml	%	% for 80% of burette volume	± ml	%	% for 80% of burette volume
1	0.01	0.01	1	1.25	0.02	2.0	2.50
2	0.01	0.01	0.5	0.625	0.02	1.0	1.25
5	0.02	0.01	0.2	0.25	0.02	0.4	0.50
10	0.02	0.02	0.2	0.25	0.05	0.5	0.625
	0.05	0.02	0.2	0.25	0.05	0.5	0.625
25	0.05	0.03	0.12	0.15	0.05	0.2	0.25
	0.1	0.05	0.2	0.25	0.1	0.4	0.50
50	0.1	0.05	0.1	0.125	0.1	0.2	0.25
100	0.2	0.1	0.1	0.125	0.2	0.2	0.25

A special problem is a verification of the real volumetric glassware compliance with the declared tolerances. In this case it is necessary to take into account the statistical uncertainty of experimental volume values [10]. As a rule, the use of experimentally

found corrections to the declared volume in the routine analysis is not valid, since the correction obtaining procedure also requires validation [10].

1.5. Personal qualification (proficiency testing)

Obviously, no modern equipment will give correct results if the personal is unskilled. This is especially important for procedures that use multiple dilutions (e.g. spectrophotometry). The difference between the relative standard deviations obtained by an experienced analyst and an analyst without experience can reach 10 times [17]. It is clear that an analyst without appropriate qualification can't carry out the validation procedure.

So a question is in what way may be checked the appropriate personal qualification (internal testing). For this purpose, different approaches may be used, for example, using test samples with known concentrations [24-25]. One of the easiest and most effective ways is to check the relative standard deviation of the water emptying from a different volume pipettes (weighing of 30 replicate emptying for each pipette). Recommended $RSD \leq 0.15$-0.20% [17].

1.6. Choice of a statistical model

A specific feature of pharmaceutical analysis is that the analytical signal is usually a function of several random variables (sample weight, pipette volume, peak areas, etc.). Thus, there is a general problem of measuring uncertainty of indirectly measured quantity, depending on the number of measured values. In particular, how to calculate a total uncertainty of an analytical procedure if you know the uncertainty of its separate components (stages)? If the measured y value is a function of n independent random variables $\boldsymbol{x_i}$, i.e.

$$y = f(x_1, x_2, ...x_n) \ , \tag{1.1}$$

and the freedom degrees of the $\boldsymbol{x_i}$ values are the same or are large enough (> 30 so that you can apply the Gaussian statistics, not the Student's), the y variance is related to the $\boldsymbol{x_i}$ variances by the correlation (rule of uncertainties propagation) [26]:

$$s_y^2 = \sum_{i=1}^{n} \left(\frac{\partial f}{\partial x_i} \right)^2 \cdot s_{xi}^2 \ . \tag{1.2}$$

In practice, however, the freedom degrees of the $\boldsymbol{x_i}$ values are usually small and are not equal to each other. In addition, typically of interest are not the dispersion (standard deviation), but confidence intervals, calculated using the equation (1.2), with small and varying degrees of freedom. Therefore, to calculate the uncertainty of the y value (Δ_y) a variety of approaches is proposed, among which there are two basic ones: Linear Model and the Welch-Satterthwaite Approach [26].

1.6.1. Linear Model [26]

If the random x_i variables are statistically independent, the confidence interval function Δ_y is related to confidence intervals of Δ_{xi} variables by the correlation (confidence intervals are taken to the same probability):

$$\Delta_y^2 = \sum_{i=1}^{n} \left(\frac{\partial f}{\partial x_i} \right)^2 \cdot \Delta_{xi}^2 \quad . \tag{1.3}$$

This ratio is a generalization of the relationship (1.2). In the compendial analysis the measured y value is usually the product or quotient of random and constant values (sample weights, dilutions, absorbances or peak areas, etc.), i.e. (K - some constant):

$$y = \frac{K \cdot x_1 \cdot x_2 \cdot \ldots x_m}{x_{m+1} \cdot x_{m+2} \cdot \ldots x_n} \quad . \tag{1.4}$$

In this case the relation (1.4) takes the form:

$$\Delta_{y,r}^2 = \sum_{i=1}^{n} \Delta_{xi,r}^2 \quad , \tag{1.5}$$

where the relative confidence intervals are used.

The ratio (1.5) is applicable for any different freedom degrees (in particular, infinite) of the x_i values. Its advantage is simplicity and clearness. The use of absolute confidence intervals leads to much more cumbersome, so it is recommended to use relative values.

When the freedom degrees number of x_i values are the same or are large enough (> 30), expressions (1.3-1.4) give:

$$s_{y,r}^2 = \sum_{i=1}^{n} s_{xi,r}^2 \quad . \tag{1.6}$$

1.6.2. Welch-Satterthwaite Approach

In this approach the variance of y (s_y^2) is calculated by the ratio (1.3), paying no attention to the differences in the freedom degrees (ν_i) of the x_i values. For the calculated variance s_y^2 an "effective" number of freedom degrees ν_{eff} (which is

usually fractional) is calculated. Then for this ν_{eff} the corresponding *t-value* (Student's coefficient) is found. Further for a given probability in common way the confidence interval of *y* value (Δ_y) is calculated.

$$\nu_{eff} = \frac{s_y^4}{\sum_{i=1}^{n} \frac{\left(\frac{\partial f}{\partial x_i}\right)^4 \cdot s_{xi}^4}{\nu_i}}. \tag{1.7}$$

In the pharmacopoeial analysis the equation (1.4) for the *y*-values usually holds. In this case the ratio (7) takes a simpler form:

$$\nu_{eff} = \frac{s_{y,r}^4}{\sum_{i=1}^{n} \frac{s_{xi,r}^4}{\nu_i}}. \tag{1.8}$$

Here $s_{y,r}^4$ value is calculated by the equation (1/6).

The Welch-Satterthwaite Approach usually produces more narrow confidence intervals than the Linear Model (examples of calculations on both models are illustrated in [26]). However, it is much more difficult to use and not allows to select the partial uncertainties of different stages (with subsequent recommendations to minimize them) as the Linear Model in the form of the expression (1.5).

The forecast of procedure uncertainty uses the parent quantities (with an infinite number of degrees of freedom). In this case, the Welch-Satterthwaite Approach coincides with the Linear Model.

Next, we will use only the Linear Model in the form of expression (1.5).

1.6.3. Correlation coefficient [26]

When conducting a validation we assess the linear relationship:

$$Y = b \cdot x + a. \tag{1.9}$$

A prerequisite for this is the evaluation of the correlation coefficient [4, 11, 12].

The linear correlation coefficient is calculated by the equation:

$$r = \frac{m\sum_{1}^{m} x_i y_i - \sum_{1}^{m} x_i \cdot \sum_{1}^{m} y_i}{\sqrt{\left[m \cdot \sum_{1}^{m} x_i^2 - \left(\sum_{1}^{m} x_i \right)^2 \right] \left[m \cdot \sum_{1}^{m} y_i^2 - \left(\sum_{1}^{m} y_i \right)^2 \right]}} \tag{1.10}$$

The linear correlation coefficient r varies between -1 to +1. Positive values of r indicate growth, and negative - reduce y with x increasing. It can be shown that the ratio (1.10) can be represented in a simpler and accessible way:

$$r = b \cdot \frac{s_x}{s_y} \tag{1.11}$$

Here the s_x and s_y are the standard deviations of the clouds of x_i and y_i values around their averages. Note the absolute x- and y-coordinates equality. This approach is correct, when we correlate two random variables x and y *with* the same number of freedom degrees of *m-1*. But in practice the x_i values are not random, they are set. In addition, in equations (1.10-1.11) the correlation coefficient is not associated with the residual standard deviation that characterizes the dispersion of the experimental points around the straight line.

The linear correlation coefficient r is a special case of the general correlation index R_c which is also applied to non-linear relationships between variables x and y:

$$R_c = \sqrt{1 - \frac{s_0^2}{s_y^2}}, \tag{1.12}$$

here: s_o – residual standard deviation,

s_y – Standard deviation of y_i values around the their average value $\bar{y}$.

It may be shown, that equations (1.10-1.11) and (1.12) will be equivalent, if in the equation (1.12) to substitute the residual standard deviation with the freedom degree of $\nu = n-2$ (as must be) for $\nu = n-1$.

The equation (1.12) is more correct, because takes into account the inequalities of x- and y-coordinates. Because of its simplicity and clarity, as well as a clear link with the residual standard deviation, the equation (1.12) is much easier to use for obtaining acceptability criteria than the ratios (1.10-1.11). Therefore further we'll use just the equation (1.12).

1.7. Proving and Confirmatory approaches

Two approaches have been proposed, reflecting the evolution of attitudes about the role of the test "Assay" in the quality control of drug substances and drug products (DP) and leading to the different requirements to the permissible procedure uncertainty. These approaches for convenience may be described as "Proving" (we ***do not know*** the true content, find it and prove that it is within the specification limits) and "Confirmatory" (***we know*** the true content and confirm it).

1.7.1. Proving approach

This approach is applicable as to drug substances and drug products.

Assumptions. The tolerances of an analyzed component contents are not connected with the procedure uncertainty. Total impurity content is not controlled. Methods of analysis: non-selective – for drug substances and drug products; selective (chromatography) - only for drug products. The assay results may match as the real concentrations of the analyzed component (using the selective method), and some conditional values (if you use a non-selective method). For convenience call them "conditional concentrations".

Consequence. The conditional concentrations are considered to be distributed to a random (Gaussian) law around 100% (or other value) within the tolerances of the specification. In particular, there are concentrations on the borders of the specification requirements.

Requirements. The assay results, without regard to the uncertainty of the procedure, should be within tolerances of the pharmacopoeial monograph or specification. Going beyond these limits means that the impurity contents exceed the tolerable levels (drug substances) or the content of the analyzed component exceeds the regulated limits (drug products).

Procedure objective. To find and prove the conditional concentrations are within the tolerances. In order to prove this, you must use the procedure with the uncertainty that is significantly less than the tolerances.

This approach raises no objection for the drug product assay, which content tolerances are related mainly with the uniformity of the active substance content, and where the assay results are essential for the material balance and stability study.

Also the approach did not produce the particular objections for drug substances if their assays used non-selective methods of analysis, and the total impurity content was not limited. However, with the introduction of the principle of transparency of the monograph into the Eur.Ph. [3, 4], the situation for the drug substance assays changed radically.

1.7.2. Confirming approach

This approach is applicable to the drug substances only. Now it is an official approach in the Eur.Ph.-SPU [13].

Assumptions. All the impurities are known and controlled by the monograph. The content tolerances for the main component in the drug substance are determined on the basis of the maximum content of impurities, assay uncertainty and taking into account the different sensitivity of the procedure for the impurities and the main component.

Consequence. The true concentrations of the basic component are distributed by the random law not within the specification limits but from (100 - total impurity content)% to 100%. Within the specification limits are distributed randomly not true concentrations but the *assay results*. Obviously, in this case the upper specification limit of the basic component in the drug substance is only associated with the procedure uncertainty.

Requirements. The assay results must be within the tolerances of the pharmacopoeial monograph or the specification. Because all impurities are controlled by other tests (the principle of the "transparency" of the monograph [3, 4]), going beyond those limits (with the impurities within the requirements of the monograph or the specifications) can only mean one thing - this drug substance is produced by the technology, not controlled by the monograph of the Pharmacopoeia, or the specification.

Procedure objective. To confirm that the main component content does not significantly differ from the interval [(100 - total impurity content)%, 100%].

As you can see, in this case an assay test loses its original meaning and, in fact, plays the role of a identification test.

The correctness of this approach for the drug substances that meets the principle of "the transparency" of the monograph has no doubt. In the case of the drug products this approach is not applicable, because the *assumptions* are not fulfilled.

1.8. Validation characteristics [4, 11, 12]

A set of investigated validation characteristics depends on a analytical method task. The typical validation characteristics are:

- Accuracy
- Precision
  - Repeatability
  - Intermediate Precision
- Specificity
- Detection Limit
- Quantitation Limit
- Linearity
- Range

Below is the definition of each of these validation characteristics.

Glossary

1. Analytical procedure. The analytical procedure refers to the way performing the analysis. It should describe in detail the steps necessary to perform each analytical test. This may include but is not limited to: the sample, the reference standard and the reagent preparations, use of the apparatus, generation of the calibration curve, use of the formulae for the calculation, etc.

2. Limit tests are such tests which regulate impurity contents low some set limits.

3. Specificity. Specificity is the ability to assess inequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc.

Lack of specificity of an individual analytical procedure may be compensated by other supporting analytical procedure(s).

This definition has the following implication:

Identification: to ensure the identity of an analyte.

Purity tests: to ensure that all the analytical procedures performed allow an accurate statement of the content of impurities of an analyte i.e. related substances test, heavy metals, residual solvents content, etc.

Assay (content or potency): to provide an exact result which allows an accurate statement on the content or potency of the analyte in a sample.

4. Accuracy. Accuracy of an analytical procedure expresses the closeness of agreement between the value which accepted either as conventional true value or an accepted reference value and the value found.

5. Precision. The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility.

Precision should be investigated using homogeneous, authentic samples. However, if it is not possible to obtain a homogeneous sample it may be investigated using artificially prepared samples or a sample solution.

The precision of analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements.

> ***5a. Repeatability*** expresses the precision under the same operation conditions over a short interval of time. Repeatability is also termed intra-assay precision.
>
> ***5b. Intermediate precision*** expresses within laboratory variations; different days, different analyst, different equipments, etc.
>
> ***5c. Reproducibility*** expresses the precision between laboratories (collaborative studies, usually applied to standardization of methodology).

6. Detection limit. The detection limit of an individual analytical procedure is the

lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

7. Quantitation limit. The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays for low levels of substances in sample matrices, and is used particularly for the determination of impurities and/or degradation products.

8. Linearity. The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample.

9. Range. The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

10. Robustness. The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

The Table. 1.6 lists those validation characteristics regarded as the most important for the validation of different types of analytic procedures. This list should be considered typical for the analytical procedures cited but occasional exceptions should be dealt with on a case by case basis. It should be noted that robustness is not listed in Table 1/6 but should be considered at an appropriate stage in the development of the analytical procedure.

Furthermore revalidation may be necessary in the following circumstances:

- changes in the synthesis of the drug substance;
- changes in the composition of the finished product;
- changes in the analytical procedure.

The degree of revalidation required depends on the nature of the changes. Certain other changes may require validation as well.

Table 1.6

The set of validation characteristics depending on the type of analytical procedure [1, 4, 12]

Characteristics	**Type of analytical procedure**			
	Identification	**Testing for impurities**		**Assay**
		Quantitative	Limits	Dissolution Measurement only

				Content/potency
Accuracy	-	+	-	+
Precision:				
Repeatability	-	+	-	+
Intermediate precision	-	+*	-	+*
Specificity**	+	+	+	+
Detection limit	-	-***	+	-
Quantification limit	-	+	-	-
Linearity	-	+	-	+
Range	-	+	-	+

- signifies that this characteristics is not normally evaluated;
+ signifies that this characteristics is normally evaluated;
* in cases where reproducibility (see Glossary) has been performed, intermediate precision is not needed;
** lack of specificity of analytical procedure, could be compensated by other supporting analytical procedure(s);
*** may be needed in some cases.

The practice, however, shows that for all types of analytical procedures (except for the identity), you must get the ***complete*** set of validation characteristics.

Indeed, how can we talk about a detection limit in the limit test for impurities, if an accuracy of this test is unknown? After all, the following test on the same sample, the detection limit can be several times more or less.

It is also impossible to talk about the detection limit, if the linearity isn't proved, as in this case, there can be multiple detection limits (for example, for the quadratic relationship).

Again, the detection limit requires the analytical range, since for the purposes of validation, the limit of detection (or quantification) is not so important as the fact that it is less than a prescribed value (see the validation of impurity tests).

If there is a standardized validation scheme, all these validation characteristics can be calculated from the same data.

As you can see, there is a need to standardize the validation scheme for the drug quality procedures.

Standardization of the validation scheme for the drug quality procedures can be divided into 4 stages:

1. Standardization of the scheme of validation characteristics acquisition.
2. Standardization of coordinates.
3. Formulation and standardization of the acceptability criteria.
4. Prognosis of the total procedure uncertainty.

2. VALIDATION OF ASSAY PROCEDURES

2.1. Standardization of the scheme of validation characteristics acquisition

Investigations of the linearity are the basic for validation of assays, because the data obtained allow calculating other metrological characteristics [6]. Criteria of linearity are defined by the number of line points, their concentrations, type of analytical procedure and specification tolerances. For drug product assays, application of reference standard methods (chromatography, spectrophotometry) is typical. Standardization of the validation methodology allows us to standardize the requirements to metrological characteristics of the linear relationship.

The linearity investigation is the most expedient to combine with precision and accuracy study. That can significantly reduce the volume of the experiment. With this in mind, as well as the requirements of the Eur.Ph.-SPU [4, 11], the minimum sufficient points number for linearity study is g = 9. These points must be distributed with the uniform step inside the analytical range (D) which is different for various tests (see Table 2.1) [4, 11, 12]. In addition, the measurements are carried out for the reference solution with an approximate nominal concentration. The concentration and analytical signal of the reference solution are used to transform sample signals into normalized coordinates according to the relationships (2.1). In this way we get 9 points, each of which is one assay according to the procedure validated.

2.2. Standardization of coordinates: normalized coordinates

During the validation studies, a concentration is usually an independent value (abscissa) and an analytical signal (the peak area or height, absorbance, etc.) is a dependent one (ordinate). The concentrations and analytical signals of different substances can be in a variety of digital ranges. It requires the criteria calculation for each particular case and takes generality and obviousness from these criteria (e.g., representation of straight lines in the real concentrations and areas of peaks). At the same time, we are typically interested in the concentrations and analytical signals to be expressed not as real values but as a percentage of nominal (or declared) value, i.e. as the so-called “normalized” coordinates.

From a practical point of view, it is appropriate to present concentrations and analytical signals just in normalized coordinates. This allows us to define uniform criteria, associated only with the tolerances but not with sample specificity.

Let C_i is a concentration of a test component in the i-th analyzed solution (or sample), C^{st}_i is a concentration of this component in the reference solution (or sample) (it is considered that C^{st}_i is very close to the nominal or declared concentration). Similarly, A_i is an analytical signal of the test component for the i-th test solution; A^{st}_{i} is an analytic signal of the same component for the reference solution. Let’s introduce the normalized coordinates X_i , Y_i and Z_i, defining them as follows [6, 9]:

$$X_i = \frac{C_i}{C^{st}} \cdot 100\%, \quad Y_i = \frac{A_i}{A^{st}} \cdot 100\%, \quad Z_i = \frac{Y_i}{X_i} \cdot 100\%. \tag{2.1}$$

During the validation studies X_i , Y_i and Z_i values have a number of advantages over the original C_i , C^{st}, A_i , A^{st} values:

1. The X_i and Y_i values, irrespective of the sample specificity, are always within the same analytical range around 100%. The Z_i value is a recovery factor (found as a per cent of the introduced value).

2. A linear relationship $Y_i = b \bullet X_i + a$, irrespective of the sample specificity, is always lies in the same range (see section 2.1). The slope (b) is always close to 1. The y-intercept (a) is insignificantly (statistically or practically - see below) differs from zero (which is not surprising, since it is considered the applicability of the reference standard method). It standardizes the representation of the linear relationship plot and makes it clear.

3. The relationship $Y_i = b \bullet X_i + a$ is characterized by the residual standard deviation of $SD_{Y,res}$. The inverse linear relationship $X_i = (1/b) \bullet Y_{ii} + (-a/b) = b' \bullet Y + a'$ is characterized by the residual standard deviation of $SD_{X,res}$. Taking in account the proximity of the straight slope (b) to 1 and insignificance of the y-intercept (a), we obtain [6, 9]:

$$SD_{Y,res} \approx SD_{X,res} = RSD_o. \tag{2.2}$$

The $SD_{Y,res}$ and $SD_{X,res}$ are *relative* standard deviations with respect to a nominal (or declared) and A^{st} and C^{st} values that is emphasized by a RSD_o symbol.

4. The cloud of points $Y(X)$ in the X-Y coordinates can be characterized by a standard deviations s_X or s_Y. Since the average values of X_i and Y_i ($\overline{X}$ and $\overline{Y}$ respectively) are close to 100%, the s_X and s_Y values are relative standard deviations (with respect to the nominal or declared values) and, in view of paragraph 2.1, close to each other, i.e. (g = 9 is the number of points) [6, 9]:

$$SD_Y \approx SD_X = RSD_{range} = \sqrt{\frac{\sum_{i=1}^{g}(X_i - \overline{X})^2}{g-1}}. \tag{2.3}$$

The RSD_{range} values for different analytical ranges are shown in Table 2.1.

5. The Z_i values (see the relationship (2.1)) (g = 9) for the regression line is characterized by a mean $\overline{Z}$ and a standard deviation of SD_Z, which, given the proximity of the value to 100%, in fact, is the relative standard deviation. Therefore,

the analytical procedure uncertainty throughout the concentration range is characterized by a confidence interval equal to a single *Z* value confidence interval:

$$\Delta_{As} = t(95\%, g-1) \cdot SD_Z = 1.860 \cdot SD_Z \le max\Delta_{As}\,, \tag{2.4}$$

where $max\Delta_{As}$ is a maximum acceptable procedure uncertainty (target uncertainty).

2.3. Formulation and standardization of the acceptability criteria

As mentioned above, the validation of an analytical procedure is the experimental proof that the procedure is suitable for the intended tasks [1, 3, 4]. Therefore the validation methodology necessarily involves formulation and justification of acceptance criteria (what is "good" and what is "bad"). It is possible to use different approaches, which can lead, in general, to different results. Set out below is based on the systematic application of the insignificance principle [27].

2.3.1. Insignificance principle

As mentioned above, the analytic signal of the absolute majority of analytical methods is a function of not one but several random variables. Herein the total uncertainty of the analytical signal is estimated by the ratio (1.5). In practice we often have to estimate the influence of one random factor on the total procedure uncertainty. This effect can be significant or insignificant from the point of view of the problem. If this effect is not significant, they can be ignored, if not, it should be taken into account.

In accordance with the insignificance principle [27], the confidence interval Δ_2 is significant at the level of probability *p*% (insignificant at the level 100-*p*%) compared with the confidence interval Δ_1 if the total confidence interval Δ_{pooled} is greater than Δ_1 not more than *p*%, i.e. we have the inequality:

$$\Delta_{pooled} = \sqrt{\Delta_1^2 + \Delta_2^2} \le (1 + \frac{p}{100}) \cdot \Delta_1 \cdot . \tag{2.5}$$

In principle, we can set any level *p* of significance. In analytical practice, the significance level of *p* = 5% (i.e. the insignificance level of 95%) is commonly accepted. In this case, the solution of the ratio (2.5) is an inequality [27]:

$$\Delta_2 \le 0.32 \cdot \Delta_1 . \tag{2.6}$$

This inequality is the basic expression of the insignificance principle for formulating the acceptability criteria of the validation characteristics [5-12].

Note that for the level of significance p = 1% (i.e. the insignificance level of 99%) the coefficient in front of Δ_1 in equation (2.6) will be 0.14, and for the level of significance p = 10% (i.e. the insignificance level of 90%) will be 0.46.

2.3.2. Criteria of acceptability of an analytical procedure

Controlling the quality of medicines, we are interested in the metrological characteristics of the analytical procedure only within the analytical range (D) and in comparison with the tolerances ($\pm B$%) of the analyzed component according to the specification.

An assay of a drug substance and a drug product has fundamental differences. Impurity content (except water) in drug substances is usually very small and not significant in comparison with the content tolerances of the main component. Therefore, the drug substance assay is not intended to find the actual concentration of the main component (this can be done, subtracting total impurities content from 100%; this impurities content may be found by other specification tests). We must confirm that the obtained main component content has no significant (usually for a probability of 95%) difference from 100% ("confirming" approach [27]). Therefore, target uncertainty for a drug substance is equal to the specification tolerances ($\pm B$%).

At the same time, for an assay of a drug product, an actual concentration of an analyzed component (which, in principle, can vary widely) is important. In accordance with the insignificance principle (2.5-2.6), a procedure uncertainty of a drug product must be insignificant in comparison with the content tolerances width ("proving" approach [27]). In this case, this uncertainty not significantly affects the decision about drug product quality.

Thus, taking into account the ratio (2.6), total relative procedure uncertainties of a drug substance and a drug product (Δ_{As} %) are connected with a symmetrical content tolerance (B) of a analyzed component by the ratios [5-12]:

Drug substance:
$$\Delta_{As}(\%) \le max\,\Delta_{As} = B. \tag{2.7}$$

Drug product:
$$\Delta_{As}(\%) \le max\,\Delta_{As} = 0.32 \cdot B. \tag{2.8}$$

2.3.3. Accuracy. Statistical and practical insignificance of a systematic error

The usual requirement for a systematic error (δ) is its statistically insignificant difference from zero i.e. it should not exceed the random component of the result uncertainty. This means that it should not exceed the confidence interval of the average value $\overline{Z}$, i.e. the inequality must be true (g = 9) [26]:

Statistical insignificance:

$$\delta\% = \left|\overline{Z} - 100\right| \le \Delta_{\overline{Z}} = \frac{\Delta_{As}}{\sqrt{g}} = \frac{\Delta_{As}}{3}. \qquad (2.9)$$

The ratio (2.9) shows that the criterion of statistical insignificance of the systematic error depends on the actual procedure uncertainty Δ_{As} decreasing with its decrease (i.e. with precision improving). However, this requirement is not correct, because the higher the precision of the analysis (for example, due to the large number of multiple sampling), the smaller δ values are statistically significant. On the contrary, roughening the results (e.g., reducing the number of multiple sampling), we can even higher δ values do insignificantly differing from zero.

A more correct validation is to use the concept of practical insignificance of the systematic error [5-6, 9]. Systematic error δ is practically insignificant to the task of quality control, if it is not significant compared with the maximum acceptable procedure uncertainty (target uncertainty) $max\Delta_{As}$ of ratios (2.7-2.8), i.e.

Practical insignificance:

Drug substance:

$$\delta(\%) \le max\,\delta = 0.32 \cdot max\,\Delta_{As} = 0.32 \cdot B. \qquad (2.10)$$

Drug product:

$$\delta(\%) \le max\,\delta = 0.32 \cdot max\,\Delta_{As} = 0.1 \cdot B. \qquad (2.11)$$

The ratios (2.10-2.11) show that the criterion of practical insignificance depends only on the content tolerance, but does not depend (as opposed to statistical insignificance) on the actual procedure uncertainty Δ_{As}. The *max* δ values are listed in Table 2.1.

The comparison of ratio (2.9) with ratios (2.10-2.11) shows that if the actual Δ_{As} is close to the limit value (2.7-2.8), then the ratio (2.9) and ratios (2.10-2.11) are almost equivalent for $g = 9$. However, if the actual Δ_{As} is much less limit value (2.7-2.8), the requirements (2.10-2.11) are considerably more liberal than (2.9).

2.3.4. Acceptance criteria for the linear relationship

Below there are the criteria for the standardized validation scheme (section 2.1), using g = 9 points, but these criteria can be obtained for any number of points.

2.3.4.1. Residual standard deviation RSD_o

The confidence interval of points variation around the linear relationship $Y_i = b \bullet X_i + a$ is equal to $t(95\%, g\text{-}2) \bullet RSD_o$. When using the standardized scheme of the section 2.1, this confidence interval is equal to a confidence interval (Δ_{As}) of the procedure uncertainty, which should satisfy the inequalities (2.7-2.8). With this in mind, as well as [26], we get:

Drug substance: $$\Delta_{As} = t(95\%, g-2) \cdot RSD_o \le max\Delta_{As} = B\,. \quad (2.12)$$

Drug product: $$\Delta_{As} = t(95\%, g-2) \cdot RSD_o \le max\,\Delta_{As} = 0.32 \cdot B\,. \quad (2.13)$$

From here we obtain the requirements to RSD_o value (g = 9):

Drug substance: $$RSD_o \le B / t(95\%, g-2) = 0.53 \cdot B. \quad (2.14)$$

Drug product: $$RSD_o \le 0.32 \cdot B / t(95\%, g-2) = 0.17 \cdot B. \quad (2.15)$$

For the "Uniformity of content" and "Dissolution" tests [1, 3] the target procedure uncertainty is $max\Delta_{As}$ = 3.0%, which corresponds to the formal tolerances of = 9.3% [28]. This value should be put to the test data in the ratio (2.15).

2.3.4.2. Correlation coefficient

The use of normalized coordinates and ratios (2.1-2.3) provides the criteria for acceptability of the correlation coefficient in the form of a ratio (1.12).

Considering in the expression (1.12) $s_o = RSD_o$, $s_y = RSD_{range}$ and taking into account the ratios (2.2-2.3), we obtain the expression for the correlation coefficient (index) R_c [26]:

$$R_c = \sqrt{1 - \frac{RSD_0^2}{RSD_{range}^2}}. \quad (2.16)$$

Substituting into the equation (2.16) the RSD_{range} values from Table 2.1 and taking into account the ratios (2.14-2.15), we obtain the critical (lowest) correlation coefficient R_c for different tests, g = 9 points and various tolerances of content (see Table 2.1).

Sometimes it is rational to validate a procedure that would be suitable for use in the "Assay" (*As*), "Uniformity of content" (*UC*) and "Dissolution" (*Ds*) tests at the same time. In this case, we must choose a minimum of *As*, *UC* and *Ds* tests acceptable RSD_o value (as a rule it is of the *As* test), critical a_c value of the *Ds* test (as having the lowest limit of the analytical range), a and critical value R_c we must calculate from these values and actual broadest analytical range (typically for *Ds*). Results of such calculations are also given in Table 2.1.

Table 2.1

Critical values of a systematic error (*max* δ), total uncertainty ($max\Delta_{As}$) of a procedure and linear relationship $Y_i = b \bullet X_i + a$ parameters for different tests, $g = 9$ points and different tolerances *B* [1]

Test	Analytical range%, step %, RSD_{range} %	*B*, %	$max\Delta_{As}$ %	*max* δ%	RSD_o , %	*min* R_c	*max a*, %
Drug substance							
As	Range = 80-120, step = 5, RSD_{range} = 13.69	1.0	1.0	0.32	0.53	0.99926	1.6
		1.5	1.5	0.48	0.79	0.99833	2.4
		2.0	2.0	0.64	1.06	0.99702	3.2
		2.5	2.5	0.80	1.32	0.99535	4.0
		3.0	3.0	0.96	1.58	0.99329	4.8
Drug product							
As	Range = 80-120, step = 5 RSD_{range} = 13.69	5	1.6	0.51	0.84	0.99810	2.6
		7.5	2.4	0.77	1.27	0.99571	3.8
		10	3.2	1.02	1.69	0.99236	5.1
		15	4.8	1.54	2.53	0.98273	7.7
		20	6.4	2.05	3.38	0.96909	10.2
UC	Range = 70-130, step = 7.5, RSD_{range} = 20.54		3.0	0.96	1.58	0.99710	3.1
Ds	Range = 50-130, step = 10, RSD_{range} = 30.43		3.0	0.96	1.58	0.99865	1.9
	Range = 55-135, step = 10, RSD_{range} = 27.39		3.0	0.96	1.58	0.99839	2.1
As + *UC* + *Ds*	Range = 55-135, step = 10, RSD_{range} = 27.39	5	1.6	0.51	0.84	0.99952	2.1
		7.5	2.4	0.77	1.27	0.99893	2.1
		10	3.2	1.02	1.56	0.99837	2.1
		15	4.8	1.54	1.56	0.99837	2.1
		20	6.4	2.05	1.56	0.99837	2.1
As + *UC* + *Ds*	Range = 60-135, step = 9.4 RSD_{range} = 25.67	5	1.6	0.51	0.84	0.99946	2.4
		7.3	2.34	0.75	1.23	0.99885	2.4
		7.5	2.4	0.77	1.27	0.99878	2.4
		10	3.2	1.02	1.56	0.99814	2.4
		15	4.8	1.54	1.56	0.99814	2.4
		20	6.4	2.05	1.56	0.99814	2.4

Table 2.1 shows that the critical correlation coefficient value R_c decreases with with the tolerance B growth and increases with broadening of the analytical procedure range. Thus the correlation coefficient is not informative enough without indicating the range and tolerances.

2.3.4.3. Y-intercept. Statistical and practical insignificance

Y-intercept (an absolute term a of the straight line) characterizes the systematical error of the reference standard procedure. In accordance with the section 2.3.3, requirements to the a value may be of 2 types:

1. The *statistically* insignificant difference from zero: the a value must not exceed the confidence interval of its uncertainty, i.e. (g = 9):

Statistical insignificance:

$$a \le t(95\%, g-2) \cdot s_a = 1.89 \cdot s_a . \qquad (2.17)$$

Here s_a is a standard deviation of the *y*-intercept (a) of the straight line, obtained by the least square method.

2. The *practically* insignificant difference from zero: the a value is practically insignificant for the task solving if the systematic error added by it does not exceed the maximal values of ratios (2.10-2.11). As was shown [5, 6], the criteria of practical insignificance of the a value for the reference standard procedure in the normalized coordinates have the form:

Practical insignificance:

$$|a| \le \frac{\max \delta}{1-(X_{\min}/100)} = \frac{0.32 \cdot \max \Delta_{As,r}}{1-(X_{\min}/100)}. \qquad (2.18)$$

Here X_{min} is a lower range limit of the procedure (in our case 80, 70 or 55%), and the Δ_{As} value must comply with the ratios (2.7-2.8, 2.12-2.13).

The ratio (2.18) we use only in the case when the criterion of statistical insignificance (2.17) is not met. The critical a values are given in Table 2.1.

2.3.5. Detection limit (*DL*) and Quantification limit (*QL*)

These values are not required for validation of an assay procedure, but they are useful as information about how a procedure range surpasses its limits ("safety margin" of the procedure). In case of impurity control obtaining of *DL* and *QL* is mandatory [4, 11].

According to the Eur.Ph.-SPU [4, 11], *DL* and *QL* may be calculated from the standard deviation s_a of the absolute term of the straight line and its slope b:

$$DL = 3.3 \cdot s_a / b \approx 3.3 \cdot s_a , \qquad (2.19)$$

$$QL = 10 \cdot s_a / b \approx 10 \cdot s_a , \qquad (2.20)$$

taking into account that in the normalized coordinates *b* value close to 1.

Use of the characteristics of the straight line for the calculation of the *DL* and *QL* is much more reliable and more correct than use of the signal-to-noise ratio [4, 11], because it takes into account not only noise, but also the uncertainty of sample preparation, which, for example, in the case of head-space gas chromatography may be very significant [4, 11].

If the straight line is plotted in the normalized coordinates (i.e. $Y_i = b \bullet X_i + a$), the *DL* and *QL* are calculated in per cent of reference standard solution concentration. It allows easily assessing the "safety margin" of the procedure.

2.3.6. Intermediate precision

It is reasonable to use the "Confirmatory" approach [27]: the confidence interval of *Z* values obtained under different conditions should not exceed target uncertainty $max\Delta_{As}$ of the ratios (2.12-2.13) and Table 2.1.

To do this, examine by the specification procedure $n = 5$ samples (weights) of the same batch of the investigational drug at the $m = 3$ different days. The studies are carried out by different analysts on different equipment (spectrophotometers, chromatographic columns, etc.). All the results obtained (Z_i) must belong to the same population. So calculate their pooled mean value (Z_{intra}), standard deviation ($SD_{Z\text{-}intra}$ %) and the relative confidence interval (Δ_{intra} %) [26]. The Δ_{intra} % value should not exceed the $max\ \Delta_{As}$ value of equations (2.12-2.13) and Table 2.1, i.e.

$$\Delta_{int\,ra} = t[95\%,(n \cdot m - 1)] \cdot SD_{Z-int\,ra} = 1.76 \cdot SD_{Z-int\,ra} \le max\Delta_{As}. \qquad (2.21)$$

If the specification procedure uses *k* samples for analysis, the $SD_{Z\text{-}intra}$ value is divide by $\sqrt{k}$ [6].

2.3.7. Solution stability study

Checking the stability of the sample and reference solutions is one of the elements of the robustness study of the procedure [11] and must be carried out before all other validation studies. Usually you need to show that solutions are stable for at least 1 hour [5]. This means that the contribution of instability systematic error δ does not exceed the limit $max\delta$ value from the Table. 2.1.

The stability criteria differ from the spectrophotometric and chromatographic assays.

In the case of a reference standard spectrophotometric assay (sample and standard solutions are prepared at the same time) it is necessary to show that the change of the ratio of sample to reference solution absorbances (in the normalized coordinates it is change of the *Y* value from the equation (2.1)) does not exceed the $max\delta$ of Table 2.1 during 1 hour. For this, measure simultaneously the absorbances of sample and reference solutions after time $t = 0$, 15, 30, 45 and 60 minutes, calculate by the equation (2.1) the Y_t values, their relative standard deviation (RSD_t) and the

confidence interval Δ_t% (one-sided *t-value* for 4 degrees of freedom and the probability of 0.95 is equal to 2.13), which must not exceed the *maxδ* value of Table 2.1, i.e.:

$$\Delta_t(\%) = 2.13 \cdot RSD_t \leq 032 \cdot \max\ \Delta_{As} = \max\ \delta\ . \qquad (2.22)$$

Here the *max* Δ_{As} value is calculated by the ratios (2.12-2.13).

In the case of the chromatographic reference standard assay above mentioned approach is not possible in principle due to the duration of the chromatographic procedure (one chromatogram often takes about 20 min). However, this has its advantages for proving the necessary stability. Indeed, if we prepared and chromatographed 10 solutions to study linearity, this time far exceeds the assay of 1-3 samples, which usually analyze in practice. Therefore, the positive results of the accuracy and precision of the obtained when studying the linearity proof needed stability. Another proof is practically insignificant difference ($\leq\sqrt{2}\cdot max\Delta_{As}$) between the *Z* values of the first and last solutions chromatographed.

2.4. Prognosis of the total procedure uncertainty

To confirm the reproducibility of the procedure in another laboratory, validation results obtained in one laboratory are insufficient, because the level of equipment in this laboratory can be much higher than the acceptable Pharmacopeia requirements. So we need in prognosis of the total procedure uncertainty in accordance with these requirements.

The predictable total procedure uncertainty should not exceed the target uncertainty $max\Delta_{As}$ (Table 2.1). The predictable total relative uncertainty is calculated from the formula [11, 26 and 27]:

$$\Delta_{As} = \sqrt{\Delta_{SP}^2 + \Delta_{FAO}^2}\ . \qquad (2.23)$$

Here: Δ_{SP} – predictable uncertainty of the sample preparation; Δ_{FAO} - predictable uncertainty of the final analytical operation.

Predictable sample preparation uncertainty Δ_{SP} is calculated by the ratio [11, 26, 27]:

$$\Delta_{SP} = \sqrt{\sum_{i}^{n} \Delta_{V,i}^2}\ , \qquad (2.24)$$

где $\Delta_{V,i}$ – the component of the uncertainty related with the specific operation of sample preparation (weighing, aliquots of small volume, filling of the volumetric flasks, etc.), expressed as one-sided confidence interval for the 95% probability. Herein we must use the limit volumetric glassware uncertainty recommended by the SPU [10, 11] (see Table 1.4-.15).

The final analytical operation uncertainty Δ_{FAO} may be calculated by different ways.

In the case of the chromatographic procedures it is rational to proceed from the limit relative standard deviation of replicate injections in the system suitability test, regulated by the specification [13].

In the case of spectrophotometric procedures, such requirements for repeated measurements of the absorbance with the cell withdrawal are usually absent, although the recommendations (not more than 0.25%) present [15]. Therefore for the prognosis of the Δ_{FAO} value should be used the relative standard deviation (RSD_A = 0.52 %) of the absorbance measurements with the cell withdrawal, obtained in extensive inter-laboratory experiment [15, 17]. This value characterizes the real precision of currently available in national control laboratories.

Taking into account presence of 2 solutions (sample and reference) and the recommendations [15] on at least 3 replicate absorbance measurements with the cell withdrawal for each solution, we obtain for the spectrophotometric analysis [5, 26]:

$$\Delta_{FAO} = \sqrt{2} \cdot \frac{RSD_A \cdot 1.65}{\sqrt{3}} = 1.34 \cdot RSD_A = 1.34 \cdot 0.52 = 0.70\%, \qquad (2.25)$$

Here: 1.65 – Gauss coefficient for the one-sided probability of 95% [26].

Expression (2.25) characterizes the final analytical operation uncertainty which is typical for the domestic system of state drug control laboratories [17].

2.5. Procedure transfer

The procedure transfer is a mandatory requirement when the validation is not carried out by the drug manufacturer itself but, for example, by a contract research institution. It should be noted that this stage is an important part of the robustness verification (since an equipment, volumetric glassware, reagents and analysts at the manufacturer and research institution laboratories are different), as well as the reproducibility, complementing the procedure uncertainty prognosis.

The procedure transfer is appropriate to carry out by a comparative analysis of 5 drug samples according to the specification using the equipment of the manufacturer.

2.5.1. Acceptance criteria

It is reasonable to use the "confirmatory" approach: all the contents of 5 samples analyzed ($Z_{i,Transfer}$) must not differ from the average value ($\overline{Z_{intra}}$) (found when conducting an intermediate precision study) more than the target uncertainty $max\Delta_{As}$ from Table 2.1:

$$\left| Z_{i,Transfer} - \overline{Z_{intra}} \right| \le \max \Delta_{As} . \qquad (2.26)$$

2.6. Example. Validation of the spectrophotometric assay of ambroxol tablets

Object of study. Ambroxol hydrochloride (AHC) tablets 0.03 g, total mass 0.100 g. Excipients: potato starch, lactose, calcium stearate. According to State Pharmacopoeia of Ukraine (SPU) requirements [30], this medicine must comply with "Assay", "Uniformity of content" [31-32] and "Dissolution" tests [33]. All these three quantitative procedures use direct spectrophotometry on ambroxol characteristic absorption at 244 nm. It allows to carry out their combined validation.

Studies have used AHC substance, starch, lactose, calcium stearate, complying with the requirements of SPU-Eur.Ph [1, 3]. The reference standard – AHC SPU RS batch 120104. Titrants and reagents conformed with the SPU requirements [1].

Analytical equipment: spectrophotometer Specord 200, met the requirements of the SPU [15]; Sartorius balances MC 210S. Volumetric glassware – Class A, complied with the SPU [1].

2.6.1. Procedures to be validated

Assay. About 0.1 g (accurate weight) of crumbled tablets powder is placed in a 100 ml measuring flask, add 50 ml of *0.01 M hydrochloric acid*, shake 10 minutes, dilute to the mark with the same acid, filter through the "Milipor" with a diameter of not more than 0.5 microns. Place 10 ml of the filtrate in a 100 ml measuring flask and dilute to the mark with *0.01 M hydrochloric acid*. Measure the absorbance of the resulting solution at 244 nm, using *0.01 M hydrochloric acid* as a compensation solution.

Sample solution. The tablet solution prepared by the procedure.

Reference solution. 0.030 g (accurate weight) of the AHC SPU RS place in a 100 ml measuring flask, dissolve in 50 ml of *0.01 M hydrochloric acid* and continue as described above for the assay.

Requirements: 92.7 – 107.3% of the stated amount of AHC in the tablets.

Uniformity of content. One tablet place in a 100 ml measuring flask and continue as described above for the assay.

Requirements: compliance with the SPU [31-32].

Dissolution. The dissolution medium - *0.01 M hydrochloric acid*, 1000 ml. Basket apparatus [33], rotation speed – 100 revolutions per minute, dissolution time – 30 minutes. Place one tablet into the basket.

In 30 min take 25 ml of the fluid and filter through the "Milipor" with a diameter of not more than 0.5 μm, discarding the first 5 ml of the solution. Measure the absorbance of the resulting solution at 244 nm, using *0.01 M hydrochloric acid* as a compensation solution.

Requirements: ≥ 80% of the stated amount of AHC in the tablets.

In accordance with the requirements of Table 2.1 to the procedures to be validated, we carry out the validation of the unified procedure that is both suitable for the assay, uniformity of content test and dissolution test for the range 60-135%. Maximum procedure uncertainty - 7.3% (as for the assay). The relevant criteria are shown in Table 2.1.

2.6.2. Specificity: background absorption uncertainty checking

In the case of chromatographic methods, specificity is determined by the degree of separation of the specified substances peaks. In the case of non-specific analytical methods (e.g. spectrophotometry), the specificity for the task is proved if the relative systematic error (δ_{noise} %) paid by the excipients and degradation products in the determination of the analyte is not significant compared with the target uncertainty (Δ_{As} %). In particular, in our case (spectrophotometry), taking into account [27], we obtain:

$$\delta_{noise}(\%) = \frac{100 \cdot \sum_{i=1}^{k} A_{\mathrm{Im}\,p,i}}{A^{st} + \sum_{i=1}^{k} A_{\mathrm{Im}\,p,i}} \approx \frac{100 \cdot \sum_{i=1}^{k} A_{\mathrm{Im}\,p,i}}{A^{st}} = \\ = \delta_{exc} + \delta_{imp} \le 0.32 \cdot \max \Delta_{As} = \max \delta. \tag{2.27}$$

The numerator is a sum of the absorbances of all impurities and excipients for their maximum acceptable concentrations in the preparation (in the procedure dilution) and the denominator is the absorbance of the reference solution in the nominal concentration. Value of $max\ \Delta_{As}$ must comply with the ratios (2.7-2.8).

The δ_{noise} can be represented as the sum of contributions associated with the excipients (δ_{exc}) and impurities (δ_{imp}). As can be seen from the equation (3), the value of δ_{noise} must not exceed the maximum acceptable (target) systematical error $max\ \delta$.

///Often the question arises, how is it possible to prepare model solutions to verify linearity, precision and accuracy without the use of excipients (placebo). This is possible when the contribution of the placebo (δ_{exc}) in the total value of background absorption (δ_{noise}) is insignificant, i.e. taking into account (2.8), the ratio holds [27]:

$$\delta_{exc} \le 0.32 \cdot \max\delta = 0.32 \cdot 0.32 \cdot \max \Delta_{As} = 0.033 \cdot B. \tag{2.28}$$

2.6.2.1. Effect of placebo

Placebo solution (blank). Prepare in the same way as the sample solution, but instead of 0.1 g tablet powder, take the mixture of 0.07 g starch, calcium stearate and lactose in appropriate proportions.

Measure the absorbance (A_{blank}) of placebo solution, making no less than three replicates with cell withdrawal. In parallel, measure the absorbance (A_{st}) of the reference solution. Found: $A_{blank} = 0.00114$; $A_{st} = 0.7322$. Contribution of the placebo in a total preparation absorbance is $\delta_{exc} = 100 \cdot 0.00114/0.7322 = 0.16\%$.

2.6.2.2. Effect of degradation products

The impurity profile study was conducted for the ambroxol hydrochloride tablets, subjected to stressful exposure. The procedure described for the AHC substance was used [1]. It had been shown that the impurity profiles for the AHC substance and tablets were the same (detailed consideration of this issue is beyond the scope of this publication). Therefore, to assess the effect of impurities on the assay results it is enough to consider the effect of the substance profile impurities, taking into account the maximum impurities limits for the AHC tablets.

All the impurities have chromophores similar to a chromophore of ambroxol [1], so the total relative impurities content found by the internal normalization (in %), using HPLC with a spectrophotometric detector at 244 nm, will correspond to their relative contribution (δ_{imp}) in the total absorbance at 244 nm for the assay. It was found: $\delta_{imp} = 0.50\%$.

2.6.3. Model solutions, performance measurements and calculations

The reference and model solutions are prepared by the same scheme, so the actual X_i value from the relationship (2.1) is equal to quotient (in %) of the actual ACH substance weights taken for the preparation of the *i-th* model solution and the reference solution. The actual $X_{i,act}$ values are represented in Table 2.3.

The absorbance of each solution with cell withdrawal was measured. Measurements were carried out according to the following scheme: the reference solution (3 times), the model solutions of 1-3 (3 times for each solution), the reference solution (3 times), the model solutions of 4-6 (3 times for each solution), the reference solution (3 times), the model solutions 7-9 (3 times for each solution) and the reference solution (3 times). In the issue, there were received 12 absorbance values for the reference solutions and 3 absorbance values for each of the 9 model solutions. There was calculated the ratio of an average absorbance for each of 9 model solutions to an average absorbance for the reference solution, getting the value of $Y_i = (A_i/A_{st}) \cdot 100$. Also it was calculated the value of $Z_i = 100*(Y_i/ X_i)$, which was a recovery factor (found in % to the introduced value). The results of the calculations are presented in Table 2.3. Criteria were taken from Table 2.1.

Calculation of the linear relationship $Y = b \cdot X + a$ were conducted by the least square method [26]. The results of the calculations – values of b, s_b, a, s_a, s_r (the residual standard deviation) and r (correlation coefficient) - are presented in Table 2.2. The linear regression obtained in the normalized coordinates is illustrated on the Figure 2.1. It is typical for all applications of the reference standard method, regardless of the procedure specificity (spectrophotometry, liquid or gas chromatography).

2.6.4. Intermediate precision

Intermediate precision was studied on 5 samples of every tablet batches. We carried out the assay for each sample (i) according to the specification, performing 3 replicates for each solution. The Z_i value was calculated by the equation (2.1) (test 1). We carried out the same assays of this batch with the same number of replicates with other analysts in 2 another days using other volumetric glassware (tests 2 and 3). Obtained Z_i values were combined, the average $\overline{Z}$ value and standard deviation SD_{intra} were calculated. They were conformed to the equation (2.21). The results are summarized in Table 2.4.

2.6.5. Stability study

The specification doesn't regulate the time after which the absorbance is measured, so we checked its stability during 1 hour [5]. For that we measured the absorbances of the test (A) and reference (A_o) solutions by three times with the cell withdrawal immediately after preparation, in 15 min, 30 min, 45 min and 60 min. The results are presented in Table 2.5. We calculated the confidence interval and checked the conformance to the requirement (2.22). In our case (see Table 2.1) $max\ \delta = 0.75\%$.

2.6.6. Results and discussion

2.6.6.1. Specificity

Using the sections 2.6.2.1 and 2.6.6.2, let's assess the total background effect. In accordance with the equation (2.27), we get: $\delta_{noise} = \delta_{exc} + \delta_{imp} = 0.16 + 0.50 = 0.66\%$. From Table 2.1 for $B = 7.3\%$ we can find: $max\ \Delta_{As} = 2.34\%$, $max\ \delta = 0.75\%$. As you can see $0.66\% \leq 0.75\%$, i.e. the requirement (2.27) is met, the background absorption is insignificant, and the procedure is characterized by acceptable specificity.

In addition, the ratio of (2.28) shows that the contribution of placebo ($\delta_{exc} = 0.16\%$) is insignificant because $0.16 \leq 0.033 \cdot 7.3 = 0.24\%$. Thus, the model solutions can be prepared without a placebo, as was done in this case.

2.6.6.2. Linearity

Table 2.2 shows that in our case the requirements of Table 2.1 to the parameters of the linear relationship are met, i.e., the linearity of the method is confirmed throughout the concentration range 60-135%.

Table 2.2

The metrological characteristics of the linear relationship $Y = b \cdot X + a$

Name	Value	Table 2.1 criteria for the tolerances 92.7-107.3%, point number 9)	Conclusion (conform or no)
b	0.9937	-	-
s_b	0.0087	-	-
a	0.775	1) $\leq 1.8946 \cdot s_a = 1.63$; 2) if 1) doesn't conform than ≤ 2.4;	Conform
s_a	0.861	-	-
s_r	0.584	≤ 1.24	-
R	0.99973	≥ 0.99885	Conform

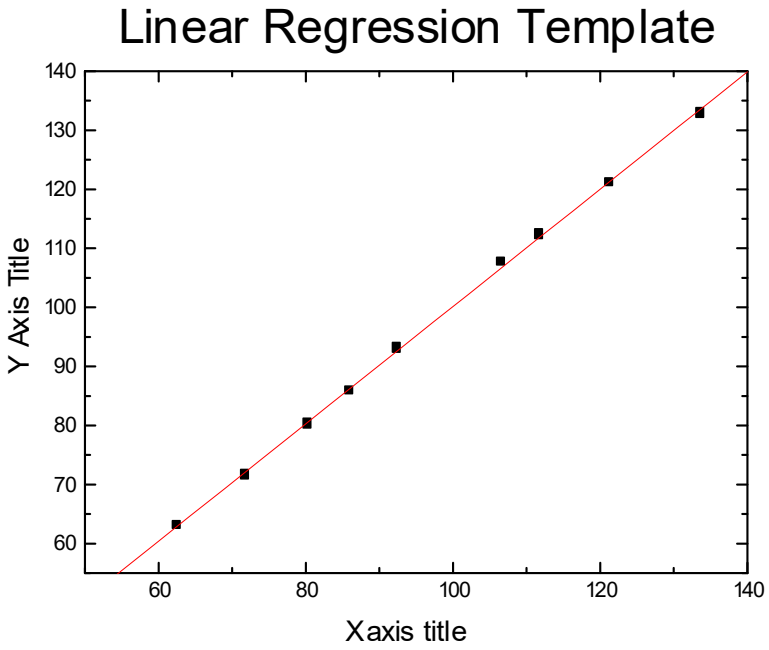


Figure 2.1. The linear relationship of the absorbance on ambroxol hydrochloride concentration in normalized coordinates.

2.6.6.3. Detection limit (*DL*) and Quantitation limit (*QL*)

Calculations of *DL* and *QL* for ACH were carried out optionally because SPU-Eur.Ph. [1] doesn't require them for an assay. We used equations (2.19-2.20) and Table 2.3 on the base of values s_a and b:

$DL = 3.3 \cdot 0.861 = 2.84$ % of nominal ACH concentration,

QL = 10·0.861 = 8.61 % of nominal ACH concentration (0.03 mg/ml – according to the specification procedure).

As you can see, these values are much less the lower range concentration limit (60%) and so cannot affect the procedure uncertainty.

2.6.6.4. Precision and accuracy

2.6.6.4.1. Repeatability and accuracy

Table 2.3 shows that the procedure has acceptable repeatability and accuracy through the whole analytical concentration range 60-135%.

Table 2.3

The results of assay of the model mixtures and their statistical processing (used the criteria of Table 2.1)

Model solution number	ACH weights, g (m_{st} =0.02974)	Introduced concentration in % to reference solution concentration – $X_{i,act}$ %	Average absorbance (A_i^{st} = 0.7322)	Found in % to reference solution concentration – Y_i%	Found in % to introduced Z_i = 100*(Y_i/ X_i) %
1.	0.01859	62.51	0.4619	63.07	100.9
2.	0.02134	71.77	0.5240	71.56	99.71
3.	0.02387	80.29	0.5871	80.18	99.86
4.	0.02555	85.92	0.6290	85.92	99.99
5.	0.02748	92.41	0.6812	93.03	100.68
6.	0.03170	106.62	0.7879	107.6	100.91
7.	0.03321	111.68	0.8224	112.31	100.56
8.	0.03607	121.29	0.8948	121.2	100.75
9.	0.03975	133.66	0.9725	132.82	99.37
Average, $\overline{Z}$ %					**100.30**
Relative standard deviation, s_z%					0.58
Relative confidence interval $\Delta\% = t(95\%,8) \cdot s_z = 1.860 \cdot s_z =$					**1.07**
Criterion for results repeatability $\Delta\% \leq$					2.34
Systematical error $\delta = \lvert \overline{Z} - 100 \rvert$					**0.30**
Criterion of systematical error uncertainty 1) $\delta \leq \Delta/3 = 1.07/3 = 0.36$; 2) If 1) doesn't met than $\delta \leq 0.73$					 Conform Conform
General conclusion about procedure:					Correct

2.6.6.4.2. Intermediate precision

Table 2.4

Results of intermediate precision study

	Z_i value		
Solution number	Test 1	Test 2	Test 3
1	99.42	99.66	99.96
2	99.57	99.76	98.87
3	97.23	96.99	99.09
4	97.53	97.63	98.61
5	99.53	99.12	98.53
Average	*98.65*	*98.63*	*99.01*
Pooled average $\overline{Z_{\text{int}\,ra}}$ %	**98.77**		
S_Z (%)	1.17	1.25	0.57
SD_Z (%)	**1.04**		
Δ_{intra}% (k = 5)	= 0.79*1.04 = **0.82** < 2.34%		

As you can see, requirements of the equation (2.21) are met, i.e. intermediate precision is confirmed.

2.6.6.5. Solution stability through time

Table 2.5

Solution stability through time

t, min	0	15	30	45	60	Average	RSD_t %	Δ_t%	$max\delta$, %
A_o	0.7560	0.7567	0.7595	0.7592	0.7618	0.7586	0.307	0.65	0.75
A	0.7522	0.7527	0.7539	0.7549	0.7567	0.7541	0.238	0.51	

As you can see, $\Delta_t \leq max\delta$ = 0.75%, i.e. test and reference solutions are stable during at least 1 hour.

2.6.6.6. Prognosis of total procedure uncertainty

To confirm the procedure correctness in another laboratory, we need in a total uncertainty prognosis.

The predictable total procedure uncertainty should not exceed the target assay uncertainty of Table 2.1 for tolerances of $\pm$ 7.3% ($max\ \Delta_{As}$ = 2.34 %). Total predictable uncertainty is calculated by the formula (2.23). In our case, the uncertainty of the final analytical operations (spectrophotometry) is known: according to the relation (2.25) it is equal to 0.70%. Therefore, let's calculate the uncertainty of sample preparation, which varies for different assays.

2.6.6.6.1. Sample preparation uncertainty prognosis

Calculations were made by the ratio (2.24), on the basis of the calculation formula of the specification and using the approach and the uncertainty limits of volumetric glassware, described in [11, 26, 27] (see Table 1.4.1.5).

Assay

Calculation formula; $$X = \frac{D_1 \cdot m_0 \cdot 10 \cdot 100 \cdot 100 \cdot b}{D_0 \cdot m_1 \cdot 100 \cdot 100 \cdot 10} \cdot \frac{P}{100}$$

Table 2.6

Calculation of the sample preparation uncertainty for the *Assay*

Sample preparation steps	Calculation formula parameters	Uncertainty [11]
Reference solution		
1. Weighing of ACH RS	m_0	0.2 mg/30 mg · 100 % = 0.67 %
2. Dilution to the mark in the 100 ml volumetric flask	100	0.12 %
3. Aliquot sampling with 10 ml pipette	10	0.25 %
4. Dilution to the mark in the 100 ml volumetric flask	100	0.12 %
Test solution		
5. Weighing of tablet sample	m_1	(0.2 мг/100 мг)·100 % = 0.2 %
6. Dilution to the mark in the 100 ml volumetric flask	100	0.12 %
7. Aliquot sampling with 10 ml pipette	10	0.25 %
8. Dilution to the mark in the 100 ml volumetric flask	100	0.12 %

$$\Delta_{SP} = \sqrt{0.67^2 + 0.12^2 + 0.25^2 + 0.12^2 + 0.2^2 + 0.12^2 + 0.25^2 + 0.12^2} = 0.82\%.$$

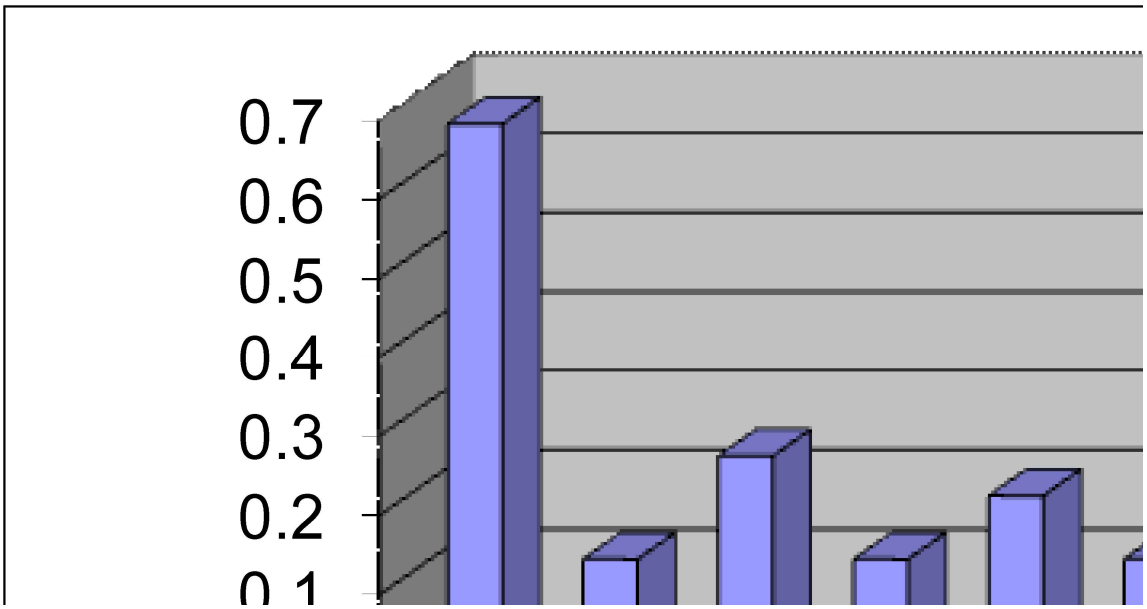


Figure 2.2. Step distribution of sample preparation uncertainty for the ACH tablet assay

From the Figure 2.2 we can see that the maximum contribution in the sample preparation uncertainty in the sample preparation is made by the step 1 - weighing of ACH RS, and steps 3 and 7 (aliquot sampling with 10 ml pipette) as well. This distribution of the sample preparation uncertainty components is sufficiently distinctive in the quality control of medicines.

Uniformity of content

Table 2.7

Calculation of the sample preparation uncertainty for the *Uniformity of content* test

Sample preparation steps	Calculation formula parameters	Uncertainty [11]
Reference solution		
1. Weighing of ACH RS	m_0	0.2 mg/30 mg·100 % = 0.67 %
2. Dilution to the mark in the 100 ml volumetric flask	100	0.12 %
3. Aliquot sampling with 10 ml pipette	10	0.25 %
4. Dilution to the mark in the 100 ml volumetric flask	100	0.12 %
Test solution		
5. Dilution to the mark in the 100 ml volumetric flask	100	0.12 %
6. Aliquot sampling with 5 ml pipette	5	0.37 %

7. Dilution to the mark in the 50 ml volumetric flask	50	0.17 %

$$\Delta_{SP} = \sqrt{0.67^2 + 0.25^2 + 0.12^2 + 0.12^2 + 0.12^2 + 0.37^2 + 0.17^2} = 0.85\%$$

Dissolution

Table 2.8

Calculation of the sample preparation uncertainty for the *Dissolution* test

Sample preparation steps	Calculation formula parameters	Uncertainty [11]
Reference solution		
1. Weighing of ACH RS	m_0	0.2 mg/30 mg·100 % = 0,67 %
2. Dilution to the mark in the 100 ml volumetric flask	100	0.12 %
3. Aliquot sampling with 10 ml pipette	10	0.25 %
4. Dilution to the mark in the 100 ml volumetric flask	100	0.12 %
Test solution		
5. Dilution to the mark in 1000 ml graduated cylinder	1000	1.0 %

$$\Delta_{SP} = \sqrt{0.67^2 + 0.25^2 + 0.12^2 + 0.12^2 + 1.0^2} = 1.24\%$$

2.6.6.6.2. Total uncertainty of the analytical procedure

Assay :

$$\Delta_{As} = \sqrt{\Delta_{SP}^2 + \Delta_{FAO}^2} = \sqrt{0.82^2 + 0.70^2} = 1.08\% \leq \max\Delta_{As} = 2.34\%.$$

Uniformity of content:

$$\Delta_{As} = \sqrt{\Delta_{SP}^2 + \Delta_{FAO}^2} = \sqrt{0.85^2 + 0.70^2} = 1.10\% \leq \max\Delta_{As} = 2.34\%.$$

Dissolution:

$$\Delta_{As} = \sqrt{\Delta_{SP}^2 + \Delta_{FAO}^2} = \sqrt{1.24^2 + 0.70^2} = 1.42\% \leq \max\Delta_{As} = 2.34\%.$$

As you can see, the predicted total uncertainties of the results for all three analytical procedures do not exceed the critical value (2.34%) i.e. the procedures will give the correct results in other laboratories.

2.6.6.7. Robustness

When testing the robustness of the spectrophotometry we should study [11, 27]: stability of solutions through time, the influence of pH, different reagents, analysts (human factor). In our case, the absorbance within $\pm$ 10% did not depend on the acidity of a solutions. Stability of solutions is confirmed in the section 2.6.6.5. Insignificance of the influence of reagents, equipment and human factor were confirmed when transferring the procedures.

Thus, the validation confirms the correctness of the quantitative procedures for the *Assay*, *Uniformity of content* and *Dissolution* of ambroxol hydrochloride tablets 0.030 g.

The considered validation scheme can without any serious changes applied to quantitative chromatographic procedures. In case of using chromatography for the *Uniformity of content* and *Dissolution* test, it is rational to use the metrologically substantiated rational scheme that allows to reduce by several times the experiment size [28] (see Annex 1).

It should be noted that the suggested approach may use the software.

3. VALIDATION OF CHROMATOGRAPHIC PROCEDURES FOR IMPURITY CONTROL IN DRUG PRODUCTS

In accordance with the requirements of SPU-Eur.Ph. [1, 3, 11] and GMP [34], procedures for control of related substances in drug product (DP) stability studies are the quantitative testing and must be validated.

Control of impurities in DP combines features of limit and quantitative tests. In the case of DP control for compliance to a specification in state control laboratories or laboratories of manufacturers we are dealing with the limit test, since the question is merely whether the impurity exceeds the specification limit or not. At the same time, impurity testing procedure, described in the specification, is also used to study the stability and control of the DP manufacturing process. In this case, it is not important only the compliance of the impurity content with the specification requirements, but the impurity content itself. I.e. we are dealing with a quantitative test. Validation criteria for both cases, generally speaking, are different.

Questions of medicine assay validation were enough discussed in the previous sections. At the same time, a systematic consideration of the issues associated with the validation of impurity control procedures in DP with a justification for the criteria that take into account the specificity of the DP, in the scientific literature, for various reasons, is quite rare. This causes certain difficulties for manufacturers which develop the registration dossier. This problem is particularly serious for the manufacturers that implement the GMP requirements, because the validation of analytical procedures is one of the prerequisites for such an implementation.

The most important method of impurity control in medicinal substances and DP is high performance liquid chromatography (HPLC), which is gradually replacing in this test all other methods. Therefore, the development and standardization of schemes for validation of HPLC impurity control procedures is an important issue for all DP manufacturers and developers of specifications.

This problem is multi-aspect and quite complex. Therefore, this section discusses the most common case – validation of impurity control procedure for a DP manufactured on the base of the pharmacopoeial medicinal substance and conformed to the pharmacopoeial requirements. It is further assumed that the procedure to be validated is included in the specification which establishes a limit for the impurity content. Note that as this procedure it is rational to use the procedure described in the European Pharmacopoeia monograph for the medicinal substance, or the procedure described in a pharmacopoeial monograph for the DP. However, since the excipients for a particular DP are not described in a Pharmacopeia, the procedure is still need of validation. In principle, this procedure can be also applied to much smaller or larger concentrations of impurities, but in this case, the requirements for its validation, generally speaking, can be very different.

This section is dedicated to the discussion of these problems. The consideration shall be carried out for the HPLC procedures; however all the findings and approaches

without any serious changes can also be applied to the gas chromatography procedures.

Unless otherwise specified, one-tailed confidence intervals for the probability of 95% are used.

Impurity control feature is that there is no nominal value for an impurity. So to obtain the normalized coordinates it is rational to use the specification impurity *ImL*, i.e. all impurity concentrations to express as a percentage of the *ImL*. This allows us to keep the basic principles of the approach to the validation, which was developed in the previous sections.

3.1. Theoretical part

3.1.1. Detection limit (*DL*)

This value is important when you use the control of impurities as a limit test, i.e. at the control of impurities for the specification compliance.

The validation of an impurity control procedure for a drug product (DP) rises a problem of requirements to a detection limit (*DL*) because the SPU [11] doesn't indicate what a value (let's call it *max DL*) is acceptable (maximum acceptable) for a correct control of the impurity according to a specification.

For example, for some limit test, the maximum acceptable concentration of a particular impurity according to the specification is *ImL* = 1.0%. The use of different analytical conditions allows getting *DL* values equal to, respectively, 0.1%, 0.2%, 0.3% and 0.4%. Which of these values are sufficient for a correct control of the impurity according to the specification?

The Technical Guide for the Elaboration of Monographs (European Pharmacopoeia) [4, p. 4] doesn't clarify this question. It recommends the analytical range from the *QL* or 50% (more of these values) to 120% of *ImL*. As there are no requirements to the *QL*, the question remains about the analytical range and the maximum acceptable *QL* or *DL* (taking into account the relationship between *DL* and *QL* [1]).

Based on the *Insignificance principle* in the wording (2.6), developed in the previous sections, *DL* is acceptable for the analysis according to the specification and significantly does not affect the quality conclusion for the limit test, if it is insignificant compared to the impurity specification limit, i.e.

Limit tests:

$$DL \le \max\ DL = 0.32 \cdot \mathrm{Im}\ L,$$
$$DL\,(\%) = 3.3 \cdot s_a = 100 \cdot \frac{DL}{\mathrm{Im}\ l} \le \tag{3.1}$$
$$\le \max\ DL\,(\%) = 32\,\%.$$

Here: *DL* (%) is a limit of detection in normalized coordinates. It is calculated on the

basis of the standard deviation (s_a) of the absolute term (a) of the linear relationship [3, 11].

In our case (ImL = 1.0%) we get $DL \leq 0.32\%$. Consequently, the indicated above values DL = 0.1%, 0.2% and 0.3% are acceptable for the control of impurities according to the specification and DL = 0.4% is unacceptable.

Thus, when we validate an impurity control procedure, we don't need to find the true DL value. It is enough to prove that DL meets the requirements of the ratio (3.1). It is much easier, because the true DL value can be tens or even hundreds of times less than *max* DL of the ratio (3.1). To find the true DL value in accordance with the requirements of SPU-Eur.Ph. [4, 11], it is necessary to conduct research in the range of concentrations, close to that DL, but often not representing any interest for the control of impurities according to the specification.

There is another important aspect. As a rule, we can't get absolutely pure active ingredient (without degradation products).Conducting the impurity control test, we must often take into consideration the unavoidable degradation of the active ingredient during the sample preparation, storage and chromatography of test and standard solutions, which leads to increased levels of detectable impurities. However, depending on the conditions, impurities can be increased many times compared to the original (this situation is common, for example, for cefuroxime discussed in the experimental part). Therefore, there is a direction to analyze freshly prepared solutions in some pharmacopoeial monographs (see, for example, Table 3.1- Cefoxitin sodium, Cefuroxime axetil). This means that for each sample, there is some minimum concentration limit below which we find not the real content of impurities in the original sample, but the content, which was formed during sample preparation, storage and chromatography. Therefore, the analysis in the range of very small impurity concentrations is fraught with large systematic errors. These shortcomings are absent for the approach based on the ratio (3.1).

3.1.2. Quantitation limit (*QL*)

Similarly to DL in the limit test, quantitation limit (QL) is acceptable for an analysis according to a specification and significantly does not affect the quality conclusion for the assay if it is insignificant compared to the impurity specification limit ImL, i.e., taking into consideration the ratio (2.6), we may have:

Quantitative tests:

$$QL \leq \max QL = 0.32 \cdot \mathrm{Im}\, L,$$
$$QL\,(\%) = 10 \cdot s_a = 100 \cdot \frac{QL}{\mathrm{Im}\, L} \leq \tag{3.2}$$
$$\leq \max QL\,(\%) = 32\,\%.$$

Here QL (%) is a quantification limit in normalized coordinates (i.e., percentage of ImL). . It is calculated on the basis of the standard deviation (s_a) of the absolute term

(*a*) of the linear relationship [3, 11].

Taking into consideration the relationship between *DL* and *QL* [11], it can be shown that the requirement (3.2) to the *QL* corresponds with the next requirement for *DL*:

$$DL \le \max\ DL = 0.10 \cdot \mathrm{Im}\ L,$$

Quantitative tests: $$DL\,(\%) \le \max\ DL\,(\%) = 10\,\%. \qquad (3.3)$$

Ratios (3.2) and (3.3) are equivalent. In practice, it is easier to operate with one *DL* value than with two ones (*DL* and *QL*). So the equation (3.3) is more suitable than (3.2).

3.1.3. Range

As can be seen from the section 3.1.1., when we conduct the impurity control according to the specification, its concentrations below *max DL* are not of interest for us. So the impurity concentration range must be chosen in such way that its lower border was not far below *max DL* (i.e. it was near *max DL* that we are looking for).

Taking into consideration the ratio (3.1), it may be offered the following range in normalized coordinates for limit tests [7]: 25-125% of the maximum acceptable level of an impurity according to the specification (*ImL*).

In a view of the ratio (3.2), the concentration range in normalized coordinates for quantitative tests would be the same, only the linearity requirements would be more stringent.

Limit test,
Quantitative test: **Range: 25 – 125% of *ImL*** . (3.4)

It should be noted that the proposed range is somewhat broader than it is recommended by the Technical Guidance of the European Pharmacopoeia [4], which for the control of a particular impurity recommends the range from *DL* or 50% of the *ImL* (the most of these values) up to 120% of the *ImL*. However, the range (3.4) seems more reasonable.

3.1.4. Requirements to an uncertainty of an analytical procedure

For quantitative testing requirements for procedure uncertainty are determined by the tolerances of analyzed component content ($\pm$ *B*% of the nominal value) - see ratios (2.7-2.8). However, in the case of a control of impurities in drug products the nominal contents and tolerances are missing – there is only an upper limit in the specification (not more than ...%). This makes it difficult to establish requirements for the target uncertainty and makes them not so unambiguous, as in the case of the assays (see previous sections). To set the requirements to the uncertainty of results of impurities control we can offer several approaches.

3.1.4.1. Approach based on a rounding error

In accordance with the SPU-Eur.Ph. general article "1.4. The Monograph. Test and assays. Limits" [1], the procedure result must be rounded up to the specified number of significant digits. For example, a procedure regulates the particular impurity content at a level not more than 0.2% and the total impurities content not more than 0.5%. This means, for example, that 0.1501 % and 0.2499% contents of the individual impurity, found experimentally, should be rounded up equally - to 0.2%, and 0.4501% and 0.5499% contents of the impurity must be rounded up to 0.5%. As you can see, in this case the quality conclusion does not change if the impurity content difference is 0.05% absolute. This value is the target uncertainty of determination of the individual impurity or total impurities content (Δ_{Imp}), expressed as a one-sided confidence interval for the probability of 95 per cent, i.e.:

$$\Delta_{\mathrm{Im}\,p} \leq 0.05\,\%\,abs \quad . \tag{3.5}$$

The disadvantages of this approach are the lack of its connection with the *DL* or *QL* and with the requirements to limit values of metrological characteristics of the linear relationship (see the section 2.3.4). In addition, errors or inaccuracies in the specification (in number of significant digits) automatically lead to the errors and inaccuracies in the acceptable Δ_{Imp} value. Therefore, in some cases, this approach can lead to unnecessarily large or small acceptable Δ_{Imp} values. As an example it may be noted the *Dequalinium Chloride* monograph [3] which regulates the maximum level of the impurity *A* at 1% (but not 1.0% that would be natural). This corresponds to a maximum acceptable uncertainty of rounding (and, accordingly, Δ_{Imp}) at 0.5% abs or 50% relative, which is clearly too much for Δ_{Imp}.

Another disadvantage of this approach is its statistical incorrectness. Indeed, for example, the ciprofloxacin hydrochloride monograph regulates the content of each impurities B, C, D, E at a level not more than 0.2%, and the total impurities content at a level not more than 0.5% [1]. If we assume that for each impurity the uncertainty Δ_{Imp} = 0.05%, the uncertainty for the total impurities content will be [26] the value $\sqrt{(4*0.05^2)}$ = 0.1%, but not 0.05% as it should be, on the basis of the rules for rounding of the value 0.5% (total impurities content). It should be noted that this deficiency, in varying degrees, is characteristic for all the other approaches, because of the summation of impurities peak areas.

Thus, this approach can be applied in some specific cases, but it cannot be recommended as a general principle for setting the limit uncertainty Δ_{Imp} of impurity control.

3.1.4.2. Approach based on the acceptable values of the correction factors

The correction factor is the ratio of the detector sensitivities to the standard substance and the examined [10].

In accordance with the approach of the European Pharmacopoeia [4, section 5.1.2], [10] where the correction factor is beyond the 0.8-1.2, it must be taken into account for the control of impurities with reference to the basic substance standardization. From here, you can see that the acceptable relative uncertainty of the particular impurity determination must not exceed 20%, i.e.:

$$\Delta_{\mathrm{Im}\ p}(relative\ ) \le 20\% . \tag{3.6}$$

Note that in this case the statistical incorrectness described in the section 1.4.1 is substantially leveled. In particular, for the same ciprofloxacin hydrochloride substance [1] (see section 3.1.4.1), each of the impurities B, C, D, E content is set at a level no higher than 0.2%, and the total impurities content is not above 0.5%. According to the ratio (6), the uncertainty of each impurity must be $\le 0.04\%$. Considering the rules of propagation of uncertainties [9], we obtain that the uncertainty of the sum equals to $\sqrt{(4 \cdot 0.04^2)} = 0.08\%$. At the same time, for the limit total impurities content of 0.5% ratio (3.6) gives the limit uncertainty of 0.1%. As can be seen, the ratio (3.6), in general, is met.

The disadvantage of this approach is that it isn't connected with *QL* and *ImL* and establishes the relative uncertainty of the impurity control regardless of its concentration (for example, determinations of the concentrations of 100% and 10% of the *ImL* have the same relative uncertainty) that is not always correct.

3.1.4.3. Approach based on disregard limits of peak areas

In many European Pharmacopoeia monographs (see Table 3.1) it is specified the disregard limit (*DRL*) of the peak area relative to the peak area of the reference standard (*RS*). This *DRL* is not taken into account in the control of impurities by HPLC. On the basis of general considerations, it is clear that the lower bound value of *DRL* cannot be less than *DL* for the limit tests and *QL* for the quantitative tests. In some cases, it is regulated the higher level of the signal-to-noise ratio (*S/N*), which allows, in view of the relationship of it with the *DL* (*DL = 2·S/N* or *DL = 3·S/N*) [11], to trace the relationship between these variables and the *DRL*.

Table 3.1

DRL values for some medicinal substance HPLC analysis [35]

№	**Monograph name**	**Page number** [35]	**Disregard limit**		***S/N* (<)**	
			In % to *RS* peak area	In % to *ImL* of specified impurity peak area	Value of *S/N*	In % to *RS* peak area
1.	*Amoxicillin sodium*	990	10	3.3-5		

2.	*Cefaclor*	1198	10	20		
3.	*Cefadroxil monohydrate*	1200	5	5	10 = *QL*	4
4.	*Cefalexin monohydrate*	1202	5	5		
5.	*Cefalotin sodium*	1203	10	10		
6.	*Cefamandole nafate*	1204	10	10		
7.	*Cefapirin sodium*	1206	5	5 - 16.7		
8.	*Cefatrizine propylene glycole*	1207	5	5		
9.	*Cefazolin sodium*	1209	5	5		
10.	*Cefixim*	1211	10	20		
11.	*Cefoperazone sodium*	1212	10	6.7		
12.	*Cefoxitin sodium**	1215	5	10		
13.	*Ceftazidime*	1218	10	20		
14.	*Ceftriaxone sodium*	1220	10	10		
15.	*Cefuroxime axetil**	1222	5	3.3-10		
16.	*Cefuroxime sodium*	1223	5	5		
17.	*Ciprofloxacin hydrochloride*	1302	25	50		
18.	*Dalteparin sodium*	1387			5	100
19.	*Dextropropoxyphene hydrochloride*	1414			5	100
20.	*Esketamine hydrochloride*	1533	20	50-100	3 = *DL*	4
21.	*Isoprenaline hydrochloride*	1839	5	5	3 = *DL*	5
22.	*Ketotifen hydrogen fumarate*	1875	25	25		
23.	*Naloxone hydrochloride dihydrate*	2080	10	10	10 = *QL*	4
24.	*Netilmicin sulphate*	2089			10= *QL* = *DRL*	
25.	*Neomycin sulphate*	2086	20%	6.7-33	10= *QL* = *DRL*	20

26.	*Pentoxyverine hydrogen citrate*	2204	10%	30	100	10
27.	*Pethidine hydrochloride*	2216	10%	10		
28.	*Phenylbutazone*	2229	25%	10-25		
29.	*Primaquine diphosphate*	2308			5 = *DRL*	
30.	*Ramipril*	2355			3 = *DL* = *DRL*	
31.	*Sodium fusidate*	2433			3 = *DL* = *DRL*	

*Solution is prepared immediately before use

Interrelation of *DRL* with *DL* and *QL* is evident on the examples of *Ramipril*, *Sodium fusidate* (*DRL* = *DL*), *Neomycin sulphate* and *Netilmicin sulphate* (*DRL* = *QL*). Thus, the lower limit of the *DRL* is really determined by *DL*. Therefore, taking into account the ratios (3.1-3.3), we can obtain such equations in the normalized coordinates:

Limit test: $$DRL = DL \leq 32\% . \quad (3.7)$$

Quantitative tests: $$DRL = DL \leq 10\% . \quad (3.8)$$

The requirements (3.7-3.8) are sufficient. However, the DRL values depend not only on the procedure sensitivity, but on the task conditions as well (for example, to ignore the peak areas that are lower not than *Dl* or *QL* but some other values). So, as you can see from the Table. 3.1, the *DRL* values vary in a quite wide range - from 3.3% (*Cefuroxime axetil*) to 100% (*Esketamine hydrochloride*) in relation to the peak area of a single regulated impurity. It sets usually more stringent requirements. Thus, for example, the *DRL* value for *Naloxone hydrochloride dihydrate* is 2.5 times more than its *QL* value (it confirms that *DRL* depends not only on the procedure sensitivity, but on other considerations as well).

In general, as can be seen from the Table. 3.1, the ratios (3.7-3.8) are met. For quantitative tests a *DRL* value is usually 5-10% to the *ImL* of the specified impurity, i.e. the (3.8) ratio is satisfied. Higher *DRL* values (for example, *DRL* = 50% for *Ciprofloxacin hydrochloride*) indicate the limit tests and the (3.7) ratio.

Taking into consideration the (3.7-3.8) ratios, we can evaluate the requirements to an uncertainty of an impurity control procedure (Δ_{Imp}). SPU-Eur.Ph. doesn't indicate directly the interrelation between *DL* and Δ_{Imp} [11], however this interrelation can be estimated. In accordance with SPU-Eur.Ph. [11], we have:

$$DL = 3.3 \cdot \sigma / b . \quad (3.9)$$

$$QL = 10 \cdot \sigma / b = 3 \cdot DL \quad . \tag{3.10}$$

Here: b is the slope of the calibration line (in normalized coordinates ($b \approx 1$); σ is the standard deviation of the signal, which can be used by the standard deviation of the blank signal or the absolute term (a) in the calibration line. Taking into account that the Gaussian coefficient for the 95% probability (accepted for the Δ_{Imp} calculations) is 1.645 [26], we can get $\Delta_{Imp} = 1.645 \cdot \sigma \approx 0.5 \cdot DL$. Then the ratios (3.9-3.10) give:

$$\Delta_{\mathrm{Im}\ p} = 0.5 \cdot DL = 0.5 \cdot DRL = 0.17 \cdot QL \quad . \tag{3.11}$$

Consider the (3.7-3.8, 3.11) equations, we can get:

Limit test: $$\Delta_{\mathrm{Im}\ p} \le 16\% \quad . \tag{3.12}$$

Quantitative test: $$\Delta_{\mathrm{Im}\ p} \le 5\% \quad . \tag{3.13}$$

Requirements (3.12-3.13) to the uncertainty of the monitoring of contaminants ((Imp) are based on ratios (3.7-3.8) and are sufficient. More stringent requirements to *DRL* are hardly feasible.

It should be noted that the real *DRL* values in the Eur.Ph. monographs (Table 3.1) do not meet the requirements to the system suitability test of the Eur. Ph. general article "Chromatographic separation techniques" [13]. In accordance with these requirements [13], shall be the inequalities: $DL < DRL$, $QL \le DRL$. But these inequalities contradict each other (considering $QL = 3 \cdot DL$ (see ratio (3.10)) and can lead to unnecessarily strict requirements for the procedure precision.

In fact, in the case of *DRL* = 5% (for example, Cefazolin sodium, Cefuroxime sodium and others in Table 3.1) the relationship (3.11) gives Δ_{Imp} = 2.5%. There are even more incomprehensible strict requirements to *QL* for the Cefadroxil monohydrate (*QL* = 4% of *ImL*). In this case the ratio of (3.11) gives Δ_{Imp} = 0.68%. At the same time, the usual uncertainty of chromatographic assays in the European Pharmacopoeia and SPU for medicinal substances is 2% [1, 3] and for drug products with tolerances of $\pm$10% (one of the most common cases) is 3.2% (see the relationship (2.8)). It should be noted that the uncertainty of 5% corresponds to the assay precision of drug products with tolerances of $\pm$ 15% (the ratio of (2.8)), that is quite sufficient for the control of impurities.

It should be noted that when controlling residual solvents by using the head-space gas chromatography procedure, Eur. Ph. - SPU allows related standard deviation of 15% for three replicated differences between test and standard solutions peaks [36]. This corresponds to a confidence interval of average values of 25%. Against this background, the uncertainties of HPLC procedures for the impurity control limit test of 16% and for quantitative testing of 5% are perfectly acceptable.

The advantage of this approach is its clear connection with the *DL, QL* and *ImL* values. It allows to tie it with the parameters of the linear relationship. In addition, the ratios of (3.12-3.13) set the uncertainty in per cent to an impurity *ImL*, regardless of the concentration of this impurity. So, for example, for a impurity concentration of $0.5 \cdot ImL$ the assay relative uncertainty, in accordance with the ratio of (3.13), will be 10% but not 5%. I.e. smaller concentrations have a greater relative uncertainty that is natural and distinguishes this approach from others.

So, summarizing, we may say that the requirements to uncertainties of impurity control procedures based on ratios of (3.11-3.13), appear to be the most reasonable, and will be used next.

3.1.5. Specificity

Control of impurities in drug products usually regulates contents of some specific impurities, contents of any other impurities and the total content of all impurities.

As mentioned above, it is assumed that the procedure for control of impurities in a drug product is a pharmacopoeial one, i.e. or is the same as for the corresponding medicinal substance, or described in the pharmacopoeial monograph for this drug product. This means that this procedure is validated and, if the system suitability test is satisfied, provides the necessary control of the substance impurities. Therefore, when checking the specificity of this procedure for the drug product you must prove the absence of effects of the matrix (excipients), i.e. to prove that:

- the peaks of all possible impurities for the test solution are separated from the principal peak (i.e. impurities content is not underestimated);
- peaks of the excipients or products of their interaction with the substance don't significantly affect the peaks of the regulated impurities.

One way for such evidence is to compare the impurity profiles of the "stress" and the original solutions of the drug product placebo, the substance and the drug product [4, p. 2.2.2].

"Stress" solutions can be obtained by degradation of drug product, substance, drug product placebo in the alkaline or acid hydrolysis, heat, oxidation, UV irradiation.

The procedure specificity can be considered to be proved if:

- For all chromatograms of "stress" placebo solutions there are no impurity peaks with the retention times that coincide with the peaks of the regulated impurities or the principal peak for the chromatograms of the substance. If these impurities are present, their content must not exceed the uncertainty of the corresponding impurity assay result of determining individual impurity (Δ_{Imp}). This means that the drug product excipients do not significantly affect the results of the impurity control according to the specification procedure.
- For the drug product "stress" chromatograms, peaks of all impurities are separated from the principal peak, and the principal peak stands the test for the chromatographic purity.

3.1.6. Robustness, system suitability test

The specificity must be confirmed in different columns (column series and size, the sorbent brand manufacturers vary). To do this, choose the "stress" drug product solution that represents the "worst" case. Similarly, choose the "worst" case for the "stress" placebo solution. For all the columns must be the specificity of the section 3.1.5.

3.1.6.1. Stability of the solutions

Checking the stability of the test and reference solutions is one of the elements of the procedure robustness study [11] and must be carried out before all other validation studies. For the assay procedures this issue was discussed in the previous sections. When checking the stability of solutions for related substances (impurities) control procedures, the *Confirmatory approach* is used [27]: the solution is considered sustainable, if any impurity content or the total impurities content through a selected period differ from the original content in the freshly prepared solution of not more than $\sqrt{2}\cdot \Delta_{Imp}$ (as a difference of two means [26]), i.e.:

$$\left|Dif\ (stab\ )\right| \le \sqrt{2}\cdot \Delta_{Im\ p}\ . \qquad (3.14)$$

3.1.7. Linearity

This characteristic is studied within the *Range* (see section 3.1.3) [11].

In accordance with the approach developed in the previous sections, it is rationally to study the linearity and other metrological characteristics in normalized coordinates (i.e. the linear relationship $Y_i = b\cdot X_i + a$). In this case the X_i concentrations (X-axis) are taken as a percentage of the maximum specification impurity concentration (*ImL*) and the peak areas (Y_i) are taken as a percentage of the peak area corresponding the 100% of *ImL*. As follows from the relation (3.4), the validation studies are conducted in the range 25-125% of the *ImL* - as for limit or quantitative tests.

For limit testing linearity verification is not required [11]. However, you must show that the sensitivity and accuracy of the procedure is sufficient for the task (limit test). This means that you need to find *Dl* or *QL* and check their insignificancy in comparison with *ImL* (equations (3.1-3.3)). The easiest way to get such results is from the linearity studies (see section 2.3.4).

As on the requirements of the SPU [11], 5 concentration levels (25, 50, 75, 100 and 125 %) are enough for linearity study. In case of limit tests it is enough for calculation of *DL* and *QL* and confirmation of the necessary correctness and accuracy. Typically, impurity control procedures are validated as quantitative tests as well - for stability studies. In this case, as shown in section 2.3.4, it is advisable to carry out research on 9 concentration levels because it makes possible to calculate the accuracy and precision in accordance with the requirements of the SPU [11].

At the same time, the requirements to the accuracy and precision of an impurity control procedure are much more liberal (see ratios (3.12-3.13)) than for an assay (Table 2.1). So it is too complex to explore 9 points, distributed evenly over the entire range (as in the case of an assay, see section 2.1). Better to get 2 points for each of 5 concentration levels in the range of 25-125% (25, 50, 75, 100 and 125%) while one of the spots, which is 100% concentration, is taken as the standard for normalized coordinates transform (see section 2.1). As a result, we have g = 9 points (25, 25, 50, 75, 50, 75, 100, 125, 125), i.e. the same as for the assay (see section 2.1).

Taking into account the ratios (3.12-3.13) and the approach developed in the previous sections, the critical value of the residual standard deviation of the linear relationship (RSD_o) may be found from the ratio of:

Limit tests:

$$RSD_o(\%) \le \Delta_{Im\,p}(\%) / t(95\%, g-2) = \\ = 16 / 1.8946 = 8.4\% \quad (3.15)$$

Assays:

$$RSD_o(\%) \le \Delta_{Im\,p}(\%) / t(95\%, g-2) = \\ = 5 / 1.8946 = 2.6\% \quad (3.16)$$

For our 9 points (25, 25, 50, 50, 75, 75, 100, 125, 125%) we have $SD_x = RSD_{range} =$ 38.41%. Taking into account the ratios (3.15-3.16) and (2.16), the critical value of the correlation coefficient R_c for our are:

Limit tests:

$$R_c \ge \sqrt{1 - \frac{RSD_o^2}{RSD_{range}^2}} = 0.9755 . \quad (3.17)$$

Assays:

$$R_c \ge 0.9976 . \quad (3.18)$$

Requirements for the linear relationship *Y*-intercept:

1) The *statistically* insignificant difference from zero, i.e. (see section (2.17)):

$$a \le t(95\%, g-2) \cdot s_a = 1.89 \cdot s_a . \quad (3.19)$$

2) If the a value is statistically significant, we check its *practically* insignificant difference from zero. Taking into account the ratios (3.12-3.13) and (2.18), we get in the normalized coordinates, i.e. for our case in % to *ImL*:

Assays:

$$|a| \le \frac{0.32 \cdot \Delta_{imp}(\%)}{1-(25/100)} = 2.1\%. \quad (3.20)$$

Limit tests:

$$|a| \le \frac{0.32 \cdot \Delta_{imp}(\%)}{1-(25/100)} = 6.8\%. \quad (3.21)$$

3.1.8. Accuracy and precision

These characteristics are calculated in the same way as for the assays (see sections 2.3.2-2.3.3.).

3.2. Example. Validation of the related substance control procedure for the *Cefuroxime Sodium*

As an object of the validation study to test the scheme we used a powder of *Cefuroxime Sodium* (CS) for injection solution preparation.

For the study we used this preparation itself and *cefuroxime sodium CRS* of the European Pharmacopoeia [3]. Reagents and volumetric solutions used conformed to requirements of the SPU [1].

Analytical equipment: accepted liquid chromatograph Agilent 1100 3D LC System, Agilent Technologies; accepted 204 AG scales, Mettler Toledo; Class A volumetric glassware of Simax, Czech Republic, meeting the requirements of SPU [1, 11].

3.2.1. Procedure to be validated

In this case for the calculation of related impurities it is used the HPLC assay procedure for cefuroxime sodium as provided by the Eur. Ph. monograph for *Cefuroxime Sodium* [3]. Consider the specificity of the analysis in each particular analytical laboratory, SPU-Eur.Ph. allows limited variations of chromatographic conditions defined in the monograph [13]. If the variations exceed these limits, it is necessary to carry out the validation of the methodology, but not fully in accordance with the established requirements, and on the most critical validation characteristics that reflect real changes.

3.2.1.1. Related substances

Test conduct by liquid chromatography in accordance with the requirements of SPU-Eur.Ph. [1, 3].

50 µl of the test solution and the solution comparison (*c*) prepared in the section "Assay", alternately chromatograph on the liquid chromatograph with a UV-detector under the conditions described in the section "Assay".

The chromatographic time of the test solution should be four time longer than retention time of the principal peak.

In the chromatogram obtained with the test solution, the peak area of the impurity A, as well as peak area of any other impurity, should not exceed the principal peak area in the chromatogram obtained with the reference solution (*c*) (not more than 1.0%); the sum of all peak areas, except the principal peak area, should not exceed more than 3.0 times the area of the principal peak in the chromatogram obtained with the reference solution (*c*) (not more than 3.0%); do not take into account the peaks with an area of less than 5% of the area of the principal peak in the chromatogram obtained with the reference solution (*c*) (0.05%).

The analysis results are considered valid if the system suitability requirements are met.

3.2.1.2. Assay

The test is conducted by liquid chromatography in accordance with the requirements of SPU-Eur.Ph. [1, 3].

Test solution. Dissolve about 0.04 g (accurate mass) of the container content in *water R* and dilute to 100.0 mL with the same solvent.

For the test "Related substances" the test solution prepare immediately before use.

For the assay the test solution use freshly made (storage time 6 hours).

Reference solution (a). Dissolve about 0.04 g (accurate mass) of the *cefuroxime sodium EP CRS* or *SPU CRS* in *water R* and dilute to 100 mL with the same solvent.

Use the solution freshly made.

Reference solution (b). Place 20 mL of reference solution (*a*) in a water bath at a 80 °C for 15 min. Cool and inject immediately.

Reference solution (c). Dilute I ml of test solution to 100 mL with *water R.*

Use the solution freshly made.

Column (column 1)*:*

- *size*: l = 0.150 m, θ = 4.6 mm;
- *stationary phase*: *Lichrospher 60 RP-select B* (5 μ).

Mobile phase: mix 8 volumes of *acetonitrile R* and 92 volumes of an acetate buffer solution pH 3.4, prepared by dissolving 6.01 g of *glacial acetic acid R* and 0.68 g *sodium acetate R* in *water R and diluting* 1000.0 mL with the same solvent.

Flow rate: 2.0 mL/min.

Detection: spectrophotometer at 273 nm;

Autosampler temperature: 10 °C.

Column temperature: 25 °C.

Injection: 5 µL loop injector; inject 3 times reference solution (*b*), then 3 times alternately test solution and reference solution (*a*).

Run time: 4 times the retention time of cefuroxime.

System suitability:

- *resolution*: minimum 2.0 between the peaks due to cefuroxime and impurity A in the chromatogram obtained with reference solution (*b*);
- *relative standard deviation*: maximum 1.30% calculated for the peak areas due to cefuroxime in the 3 chromatograms obtained with reference solution (*a*);
- *column efficiency*: minimum 2500 theoretical plates calculated for the peak due to cefuroxime in the chromatogram obtained with reference solution (*a*);
- *symmetry factor:* maximum 2.0 calculated for the peak due to cefuroxime in the chromatogram obtained with reference solution (*a*).

Such changes were introduced into the compendial procedure:

- Compendial stationary phase *hexylsilyl silics gel for chromatography R* was changed to *Lichrospher 60 RP-select B;* it entailed a correction of the chromatographic conditions – increase of the acetonitrile fraction in the mobile phase.
- Sample preparation was optimized to reduce its uncertainty but the quantity of the chromatographed sample remained the same as in the Eur.Ph, monograph.
- Autosampler temperature was set 10 ^{0}C to reduce cefuroxime degradation at the room temperature (it degrades to the impurity A mainly).

In view of these changes, as well as the related substances control procedure will also be used to control the content of impurities in studying the stability of the drug, the procedure requires validation by the most critical validation characteristics: specificity, robustness, linearity, accuracy, precision (repeatability), quantitation limit (QL) and the detection limit (DL).

Uncertainty of the result of determining individual impurity and sum of impurities (Δ_{Imp}), expressed as a one-sided confidence interval for 95% probability, must meet the ratio (13), i.e. does not exceed 5% of the maximum content of impurities (*ImL*) that in absolute units for content of impurities and other individual impurity will be $\Delta_{Imp} \leq 0.05\%$ and for sum of impurities $\Sigma\Delta_{Imp} \leq 0.15$.

3.2.2. Specificity and robustness

3.2.2.1. Specificity

The test solution was subjected such “stress” effects: acid hydrolysis, alkaline hydrolysis, heating, UV-exposure.

The treated solutions were chromatographed under above–mentioned conditions. To get a “representative” solution, we mixed equal volumes of “alkaline stress” and “UV stress” solutions.

To select the optimal chromatographic conditions, we chromatographed the "representative" solution under above-mentioned conditions, modifying the content of acetonitrile from 4% to 10%.

In optimal conditions, "representative" solution we chromatographed on another column (column 2): size 4.6 × 150 mm, filled with sorbent Symmetry C-18 (5 μ).

3.2.2.2 Solution stability study

The time during which the solutions are stable, must be sufficient when used in routine analysis of the chromatograph autosampler, i.e. it shall be not less than 6 hours. To verify this, the test solution was chromatographed immediately after preparation of solutions and through 6 hours.

The cefuroxime sodium solution is not stable by reason of hydrolysis. The principal product of hydrolysis is impurity A of cefuroxime [3]. Intensive accumulation of this impurity is already observed at room temperature of the solution and increases with increasing temperature. To slow the formation of impurity A in the solution, autosampler thermostating at 10 ^{0}C was introduced into the procedure (see results in Table 3.2).

There were also simulated "worst-case" chromatographic conditions conducive to the accumulation of impurity A in a solution (room temperature of the autosampler, column temperature 35 °C, the content of acetonitrile in mobile phase of 3%), and received the results of the verification of the stability of the test solution (results are in Table 3.3).

In the section 3.2.1 it was shown that$\Delta_{Imp} \leq 0.05\%$ (abs) and $\Sigma\Delta_{Imp} \leq 0.15\%$ (abs). According to the relation (3.14), a deviation from its original value should not exceed: $Dif\ (stab) \leq \sqrt{2} \cdot 0.05 = 0.07\%$ (abs) for an individual impurity content and $\Sigma Dif(stab) \leq \sqrt{2} \cdot 0.15 = 0.21\%$ (abs) for the total impurity content.

3.2.3. Model solutions, measurements and calculations

Table 3.5 shows the theoretical $X_{i,theor}$ and the actual $X_{i,act}$ values (normalized) of the cefuroxime sodium content in model solutions. The model solutions and reference solution were prepared from individual weights with use of gravimetric aliquot sampling.

Measurements were carried out in the following order: *3 measurements of the 1-st model solution; 3 measurements of the 2-nd model solution 2; … 3 measurements of the i-th model solution;… 3 measurements of the 9-th model solution*. Between the model solutions we chromatographed the reference solution, obtaining a total of at least 3 replicate chromatograms.

There were calculated the ratios of a mean of the cefuroxime peak area of each of 9 model solutions to a mean cefuroxime peak area of the reference solution to get the value of $Y_i = (S_i/S_{st})*100$. Then there were calculated the value of $Z_i = 100 * (Y_i/X_i)$, which are the found concentrations calculated as a percentage of the input concentra-

tions. The results of the calculations are presented in Table 5. The criteria are based on the approach [4,5].

Calculations of the linear relationship $Y = b * X + a$ were conducted by use of the least square method [26]. The results of the calculations – values of b, s_b, a, s_a, s_r (the residual standard deviation) and r (correlation coefficient) - are presented in Table 4, the resulting linear relationship in normalized coordinates - in Figure 3.6.

3.2.4. Results and discussion

3.2.4.1. System suitability

The optimum acetonitrile concentration in the mobile phase is 8%, because in this case the run time significantly shortens, the cefuroxime peak is separated from the closest impurity peaks and impurity peaks are separated from each other (see Figure 3.1).

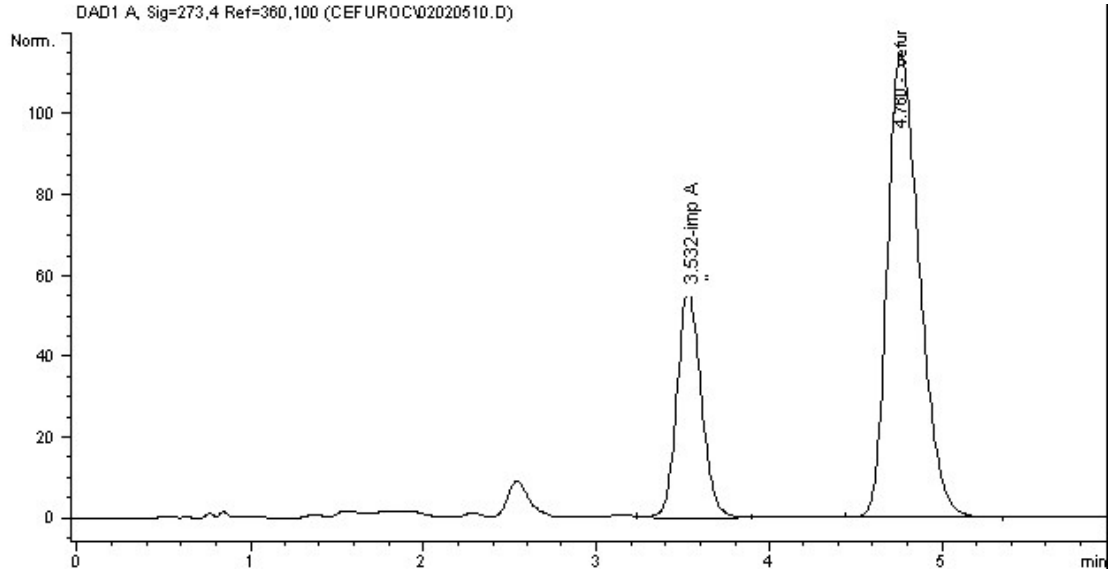


Рис. 3.1. A typical chromatogram of the reference solution (*b*) for 8% of acetonitrile content in the mobile phase.

3.2.4.2. Specificity

As we can see from Figures 3.2-3.3, impurity profiles in the chromatograms of the "representative" solution, obtained in different columns, are similar, peaks of impurities are separated from the peaks of cefuroxime and impurity A (specified in the specification), a test for the peak purity is met for the cefuroxime peak in the chromatogram of the “representative” solution. So the procedure is specific.

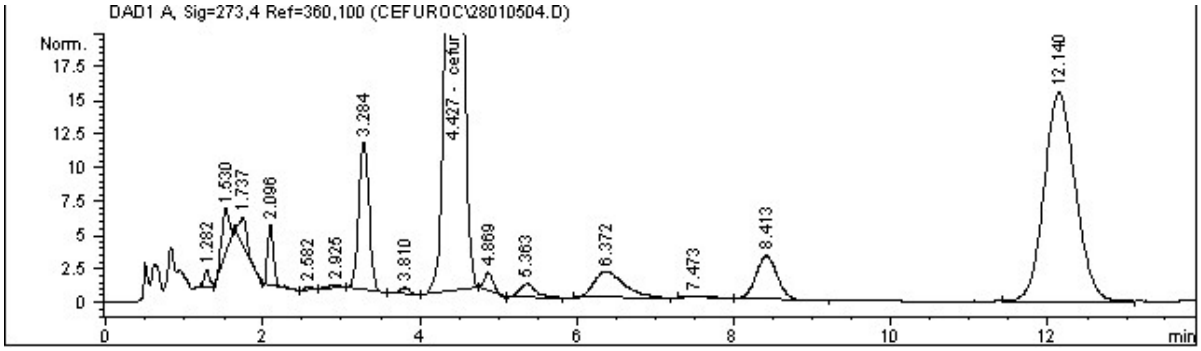


Figure 3.2. A typical chromatogram of the "representative" solution in the column 1.

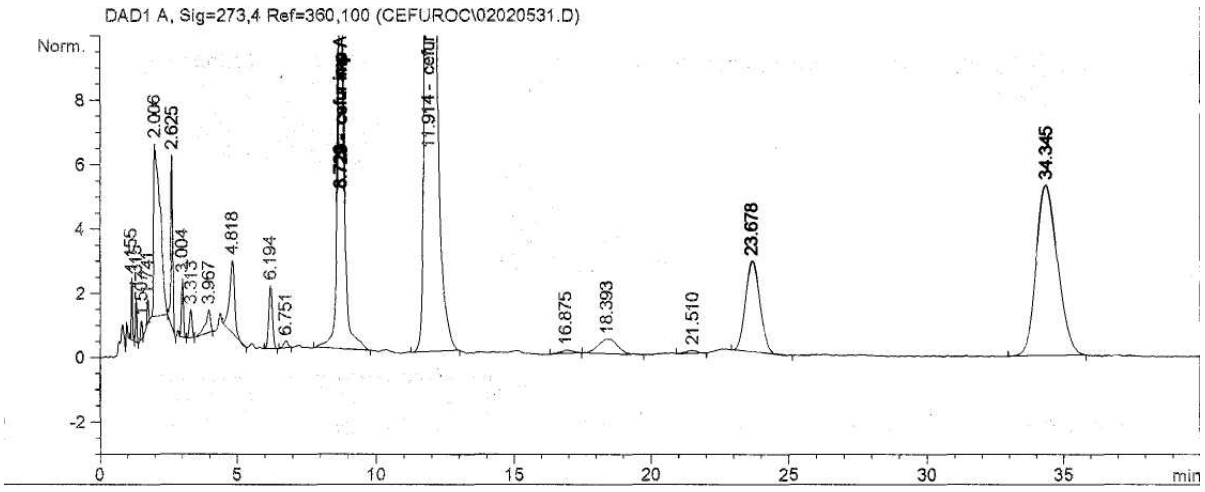


Figure 3.3. A typical chromatogram of the "representative" solution in the column 2.

3.2.4..3. Stability of solutions through time

Calculations of impurity content in the freshly made test solution and through 6 hours after preparation are presented in Table 2. 3.2. A typical chromatogram is presented in Figure 3.4.

Table 3.2

Results of the stability study of the test solution under specification conditions (average cefuroxime peak area of the chromatogram of the reference solution (*c*) = 230.1)

Freshly made test solution		
Impurity retention time, min	Average impurity peak area	Impurity content, % of average cefuroxime peak area of reference solution chromatograms
1.1	1.48	0.01
1.3	1.32	0.01
1.7	0.64	0.00

1.9	4.06	0.02	
2.3	3.22	0.01	
2.8	8.06	0.04	
3.3 (impurity A)	14.59	0.06	
5.7	10.34	0.04	
8.9	9.42	0.04	
10.2	2.95	0.01	
12.3	30.74	0.13	
14.4	3.66	0.02	
Total impurity content	89.75	0.39	
Test solution after 6 hours			
Impurity retention time, min	Average impurity peak area	Impurity content, % of average cefuroxime peak area of reference solution chromatograms	$\lvert Dif(stab) \rvert \leq 0.07\%$ $\lvert \Sigma Dif(stab) \rvert \leq 0.21\%$
1.1	1.40	0.01	0.00
1.3	1.71	0.01	0.00
1.7	0.54	0.00	0.00
1.9	4.02	0.02	0.00
2.3	10.91	0.05	0.04
2.8	8.79	0.04	0.00
3.3 (примесь А)	42.82	0.19	$0.13 \geq 0.07$
5.7	9.47	0.04	0.00
8.9	9.73	0.04	0.00
10.2	2.56	0.01	0.00
12.3	31.59	0.14	0.01
14.4	3.80	0.02	0.00
Total impurity content	126.30	0.55	$0.16 \leq 0.21$

As we can see from the Table 3.2, the contents of individual impurities and their total content in the test solution after 6 hours are within acceptable limits, i.e. satisfy the ratio (3.14). The exception is the cefuroxime impurity A (retention time of 3.3 min)

for which the ratio (3.14) is not running. However, its content is less than the critical value of the ratio (3.2) for the limit of quantification ($QL \leq 0.32\%$). Total impurities content after 6 hours also remains within the acceptable limits of the specification ($\leq$ 3.0%), so the growth of the impurity A in the test solution through the study time can't affect a positive conclusion about the quality of the medicine during the routine control. But if the original impurity A content in the medicine exceeds 0.32%, it is possible that through 6 hours the medicine will not meet the requirements of the specification.

Based on the stability study, the specification was supplemented with the instruction to prepare a test solution for related impurities directly before chromatography to avoid accumulation of the impurity A in the solution.

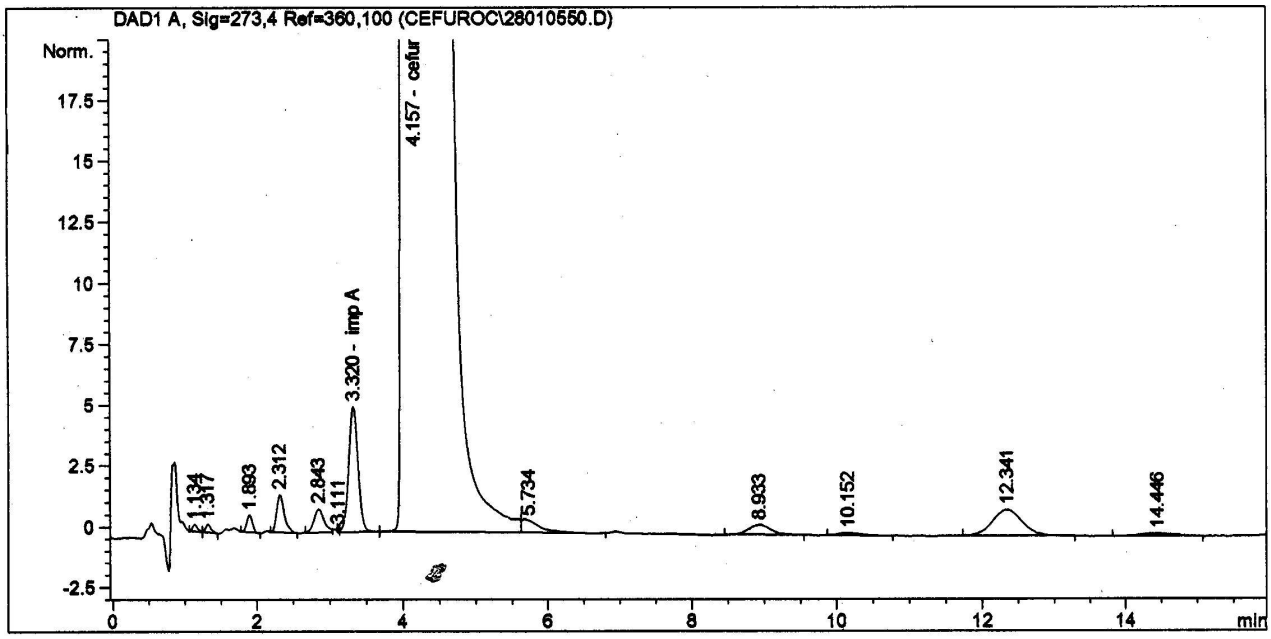


Figure 3.4. A typical chromatogram of the test solution obtained during its stability study under the specification condition.

The results of stability study reveal a high risk to get a wrong content of the impurity A in a test preparation because of cefuroxime decomposition through the analysis process.

In order to determine whether it may lead to the wrong conclusion about the quality of the drug, we have simulated the conditions under which is intensive accumulation of cefuroxime impurity A: room temperature of the autosampler, column temperature of 35 °C, acetonitrile content in the mobile phase of 3%.

Table 3.3 shows the calculations of impurities content in the freshly prepared test solution and through 2.5 hours after preparation. A typical chromatogram is presented in Figure 3.5.

Table 3.3

Results of the stability study of the test solution with intensive accumulation of impurity A (average area of cefuroxime peak of the reference solution (*c*) chromatograms is 218.5)

Freshly made test solution			
Impurity retention time, min	Average impurity peak area	Impurity content, % of average cefuroxime peak area of reference solution chromatograms	
1.5	2.00	0.01	
2.6	6.67	0.03	
3.8	4.91	0.02	
4.2	9.67	0.04	
4.9	49.55	0.23	
7.6 (impurity A)	145.84	0.67	
9.3	18.82	0.09	
31.5	23.21	0.11	
38.0	27.04	0.12	
Total impurity content	274.24	1.26	
Test solution after 2.5 hours			
Impurity retention time, min	Average impurity peak area	Impurity content, % of average cefuroxime peak area of reference solution chromatograms	$\lvert Dif(stab) \rvert \leq 0.07\%$ $\lvert \Sigma Dif(stab) \rvert \leq 0.21\%$
1.5	3.87	0.02	0.01
2.6	11.02	0.05	0.02
3.8	4.93	0.02	0.00
4.2	8.54	0.04	0.00
4.9	91.67	0.42	$0.19 \geq 0.07$
7.6 (impurity A)	247.59	1.13	$0.46 \geq 0.07$
9.3	18.28	0.08	0.01
31.5	22.20	0.10	0.01
38.0	26.98	0.12	0.00
Total impurity content	421.45	1.93	$0.67 \geq 0.21$

As can be seen from the Table 3.3, variations of the individual impurities contents in the test solution through 2.5 hours are within an acceptable limits of the ratio (3.14) for most of the impurities except of the cefuroxime impurity A (retention time of 7.6 min) and an impurity with the retention time of 4.9 min. The change of the total impurity content after 2.5 hours also exceeds the requirements of the ratio (3.14), i.e. the test solution is unstable for 2.5 hours under selected chromatographic conditions.

The content of impurity A in the freshly prepared solution exceeds the critical value (0.32 %) of the ratio (3.2) for the *QL*. In 2.5 hours after the preparation, the content of impurity A exceeds the maximum acceptable value of the specification (not more than 1.0%), i.e. in a routine quality control analysis the drug would fail the specification requirements.

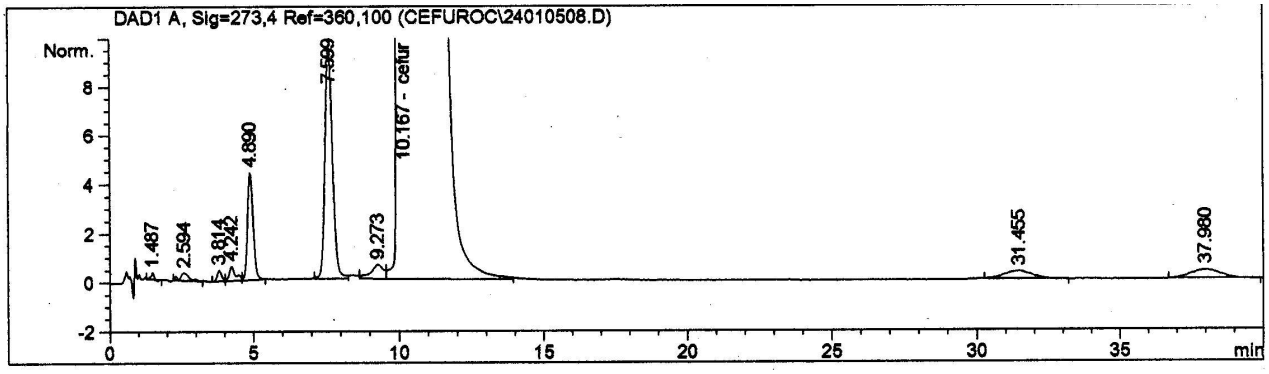


Figure. 3.5. A typical chromatogram of test solution obtained during stability study of the test solution at autosampler room temperature and column temperature of 35 ^{0}C.

The results of stability study (Table 3.2-3.3) clearly show in this case the futility of finding the true values of *QL* and *DL* - these values are unattainable because of the decomposition of cefuroxime and accumulation of impurity A during the analysis. At the same time, the proposed approach, based on confirmation of compliance of *QL* and *DL* to maximum acceptable values (ratios (3.1-3.2)), enables us to correctly evaluate the quality of the products.

3.2.4.4. Linearity

Evaluation of the linearity is conducted in accordance with the scheme described earlier in the section 2.3.4. The results are presented in Table 3.4, the regression line is in Figure 3.6. As can be seen from the Table 3.4, in our case, the requirements to the parameters of a linear relationship are met, i.e. the linearity of the procedure is confirmed throughout the range of concentrations of 25-125%.

Table 3.4

Metrological characteristics of the linear relationship $Y = b \cdot X + a$

Characteristic	Value	Criteria (target uncertainty of the analysis results is 5%, point number is 9 in the range 25-125 %)	Conclusion (complies or not)
b	0.981	-	-

Character-istic	Value	Criteria (target uncertainty of the analysis results is 5%, point number is 9 in the range 25-125 %)	Conclusion (complies or not)
s_b	0.0069	-	-
a	1.28	(1) $\leq$ 1.8946* s_a = 1.07; if (1) doesn't comply, then (2) $\leq$ 2.1;	Complies with criterion (2)
s_a	0.57	-	-
s_r	0.78	$\leq$ 2.6	complies
r	0.9998	$\geq$ 0.9976	complies

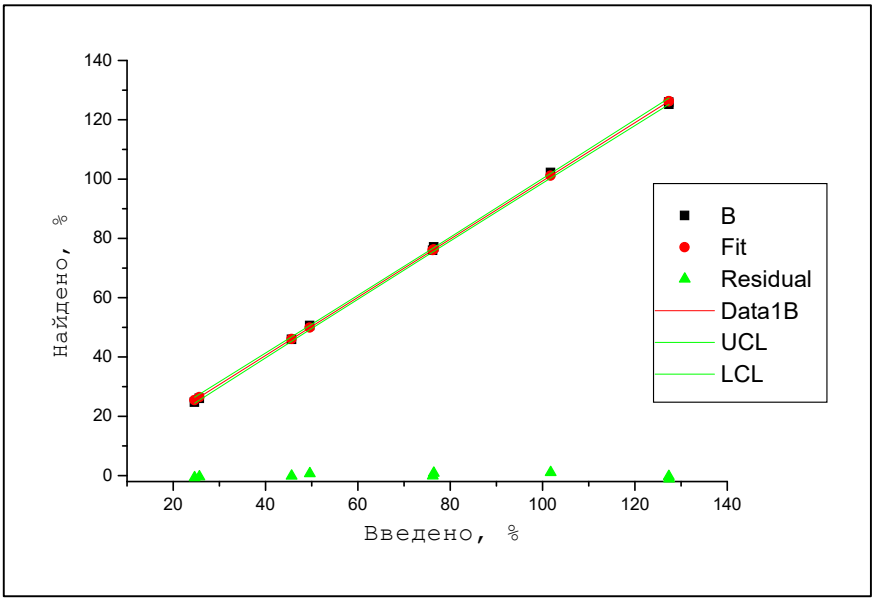


Figure 3.6. The linear relationship of the cefuroxime hydrochloride peak areas (model solution peak areas in % of reference solution peak area) versus model solution concentrations (entered in % of reference solution concentration) in normalized coordinates.

3.2.4.5. Repeatability and accuracy

Evaluation of repeatability and accuracy is carried out in accordance with the scheme described earlier in sections 2.3.2 - 2.3.3 and ratio (3.13). The results are presented in Table 2.3.5.

Table 3.5 shows that the procedure is characterized by an acceptable repeatability and accuracy throughout the range of concentrations of 25-125%.

Table 3.5

The results of the cefuroxime sodium model solutions analysis and their statistical processing (used criteria [5])

Model solution number	Theoretical solution concentrations, $X_{i.theor.}$%	Actual solution concentration in mg/g (C_i^{st} = 0.3952)	Actual concentrations in % of reference solution concentration, $X_{i.act.}$%	Average peak areas (S_i^{st} = 212.95)	Found in % of the cefuroxime peak area in reference solution, Y_i%	Found in % of entry concentration $Z_i = 100 \cdot (Y_i / X_i)$ %
1.	25	0.1012	25.66	55.50	26.06	101.81
2.	25	0.0971	24.62	52.70	24.75	100.77
3.	50	0.1804	45.66	97.85	45.95	100.64
4.	50	0.1955	49.59	107.78	50.61	102.32
5.	75	0.3006	76.44	164.23	77.12	101.39
6.	75	0.3005	76.21	161.81	75.98	99.95
7.	100	0.4002	101.77	217.63	102.19	100.92
8.	125	0.5045	127.33	268.23	125.96	98.67
9.	125	0.5048	127.41	266.60	125.19	98.02
					Mean, $\overline{Z}$ %	100.50
					Relative standard deviation, s_z%	1.41
					Relative confidence interval $\Delta\% = t(95\%.8) * s_z = 1.860 * s_z =$	2.62
					Critical value for results repeatability $\Delta_{Imp}\% \leq$	5.0
					Systematic error $\delta = \lvert \overline{Z} - 100 \rvert$	0.50
					Criteria for systematic error insignificance: 1) $\delta \leq \Delta/3 = 2.62/3 = 0.87$; 2) if 1) doesn't comply then $\delta \leq 5*0.32 = 1.6$	Complies Complies
					General conclusion about the procedure:	Correct

3.2.4.6. Limit of quantitation and limit of detection

Calculations of quantitation and detection limits are presented in Table 3.6. The assessment is carried out in accordance with the ratios (3.9-3.10) and criteria (3.1-3.2).

Таблица 3.6

s_a	*QL*, %	Critical value for *QL*, %	*DL*, %	Critical value for *DL*, %
0.5652	5.7	32	1.9	10

As you can see, the calculated *DL* and *QL* values are far below its critical values, and that is proof of the correctness of impurities control by using this procedure.

4. VALIDATION OF PROCEDURES FOR THE RESIDUAL SOLVENTS CONTROL IN DRUG PRODUCTS BY GAS CHROMATOGRAPHY

In previous sections we considered standardized schemes of validation of assays and related impurities control procedures. Control of residual solvents (RS) has a lot of similar points with the control of related impurities. However, due to a limited set of these solvents, it can be standardized. In particular, the State Pharmacopoeia of Ukraine (SPU), recommends to carry out the identification and control of RS with using unified standardized head space [37] gas chromatographic procedure (SPU general chapter 2.2.24 [36]). Chromatography was carried out with use of capillary column (30 m) to achieve the necessary efficiency of separation for most of the 59 residual solvents described in SPU [38]. With a view of leveling the matrix effects (dissolved test substance affects the concentration of RS in the vapor phase), analysis is carried out with the standard addition method [36].

The SPU general article 2.4.24 is harmonized with the European Pharmacopoeia (Eur.Ph.) [3].

According to the SPU, this pharmacopoeial uniformed procedure [36] may be used in the following cases:

1) to identify the most of the RS of 1 and 2 Classes of toxicity in substances, excipients and finished medicinal products, if these solvents are unknown;
2) as a limit test for RS of 1 and 2 Classes of toxicity if they are present in substances, excipients and finished medicinal products;
3) to quantify RS of 2 Class of toxicity, if their content exceeds1000 ppm (0.1%), or, if necessary, to quantify RS of Class 3 of toxicity.

As we can see, this procedure can also be used as limit (p. 2) and quantitative (p. 3) test. In the case of the pharmacopoeial limit test, its validation is not required. In the case of quantitative test for a particular substance validation is required [36]. Validation of a procedure is also needed in the case of its significant modification [11].

In practice, the application of the 2.2.24 procedure raises a number of issues:

1. The recommendation of the general article 2.2.24 [36] to use this procedure for identification of RS is not entirely clear. RS profile is known from a manufacture technology of a drug substance, excipient or a drug product. If this is not the case, such products should not get the market authorization and therefore there is no need to identify and monitor their RS. Problems of RS identification for them sometimes occur, but this is not the pharmacopeial analysis.
2. Application of the 2.2.24 general article procedure requires quite a long time. Thus, the duration of one chromatogram of Class 2 RS for system A reaches 40 min, and the procedure requires at least six chromatograms.
3. The procedure of 2.2.24 general article uses quite reactionary solvents: water, dimethyl sulfoxide, dimethyl formamide, dimethyl acetamide. Many of the RS (such as chlorinated hydrocarbons and esters) in such solvents (especially when

those small concentrations that occur in practice) can be subjected to solvolytic degradation, especially in view of the duration of the analysis.

4. The 2.2.24 procedure has too much generality, which makes it difficult to use in practice. Indeed, if, for example, there is a need to control only 2-3 RS in a drug substance, then what's the need to use a standardized procedure to control 59 solvents?
5. What is the accuracy of the 2.2.24 procedure? This issue is quite relevant when comparing results across different test laboratories [17].

Given all of this, as well as the high cost of equipment for head-space gas chromatography, in practice we quite often deal with the case where the conditions of 2.2.24 [36] have to be modified or completely changed. Often, it is reasonable to develop a simple gas chromatographic procedure without head-space, including the packed columns. Such an analysis can be performed both by the standard addition method, and the usual methods of absolute calibration (external standard) or internal standard [37]. In all these cases, developed procedures should be validated in accordance with the requirements of the SPU [11]. This raises issues related to the acceptability criteria of the results and the standardization of the validation scheme. It is also interesting to compare the application of the reference method and standard addition method.

In this section control of RS is considered only as a limit test, since quantitative analysis of RS does not refer to the quality control and is very rare in the compendial analysis .

4.1. Theoretical part

Control of residual organic solvents is the limit test [36]. In accordance with the SPU general article [11], for limit tests you must demonstrate just the limit of detection (*DL*) and specificity. The correctness of this requirement, however, is highly questionable.

Indeed, the limit test is used for quality control of drug substances, excipients and drug products. Based on the results of this test you make a decision on their quality (reject or accept). This conclusion must be reproduced in this laboratory and in other laboratories. Of course, the manufacturer shall provide such RS contents to guarantee a positive result in different laboratories (i.e. apply the so-called "guaranteeing tolerances" [39]), but you need to know the maximum total procedure uncertainty (including random and systematic components). In addition, this uncertainty you must know at different concentration levels. The maximum total procedure uncertainty is characterized, in large part, by *DL*, but only if *DL* is calculated from the parameters of the linear relationship (see section 3.1.1).

In general, limit tests conceptually differ little from assays with unilateral borders [14]. Thus, the validation of these tests differs little from the validation of the assays. The difference, in fact, is bigger value of the target uncertainty.

4.1.1. Target uncertainty of the procedure

The starting point for the development of all validation criteria is to find out what value of procedure uncertainty is acceptable. The pharmacopoeial procedure for RS control

[36] has system suitability criteria that seemingly allow us to find out what the procedure limit uncertainty is acceptable. However, the concept of the "procedure limit uncertainty" is rather vague in the case of the pharmacopoeial procedure for RS control [36].

Control of RS in the pharmacopeial procedure is carried out with the standard addition method. RS amounts, corresponding to their SPU limit permissible concentrations (*ImL*), are added to a test solution. This solution is used as a reference solution [38]. Vapor phases above the test and reference solutions are chromatographed (3 chromatograms for each solution).

Calculate the average peak areas of the analyzed RS from the chromatograms of test ($S_{test,j}$) and reference ($S_{ref,j,}$) solutions. The following inequality should be performed for each (j) RS:

$$K_{Im,j} = \frac{S_{test,j}}{S_{ref,j}} \le 0.5 \cdot . \tag{4.1}$$

Calculate 3 pairwise peak area differences $dif_j = S_{ref,j} - S_{test,j}$. The relative standard deviation ($RSD_{dif,j}$) of these 3 differences for each (j) RS must not exceed 15% (the system suitability test) [36]:

$$RSD_{dif,j}(3) \le 15\% . \tag{4.2}$$

Given that $t(0.95, 2) = 2.92$ [26], the relative confidence interval of the mean of the peak area differences (Δ_{dif}), corresponding to the inequality (4.2), is equal to [26]:

$$\Delta_{dif} \le \frac{2.92 \cdot 15}{\sqrt{3}} = 25.3\%. . \tag{4.3}$$

The ratio of (4.1) is a criterion of acceptability of the RS content in the test sample. It regulates the maximum acceptable value of the *ratio* (K_{Im}) of the peak areas of the test and standard solutions. At the same time, the accuracy of the procedure is regulated by the inequality of (4.2) that establishes the requirements to the maximum acceptable precision of the *difference* between the peak areas of standard and test solutions. There is no simple relationship between inequalities of (4.1) and (4.2), so the value of Δ_{dif} cannot be considered as the procedure uncertainty. Seemingly, the procedure uncertainty can be regarded as the uncertainty of the ratio of K_{Im} (see below for the calculation). However, the uncertainty of the K_{Im} value characterizes little the RS content in the analyzed sample as much weaker depends on the RS concentration than the RS peak area itself (twice weaker near the *ImL* content, in other content range even weaker).

The questions of interest are what the uncertainties in the peak areas of the reference and the test solutions correspond to a ratio (4.2), and what is the uncertainty of the K_{Im} value.

The reference solution is a test solution with the addition of RS with concentrations corresponding to the SPU maximum permissible values (ImL), for parent population values

of the *j-th* RS can be written:

$$S_{ref,j} = S_{test,j} + S_{ImL,j}\ . \tag{4.4}$$

Here indices "*ref*" refers to the reference solution, *"test"* to the test solution, and *"ImL"* - to the theoretical peak area corresponding to the *ImL*.

From here, we get:

$$S_{ImL,j} = dif_j = S_{ref,j} - S_{test,j}\ . \tag{4.5}$$

The related standard deviation of the dif_j values ($RSD_{dif,j}$) is determined by the ratio (4.2). On the other hand, given the (4.5), in accordance with [26], we get:

$$RSD^2_{dif,j} = \frac{1}{S^2_{ImL,j}} \cdot (S^2_{ref,j} \cdot RSD^2_{ref,j} + S^2_{test,j} \cdot RSD^2_{test,j}). \tag{4.6}$$

Of greatest interest is the critical range when the concentration of the RS is close to the *ImL* value. In this case the S_{ref} and S_{test} areas not really vary greatly (twice), so can be considered:

$$RSD_{test,j} = RSD_{ref,j}\cdot \tag{4.7}$$

Then, given the (4.4), equation (4.7) comes to the expression:

$$RSD^2_{dif,j} = \frac{RSD^2_{ref,j}}{S^2_{ImL,j}} \cdot [(S_{ImL,j} + S_{test,j})^2 + S^2_{test,j}]. \tag{4.8}$$

S_{ImL} doesn't depends on the S_{test} so we can see from the the equation (4.8) that RSD_{dif} increases with S_{test} growth, i.e. with an increase of the RS content in the sample. Therefore, the requirement of $RSD_{dif} \le 15\%$ (4.2) [36] is uncertain without indication of S_{test} or what is the same, the RS concentration in the test sample. It is advisable to regulate the RS concentration at the level of the maximum permissible content *ImL* (this case is of most practical interest). Then $S_{test} = S_{ImL}$, and the equation of (4.8) comes to a simple type (index *"j"* is omitted as the limit values of RSD_{ref} are the same for all RS):

$$RSD_{dif} = \sqrt{5} \cdot RSD_{ref}\ . \tag{4.9}$$

Bearing in mind the SPU requirements of (4.2) to the RSD_{dif} values for the number of pairwise chromatogram $n = 3$, that is, the number of degrees of freedom of $f = 2$, we get requirements to the RSD_{ref}:

$$RSD_{st}(f = 2) = \frac{RSD_{dif}}{\sqrt{5}} \le \frac{15}{\sqrt{5}} = 6.7\%\cdot \tag{4.10}$$

Equation (4.10) allows us to define the requirements for the relative confidence interval of repeatability of areas of replicated chromatograms. According to the relation of (4.7), they are the same for the test and references solutions [26]:

$$\Delta_{st} = \Delta_{test} \le \frac{2.92 \cdot 6.7}{\sqrt{3}} = 11.3\%. \tag{4.11}$$

When carrying out the validation we obtain the results with the larger number of degrees of freedom/ It allows us to reduce the *t-value*. The average value, in accordance with [36], is found from 3 pairwise injections. The requirements RSD_{st} are determined by the ratio [17, 40]:

$$RSD_{st}(f) \le \frac{t(95, f=2)}{t(95, f)} \cdot RSD_{st}(f=2) \cdot \tag{4.12}$$

Here $t(95, f=2) = 2.92$ is a *t-value* for probability of 95% and the number of degrees of freedom of $f = 2$; $t(95, f)$ is a *t-value* for probability of 95% and the number of degrees of freedom f. The requirements of (4.11) to the confidence interval of repeatability of the peak areas remain the same.

From equation of (4.12) we can get the RSD_{st} values for the most common numbers of degrees of freedom of $f = 2$, 3 and 4, as well as the parent population value ($f = \infty$):

$$\begin{aligned} RSD_{st}(f=2) &\le 6.7\% \\ RSD_{st}(f=3) &\le 8.3\%, \\ RSD_{st}(f=4) &\le 9.2\%, \\ RSD_{st}(f=\infty) &\le 11.9\%. \end{aligned} \tag{4.13}$$

This approach is common for the SPU [13].

Equations of (4.10-4.13) allow us to set such requirements to the RSD_{st} of reference solution, that ensure compliance with the requirements of (4.2) SPU [36] (or specification), as well as to establish requirements for the parameters of the linear relationship.

Ratios (4.1) and (4.11) allow getting the relative uncertainty of the K_{Im} value [26]:

$$\Delta_{Im} = \Delta_{K,Im} = \sqrt{\Delta_{test}^2 + \Delta_{st}^2} = \sqrt{2} \cdot \Delta_{ref} \le \sqrt{2} \cdot 11.3 = 16.0\%. \tag{4.14}$$

Statistically insignificant difference between the results of two laboratories will be in $\sqrt{2}$ times more [26]. Thus, if the first laboratory gets $K_{Im} \ge 0.5$ (which corresponds to the RS normalized concentration $X \ge 100\%$ of *ImL*, i.e. rejects), then the second laboratory (running strictly accordingly to the SPU requirements [36]) can gets:

$$K_{Limit} \geq 0.5 \cdot 100/(100 + \sqrt{2} \cdot \Delta_{K,\mathrm{Im}}) = 0.41, \qquad X_{Limit} \geq 69\%. \tag{4.15}$$

i.e. compliance with the SPU requirements. At the same time, the values $K_{Im} = 0.5$ and $K_{Im} = 0.41$ and the corresponding normalized concentration (see previous section 3) $X = 100\%$ and $X = 69\%$ of I_{mL} are statistically indistinguishable.

The values $K_{Limit} = 0.41$ and $X_{Limit} = 69\%$ from the equation (4.15) can be seen as lower limits of these quantities at product release, that ensure positive results in control laboratories. Otherwise, these laboratories may have the different conclusions on the quality of the test sample though have obtained statistically indistinguishable (accordingly to SPU) results. As we can see, even with the $X = 70\%$ of *ImL*, there is a statistically significant risk of rejecting of the test material for RS content.

The expression of (4.14) characterizes the maximum uncertainty ($\Delta_{K,Im}$) of the uniformed pharmacopoeial procedure of RS control, which is carried out with the standard addition method [36]. The question arises, what is the target uncertainty of the procedure (Δ_{Im}) when using the regular reference standard (external or internal) method [37]. Given the ratio (4.7), it is not difficult to see that this uncertainty coincide with the $\Delta_{K,Im}$, as noted in the expression (4.14).

It should be noted that the requirements derived for the uncertainty of RS control procedures ($\Delta_{Im} \leq 16\%$) coincide with the requirements to the uncertainty of HPLC impurity control procedures obtained previously entirely from other considerations, - see the ratio (3.12). It confirms sufficient commonality of these requirements.

4.1.2. Detection limit

In the study of the accuracy and precision of the procedure, finding of the detection limit (*DL*) and the quantitation limit of (*QL*), the linearity study are basic (see previous sections 1-2).

Control of RS is the limit test [36]. In accordance with the SPU requirements [11], in the validation of such tests we need to find the *DL* only.

In accordance with the SPU requirements [11], the *DL* value we can find by two ways - from the signal-to-noise ratio and using the characteristics of the linear relationship. The RS control using the 2.2.24 gas chromatographic procedure [36] is a clear example of a small application of the signal-to-noise ratio for calculation of the *DL* value, since this approach takes into account only the chromatographic components of the procedure uncertainty and does not take into account the uncertainty of sample preparation and gas sample injection.

At the same time, sample preparation contributes substantively to the procedure uncertainty in the case of liquid chromatography with liquid samples [27]. Sample preparations in the case of the gas (regular or head-space) and liquid chromatography are not different and their precisions are similar, so the same could be said for regular gas

chromatography. But in the case of head-space gas chromatography we have additional and very important factors contributing to the procedure uncertainty. They are gas sample injection and a highly volatile RS evaporation from water solutions. Therefore, the calculation of the *DL* values from the parameters of the linear relationship is considerably more reliable and objective, because it takes into account both chromatographic and not chromatographic factors.

*DL c*alculation (in per cent of the *ImL* value) is carried out according to the SPU [11]:

$$DL = 3.3 \cdot SD_A / b. \tag{4.16}$$

Here SD_A is the standard deviation of the *Y*-intercept of the calibration line; *b* is the slope of the calibration line.

The linear relationships are plotted in the normalized coordinates so SD_A и *DL* are evaluated in per cent of the maximum permissible RS value according to the specification (*ImL*).

Principled position is that we are not looking for the real limit of detection, but confirm that it does not exceed the acceptable limit of our procedure. This corresponds to the general approach to quality control and validation of drug quality control procedures (see ratios (3.1.1, 3.1.2)).

In the case of limit tests, relative limit of RS detection (*DL*) should be insignificant [17] compared to the maximum permissible RS concentration (*ImL*) which is assumed to be 100% in normalized coordinates. As shown in section 3.1.1, in normalized coordinates must be the ratio (3.1), i.e.:

$$DL \le \max\ DL = 32\%. \tag{4.17}$$

Interestingly, the upper *DL* bound of roughly coincides with a statistically insignificant difference of normalized concentrations in different laboratories (100-69 = 31%) – see the ratio (4.15), obtained from an entirely different considerations. It indicates sufficient commonality of the ratios (4.14) and (4.17).

4.1.3. Procedure and range

In accordance with the SPU requirements [11], the linearity verification needs use of at least 5 points.

As shown in section 2.1, optimal number of points for quantitative tests is 9 (plus 1 for the standard to transfer data into normalized coordinates). Quantitative tests are fairly common for the control of impurities by HPLC. This is due to the accumulation of impurities (degradation products) in the course of storage, which necessitates controlling this process for determining the expiration date. Therefore, HPLC impurities control procedures are generally validated as quantitative tests (see section 3).

In the case of RS, no their accumulation during storage can be because the RS contents during storage can only diminish by evaporation. Accordingly, there is no need to ex-

amine quantitatively the changes or to establish the shelf life. Therefore, methods of RS control are usually only limit tests. Requirements to procedure uncertainties for limit tests are much liberal than for assays. Therefore, when studying the linearity of these procedures, there is no need for such a big (9) number of points. Usually it is enough 5 points (plus 1 for the standard for transferring data into normalized coordinates).

For every solution, indicated in the specification number of replicate chromatograms is carried out.

The procedure range depends on the standardization method – is it a standard addition method or reference standard (external or internal) method [37].

4.1.3.1. Reference standard method

As shown earlier in section 3.1.7, it is reasonable to study 5 model solutions with concentrations of 25%, 50%, 75%, 100% and 125% of the *ImL*. In contrast to quantitative impurities control (see section 3), these concentrations (model solutions) are prepared in one rather than two times. Model solutions are prepared with using pre-dried (for RS removal) test material in the same concentration as in the procedure. To confirm that the solution of the dried test material does not give the chromatographic peaks, which may interfering with RS peaks, prepare also a blank solution (*0*) that is a solution of the test material in the same solvent, and in the same concentration as in model solutions (and in preparation). We must also get a chromatogram of the solvent (*00*) to confirm that there are no peaks on it, interfering with the analyzed RS.

Note that if there are no interfering peaks in the chromatogram of the blank solution (0), the need to study the solvent chromatogram (0000) is already not (because the solvent is also included in the blank solution). But if there are the interfering peaks in the chromatogram of the blank solution, then the chromatogram of the solvent is required.

In addition, one more solution (*st*) with 100% RS concentration is required for transferring into the normalized coordinates. Unlike the model and blank solutions, *st* is prepared without the use of the test material (as in real analysis by the reference standard method).

4.1.3.2. Standard addition method [36]

A feature of the standard addition method is that it requires the linearity in a much broader range than the conventional reference standard method, as it involves the addition of RS in nominal concentrations to the test sample [36]. Thus, if the range of the reference standard method is 25-125% of the *ImL* (see above), then the standard addition method expands the range up to 25-225%, and 200% corresponds to a standard solution with RS in the test sample, equal to 100% of the *ImL*. Given that the number of solutions is equal to 5, to study linearity should take the model solutions with concentrations of 25%, 75%, 125%, 175%, 225%.

As in the reference standard method (see above), model solutions are prepared using pre-dried (for RS removing) test material in the same concentration as in the procedure. The pre-dried test material is also used for the preparation of the blank solution (*0*) - to

verify the absence of the interfering peaks of the test material in the chromatogram. We must also get a chromatogram of the solvent (*00*) to confirm that there are no peaks on it, interfering with the analyzed RS.

For transferring into the normalized coordinates, we need also another solution with RS 100% concentrations (*st*). In contrast to the reference standard method, it is also prepared using the test material (as in real analysis by the standard addition method).

The wider the range, the more difficult it is to achieve the necessary linearity and precision. So for usual (not head-space) chromatography, the standard addition method is less precise than the reference standard method, and its application is usually inappropriate.

4.1.4. Specificity: effect of interfering peaks of the substance and the solvent

Preparation of model solutions to check the effects of the matrix requires the use of the test substance. This substance contains unknown RS concentrations. It doesn't allow preparing model solutions with known RS concentrations. Therefore, the preparation of the model solutions requires removing RS from the substance. This is usually done by drying in a vacuum. However, even after this removal, during validation we have often to face with extraneous peaks interfering with RS peaks, which affect the specificity of the procedure. To control these peaks we need to obtain chromatograms of the blank solution (*0*) and the solvent used for the procedure (*00*). Extraneous peaks prevents from the validation, as well as the control of RS. The emergence of interfering peaks can be due to the following reasons:

1. RS are not completely removed after drying from the test material used for the preparation of the model solutions. It leads to an overstatement of the actual content of the RS in model solutions and to worsening of the validation characteristics.
2. Interfering peaks are peaks of the impurities of solvent used for analysis.
3. Interfering peaks are impurities or degradation products of the analyzed substance and/or products of its interaction with the solvent.

4.1.4.1. Effect of irremovable rests of RS

Case 1 occurs only at the stage of validation. It complicates the obtaining of metrological characteristics, but did not affect the results of the RS control and deciding on quality. For the leveling influence of interfering peaks (which are the irremovable rests of the RS) in this case, during the validation (but not the quality control itself) we have simply to subtract these RS peak areas in the blank solution chromatogram (*0*) from the peak areas in the chromatograms of the standard solution (in the standard addition method but not in the reference standard method) and model solutions.

In this case the metrological characteristics of the validated procedure are deteriorating compared to the true values. In particular, we can see from the equation (4.8) that the relative standard deviation of the peak areas of the model solutions increase by about as much per cent as per cent are in normalized coordinates the RS peak areas in the chromatogram of the blank solution (*0*). For example, if the area of the interfering peak in

the chromatogram of the blank solution is, in normalized coordinates, 10% of the peak area corresponding to the *ImL*, the *RSD* of peak areas of model solutions will grow by 10% against the true values. However, if the above developed criteria are satisfied, the procedure can be considered as validated, because its actual metrological characteristics are even better.

Demonstration that interfering peaks belong to irremovable RS can be done in a variety of ways, for example, by repeated drying of the test material. The peak reducing shows in favor them to be the irremovable RS.

4.1.4.2. Effect of impurities in the solvent and the test substance

In cases 2-3 the situation is different. These cases occur at the stage of the procedure validation and RS control. We do not know a priori the peak areas of impurities in real objects. So we have no right to deduct them from the peak areas of the model and reference solutions when we carry out the procedure validation.

The presence of interfering peaks in the solvent chromatogram (00) indicates the presence of interfering impurities in the solvent used for the analysis (they can be also formed by degradation of the solvent in the process of analysis). The presence of interfering peaks (which are not the irremovable RS) in the chromatogram of the blank solution indicates the presence of the interfering impurities in the original test material or their formation during the chromatographic process and the interaction with the solvent.

In the ideal case, the interfering peaks must be absent in the chromatograms of the blank solution (0) and the solvent (00). However, in practice, they are often present. The question arises as to how these interfering peaks affect the validation process and quality control, and what their peak areas are acceptable.

The interfering peaks characterize the systematic error of the procedure (δ_j) that, in order not to influence decisions about quality, should be insignificant compared with the target uncertainty of the procedure Δ_{Im} (see ratios (2.10-2.11)), i.e., given (4.14):

$$\delta_j \le max\,\delta = 0.32 \cdot \Delta_{Im} = 0.32 \cdot 16 = 5.1\%. \tag{4.18}$$

The interfering peaks of the solvent and the test material affect the validation process and quality control in different ways for the reference standard method and for the standard addition method.

4.1.4.2.1. Standard addition method

In the standard addition method, concentrations of the test material and solvent (and therefore their corresponding impurities) in the test (or model) solution and reference solution are the same. But the concentrations of analyzed RS in the test and reference solutions differ at *ImL*. So for areas of *j-th* RS in the test and reference solutions we can write such ratios:

Standard addition method:

$$S_{ref,j} = S_{ImL,j} + S_{test,j} = S_{ImL,j} + (S_j + S_{oj}).$$
$$S_{test,j} = S_j + S_{oj}. \quad (4.19)$$

Here the indices refer: "*0*" - to the blank solution, "*ref*" - to the reference solution, "*test*" - to the test solution, "*ImL*"-to the theoretical peak area corresponding to the *ImL*;

S_j is a theoretical peak area corresponding to the actual content of the *j*-th RS in the test solution.

In the pharmacopoeial standard addition method, the systematic error of *j-th* RS content determination (δ_j), caused by the influence of impurities in the solvent and the analyzed substance, is a change of the quotient K_{Imp} from the ratio (4.1), caused by the presence of these impurities. Quantity δ_j depends on the RS concentration in the test material, decreasing with its growth. We are dealing with the limit test so it is reasonable to regulate the δ_j value for the critical case - when the RS concentration in the test material is *ImL* (i.e. $S_j = S_{ImL,j}$) and the theoretical value $K_{Imp} = 0.5$. Then, given the correlations (4.1, 4.18-4.19), we obtain requirements for the peak area of the *j-th* RS in the blank solution chromatogram (S_{0j}):

Standard addition method:

$$\delta_j = \frac{100}{0.5}\cdot\left(\frac{S_{test,j}}{S_{ref,j}} - 0.5\right) = \frac{100}{0.5}\cdot\left(\frac{S_{ImL,j} + S_{oj}}{2\cdot S_{ImL,j} + S_{oj}} - 0.5\right) =$$
$$= \frac{100\cdot S_{oj}}{2\cdot S_{ImL} + S_{oj}} = \frac{100\cdot S_{oj}}{S_{ref}} \le max\,\delta = 5.1\%. \quad (4.20)$$

4.1.4.2.2. Reference standard method

In this case, the solvent concentration (and therefore contents of impurities associated with it) in the test and reference solutions can be considered the same, but the test material (and related impurities) is present in the test and is not in the reference solutions. Expressions for the peak areas of the *j-th* RS are similar to (4.19) and have such forms (the index "00" refers to the solvent chromatogram):

Reference standard method:

$$S_{ref,j} = S_{ImL,j} + S_{ooj}.$$
$$S_{test,j} = S_j + S_{oj}. \quad (4.21)$$

Like the standard addition method, the systematic error of the *j-th* RS content determination (δ_j) in the reference standard method depends on the RS concentration in the test material, decreasing with its growth. So it is also reasonable to regulate this δ_j value for the critical case - when the RS concentration in the test material is *ImL* (i.e. $S_j = S_{ImL,j}$). In this case the theoretical value of the quotient of the peak areas in the chromatograms

of the test and reference solutions is 1. Therefore, given the ratios (4.18 4.21), we obtain, like ratio (4.20), the requirements to the peak areas of the *j-th* RS in the chromatograms of the blank solution (S_{0j}) and solvent (S_{00j}) in the reference standard method:

Reference standard method:

$$\delta_j = 100 \cdot \left(\frac{S_{test,j}}{S_{ref,j}} - 1 \right) = 100 \cdot \frac{S_{oj} - S_{ooj}}{S_{ref,j}} \le max\,\delta = 5.1\%. \quad (4.22)$$

Given the ratiod (4.19) and (4.21), it is easy to see that the reference standard method for the critical case (the RS content in the test material equals to *ImL*) and in the absence of the interfering impurities in the solvent is approximately two times more sensitive to the test material interfering peaks than the standard addition method – because of two times less concentration of the reference solution.

4.1.5. Requirements to linearity

As shown in the section 2.2, it is convenient to carry out the linearity studies for the accuracy and precision in the normalized coordinates. In the case of the impurity control (see section 3) the concentration is expressed as a percentage of the maximum permissible concentration of the impurity according to the specification *ImL* (in this case, the impurity is the RR), and the area (or height) of the peak is expressed as a percentage of the peak area corresponding to the ImL.

The main metrological characteristics of the linear relationship $Y = a + b \cdot X$ are: the residual standard deviation (RSD_o), the correlation coefficient (R_c) and the *Y*-intercept (a).

The residual standard deviation RSD_o is determined exclusively by the procedure uncertainty Δ_{Im} and the number of the line points (g) (see ratios (3.15 3.16)). In this case, g = 5, Δ_{Im} = 16.0% (see equation (4.14)), so:

$$RSD_o \le \Delta_{Im} / t(95\%, g-2) = 16 / 2.35 = 6.8\%\,. \quad (4.23)$$

The requirements to the correlation coefficient (R_c) and *Y*-intercept (a) depend on the range that is different for the reference standard method and the standard addition method. Therefore the requirements for these methods are different.

4.1.5.1. Reference standard method

4.1.5.1.1. Correlation coefficient

The studied concentrations (25, 50, 75, 100 and 125 per cent) have a standard deviation of $SD_x = RSD_{range}$ = 39.53%. As shown (see the relation (3.17)), the requirements to the correlation coefficient in this case are given by the ratio:

Reference standard method:

$$R_c \geq \sqrt{1 - \frac{RSD_o^2}{RSD_{range}^2}} = 0.9851 \; . \qquad (4.24)$$

4.1.5.1.2. Requirements to Y-intercept

1) A statistically insignificant difference from zero, i.e. for $g = 5$:

Reference standard method:

$$a \leq t(95\%, g-2) \cdot s_a = 2.35 \cdot s_a \; . \qquad (4.25)$$

2) In a case of inequality of (4.25) (i.e. a statistically significantly differs from zero), we may use the practical insignificance. In our case, given the (4.12-4.13), we obtain in the normalized coordinates the following ratio (compare with the equation (2.18)):

Reference standard method:

$$|a| \leq \frac{0.32 \cdot \Delta_{imp}}{1 - (25/100)} = \frac{0.32 \cdot 16}{0.75} = 6.8\%. \qquad (4.26)$$

4.1.5.2. Standard addition method

4.1.5.2.1. Correlation coefficient

The studied concentrations (25, 75, 125, 175, 225) have a standard deviation of $SD_x = RSD_{range} = 79.1\%$. As shown in the section 2.3.4.2, the requirements to the correlation coefficient in this case are given by the ratio:

Standard addition method:

$$R_c \geq \sqrt{1 - \frac{RSD_o^2}{RSD_{range}^2}} = 0.9963 \; . \qquad (4.27)$$

4.1.5.2.2. Requirements to Y-intercept

1) A statistically insignificant difference from zero – see the ratio (4.25).

2) In a case of inequality of (4.25), Y-intercept a in the relationship $Y = a + b \cdot X$ is statistically significant and cause the systematic error. A feature of content determination by the standard addition method is that our interest is not the systematic error of the concentration found but is the systematic error of K_{Im} value from the equation (4.1). In the normalized coordinates for the standard addition method, dependences of a peak area on a concentration for the test and reference solution have the form ($ImL = 100\%$):

Standard addition method:

$$Y_{test} = a + b \cdot X_{test}\ ,$$
$$Y_{ref} = a + b \cdot (X_{test} + Im\ L) = a + b \cdot (X_{test} + 100). \quad (4.28)$$

Given the ratio (4.28) and the requirements to the systematic error (4.15) as well, the relative error (δ_a), caused by the *Y*-intercept into the ratio (4.1) calculation, is:

$$\delta_a(\%) = 100 \cdot \left[\frac{a + b \cdot X_{test}}{a + b \cdot (X_{test} + 100)} - \frac{X_{test}}{X_{test} + 100} \right] \cdot \frac{X_{test} + 100}{X_{test}} =$$
$$= \frac{100 \cdot 100 \cdot a}{Y_{ref} \cdot X_{test}} \le max\ \delta = 5.1. \quad (4.29)$$

The equation (4.29) shows that δ_a decreases with increasing RS concentration in the test solution (X_{test}). Using the ratios (4.28-4.29), proximity of the slope *b* to the unit in the normalized coordinates, as well as a small free term *a*, we can get the requirements to the *Y*-intercept in the standard addition method for different concentrations:

Standard addition method:

$$X_{test} = 25\% : \ |a| \le 1.6\%,$$
$$X_{test} = Im\ L = 100\% : \ |a| \le 2.6\%. \quad (4.30)$$

As we can see, even for the critical case $X_{test} = ImL$ the standard addition method determine (2.6%) more than 2.5 times stricter requirements for the practically insignificant value (see the section 2.3.3) of free term *a* than the ratio (4.26) of the reference standard method (6.8%)/ For the concentration of the X_{test} = 25% of the *ImL* (for this concentration, as the lower range limit, are requirements (4.26)) the ratio (4.30) gives unrealistic in practice the value *a* = 1.6%.

Thus, in contrast to the reference standard method (4.26), extremely stringent requirements (4.30) to practical insignificance of the free term *a* in the standard addition method are useless compared to the statistically insignificance of the ratio (4.25). This is due to the very large differences in the areas of the test and standard solutions (at least twice).

4.1.6. Accuracy and precision

These characteristics are calculated the same as for the assays - from the linearity study results (see section 2.3.4).

4.1.7. Robustness

Specificity must be confirmed in different columns, on which the requirements to the specificity of section 4.1.4 must be satisfied.

4.1.7.1. Stability of the solutions

Check the stability of test and reference solutions is one of the elements of a procedure robustness study [11] and must be carried out before all other validation studies. In the case of RS control, the stability study is not as important as for assays (see section 2) and, particularly, for related substance control procedures by HPLC (see section 3), since, unlike the latter, residual solvents during storage are not accumulated, and the RS are usually sufficiently stable. With this in mind, to confirm the stability of the solutions we can use linearity study results. If all model solutions are prepared at the same time, their analysis duration is several times more than the analysis duration of the test material under the specification. Therefore, the conformity with the requirements to linearity, accuracy, and precision is proof of sufficient stability of solutions for RS control under the specification.

In a case, when we must check the stability of the solutions through long time, we can use the same approach as for relative substance control procedures (see the section 3.3.2): the solution is considered to be stable if the RS content of any RS in it through the specified time interval differs from this RS original content not more than $\sqrt{2} \cdot \Delta_{Imp}$.

4.1.8. System suitability test

Linearity study data may be used for substantiation of the system suitability requirements to repeatability of the replicate injections. According to the standardized scheme of validation characteristics acquisition (see the section 2.1), every point of the linear relationship is obtained under the specification conditions with a number of replicate injections for each point (of a model solution) of n_i (for the pharmacopeial procedure [36] $n_i = 3$) and the relative standard deviation of RSD_i. As the number of points equals g (in this case $g = 5$), and number of replicate injections for the reference solution equals n_{ref} (usually $n_{ref} = 3\text{-}5$), from the study of linearity we can get the combined relative standard deviation of RSD_{tot} with the number of degrees of freedom f_{tot} [26]:

$$RSD_{tot} = \sqrt{\frac{(n_{ref} - 1) \cdot RSD_{ref}^2 + \sum_{i=1}^{g} (n_i - 1) \cdot RSD_i^2}{f_{tot}}}, \qquad (3.31)$$

$$f_{tot} = (n_{ref} - 1) + \sum_{i=1}^{g} (n_i - 1).$$

In particular, for the pharmacopeial procedure number of replicate injections $n_i = 3$ [36] and the recommended $n_{ref} = 5$, we obtain $f_{tot} = 14$.

System suitability requirements to a relative standard deviation of replicate injections can be considered to be confirmed if all values RSD_i and RSD_{ref} meet to requirements (4.10, 4.13) and the combined relative standard deviation RSD_{tot} is significantly (by the Fisher criterion on level 95% [26]) less than the value of the parent population RSD ($f = \infty$) = 11.9%, i.e.:

$$
\begin{aligned}
&RSD_{tot} \le RSD(f = \infty) / \sqrt{F(95\%, \infty, f_{tot})}, \\
&RSD_{tot}(f_{tot} = 14) \le 8.2\%, \\
&RSD_{tot}(f_{tot} = 16) \le 8.4\%, \\
&RSD_{tot}(f_{tot} = 20) \le 8.8\%, \\
&RSD_{tot}(f_{tot} = 22) \le 8.9\%.
\end{aligned}
\tag{4.32}
$$

An important characteristic of the chromatographic system suitability is also a resolution (R_s) of a critical pair of peaks. In accordance with the requirements of [37], should be $R_s \ge 1.0$, however, a resolution of greater than 1.5 corresponds to baseline separation [13]. So this value ($R_s \ge 1.5$) can be recommended for introduction into the chromatographic system suitability test. To verify this criterion, take the "worst case". In the case of strongly asymmetric peaks (symmetry factor A_s [37] is outside the recommended range 0.8-1.5 [13]), the R_s should be increased accordingly. In particular for "tailed" peaks approximate increase of R_s value is A_s - 1. For example, for A_s = 2.5 it is possible to recommend the value of $R_s \ge 1.5 + (2.5 - 1) = 3.0$.

The symmetry factor A_s characterizes the specificity of the procedure, and *RSD* of peak areas of replicated chromatograms – the repeatability of the results. Therefore, these requirements are the principal requirements of the chromatographic system suitability test.

In addition, there are also the general requirements to the system suitability [13, 37]: symmetry factor A_s must be within 0.8-1.5, column performance (apparent number of theoretical plates) must be within the specified limits. These values characterize the quality of the chromatography and have no independent meaning. However, they affect the resolution R_s and *RSD* of peak areas of replicate chromatograms. If R_s and *RSD* meet the necessary requirements, the requirements for these characteristics can be liberalized in comparison with the recommendations of the Pharmacopoeia. At sufficiently high sensitivity there may be some peaks in the chromatogram, caused by noise. For these and other reasons (caused by task conditions) some extra characteristics are also regulated: the signal/noise ratio (typically, S/N ≥ 3-10) and disregard limit of a peak area (for quantitative tests usually *DRL* ≤ 5-10%, for limit tests usually *DRL* ≤ 32% of the *ImL* - see ratios of (3.7-3.8)).

4.2. Example. Validation of the control procedure of toluene and isopropanol residuals in fensuccinal medicinal substance

To check the proposed validation scheme we used the gas chromatographic procedure of toluene and isopropanol in a new original medicinal substance "Fensuccinal". Accordingly to the SPU requirements [38], limits of their concentrations does not exceed 0.089% and 0.5% respectively.

Reagents and volumetric glassware met the SPU requirements [1].

4.2.1. Procedure to be validated

Chromatographic conditions:

- gas chromatograph «Chrom-5» (Check Republic) with a flame-ionization detector,
- glass packed column 240 cm × 0.3 cm;
- stationary phase – sorbent "Chromaton AW DMCS" of particle size of 0.20-0.25 mm (Check Republic) impregnated with 10 % of OV-1;
- column temperature – 80 ^{o}C;
- injector temperature – 135 ^{o}C;
- detector temperature – 150 ^{o}C;
- carrier gas (argon) flow – 30 ml/min;
- injectable volume - 1 мкл, microsyringe M-1H (Russian Federation);

Order of appearance in the chromatogram: toluene, isopropanol, dimethyl sulfoxide.

System suitability requirements:

- symmetry factor [37] for the toluene peak in the reference solution chromatogram does not exceed 2.5;
- resolution [37] of toluene and isopropanol peaks, calculated for the reference solution chromatogram, is not less than 3.0;
- relative standard deviation [37], calculated for toluene and isopropanol peaks from the 5 reference solution chromatograms, does not exceed 9.2% (see ratio (4.13));
- apparent number of theoretical plates [37], calculated for the toluene peak in the reference solution chromatogram, is not less 1500.

The validation of the procedure was conducted by the reference standard method and standard addition method under normal gas (without head-space) chromatography [37]. 1 μL alternately test solution and reference solutions (solvent - dimethyl sulfoxide) were chromatographed, getting at least 3 chromatogram for each solution. To verify the system suitability, the reference solution was previously chromatographed 5 times. To verify the absence of interfering impurities in the test substance and the solvent, the substance solution (*0*) and the solvent (*00*) were chromatographed as well.

4.2.2. Preparation of model and reference solutions

For the preparation of the model solution, the fensuccinal substance is previously dried in the oven at 105 ^{o}C for 4 hours.

Reference solution 00: solvent (dimethylsulfoxide, DMSO).

Reference solution 0: 1.0 g of the previously dried fensuccinal substance place in a measuring flask with a capacity of 10 ml, dissolve in DMSO and dilute to the mark with the same solvent.

The stock RS solution.

A. About 0.088 g (accurate weight m_T) of toluene and about 0.51 g (accurate weight m_{IP}) of isopropanol place in a weighed measuring flask with a capacity of 10 ml, dilute to the mark with DMSO and weigh. Find the mass of the solution A (*m(A)*).

B. About 2.67 g (accurate weight $m_A(B)$) of the solution A place in a weighed measuring flask with a capacity of 25 ml, dilute to the mark with DMSO and weigh. Find the mass of epy solution B (*m(B)*). The resulting solution contains about 0.8 mg/g of toluene and about 4.7 mg/g of isopropanol.

Model solutions. Weights of the stock RS solution, specified in the Tables 1-2, place in measuring flasks with a capacity of 10 ml, add 1.0 g of the dried fensuccinal substance, dissolve in DMSO and dilute to the mark with DMSO. Model solutions for the reference standard method (25, 50, 75, 100 and 125% in theory) and standard addition method (25, 75, 125, 175, 225% in theory) are prepared in the same way.

Reference solution for reference standard method (RSM). The weight $m_{st}(RSM)$ of the solution B, corresponding to the nominal concentrations of RS in the model solutions, place in a measuring flask with a capacity of 10 ml and dilute with DMSO to the mark.

Reference solution for standard addition method. The weight $m_{st}(SAM)$ of the solution B, corresponding to the nominal concentrations of RS in the model solutions, place in a measuring flask with a capacity of 10 ml, add 1.0 g of the dried fensuccinal substance and dilute with DMSO to the mark.

Normalized concentrations of toluene (T) and isopropanol (IP) in the *i*-th model solution calculate on the formula (see the relation (2.1)):

$$X_{i,T}\% = X_{i,IP}\% = 100 \cdot 10 \cdot (m_{B,i} / m_{st}), \qquad (4.33)$$

where m_{st} – a weight of the solution B, taken to prepare an appropriate (for the reference standard method or for the standard addition method) reference solution.

Table 4.1

Characteristics of the model solutions for the reference standard method (m_T= 0.0874 g, m_{IP} = 0.5177 g, *m(A)*= 10.6729 g, $m_A(B)$= 2.6878 g, *m(B)* = 27.1129 g)

Solution number	Weights of the solution B, $m_{B,i}$, g	Weight of RS in 10 ml of model solution, mg		Normalized concentrations, X_i, %	
		Toluene	Isopropanol	Theory	Fact
1	0.2709	0.2298	1.2816	25	24.8
2	0.5446	0.4619	2.5765	50	49.9
3	0.8101	0.6871	3.8331	75	74.2
4	1.0902	0.9247	5.1585	100	99.9
5	1.4169	1.2018	6.7034	125	129.8
St(MS)	1.0913	0.9256	5.1637	-	-

Table 4.2

Characteristics of the model solutions for the standard addition method (m_T= 0.0885 g, m_{IP} = 0.5055 g, *m(A)*= 10.7611 g, $m_A(B)$= 2.6659 g, *m(B)* = 27.3783 g)

Solution number	Weights of the solution B, $m_{B,i}$, g	Weight of RS in 10 ml of model solution, mg		Normalized concentrations, X_i, %	
		Toluene	Isopropanol	Theory	Fact
1	0.2728	0.2185	1.2477	25	25.0
2	0.8130	0.6511	3.7183	75	74.4
3	1.3761	1.1020	6.2937	125	125.9
4	1.9184	1.5363	8.7740	175	175.5
5	2.5263	2.0231	11.5543	225	231.1
St(MS)	1.0932	0.8754	4.9999	-	-

4.2.3. Chromatographic results

The typical chromatogram of *the reference solution for the reference standard method* is presented in the Figure 1.

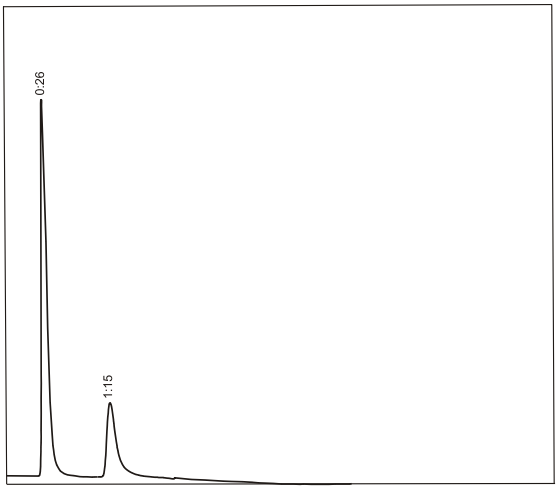


Figure 4.1. The typical chromatogram of *the reference solution for the reference standard method*

No interfering peaks were detected in the solvent (reference solution *00*) (see table 4.3-4.4). Only residual peaks of isopropanol were detected in the dried substance solution (reference solution *0*) (which were decreased with further drying). Therefore the average isopropanol peak area in the reference solution *0* (S^0_{IP}) were subtracted from the isopropanol peak areas found for the model mixtures. This subtraction was also carried out for the reference solution for the standard addition method. For the reference standard method this operation is not carried out, because in this case there is no test substance in the reference solution.

Normalized area values (Y_i) of toluene (T) and isopropanol (IP) are calculated according to the formulas (see the relation (2.1)):

Reference standard method:

$$Y_{i,T}(MS) = 100 \cdot S_{i,T} / S^{st}_{i,T}(MS),$$
$$Y_{i,IP}(MS) = 100 \cdot (S_{i,IP} - S^{0}_{IP}) / S^{st}_{i,IP}(MS). \quad (4.34)$$

Standard addition method:

$$Y_{i,T}(AD)\% = 100 \cdot S_{i,T} / S^{st}_{i,T}(AD),$$
$$Y_{i,IP}(MS)\% = 100 \cdot (S_{i,IP} - S^{0}_{IP}) / [S^{st}_{i,IP}(AD) - S^{0}_{IP}]. \quad (4.35)$$

To express the found concentrations in per cent of the input concentrations, calculate the Z values (see the ration (2.1)) величину Z:

$$Z_i\% = 100 \cdot (Y_i / X_i). \quad (4.36)$$

Chromatographic results for investigated objects are presented in Tables 4.3 and 4.4. The results of the calculations are presented in the Table 4.6. The criteria are calculated on the basis of the approach outlined in the section 2.3. The values of RSD_{tot} are calculated according to the formula (4.31) and the critical values for them - according to the ratio (4.32).

Calculations of the parameters of the linear relationship of $Y = b \cdot X + a$ are conducted by the Least Squares Method [26]. The results of the calculations – values of b, s_b, a, s_a, s_r (the residual standard deviation) and r (correlation coefficient) - are presented in the Table 4.5. The resulting straight line in normalized coordinates is presented in the Figure 4.2.

Table 4.3

Chromatographic results for the model solutions. Reference standard method

Model solution number	Toluene peak areas	Average area $S_{i,T}$ (RSD_i%)	$Y_{i,T}$%		Isopropanol peak area	Average area $S_{i,IP}$ (RSD_i%)	$S_{i,IP} - S^0_{IP}$	$Y_{i,IP}$ %
1	93.2 96.1 95.3 94.2	94.7 1.3 %	24.1		306.8 286.4 296.4 289.6	294.8 3.1 %	240.3	24.3
2	286.0 304.3 268.9	286.4 6.2 %	73.0		795.7 732.0 801.8	776.5 5.0 %	722.0	73.0
3	486.4 469.0 504.5	486.6 3.6 %	124.0		1277.9 1295.3 1310.8	1294.7 1.3 %	1240.2	125.3
4	671.6	689.2	175.6		1913.1	1824.9	1770.4	178.9

	691.4 704.5	2.4 %			1807.5 1754.2	4.4 %		
5	894.8 945.2 828.4	889.5 6.6 %	226.6		2527.1 2306.2 2245.1	2359.5 6.3 %	2305.0	232.9
st(MS)	370.8 426.3 392.4 380.4	392.5 6.2 %	-		1104.1 1065.1 1003.9 1002.9	1044.0 4.7 %	989.5	-
Reference solution *00* (S_{00})	0				0			
Reference solution *0* (S_o)	0 0 0				54.2 55.2 54.0	54.5 1.2 %		
Pooled RSD_{tot}%		**1.8** (f_{tot}= 13)				**2.9** (f_{tot} = 15)		
Critical values of RSD_{tot}%		**8.0**				**8.3**		

Table 4.4

Chromatographic results for the model solutions. Standard addition method

Model solution number	Toluene peak areas	Average area $S_{i,T}$ (RSD_i%)	$Y_{i,T}$%		Isopropanol peak area	Average area $S_{i,IP}$ (RSD_i%)	$S_{i,IP} - S^0_{IP}$	$Y_{i,IP}$ %
1	93.2 96.1 95.3 94.2	94.7 1.3 %	24.1		306.8 286.4 296.4 289.6	294.8 3.1 %	240.3	24.3
2	286.0 304.3 268.9	286.4 6.2 %	73.0		795.7 732.0 801.8	776.5 5.0 %	722.0	73.0
3	486.4 469.0 504.5	486.6 3.6 %	124.0		1277.9 1295.3 1310.8	1294.7 1.3 %	1240.2	125.3
4	671.6 691.4 704.5	689.2 2.4 %	175.6		1913.1 1807.5 1754.2	1824.9 4.4 %	1770.4	178.9
5	894.8 945.2	889.5 6.6 %	226.6		2527.1 2306.2	2359.5 6.3 %	2305.0	232.9

	828.4				2245.1			
st(AD)	370.8 426.3 392.4 380.4	392.5 6.2 %	-		1104.1 1065.1 1003.9 1002.9	1044.0 4.7 %	989.5	-
Reference solution *00* (S_{00})	0				0			
Reference solution *0* (S_o)	0 0 0				54.2 55.2 54.0	54.5 1.2 %		
Pooled RSD_{tot}%		**4.8** (f_{tot}= 14)				**3.4** (f_{tot} = 16)		
Critical value of RSD_{tot}%		**8.2**				**8.4**		

4.2.4. Results and discussion

4.2.4.1. Repeatability of replicate injections

As can be seen from the Tables 4.3-4.4, the relative standard deviations of replicate injections RSD_i for isopropanol and toluene peak areas for all model solutions satisfy the requirements (4.13), i.e. does not exceed 6.7 and 8.3% for the numbers of degrees of freedom $f = 2$ and 3 respectively. Pooled standard deviation RSD_{tot} also does not exceed the critical values of the ratio (4.32). Therefore the requirements of (4.10, 4.13) of chromatographic system suitability in terms of “relative standard deviation" can be considered justified.

4.2.4.2. Specificity

No peaks were observed in the solvent chromatogram (*solution comparison 00*), having retention times, which are the same (or close) to the retention times of toluene and isopropanol peaks.

There was a peak in the chromatogram of the *reference solution 0*, coinciding with the peak of retention time of isopropanol. Its area (S^0_{IP} = 54.5) is 5.2% of the isopropanol peak area of the *reference solution for the standard addition method.* That exceeds the requirements of (4.20) (≤ 5.1%). But the area of this peak decreased with further drying, so we can take it that it was the peak of isopropanol, which was not completely removed when drying in processing. Therefore this peak area was subtracted from the peak areas of isopropanol for all model solutions and *reference solution for the standard addition method*, except for the *reference solution for the reference standard method*.

Peak resolutions in the all investigated chromatograms exceed 5.5, i.e. meet the system suitability requirements ($R_s > 3.0$).

Column performance for the peak of toluene in all chromatograms and columns exceeds 1500 theoretical plates. Because there were satisfactory metrological characteristics, then this may be offered for an introduction to the system suitability test.

Therefore, the specificity of the procedure can be considered confirmed.

4.2.4.3. Linearity and detection limit

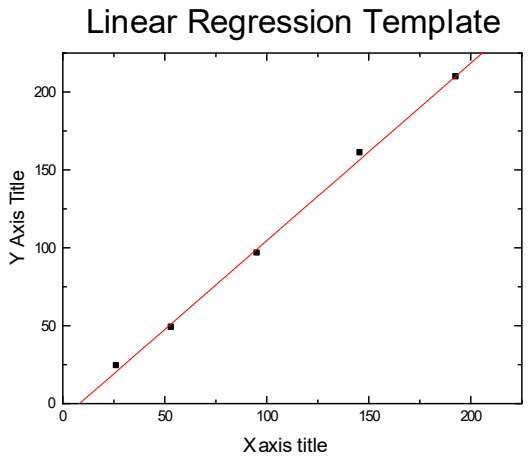


Figure 4.2. The typical linear regression for toluene in the normalized coordinates

Table 4.5

Results of processing of X_{ij} and Y_{ij} values by the Least Squares Method on the straight line $Y = A + B \cdot X$ (toluene – T, isopropanol - IP)

RS	A	SD_A	2.353* SD_A	Practical insignificance A	DL ≤ 32%	B	SD_B	RSD_o ≤ 6.8	$R \geq$ 0.9851
Reference standard method									
T	0.70	3.48	8.19	2.6	12.0	0.961	0.041	3.41	0.9972
IP	4.30	4.00	9.41	2.6	14.1	1.004	0.048	3.91	0.9966
RS	A	SD_A	2.353* SD_A	Practical insignificance A	DL ≤ 32%	B	SD_B	RSD_o ≤ 6.8	$R \geq$ 0.9963
Standard addition method									
T	-0.27	1.50	3.53	6.8	5.0	0.989	0.010	1.67	0.9998
IP	-1.90	1.36	3.20	6.8	4.4	1.019	0.009	1.52	0.9999

As shown in Table 4.5, linearity is satisfied for both residual solvents.

It must be noted that the requirement of the practical insignificance of the Y-intercept (≤ 2.6%) is useless for the standard addition method (see the section 4.1.5.2.2)..

The detection limit for both residual solvents meets the criterion (4.17) (≤ 32%).

4.2.4.4. Accuracy and precision

Table 4.6

Accuracy and precision check

№	Reference standard method					Standard addition method				
	X_i	$Y_i(T)$	$Y_i(IP)$	$Z_i(T)$	$Z_i(IP)$	X_i	$Y_i(T)$	$Y_i(IP)$	$Z_i(T)$	$Z_i(IP)$
1	24.8	23.3	25.6	94.0	103.2	25.0	24.1	24.3	96.4	97,2
2	49.9	47.8	51.6	95.8	103.4	74.4	73.0	73.0	98.8	98,3
3	74.2	74.4	75.5	100.3	101.8	125.9	124.0	125.3	99.1	99,5
4	99.9	101.3	103.3	101.4	103.4	175.5	175.6	178.9	100.1	101.9
5	129.8	122.7	125.2	94.5	96.5	231.1	226.6	232.9	96.8	100.8
Average, $\overline{Z}$ %				**97.2**	**101.7**				**98.2**	**99.54**
SD%				**3.5**	**2.9**				**1.58**	**1.88**
t(95%,3)				**2.13**	**2.13**				**2.13**	**2.13**
Δ_{Im} (≤ 16%)				**7.5**	**6.2**				**3.37**	**4.00**
Systematic error $\delta = \lvert \overline{Z} - 100 \rvert$				**2.8**	**1.7**				**1.8**	**0.5**
$\Delta_{Im}/\sqrt{5}$				**3.3**	**2.8**				**1.51**	**1.8**
max δ				**5.1**	**5.1**				**5.1**	**5.1**
Insignificance of δ: (a) $\delta \leq \Delta_{Im}/\sqrt{5}$; (b) if (a) is not met, than $\delta \leq max\ \delta = 5.1$				Yes **Yes**	Yes **Yes**				Yes **Yes**	Yes **Yes**

As shown in the Table 4.6, the repeatability complies with the requirements of (4.14) for both residual solvents. Systematic error (accuracy) of RS meets the requirements of statistical and practical insignificance (see section 2.3.3) for both RS for the reference standard method. In the case of the standard addition method the systematic error is significant statistically for toluene, but is insignificant practically. For isopropanol the systematic error is not significant statistically and practically.

Thus, precision meets the necessary requirements for both residual solvent and both approaches.

In addition, as shown in the section 4.1.7.1, compliance with the requirements for linearity, accuracy, and precision is proof of sufficient stability of the solutions for the RS control under the specification.

5. VALIDATION OF TITROMETRIC ASSAY PROCEDURES FOR MEDICINE CONTROL

The previous sections have discussed the standardized validation schemes for assays, chromatographic procedures for related substances and residual solvents control in medicines. These developments have proved themselves for the principal pharmacopoeial comparative methods - chromatography and spectrophotometry. They were included in SPU [11] and methodical Guidance of the Russian Federation [12].

However, their application to such an important and seemingly simple pharmacopoeial direct quantitative method as titration, has encountered some difficulties caused by a great variety of factors influencing the accuracy and precision of titrometric procedures. The matter was investigated for conditions of pharmacies and control laboratories [41]. However, this material is so extensive that to cover all the issues at once is hardly possible. In addition, the conditions of the titrometric procedures and the principles of their validation in the pharmaceutical industry are substantially different from those of pharmacies.

This section gives a systematic consideration of these questions for the laboratories of pharmaceutical manufacturers and related government control laboratories, with the objective of developing a standardized scheme of validation of the titrometric procedures. The consideration is carried out for the direct acid-base titrometric procedures, but the findings are largely applicable to other titrometric procedures, as well as for the back titration.

5.1. Pharmacopoeial requirements to validation of assay procedures

The objective of validation of an analytical procedure is to demonstrate that it is suitable for an intended purpose [4, 11].

Assays need to control [4, 11]: accuracy, precision, specificity (lack of specificity of the test could be compensated by other additional tests), linearity, analytical range. It should be noted that the analytical range (and, consequently, the range within which the linearity is verified) is, unless there are other indications, 80-120% of the nominal value [4, 11].

5.1.1. Range and content tolerances

Titration in pharmacopoeial analysis is the main direct method for assay of medicinal substances [1].

Typical tolerances are of 99.0-101.0% (such as *Oxazepam*), but one can give examples of substances with tolerances of 99.5-100.5% (anhydrous citric acid), 99.0-100.5% (*Ketoprofen*), 98.5-100.5% (Indometacin), 98.5-101.0% (*Artikaine hydrochloride*), 98.5-101.5% (*Caffeine*) and 98.0%-102.0 (*Nifedipine*) (see Table 1) [1].

In some special cases, the SPU applies titration to assays of complex substances like Theophylline-ethylenediamine with tolerances of theophylline 84.0-87.4% and ethylenediamine 13.5-15.0% [1].

The SPU also describes the use of titration for assays of some drug products (DPs) [8]. Tolerances are usually 95.0-105.0% of nominal contents (for example, *Boric acid solution*; *Iodine solution, alcoholic; Salicylic acid solution, alcoholic*) [1].

5.1.2. General requirements to a titrometric assay uncertainty

A general expression for the calculation of substance content (%) in the sample analyzed by titration is of the form:

$$X = K_T \cdot T \cdot \frac{(V - V_0)}{m} \cdot 100\%, \qquad (5.1)$$

где: K_T - titer correction coefficient,
V - total titration volume, ml,
V_0 - titration volume for the blank experiment, ml,
T - amount of analyzed substance (g) equivalent to 1 ml of the titrant of the nominal concentration,
m - weight of the test sample, grams.

When the titration is carried out without the blank experiment, in the expression (5.1) we can consider $V_0 = 0$. In the case of potentiometric titration, the titration volume is usually calculated as the difference between the two potential jumps [1, 3], i.e., the $(V - V_0)$ value is immediately found, so here you can also consider $V_0 = 0$.

It should be noted that refusal of the indicator (visual) titration procedures and transition to the potentiometric titration procedures is the strategic direction of the Eur.Ph. (and thus of the SPU). Now for titration assays of medicinal substances the indicator (visual) titration procedures in Eur.Ph. and SPU is used very rarely [1, 3]. This is connected both with some subjectivity of the indicator (visual) titration and simplicity of use in the potentiometry of the back titration (allowing titrating hydrochlorides of medicinal bases) and determination of the titration volume as the difference between the two potential jumps.

In general case, a relative uncertainty of a titration procedure (and any other analytical procedure) Δ_{As} may be presented as a sum of two components that during the titration act as a systematic error Δ_S and a random uncertainty Δ_R [26]:

$$\Delta_{As}^2 = \Delta_S^2 + \Delta_R^2. \qquad (5.2)$$

The random uncertainty Δ_R is defined by the uncertainty components of titration of the sample solution itself, i.e.: uncertainty of the titration volume of the test solution (including determination of the equivalence point) and repeatability of the sample weighing. The systematic component Δ_S is connected with the uncertainty of values that act as invariable during the titration of the test solution (although perhaps random with respect to each other). They are: the correction coefficient to the nominal concentration of the titrant (K), the titration volume of the blank experiment (V_o) and the accuracy of weighing for determining the titer. The relative uncertainty of the correction coefficient is equal to the relative uncertainty of the titrant concentration.

In accordance with the SPU [11], the target assay uncertainty $max\ \Delta_{As}$ is defined by the ratios:

Medicinal substance : $$\Delta_{As} \le \max\Delta_{As} = B_H - 100\%. \quad (5.3)$$

Drug product (DP): $$\Delta_{As} \le \max\Delta_{As} = \frac{B_H - B_L}{2} \cdot 0.32. \quad (5.4)$$

Here B_H and B_L are respectively the upper and lower tolerances of the target component in the analyzed sample accordingly to the specification or pharmacopoeial monograph.

In general, it is recommended that the systematic component Δ_S of the uncertainty was insignificant compared with the target uncertainty $max\Delta_{As}$ of the whole analytical procedure, i.e., in accordance with the *Insignificance Principle* (see section 2.3.1), the ratio should be performed:

$$\Delta_S \le 0.32 \cdot \max\Delta_{As}. \quad (5.5)$$

In this case, the systematic component significantly does not affect the decision about the quality and may not be taken into account when setting the requirements for the repeatability of the replicate titrations of the test sample. Otherwise such taking into account is needed.

5.2. Uncertainty of a titration final analytical operation

5.2.1. Uncertainty of weighing

In accordance with the requirements of the SPU [11], based on the Guideline of the European Pharmacopoeia [4], the uncertainty of weighing should not exceed 0.2 mg. However, it is unclear what kind of uncertainty they have in mind - the systematic, random or totall.

The Methodical Instruction [42] clearly distinguishes between the acceptable error (i.e. the total uncertainty of weighing) and the standard deviation (*SD*) for 10 replicate

weight measurements (which characterizes the random component of the weighing uncertainty). The difference between them is usually 3-5 times [42].

If the complete uncertainty of weighing does not exceed the confidence interval of the random component, then this means that the Instruction [42] does not imply the significant systematic error of weighing. This is important from a practical point of view, because the random component of the total uncertainty can be reduced by the increase of the number of concurrent weight measurements and systematic one cannot [26].

The confidence interval for a single measurement is equal to the product of the *t-value* for the corresponding probability and degrees of freedom at *SD* [26]. The *t-value* characterizes the rate of statistically insignificant distinction between *SD* and limits of the acceptable error. In analytical practice commonly the probability of 0.95 (95%) is used. Let's calculate the *t-value* for a single weighing with the probability 0.95.

In accordance with the Instructions [42], the *SD* is determined for 10 replicate weightings (i.e. $\nu = 9$ degrees of freedom). The limits of the acceptable error for the loaded balances are defined from these same 10 weightings for the whole range of weighing. Each single weighing shall not exceed the specified values. If it is considered that this requirement shall be met at all 10 weight measurements with probability 0.95, the probability for a single weighing is equal to [26]:

$$0.95^{(1/10)} = 0.99488.$$

A *t-value* for a given probability and 9 degrees of freedom is equal to $t(0.99488, 9) = 3.24$ [26]. If all of these calculations to carry out for the original probability 0.99, we can get $t(0.998995, 9) = 4.29$.

Thus, we cannot talk about a systematical error, if the difference between *SD* of the single weighting and its acceptable error limit of not more than 3.24 (with probability 0.95) or 4.29 (with probability 0.99). Given that, this difference is usually set within 3-5 [42], it can be concluded that the Instruction do not imply a significant systematical error for balances. Thus, during the titration *the weighing uncertainty can be reduced by the increase in the number of replicate weightings*.

The upper limit of the acceptable *SD* for analytical balances is usually the size of 0.1 mg [42]. Given that the *t-value* for probability of 0.95 and 9 degrees of freedom equal to 1.83, get that confidence interval of a single weighing with probability 0.95 does not exceed the value $max\Delta_m = 0.18$, i.e. single weighing precision of such balances (with $SD \leq$ 0.1 mg) satisfies the pharmacopoeial requirements (≤ 0.2 mg) [4, 11].

Summary on titrometric assays of some medicinal substances is presented in Table 5.1.

For titrometric assays of the medicinal substances SPU typically uses weights above 200 mg (see Table 5.1) that corresponds to the value of $max\Delta_m \leq 0.1\%$. This quantity even for tolerances of 99.5-100.5% does not exceed the maximum acceptable (target) value of the systematical error (0.16 %) from the equation (5.5). Given that, in practice, at least 3 replicate weights is used for a titrometric assay, we can get that *the uncertainty*

added by the weighting is not significant for titrometric assays of the medicinal substances.

Table 5.1

Characteristics of acid-base titration of some medicinal substances depending on the tolerances of the principal component [8]

Content tolerances, %	$max\Delta_{As}$ % [1]	$max\Delta_S$ % [1]	Medicinal substance	Weight for assay, g	$max\Delta_m$* %	Nominal titration volume V_{nom}, ml	% of burette capacity of 10 ml
99.0-101.0	1.0	0.32	Oxazepam	0.25	0.080	8.72	87.2
99.5-100.5	0.5	0.16	Citric acid	0.55	0.036	8.59	85.9
99.0-100.5	0.5	0.16	Ketoprofen	0.20	0.100	7.86	78.6
98.5-100.5	0.5	0.16	Indometacin	0.30	0.067	8.38	83.8
98.5-101.0	1.0	0.32	Articaine hydrochloride	0.25	0.080	7.79	77.9
98.5-101.5	1.5	0.48	Caffeine	0.17	0.118	8.75	87.5
98.0-102.0	2.0	0.64	Pheniramine maleate	0.13	0.154	7.51	75.1
Average						**8.23**	**82.3**

* calculated on the base of the SPU requirements $max\Delta_m$ = 0.2 mg.

Note that DP content tolerances in SPU, as already mentioned above, are 95-105%, which, in accordance with the equations (5.4-5.5), gives: $max\Delta_{As}$ = 1.6%, $max\Delta_S$ = 0.5%.

5.2.2. Uncertainty of a burette delivery volume

In accordance with the requirements of the SPU [1, 11], for assay should be used volumetric glassware of Class A [43-45], the requirements of which are harmonized with the requirements of GOST [18-20] to Class 1 glassware. In accordance with these requirements, burettes are of two types:

Type 1: Burettes for which no waiting time is specified; class A and B (ISO); no time is required to allow the liquid on the inner walls of the burette to flow down before the reading is taken.

Type 2: Burettes for which a waiting time is specified; Class A only (ISO), with a clear indication of the “waiting time" on the label (for example, Ex +30 s). The ISO standard for Class A burettes requires the time of 30 sec to allow the liquid on the inner walls of the burette to flow down before the reading is taken.

Burette delivery volume errors shall not exceed the specified values (Table 5.2) [18-20, 43-45]. Permissible error limits are the maximum permissible error at any point of the scale and the maximum permissible error of the difference between any two points of

the scale. The requirements for Class A burettes are presented in the Table 5.2. For comparison the requirements for Class B burettes (Class 2 according to GOST [18-20]) are presented as well. The requirements for the target values of the systematic error $max\Delta_S$ versus the target total uncertainties of the analytical procedures are taken from the Table 5.1 and are given as well.

Table 5.2

Permitted deviation from the nominal capacity of burettes

Nominal capacity, ml	Lowest graduation value	Permissible error ($max\ \Delta_{bur}$)					
		Class A			Class B		
		± ml	%	% for 80% burette capacity	± ml	%	% for 80% burette capacity
1	0.01	0.01	1	1.25	0.02	2.0	2.50
2	0.01	0.01	0.5	0.625	0.02	1.0	1.25
5	0.02	0.01	0.2	0.25	0.02	0.4	0.50
10	0.02	0.02	0.2	0.25	0.05	0.5	0.625
	0.05	0.02	0.2	0.25	0.05	0.5	0.625
25	0.05	0.03	0.12	0.15	0.05	0.2	0.25
	0.1	0.05	0.2	0.25	0.1	0.4	0.50
50	0.1	0.05	0.1	0.125	0.1	0.2	0.25
100	0.2	0.1	0.1	0.125	0.2	0.2	0.25

Target values of the systematical error $max\Delta_S$	
$max\Delta_{As} = 0.5\%$	$max\Delta_S = 0.16\%$
$max\Delta_{As} = 1.0\%$	$max\Delta_S = 0.32\%$
$max\Delta_{As} = 1.5\%$	$max\Delta_S = 0.48\%$
$max\Delta_{As} = 2.0\%$	$max\Delta_S = 0.64\%$

A natural question arises: specified in the Table 5.2 the maximum permissible errors are systematic or random? Considering that the maximum permissible errors are specified for the difference between any two scale marks (in particular, between zero and any other burette scale mark) [18-20], we can assume that for different delivery volumes (for example, 2, 3, 6 ml, etc) these errors can have a different sign, i.e. be random in nature. In this case, this uncertainty will decrease (as $1/\sqrt{n}$) when you increase the number (n) of the replicate titrations (because the volumes will be different). If the maximum permissible errors are systematic (i.e. have the same sign for the different volumes), these errors may not be decreased by increase of the number of the replicate titrations/ It needs to be taken into account in the prognosis of the total assay uncertainty of the titration procedures.

5.2.2.1. Control of a burette limit uncertainty

It should be noted that the control of the limit errors (Table 5.2) can be made in different ways. In the domestic GOST [16-18], corresponding to the international standards ISO [43-45], and in the general ISO standard on laboratory glassware [46] as well, procedures for the control of these errors with the necessary criteria (like a similar procedure for balances [42]) are not allowed. In [18] it is only specified they the errors need to control by “the specification approved in established order". Such procedures are described in the literature.

For example, the volume of burette is divided into 5 parts; each of them shall conform to the requirements of the Table 5.2 [47]. The corresponding delivered water volume is weighed and the weight is translated to the volume, taking into account the tabular density of water at a given temperature [46]. The procedure is repeated, the results of the differences between actual and nominal volumes are averaged and rounded to the nearest 0.01 ml and round-off results are compared with the requirements of the Table 5.2. In order to obtain an accurate delivered volume, it is recommended to use a magnifying glass [47]. A typical example of this calculation is shown in the Table 5.3 [47].

It is easy to see that in this case for testing the burette we use the "*Confirming approach*" (see the section 1.7). I.e. we suppose that for the "transitional volumes" of the burette (those which are not covered by the calibration) requirements of the Table. 5.2 are satisfied if they are satisfied for the calibrated volumes. The *Confirming approach* is justified in this case, if a quality assurance system is used in burette manufacture.

Table 5.3

The study of the actual volume of Class A burette of 25 ml capacity with the lowest graduation value of 0.1 ml (t = 23°C, 1 g of water = 1.0035 ml) [47]

Read burette volume, ml	Bulb weight with delivered water, g	Weight of delivered water, g	Actual delivered water volume, ml	Correction to read burette volume, ml (≤ 0.05 ml)
0.02*	41.153	0.000	0.00	0.00
5.01	46.160	5.007	5.02	+0.03*
10.00	51.136	9.983	10.02	+0.04
15.03	56.125	14.972	15.02	+0.01
20.01	61.096	19.943	20.01	+0.02
24.98	66.023	24.870	24.96	0.00

* The initial read volume is not a zero and should therefore deducted from the subsequent read volumes

But practical application of the procedures of [47], however, raises some questions. In particular, how many times to repeat the procedure? What is the criterion of adequacy of the number of replicate definitions? When we calibrate the burette we deal with two types of errors. One of them is an error of the burette volumes Δ_{bur} (that we want to check) and is defined as the difference between the experimentally found volume and

nominal burette volume. The second (Δ_{oper}) is a random error of determination of delivered volume by the analyst and is defined as a confidence interval of the replicate volume definitions. On the basis of the *Insignificance principle* (see the section 2.3.1), the Δ_{oper} value significantly does not affect the assessment of the burette volume error when running the ratio:

$$\Delta_{oper} \leq 0.32 \cdot \max\Delta_{bur}. \tag{5.6}$$

Here: *max* Δ_{bur} is a maximum permitted (by GOST [11, 20]) the deviation of the actual volume of burette from the nominal value. In particular, for Class A burettes with the capacity of 25 ml and the lowest graduation value of 0.1 ml we have *max* Δ_{bur} = 0.05 ml (see Table 5.2). In this case the operator work-related uncertainty (half-width of the confidence interval for the mean volume), in accordance with the ration (5.6), shall not exceed Δ_{oper} = 0.016 ml. Similarly, for burettes with capacity of 10 ml we can get $\Delta_{oper} \leq$ 0.0064 ml. Only in this case, you can make a right conclusion about the burette quality by the delivered volume. Otherwise, the number of replicate volume definitions should be increased. For calculation of Δ_{oper} it is reasonable to use the absolute standard deviation of replicate weight measurements, pooled for all 5 volumes of the burette (SD_{pool}) [26]. It should be noted that the Guidance [47] provides approximately the same requirements: discrepancy between the replicate definitions of the correction to the actual volume of a burette with a capacity of 25 ml should not exceed 0.03 ml.

If we carry out 5 replicate weight measurements for each volume of a burette, we can calculate what the universe absolute standard deviation (SD_{oper}) of the delivered volume (operator error) for the burettes with capacity of 10 ml and 25 ml corresponds to inequalities (5.6):

For the nominal burette capacity of 10 ml

$$SD_{oper} \leq \frac{0.0064 \cdot \sqrt{5}}{1.64} = 0.0087 \ ml. \tag{5.7}$$

For the nominal burette capacity of 25 ml

$$SD_{oper} \leq \frac{0.016 \cdot \sqrt{5}}{1.64} = 0.022 \ ml.$$

These SD_{oper} values correspond to the relative standard deviation RSD_{oper} = 0.087% for the total volume of both burettes. It is interesting to note that this is almost the same as the relative standard deviation of the 30 replicate weightings (*RSD* = 0.082%) obtained by the experienced analyst during the determination of the total volume of the pipette with capacity 10 ml [17]. Thus, it can be concluded that the requirements (5.7) correspond to the usual analytical practice. It should be noted that verification (qualification) of a burette requires sophisticated skills of the operator. Otherwise, the generated values can significantly exceed the *RSD* acceptability criteria of (5.7) [17].

The procedure [47] is intended for calibration and checking of a burette for later its use in the general case - for any titration. It should be distinguished from the procedure of qualification of a burette for the pharmacopoeial analysis, since the qualification of analytical equipment is experimental evidence that it (in this case the burette) is able to perform analysis with the necessary precision and accuracy. Since the maximum permissible error of the burette max Δ_{bur} is specified in ml (see Table 5.2), the more the delivered volume against the total burette volume, the smaller the relative uncertainty. For example, for Class A burettes with capacity of 10 ml (standard burette for the pharmacopoeial analysis) *max* Δ_{bur} = 0.02 ml. For the delivered volume of 2 ml this corresponds to a maximum relative error of 1.0%, and for the total volume of 10 ml is 0.2%. So in the pharmacopoeial analysis, the nominal delivered volume is usually selected within 70-90% from the total burette volume (see Table 5.1).

In the Table 5.1 substances for relevant content tolerances are taken randomly, so the calculated average values of volume V_{nom} and percentage of burette capacity are for information only. However, they accurately reflect the approach of the European Pharmacopoeia (Eur.Ph.) [4] and SPU [1] to titration procedures: the nominal delivered volume must be about (80 $\pm$ 10) % of the capacity of the burette used for titration.

The procedure of control of limit errors of a burette raises many questions and therefore requires a separate consideration. At the same time, an important conclusion from the Table 5.3 is that the deviations from the nominal volume of burette are of the same sign, i.e. are systematic (our own studies also confirm this). Therefore, generally, guaranteed by GOST [18-20] *maximum permissible error of the burette cannot be reduced by increasing the number of replicate definitions and should be regarded as systematic error in an uncertainty prognosis*.

5.2.2.2. Corrections to the burette nominal volume

One of the discussion points is the possibility of introducing corrections to the nominal volume of the burette, obtained under the calibration (see Table 5.3) [47]. This would significantly reduce the error of their actual volume. This approach is quite applicable in scientific researches, however, it is not possible to use it in the compendial routine quality control of medicines, since generates more problems than it solves.

First, in this case, becomes totally unnecessary the requirement of the SPU [1, 11] about the mandatory use of the Class A volumetric glassware in the quantitative analysis. After all, we use the corrections to the nominal burette volumes. Secondly, the "*Confirming approach*" (see section 1.7) is not applied in this case, and the correction must be used for *each* graduation mark of the burette (possibility of extrapolation is necessary to prove). In the case of the burette of 10 ml capacity with graduation value of 0.02 ml we have to use 500 corrections. Thus, in fact, the analyst performs the work of the burette producer. Thirdly, it is not clear what the accuracy of these corrections is and how they may change through time. In addition, the calibration procedure must be validated

(how?). There are other objections, but it is clear that much more reliable and more accurate to use the approach based on the maximum errors of GOST [18-20].

From the Table 5.2 and the finding about impossibility to reduce the systematic error of the burette by increasing the number of replicate titration we can get an important practical conclusion: the burettes with a capacity of 1 and 2 ml are not suitable for the compendial titrometric analysis of medicinal substances because they bring about the systematic error that exceeds the limit values of $max\Delta_S$ for any content tolerances.

5.3. Volumetric solutions

5.3.1. Requirements to the uncertainty of the titrant concentration

Titration uses volumetric solutions. The uncertainty of their concentrations (molarities) acts during the titration as the systematic error of $\Delta(titr)$, which should not exceed the limit value $max\Delta_S$ from the equation (5.5) and the Table 5.1, i.e.

$$\Delta(titr) = \Delta_S \le 0.32 \cdot \max\Delta_{As}. \qquad (5.8)$$

In this case, it is significant shall not affect decisions about quality. Otherwise the actual value of $\Delta(titr)$ should be taken into account when setting the requirements to the final analytical operation that is to the test sample titration. What the requirements set the Eur.Ph. (and the SPU respectively) to the uncertainty of the titrant concentration?

As indicated in the SPU [1, 2001, p. 290] and Eur.Ph. 3.0 [48], the concentrations of volumetric solutions should not differ from the nominal one by more than 10% relative and shall be determined with an accuracy of 0.2% relative. The weak point of this wording is that the uncertainty of the titrant concentration is not associated with the current task. As can be seen from the Table 5.1, for target procedure uncertainty of $max\Delta_{As}$ = 0.5%, the titer uncertainty requirements of $\Delta(titr) \le 0.2\%$ are insufficient (must be $max\Delta_{As} \le 0.16\%$), and for $max\Delta_{As} = 2.0\%$ are redundant (must be $max\Delta_{As} \le 0.64\%$). The conclusion is obvious: the uncertainty of the titer must be linked to the current task.

Perhaps, therefore, the position of the Eur.Ph. to the uncertainty of the titer has changed. So, in the Eur.Ph 6.0 [3] section 4.2.2 says: "The molarity of the volumetric solutions is determined by an *appropriate number of titrations*. The repeatability does not exceed 0.2 per cent (relative standard deviation)".

The meaning of the phrase is that the determination of the titer should be conducted with the repeatability of replicate titrations that is typical for a regular analytical practice for titration of strong acids and bases, for which the relative standard deviation of *RSD* = 0.2% is completely reachable. The required confidence interval of this repeatability is governed by a number of replicate titrations and is defined by the user, based on the requirements of the task.

As we can see, the wording of the Eur.Ph. 6.0 ($RSD \leq 0.2\%$) is, like, a more correct than the wording of the SPU-2001 [1] and the Eur.Ph. 3.0 [48] ($\Delta(titr) \leq 0.2\%$), as the titer uncertainty is linked to the current task.

However, the wording of the Eur.Ph. 6.0, unlike the SPU-2001 Eur.Ph. 3.0, governs only the *repeatability of the replicate titrations* under the determination of the titrant concentration. At the same time, the total titer uncertainty also includes other components, in particular: the uncertainty of weighting for the titer determination (this is important if the sample weight is too small), thermal factor (difference in temperature when setting the titer and titrating test sample), the uncertainty of the delivered volume of the burette, etc. These factors can be very substantial in some cases. However, the Eur.Ph. leaves it to the discretion of a user, concentrating only on the repeatability of the replicate titrations, i.e. the random uncertainty of titer. This approach seems to be not sufficiently correct.

What the minimum guaranteed uncertainty can be obtained for the concentrations of the volumetric solutions? Consider the influence of key factors – temperature and uncertainty of the burette.

5.3.2. Effect of temperature

In accordance with the requirements of the SPU [1, 2008, s. 34], harmonized with the Eur.Ph. [3], the room temperature may vary within 15-25 °C. The corresponding volume expansion may vary from 0.19% for water titrants to 1.24% for methanol ones (see Table 5.4). On the basis of the thermal expansion, the total systematic ($\Delta_S(titr)\%$) and total ($\Delta(titr)\%$) uncertainty of the titrant concentration may be calculated (see below). For comparison, in Table 5.4 there are also requirements of the ratio (5.5) and Table. 5.1 to the maximum value of the systematic error $max\Delta_S$ for different values of the maximum total procedure uncertainty $max\Delta As$ for most used pharmacopoeial solvents.

Table 5.4

Thermal expansion and total systematic uncertainty for various titrants

Solvent	Examples of the pharmacopoeial titrants	Thermal expansion [49] Δ_{therm}, %		$\Delta_S(titr)\%$		$\Delta(titr)\%$	
Temperature difference between titer determination and sample titration (Δt) →		10 °C	2 °C	10 °C	2 °C	10 °C	2 °C
Water	HCl, NaOH, Iodine	0.19	0.04	0.25	0.16	0.30	0.23
Methanol	Na and Li methylates	1.24	0.25	1.25	0.29	1.26	0.34
Ethanol	KOH, NaOH, HCl	0.82	0.16	0.83	0.23	0.85	0.28
n-Propanol	Tetrabutyl ammonium hydroxide (TBAH)	0.94	0.19	0.96	0.25	0.97	0.30
Benzene		1.22	0.24	1.23	0.29	1.24	0.33

Ethyl benzene		0.95	0.19	0.96	0.25	0.98	0.30
Toluene*	TBAH in methanol – toluene	1.08	0.22	1.09	0.27	1.11	0.31
Formic acid		1.01	0.20	1.02	0.26	1.04	0.31
Acetic acid	Perchloric acid	1.05	0.21	1.07	0.26	1.08	0.31
Chloroform		1.25	0.25	1.26	0.30	1.27	0.34

Target values of the systematical error Δ_S	
$max\Delta_{As} = 0.5\%$	$max\Delta_S = 0.16\%$
$max\Delta_{As} = 1.0\%$	$max\Delta_S = 0.32\%$
$max\Delta_{As} = 1.5\%$	$max\Delta_S = 0.48\%$
$max\Delta_{As} = 2.0\%$	$max\Delta_S = 0.64\%$

*interpolation on benzene and ethyl benzene [49]

As can be seen from the Table 5.4, effect of temperature on the uncertainty of the concentration of non-aqueous volumetric solutions is very substantial. Even for aqueous titrants for the maximum temperature range 10 °C this effect (0.19%) is significant in comparison with the limit uncertainty of an analytical procedure $max\Delta_{As}$ = 0.5%. For non-aqueous titrants this effect is 5-6 times more. Note that SPU [1] (and the Eur.Ph. [3]) for the volumetric solutions of perchloric acid in acetic acid requires taking into account the exact temperature difference between the titer and the test sample titration [1, 2001, s. 296]. At the same time, for other non-aqueous titrants such guidance, unfortunately, is absent.

However, in the context of domestic analytical laboratories to record temperature is not always possible. If the air temperature in the laboratory in the morning was 15 °C, and through 1 hour is 20 °C (air conditioning was switched on), this does not mean that the temperature of the titrant too became 20 °C. Most likely, it will be a little different from the 15 °C, since the heating of the volumetric solution takes time, and the air specific heat is low. Therefore, to determine the correct temperature amendment under such conditions, it is not always possible.

One of the obvious way out of this situation is the more stringent regulation of the temperature of the air space for titrometry, especially for nonaqueous titration. The Table 5.4 shows that regulation of the temperature within 2 °C allows us to eliminate the effect of temperature for all titrants when the target procedure uncertainty is $max\Delta_{As}$ = 1.0% or more. In case of a target procedure uncertainty $max\Delta_{As}$ = 0.5% it is more reliable to use only aqueous titrants. It should be noted that the SPU [1] and the Eur.Ph. [3] do that.

Another approach is to use the reference standard method: the titer determination and the test sample titration are conducted from the same burette at the same time.

5.3.3. Effect of differences between burettes used for the titer determination and the assay

The Table 5.5 shows that to determine the titer, the Pharmacopoeia usually uses a burette with a capacity of 25 ml and for titration of substances it uses a burette with a capacity of 10 ml (see Table 5.1). This is due to the desire to reduce the uncertainty of titer determination as the burette with a capacity of 25 ml has a lower certified uncertainty (0.12% of the total volume for the burette with a graduation mark of 0.05 ml) than the burette with a capacity of 10 ml (0.20% of the total volume) (see Table 5.2). However, the use of different burettes for determining titer and the actual titration significantly increases systematic uncertainty because in this case it includes the uncertainty (Δ_{bur}) of both burettes.

Table 5.5

Characteristics of methods of determining the molarity of some volumetric solutions [1]

Nominal molarity	Titrant, solvent	Weight or volume used for molarity determination	$max\Delta_m$% ($max\Delta_m$ = 0.2 mg [1,11])	Nominal titration volume V_{nom}, ml	% of capacity of 25 ml burette
1.0	Nitric acid, water	1.00 g Na_2CO_3	0.020	18.9	75.5
1.0	Hydrochloric acid, water	1.00 g Na_2CO_3	0.020	18.9	75.5
1.0	Sodium hydroxide, water	20 ml of titrant titrate with 1.0 M HCl		20	80
0.5	Sulfuric acid, water	1.00 g Na_2CO_3	0.020	18.9	75.5
0.1	Hydrochloric acid, water	0.10 g Na_2CO_3	0.200	18.9	75.5
0.1	Perchloric acid, acetic acid	0.35 g, potassium hydrophthalate	0.057	17.1	68.6
0.1	Sodium hydroxide, water	20 ml of titrant titrate with 0.1 M HCl		20	80
0.050	Iodine, water	0.080 g of As_2O_3	0.250	16.2	64.7
0.020	Sodium edetate, water	0.100 g of zinc	0.200	19.1	76.5
Average				**18.7**	**74.6**

5.3.4. Total uncertainty of the titer concentration

The expression for the total uncertainty of the titrant concentration $\Delta(titr)$, by analogy with the equation (5.2), can be written as:

$$\Delta^2(titr) = \Delta_S^2(titr) + \Delta_R^2(titr). \tag{5.9}$$

The random component $\Delta_R(titr)$ represents the repeatability of the results in the titer determination and can be reduced by increasing the number of replicate titrations. For $\Delta_R(titr)$ evaluation it can be used the Eur.Ph. requirements: relative standard deviation of the results repeatability in determining titer must not exceed 0.2% [3], i.e., $RSD_{rep} \leq 0.2\%$. As for the RSD_{rep} determination must be used at least 6 replicate titrations ($n \geq 6$) [4], then, consider $\Delta_R(titr)$ as a one-tailed confidence interval (for probability of 95%) for the average value, we obtain [11]:

$$\Delta_R(titr) \leq \frac{2.015 \cdot 0.2}{\sqrt{6}} = 0.16\%. \tag{5.10}$$

The systematic component $\Delta_S(titr)$ cannot be reduced by increasing the number of replicate titrations to determine titer and connected, above all, with the volume change due to temperature differences in determining titer and sample titration (Δ_{therm}), as well as the certified uncertainty of the burette [18-20] (Δ_{bur}) (other components are much less important, and they can be neglected). These two components, acting as a constant in the analysis of samples, are random in relation to each other. Therefore, a complete systematic titer uncertainty $\Delta_S(titr)$ can be represented as [11]:

$$\Delta_S^2(titr) = \Delta_{therm}^2 + \Delta_{bur}^2. \tag{5.11}$$

Notice that in the titer determining the nominal volume is not 80% (as in the titration of the medicinal substances - see Table 5.1), and about 75% (see Table 5.5). Therefore, the relative uncertainty of the burette with a capacity of 25 ml in the tier determining is equal to $\Delta_{bur} = 0.12 \cdot 100/75 = 0.16\%$. With this in mind, and taking into account the thermal expansion of the various solvents, using equation (5.11), we can calculate the values of $\Delta_S(titr)$, which are presented in the Table 5.4.

Using the ratios (5.9-5.11), we can calculate the total uncertainty of the concentration of epy volumetric solution $\Delta(titr)$ which shall satisfy the requirements (5.8). Values of $\Delta(titr)$ for different titrants are listed in the Table 5.4.

5.3.5. Effect of a weight size and purity of primary standards

For volumetric solutions there is another source of uncertainty - the original purity of standard materials (primary standards). The SPU usually does not regulate the content of the base material in them [1, 2001, p. 290]. It is assumed the content to be of 100% that is unlikely always correctly. Only for zinc the SPU regulates the base material content of not less than 99.9%, i.e. the uncertainty of not more than 0.1%. Apparently, this value must be considered for other primary standards. As you can see, it (0.1%) does not exceed $max\Delta_S$ for any content tolerances (see Table 5.4).

In addition to the content of the base material, the presence of the residual water is one of the possible factors of the uncertainty. In particular, some of the most common primary standards - sodium carbonate and potassium hydrophthalate (see Table 5.5) – are dried before using to a constant weight [1, 2001, p. 290]. The constant weight is the weight when a difference of two consecutive weight measurements does not exceed 0.5 mg [1, 2008, p. 34]. This means that if the primary standard weight, taken for drying, is over 1 g, the uncertainty of the water content does not exceed 0.05%, that does not exceed the $max\Delta_S$ for any content tolerances (see Table 5.4).

The effect of the primary standard weight size is not significant in determining titer when content tolerances are 99.0-101.0% and wider. Given that the titer is determined by the results of k replicates, values of $max\Delta_m$, presented in the Table. 5.5, will be $\sqrt{k}$ times still smaller.

5.3.6. Secondary titrant standardization

More significant is the impact of secondary standardization. For example, some of the most common titrants - 1 M and 0.1 M sodium hydroxide volumetric solutions - are standardized on, respectively, 1 M and 0.1 M volumetric solutions of hydrochloric acid [1, 2001, pp. 293], i.e. they are secondary titrants. In the titer determining, 20 ml of a solution of sodium hydroxide is titrated with the hydrochloric acid volumetric solution.

In accordance with the requirements of ISO and GOST [50], the uncertainty of a pipette with one graduated mark of Class A with a capacity of 20 ml (such pipettes must be used) shall not exceed $\Delta_{20} = 0.15\%$. This value does not exceed the $max\Delta_S$ values for any content tolerances (see Table 5.4). Note that using pipettes with one graduated mark of Class B or graduated Class A pipettes increases uncertainty, respectively, to 0.3% and 0.5% [50] and makes it significant (see Table 5.4). The uncertainty of taking aliquots (Δ_{20}) cannot be reduced by increasing the number of replicates and act in the determining titer as the systematic error.

In the secondary standardization, the total uncertainty of the concentration of the titrant, used for the secondary standardization, acts as a systematic error $\Delta(titr,1)$. The uncertainty of the pipette with a capacity of 20 ml (Δ_{20}) is a systematic error as well. Accordingly, the expression (5.11) for the systematic error and total uncertainty (5.9) in the case of secondary standardization takes the form of:

$$\Delta_S^2(titr,2) = \Delta^2(titr,1) + \Delta_{therm}^2 + \Delta_{bur}^2 + \Delta_{20}^2. \quad (5.12)$$

$$\Delta^2(titr,2) = \Delta_S^2(titr,2) + \Delta_R^2(titr). \quad (5.13)$$

Equation (5.10, 5.12, 5.13) and the Table 5.5, allow to estimate the uncertainty of the titer in the secondary standardization. This is done in the Table 5.6 for 0.1 M volumetric solution of sodium hydroxide. For comparison, the corresponding figures are given for 0.1 M hydrochloric acid volumetric solution (primary standard) and the target systematic errors for different limits of the total procedure uncertainty.

Table 5.6

Comparison of titrant concentration uncertainties for primary and secondary standardizations

	$\Delta_S(titr)\%$		$\Delta(titr)\%$	
Temperature difference between titer determining and sample titration (Δt) →	10°C	2°C	10°C	2°C
Primary standardization - 0.1 M and 1 M HCl	0.25	0.16	0.30	0.23
Secondary standardization - 0.1 M and 1 M NaOH	0.42	0.32	0.45	0.36

Target values of systematic errors Δ_S	
$max\Delta_{As} = 0.5\%$	$max\Delta_S = 0.16\%$
$max\Delta_{As} = 1.0\%$	$max\Delta_S = 0.32\%$
$max\Delta_{As} = 1.5\%$	$max\Delta_S = 0.48\%$
$max\Delta_{As} = 2.0\%$	$max\Delta_S = 0.64\%$

In general, the total titer uncertainty $\Delta(titr)$ for the secondary standardization is approximately 1.5 times higher than for the primary standardization. It is almost entirely determined by a systematic error $\Delta_S(titr)$, i.e. it cannot be reduced by increasing the number of the replicate titrations in the secondary standardization. It should be noted that the titer uncertainty in this case does not depend on its concentration, as components of the equations (5.12-5.13) do not depend on the concentration.

From the Table 5.6 you can see that the total uncertainty of the titer of 0.1 M and 1 M sodium hydroxide volumetric solutions is significant for the target uncertainties of 0.5% and 1.0% for temperature regulation as $\pm$ 5 °C (Δt = 10 °C) and $\pm$ 1 °C (Δt = 2 °C). At the same time, 1 M and 0.1 M sodium hydroxide volumetric solutions use for the assay

of citric acid with content tolerances of 99.5-100.5% ($max\Delta_{As}$ = 0.5%) and a large number of medicinal substances with $max\Delta_{As}$ = 0.5-1.0% (see Table 5.1 and [1]). Such the assays are not always a metrologically correct. So it is better to determine the concentration of the volumetric solutions of sodium hydroxide like a primary standardization - on potassium hydrophthalate.

5.4. Effect of a blank experiment

The general expression (5.1) for the calculation of the content of the test sample component analyzed with the method of titration contains the V_0 value that is the titration volume of the blank experiment. As noted above, the V_0 value is important for indicator (visual) titration (where it always presents), since for potentiometric titration the titration volume is usually calculated as the difference between the two potential jumps.

The blank volume is associated with different factors, in particular:

The first factor is the titration of the indicator itself, various acid-base forms of which have a different color. Sometimes the titration volume connected with the titration of the indicator can be significant. Thus, the assay of *Salicylic acid solution, alcoholic* (1%) is carried out by titration with *0.1 M sodium hydroxide* in the presence of 0.5 ml of *phenolphthalein solution R1* (10 mg/ml solution of phenolphthalein (M_r 318.3) in 95% alcohol) [1]. It is easy to see that the titration of 0.5 ml of *phenolphthalein solution R1* must consume (0.5·10/318.3)/0.1 = 0.16 ml of *0.1 M sodium hydroxide*, that is 2.2% of the nominal titration volume (7.2 ml). Taking into account the tolerances (+ 5%) of the content; the indicator impact in this case is very substantial. Generally, it is at least an order of magnitude lower through the use of more dilute indicator solution (in particular, the *phenolphthalein solution R* with concentration of 1 mg/ml), reducing its volume (up to 0.05-0.10 ml), and the increase of the titrant concentration (for example, up to 1 M) [1]. This calculation, however, indicates that an assessment of the impact of the titration of the indicator itself must be always done.

The second factor is the impact of acid-base impurities in the solvent. For example, in the case of basic solvents (e.g. dimethylformamide) we always have to reckon with the acidity, caused by the absorption of carbon dioxide in the air.

When the indicator (visual) titration there are two options for taking into account the titration volume of the blank experiment:

1. Neutralization of the solvent on the indicator with the subsequent dissolution of the test sample and its titration on already added indicator.

Neutralization can be done in two ways. The first (typical): to a standard volume of a solvent, taken for titration of the test sample, add the indicator and neutralize. In this case the uncertainty of the blank volume goes into the category of a random uncertainty and, therefore, can be reduced by increasing the number of replicates. This way is typical for the Eur. Ph. [3] and related SPU [1]. The second way is to neutralize a large amount of a solvent (to reduce the uncertainty of titration), followed by the use of a

standard volume of the neutralized solvent for titration of the test sample. In this case the uncertainty of the blank volume remains a systematic error. This approach is generally less correct, especially for basic solvents, since it does not take into account the possible absorption of carbon dioxide in the air.

2. Simultaneously with the titration of the test solution (volume V), the titration of the blank experiment is carried out. The blank volume (V_o) is then subtracted from the titration volume of the test solution (V) - see formula (5.1). In this case the uncertainty of the blank volume is a constant component.

The first option is the most close to the potentiometric titration and is more correct than the second option. This is because the blank volume (V_o) is usually small (tenths and hundredths of milliliter) and it is difficult to carry out its precise indicator (visual) titration because of the subjective factor. When using the first option, the subjective factor is much less. At the same time, the uncertainty of the blank volume in this option also affects the results of the analysis (it is included in the V-V_o value, which experimentally determined in the option 1).

5.5. Reference standard method

A comparative titration method (a reference standard method) is a simultaneous titration of the test sample and primary standards (to determine the titer) using the same burette and titrant. Weight of the primary standards is chosen so that it matches the nominal titration volume. Number of replicates for determining the titer and test sample solution titration are equal, which emphasizes their equal contribution to the total procedure uncertainty. In this version the titration, in fact, does not technically differ from the spectrophotometric or chromatographic analysis using reference standard method [1].

It is easy to see that the titration reference standard method (as in spectrophotometry and chromatography) eliminate the main systematic errors: the burette uncertainty (Table 5.2) and the temperature factor (Table 5.4). Impact of the blank volume is eliminated as well. Other factors, to a large extent, are moving in random errors, which can be reduced by the increase in the number of replicate titrations.

The question arises: why the Pharmacopoeia [1, 3, 4] does not use the reference standard method of titration? The main disadvantage of the titration reference standard method is that the titer in it must be redeterminated for each new sample titration. In the case of the State control (which is usually a one-time) and scientific researches it does not cause any difficulties. However, the use of the reference standard method in the routine control at the manufacturer laboratories (when every day the different series of the same sample are titrated with the traceability of the analysis) significantly extends the analysis. In this case the usual Pharmacopoeial approach (described above) has its advantages. For the State control laboratory we can recommend to develop an appropriate SOP which justify the use of the reference standard method.

5.6. General conclusions about the titration procedure uncertainty

1. As can be seen from the Table 5.4, we must regulate the temperature within $\pm$ 1°C (Δt = 2 °C). Only in this case the total uncertainty of the titrant concentration $\Delta_S(titr)$ can meet the requirements of the ratio (8). When the fluctuations of temperature are within $\pm$ 5°C (Δt = 10 °C) we may use only the water titrants and only when $max\Delta_{As} \geq 1.0\%$.

2. The main factor of the titer uncertainty for non-aqueous titrants is the thermal expansion. For water titrants for the regulation of temperature within $\pm$ 1 °C the systematic uncertainty of the burette is substantial as well.

3. Secondary standardization significantly increases the titer uncertainty and makes it significant for the limit procedure uncertainty of 0.5 and 1.0%. The metrological correctness of the titrometric assay using a secondary titrant for the target procedure uncertainty of $max\Delta_{As} = 0.5\%$ is highly questionable.

4. In general, to obtain the total titer uncertainty within 0.2%, as indicated in SPU [1], is impossible for the routine analysis, although the actual (but unknown) titer error may be less.

5. The systematic uncertainty of the titration can be insignificant compared with the total target uncertainty of the analytical procedure $max\Delta_{As}$ for each of the many factors, but to assure its insignificance compared with the combination of all these factors is very difficult.

6. At the same time, titrometric procedures are usually characterized by high repeatability of the results, i.e. the uncertainty of the final analytical operations is small compared to the target uncertainty $max\Delta_{As}$. Therefore, the principle of insignificancy of the systematical error, which has been successfully used in the validation of chromatographic and spectrophotometric analytical procedures (see sections 1-3), in the case of titrometric procedures is not effective because it can be implemented rather seldom. There are other approaches, one of which is offered by the European Pharmacopoeia (Eur. Ph.) [4, p. 3.3.7].

5.7. The European Pharmacopoeia approach to the validation of titration procedures

The main principle, which the Eur. Ph. implements in its Guide [4, p. 3.3.7] for titrometric assay procedures for quantitative determination is that the main part of the total procedure uncertainty is just the systematic error, which in principle could not be deleted. This approach is fundamentally different from the principle of insignificancy of the systematic error (see section 2.3.3), that is common in analytical practice, and is successfully applied in validation of chromatographic and spectrophotometric analytical procedures (see sections 1-4).

The Eur. Ph. approach is this [4].

When developing a new volumetric method, it is recommended to titrate at least seven different quantities under the prescribed conditions in a randomized order to give end point volumes in the range of 20 per cent to 90 per cent of the volume of the burette employed.

The relative error in reading of the weight on a balance and of the volume at the end-point is to be less than 0.5 per cent of the values found.

Calculate the linear regression by least squares method:

$$V_i = a_{obs} + b_{obs} \cdot m_i. \tag{5.14}$$

Here V_i – end-point volume in ml for weight m_i in mg (calculated on the anhydrous substance – in the Eur. Ph. Technical Guide ЕФ [4] it is missed). For the assessment of the calculated metrological characteristics, such criteria are used.

1st Criterion – Proportional Systematic Error (Bias).

The calculated slope b_{obs}, taking into account the titer of the standardized of the volumetric solution, is within 0.3% for potentiometric titrations (0.5% for visual titrations) compared to the theoretical value given as titration standard b_{theor}, i.e.

Potentiometric titrations:

$$100 \cdot \left| \frac{b_{obs} - b_{theor}}{b_{theor}} \right| \leq 0.3\%. \tag{5.15a}$$

Indicator (visual) titrations:

$$100 \cdot \left| \frac{b_{obs} - b_{theor}}{b_{theor}} \right| \leq 0.5\%. \tag{5.15b}$$

where:

$$b_{theor} = \frac{Z}{M_r \cdot C_r}. \tag{5.16}$$

M_r is the relative molecular mass of the analyzed substance, Z is the stoichiometric factor of the chemical reaction and C_r is the molar concentration of the titrant.

2nd Criterion – Additional Systematic Error (Bias)

The extrapolated intercept a_{obs} is less than 0.4% for potentiometric titrations and 0.6% for indicator (visual) titrations of the expected or target titration volume, i.e.:

Potentiometric titrations:

$$100 \cdot \left| \frac{a_{obs}}{V_T} \right| \leq 0.4\%. \tag{5.17a}$$

Indicator (visual) titrations:

$$100 \cdot \left| \frac{a_{obs}}{V_T} \right| \leq 0.6\%. \tag{5.17b}$$

Here V_T is the expected or target titration volume.

3rd Criterion – Precision (Statistical Error)

The remaining estimated standard deviation *sdv(V)* is less than 0.3% for potentiometric titrations (0.5% for visual indicator titrations) of the mean titration volume of the end-point using the titration procedure to be introduced in the specification [9], i.e.:

Potentiometric titrations:

$$100 \cdot \frac{sdv(V)}{V_T} \leq 0.3\%. \tag{5.18a}$$

Indicator (visual) titrations:

$$100 \cdot \frac{sdv(V)}{V_T} \leq 0.5\%. \tag{5.18b}$$

4th Criterion – Practical Relative Error

Some titration procedures may not fulfill the *1st* and *2nd Criteria* but exhibit low and acceptable bias at the target titration volume (8 ml $\pm$ 1 ml for a 10 ml burette). Thus the *1st* and/or the *2nd Criteria* given above are not met, then calculate the relative accuracy at the target titration volume by the formula:

$$\delta_{RL} = \left| \frac{a_{obs}}{V_T} + \frac{b_{obs} - b_{theor}}{b_{theor}} \right| \cdot 100\%. \tag{5.19}$$

The δ_{RL} value is not more than δ values given in the Table 5.7.

However, when the volumetric titration procedure is well established it is sufficient to verify that the repeatability (relative standard deviation $RSD_{rep}\%$ [26]) and accuracy (δ) of the titration (a minimum of six replicates) are not greater than the limits given in the Table 5.7. In this case the accuracy is calculated in a common way [26]:

$$\delta = \left| \frac{x - x_{theor}}{x_{theor}} \right| \cdot 100\%. \qquad (5.20)$$

Table 5.7

Validation requirements to the metrological characteristics of titrometric procedures accordingly to the Eur. Ph. [4]

№	Volumetric titration	Content limits ($max\Delta_{As}$%), ±	RSD_{rep}, %, ≤	$\Delta_R(p=0.95; n=5)$	Relative accuracy, δ %, ≤
1.	Acid/base	1.0	0.33	0.31	0.67
2.	Non-aqueous	1.0	0.33	0.31	0.67
3.	Conjugate acid of base	1.0	0.33	0.31	0.67
4.	Redox	1.5	0.50	0.48	1.0
5.	Argentometric	1.5	0.50	0.48	1.0
6.	Complexometric	2.0	0.67	0.64	1.33

5.8. Consideration of the European Pharmacopoeia approach

The above described approach of the Eur. Ph. [4] to the validation of the titrometric procedures, despite its simplicity and seemingly consistency, does not meet the requirements for the validation of the analytical procedures described in the same Guide [4] (and thus in SPU [11]).

The objective of validation of an analytical procedure is to demonstrate that it is suitable for an intended purpose [4, 11]. In our case the intended purpose is an assay of the medicines by titrometry. The necessary criteria follow from this.

5.8.1. Range

The validation of the procedure is held within the range of its applications. To assay substances and dosage forms, the analytical range must be at least 80-120% of the nominal content [11]. Given that the nominal titration volume of the burette with a capacity of 10 ml (such the burettes are usually used in the pharmacopoeial analysis [1-3]) is 80% of its capacity [11], we get a range of 6.4-9.6 ml. However the Guide [4] carries out the validation in a range from 20 to 90% of the burette volume, i.e. from 2 up to 9 ml. This range, on the one hand, is unduly broad, and on the other hand does not cover the required [4, 11] value of 9.6 ml.

The narrower the range, the easier it is to achieve the necessary linearity, accuracy, and repeatability. Thus, the Guide [4] unduly restricts demands to metrological characteristics of the titration procedure, presenting to them the requirements in those areas (< 6.4

ml), where the application of the procedure is not meant to be. On the other hand, the linear regression can distort (for example, because of the acylation of NH_2 groups by acetic anhydride). For the analytical range of volumes (64-96%) this can significantly affect the extrapolated intercept and the slope, leading to systematic errors. At the same time, the plotting of the calibration line and its processing by the least squares method in a wide range of volumes (20-90%) can neutralize the impact of this distortion on the parameters of the regression line and thereby do not reveal the systematic error.

5.8.2. Procedure linearity verification

Criteria 1-3 characterize the linearity of the procedure and *4th Criterion* characterizes the repeatability and accuracy.

In the case of comparative methods - chromatography and spectrophotometry (in the version of the reference standard method) – the extrapolated intercept of the linear regression (a_{obs}) characterizes the systematic error of the procedure and therefore is limited during the evaluation of linearity (see sections 2.3.4.3). The slope (b_{obs}) does not play any role, and requirements for it are not regulated.

In the case of a direct method (titration) the situation has cardinally changed. Here the systematic error of the procedure is affected both parameters of the linear regression - deviation of the slope (b_{obs}) from the theoretical value (b_{theor}) (regulated by the *1st Criterion*) and a statistically significant value of the extrapolated intercept (a_{obs}) (regulated by the *2nd Criterion*). This effect may have a different character, and may be mutually compensated for the analytical volume range of the burette (about 8 ml [4]), so the systematic error is characterized by their algebraic sum – *4th Criterion*. Therefore, generally speaking, a separate regulation of the slope b_{obs} and extrapolated intercept a_{obs} of the linear regression for the purpose of validation is not required, and, therefore, the *Criteia 1-2* are useless (this, in fact, the *4th Criterion* explicitly states). The specific values of these criteria - (5.15 a) (5.15 (b)), (5.17 a) and (5.17 b) - are clearly artificial in nature and are not associated with a validation of a specific titration procedure.

It draws objections the formulations of the *Criteria 1-3* separately for potentiometric and indicator titration. In the summary Table 5.7 requirements to metrological characteristics and tolerances of titration are not specified separately for these methods to determine the equivalence point. As in the Table 5.7 requirements to metrological characteristics of methods are defined only by the tolerances of the content, no matter how point of equivalence is found, if the tolerances are the same.

It should be noted that the *Criteria 1-3* are not associated with the content tolerances, i.e. they are the same, for example, for the tolerance $\pm$ 1% and $\pm$ 2%, while it is clear that the requirements for metrological characteristics here differ. In particular, the *3rd Criterion* (requirements to the residual standard deviation) is directly related to the repeatability of the procedure results (see section 2.3.4.1). In addition, there are no requirements

for the correlation coefficient. At the same time in accordance with the requirements [4, 11], the correlation coefficient should be submitted (and evaluated).

Criterion 1 checks the closeness of the actual equivalent of the titrated substance to its theoretical value. The discrepancy between theoretical and observed equivalents could be due to the incorrect determination of the equivalence point, decomposition of substances, chemical reactions etc. These factors (related to the validation methodology) are verified by this criterion.

However, the deviation of the actual equivalent from the theoretical value can be also caused by other reasons that have nothing to do with the procedure validation and totally ignored by the *1st Criterion*. The most important of these causes are impurities in substances and accuracy in determining actual concentration of the volumetric solution.

Thus, in *Ranitidine Hydrochloride* (where the assay is conducted by potentiometric titration with a sodium hydroxide volumetric solution) [1-3] the related impurities content shall not exceed 1.0% (0.5% of impurity A and 0.5% of the sum of other impurities), with tolerances of 98.5-101.5%. The influence of such a large (but allowed by the Pharmacopeia) amounts of impurities on the regression line slope (b_{obs}) can be higher than the requirements (5.13a) and (5.13b) of the *1st Criterion*.

In general, if impurities are identified (the principle of "transparency" of monographs is one of the main principles of the Eur. Ph.) and quantified (by the relevant section of the Eur. Ph. monograph), their contribution to the titration can be taken into account. This is Eur. Ph. when setting tolerances content [51-52]. Another possible approach is to use the *Insignificance principle* (section 2.3.1): if impurities below a certain limit, they do not significantly affect the validation process.

Another obvious factor influencing the deviation of the actual equivalent from the theoretical value, is the accuracy of determining the concentration of the volumetric solution, which, as can be seen from the Table 5.4, can be quite comparable (especially for non-aqueous or secondary titrants) with the requirements of (5.15a) and (5.15 b) of the *1st Criterion*.

So the fact of compliance or non-compliance with the requirements of the *1st Criterion* does not demonstrate yet the acceptable proximity of the actual and theoretical equivalents for the test material.

The second objection against the application of the *1st Criterion* is that it completely ignores the statistical uncertainty of b_{obs}, obtained by means of the least squares method. At the same time, the relative standard deviation of this value can reach 0.9% relative (see Table 2.2) when all the requirements for linearity are kept. Therefore the requirements (5.15a) and (5.15b) for the deviation from the theoretical value to be of not more than 0.3% (potentiometry) or 0.5% (visual indicator titration) are not always statistically correct.

Similarly, the requirements (5.17a), and (5.17b) of the *2nd Criteria* are statistically incorrect because such values of the extrapolated intercept (0.4% for potentiometry and 0.6% for the visual indicator titration) may well be statistically insignificant (see Table 2.2).

Thus, the *Criteria 1-3* (and the whole approach of the Guide [4] in general) do not check the acceptability of the linearity for the task purpose – the assay of the specific drug product by means of the titration method (and this is the purpose of validation). They evaluate it for some common (it is unclear which) case.

5.8.3. Repeatability and precision verification

To test the repeatability, we could use the residual standard deviation around the regression line (*3rd Criterion*) (see section 2.3.4.1) because it is a direct characteristic of the procedure repeatability for the different titration volumes. However, for assessing the procedure repeatability, the Guide [4] uses the relative standard deviation (*RSD*) of not less than 6 replicate titrations. The *RSD* for different methods of the titration shall meet the requirements of Table 5.7. These requirements (from 0.33% to 0.67%) and have nothing to do with the requirements to the maximum residual standard deviation (5.18a) and (5.18b) of the *3rd Criterion* (0.3% for potentiometric and 0.5% for visual indicator titration), although, in general, it is one and the same uncertainty.

The Table 5.7 reflects the Guide's approach [4] to the repeatability-accuracy ratio for validation of titrometric assays. It is that the symmetric content tolerances (which in the case of substances are confidence intervals of the target total uncertainty of the analytical procedure - see relation (2.7)) are divided into 3 parts: 1/3 of the tolerance is the maximum *RSD* of repeatability of not less than 6 replicate titrations, and 2/3 of the tolerance is the target systematic error.

This approach characterizes the philosophy of the Eur. Ph. to the validation criteria of a titrometric assay of medicines. The Eur. Ph. considers that the systematic error of the titrometric procedures, because of the large number of uncontrollable factors (see section 5.2), is irremovable on principle and considerably exceeds the random component of the total uncertainty. The latter is a smaller portion of the total uncertainty of the analytical procedure.

Formally, it is incorrect to add *RSD* to the systematic error, which is the confidence interval. But for *RSD* = 0.33% the one-sided confidence interval of the average result of 5 replicate titrations (the most typical number of replicates in analytical practice) will be $\Delta_R = 2.13 \cdot 0.33/\sqrt{5} = 0.315 \approx 0.33$ % (same as for *RSD* = 0.50 and 0.67% = see the table 5.7). So, practically, there is no controversy. It is only necessary to indicate that the number of the replicate titrations in the procedure must be at least 5.

As shown above, the systematic error can be caused by impurities. About the influence of impurities on the analysis, the Guide [4] only states that they should be present in low concentrations, otherwise, you must use other assay procedures. However, the Guide does not give the criteria of the "smallness". At the same time, it is obvious that it is im-

possible to talk about the proper validation of the assay procedure without assessment and regulation of the impurity impact.

It should be noted one important fact: as can be seen from the Table 5.7 (as well as of the *Criteria 1-4*), the Guide does not consider at all the validation of the titrometric procedures with tolerances of 0.5%, perhaps believing that for such procedures in routine analysis within pharmacopoeial approach (titer determination and analysis itself are conducted using different burettes and often at different times) it is impossible to achieve the necessary metrological characteristics. In view of the above discussion on the uncertainty of the titer, it is difficult to disagree with this. But how about those pharmacopoeial procedures (see, for example, Table 5.1 - citric acid), which has already set such tolerances?

One effective way out of this situation is the application of the reference standard method to these procedures, i.e. to conduct the titer determination and test titration simultaneously using the same burette and close titration volumes. In this case the titer thermal expansion and systematic error of the burette are eliminated. This approach is not described in the Pharmacopoeia; however, its use is justified by the lack of approaches to the validation of such procedures in the Guide [4].

5.9. Suggested approach to the titrometric procedure validation

5.9.1. Formulation of the problem

Titrometry, unlike the chromatography, is a non-specific method of quantitative determination. Therefore, a task of a titrometric assay of a medicinal substance is not to determine the base material content, but to make sure that it is not significantly different from 100%. The base material content may be defined as (100% - impurities content). Impurities in the monograph are controlled by other tests (usually by liquid chromatography), that provides the necessary specificity of the assay.

Therefore, the task of validation of a quantitative titrometric procedure is to make sure that in the process of titration of the substance, containing impurities within the requirements of the specification, the assay results obtained with the necessary accuracy and precision do not exceed the specification tolerances. The impurities may be also titrated, distorting the results of the actual content of the base material. There is nothing to worry about until those results are within the tolerances of the specification. It should be noted that this approach we applied to the certification of reference standards for the quantitative spectrophotometric analysis [16].

As you can see, the problem with the validation of the titrometric procedures is quite different from the problem in the validation of chromatographic procedures (see sections 1-4). Accordingly, the validation criteria should be changed as well.

In accordance with the approach of the Eur. Ph. (see Table 5.7, *Criteria 1-4*), the approach set forth is applied to the validation of the titrometric procedures only for sub-

stances and drug products with tolerances of ± 1% or more. This is consistent with the findings of the section 5.6. For the tolerances of ± 0.5% it is appropriate to use titrometric procedures in the version of reference standard method with corresponding changes in the validation scheme.

5.9.2. Requirements to purity of a substance used for titrometric procedure validation

The validation of an analytical procedure based on the comparative methods (chromatography, spectrophotometry, etc.) has no problem with evaluation of a systematic error of the procedure - it's easily estimated from the linearity study (see, for example, Table 2.3). In this case, the influence of impurities content within specification limits is largely compensated by the reference standard (or this influence is easily estimated). This is especially true for chromatography. Titration is a direct method, which causes difficulties in the procedure systematic error assessment. The main reason is the unavoidable presence of impurities that cause a systematic error, not connected with the systematic error of the titrometric procedure. To evaluate the last one, it can be used the following approaches:

1) using reference standards with 100% basic substance content;

2) using analysis results obtained by another validated procedure;

3) quantitation of all impurities, followed by an assessment of their influence on the titration.

All these three approaches have their limitations and shortcomings.

The use of reference standards with 100% basic substance content (the approach 1) to carry out the validation is very expensive. In addition, you must still take into account the residual (or acquired in the procedure process) water content. The main disadvantage is the need for mandatory assessment of the influence of impurities content in real test samples (in particular, medicinal substances) on the results of the titration (i.e., in fact, use of the approach 3). As the procedure is not validated for analysis of an ultrapure material. It is validated for analysis of a real medicinal substance. But we can direct use the approach 3.

Use of analysis results received by another validated procedure (the approach 2), despite its apparent simplicity and consistency, almost is not applicable for evaluating a systematic error of a titrometric assay of a medicinal substance. This is due to the statistical uncertainty of the results and sufficiently strict content tolerances for medicinal substances (see Table 5.1). So, if the titrometric procedure received the main content in *Oxazepam* of (100.5 + 0.6) % (tolerances are 99.0-101.0% - see the Table 5.1), and another method received of (99.6 + 0.7) %, then we cannot talk about a systematic error in the titrometric procedure, although it can be significant.

The approach 3 is the official approach of the Eur. Ph. when establishing tolerances for medicinal substances by titration method [51-52] and, like, solves all problems. But it requires the identification of all impurities and their quantification in a particular sample used for the validation. But this is not always possible.

It is easy to see that neither of these approaches estimates the significance of the influence of impurities on the titration results. A target uncertainty of an assay procedure $max\Delta_{As}$ shall satisfy the requirements of ratios (5.3-5.4). The error, contributed by impurities, is a systematic one. If this error satisfies the ratio (5.5), the impurities do not have a significant impact on the results of the titrometric assay (see the section 5.1.2). If the concentration of the *i-th* impurity is Im_i, its equivalent is Eq_i and the equivalent of the base component is Eq_s, then, similar to the attestation of the reference standards for the spectrophotometric analysis [16] and taking into account the ratio of (5.5), we can define the requirements for the insignificant content of impurities:

$$\left|\sum_i \mathrm{Im}_i \cdot [\frac{Eq_s}{Eq_i} - 1]\right| \le 0.32 \cdot \max\Delta_{As}. \tag{5.21}$$

In practice, some impurities have the equivalent less than that of the base material (overstating the results of the titration), others are smaller (thus understating the results). If the equivalent of the impurity is the equivalent of the base material, this matter does not affect the results. Unfortunately, the equivalents of impurities and a content of each specific impurity are quite often unknown, making it difficult to apply the ratio (5.21). In view of the extremely rare case where the equivalents of all the impurities are less than half the size of the equivalent of the base material, we can write:

$$\left|\sum_i \mathrm{Im}_i \cdot [\frac{Eq_s}{Eq_i} - 1]\right| \le \sum_i \mathrm{Im}_i. \tag{5.22}$$

With that in mind, we can write a more stringent, but a simple ratio of the insignificant content of impurities in the substance for the validation:

$$\sum_i \mathrm{Im}_i \le 0.32 \cdot \max\Delta_{As}. \tag{5.23}$$

The total impurities content ΣIm_i is commonly known and regulated by the specification, which makes the relationship (5.23) easy to use. If the ratio of (5.23) is not complied with, it should follow a more general relationship (5.21).

Naturally, the ratios (5.21-5.23) assume a preliminary accounting of loss on drying.

5.9.3. Normalized coordinates

The standardized validation schemes for drug quality control procedures, developed earlier in the sections 1-4 for the comparative methods, are based on the use of the normalized coordinates, which makes it possible to formulate the uniform criteria that do not depend on the specifics of the validated procedures. It is therefore advisable to apply these coordinates for the validation of the titrometric procedures. This allows us to use also the criteria obtained earlier.

When testing the linearity, the titration volume (V_i) is the ordinate axis and the sample weight, taken for titration (m_i), is the abscissa axis. The validated procedure specifies the nominal sample weight m_T (in grams or milligrams). The nominal sample weight corresponds to the nominal titration volume V_T (in ml) that equals to:

$$V_T = \frac{m_T}{K_T \cdot m_{1mL}} \cdot (1 - \frac{LD}{100}). \qquad (5.24)$$

Here m_{1mL} is the number of grams (milligrams) of titrated substance corresponding to 1 ml of the volumetric solution of the nominal concentration, K_T is the correction factor to the nominal concentration of the volumetric solution, LD is the loss on drying (or the water content) as a percentage.

There is no reference standard in the conventional pharmacopoeial titration, unlike the chromatography and spectrophotometry. So, to transform the sample weight to the normalized coordinates, it is reasonable to divide it by the nominal sample weight m_T specified in the procedure. Accordingly, we get the following normalized coordinates (compare with the ratios 2.1):

$$X_i\,(\%) = \frac{m_i}{m_T} \cdot 100; \quad Y_i\,(\%) = \frac{V_i}{V_T} \cdot 100; \quad Z_i\,(\%) = \frac{Y_i}{X_i} \cdot 100. \qquad (5.25)$$

Values of X_i, Y_i and Z_i have the same meaning as for the chromatographic procedures by the reference standard method (see the section 2.2). In particular, Z_i is a recovery factor, i.e. the relationship (found/introduced) %.

5.9.4. Range

As for the chromatographic and spectrophotometric procedures (see sections 1-4), it is reasonable to obtain all the validation characteristics from the linearity study. In principle, this is quite enough 7 points, as the *Eur. Ph. Guide* recommends [4]. However, to remain invariable the approach, already developed in the sections 1-4, it is reasonable to take 9 points (it is the formal requirement for the precision study), thus providing a less strict criteria (by increasing the number of degrees of freedom).

In accordance with the SPU requirements [11], the range must be at least 80-120% of the nominal value. As already mentioned in the section 5.4.1, the nominal titration volume for the burette with a capacity of 10 ml (such the burettes are commonly used in the pharmacopoeial analysis [1-3]) is 80% of its capacity [4]. Accordingly, we get the range of 6.4-9.6 ml. When dividing this range on 9 points, we get the following points (with a step 5% = 0.4 ml): 6.4, 6.8, 7.2, 7.6, 8.0, 8.4, 8.8, 9.2, 9.6 ml. Of course, these values can vary within $\pm$ 0.05 ml. The sample weights, taken for titration, must comply with these burette volumes. For this range (80-120%) the calculated value of s_Y is 13.69 % (see the Table 2.1).

5.9.5. Linearity criteria

In the normalized coordinates we study the linear relationship $Y_i = a + b \bullet X_i$ [1-6]. Since we are using the normalized coordinates, the developed earlier approaches (see the section 2.3.4.1) for the regression parameters estimation remain valid. The titration, however, has its own characteristics, leading to some other acceptability criteria.

5.9.5.1. Systematical error

Taking into account the analysis of the factors affecting the metrological characteristics of the titrimetry, the Eur. Ph. approach to accuracy of the titrometric procedures (Table 5.7) is correct. The Eur. Ph. requirements for the systematic error (δ) (Table 5.7) can be written as:

$$\delta\% \le \frac{2}{3} \cdot \max\Delta_{As}. \tag{5.26}$$

In the titration of the medicinal substances (and this is the main application of the titrimetry) $max\Delta_{As}$ coincides with the symmetrical content tolerances.

In the case of titrometric procedures, the requirements to the extrapolated intercept a are replaced by the requirements to the value of δ_{RL} (accuracy of the linear regression) from the ratio of (5.19), which is a relative systematic error of the calculation by the linear regression. This error is considered by the Eur. Ph. [4] as a deviation of the actual line points from the theoretical line for the nominal volume V_T. This interpretation is objectionable because the error for the nominal volume can be zero (by reason of mutual compensation of errors), but for other range volumes it can be substantial.

For example, the equation $V_i = 2 + 30 \bullet m_i$ for the nominal sample weight $m_i = m_T = 0.2$ g and the nominal titration volume $V_T = 8$ ml gives a zero error. At the same time, for the sample weight $m_i = 0.16$ g (80% of the nominal weight m_T) we obtain $V_i = 6.8$ ml, while the theoretical value is $8 \bullet 0.8 = 6.4$ ml, i.e. the error is 0.4 ml or more than 6%. Therefore the error of the line should be checked not for the nominal value but for the worst case - on the edges of the range (80 or 120% of the nominal value).

In normalized coordinates the theoretical linear regression is $Y_i^{theor} = X_i$, i.e. the directly proportional dependence with a slope of $b_{theor} = 1$. Therefore, for the arbitrary normalized volume Y_i^{theor}, the relative deviation (in percentage) of the actual linear regression from the theoretical one is:

$$\delta_{RL,i}(\%) = 100 \cdot \left| \frac{Y_i - Y_i^{theor}}{Y_i^{theor}} \right| = 100 \cdot \frac{\left| a + b \cdot X_i - X_i \right|}{X_i} = 100 \cdot \left| \frac{a}{X_i} + (b-1) \right|. \quad (5.27)$$

The value of δ_{RL} is the systematic error and must therefore satisfy the requirements (5.26) for the worst case – on the range borders. For the X_i values they are $X_i = 120$ and 80% of the nominal volume, i.e.:

Practical insignificance:

$$\delta_{RL,80} = 100 \cdot \left| \frac{a}{80} + (b-1) \right| \le \frac{2}{3} \cdot \max\Delta_{As}.$$
$$\delta_{RL,120} = 100 \cdot \left| \frac{a}{120} + (b-1) \right| \le \frac{2}{3} \cdot \max\Delta_{As}. \quad (5.28)$$

Critical values of $\delta_{RL,80}$ and $max\ \delta_{RL,120}$ are presented in the Table 5.8.

The ratio of (5.28) is a requirement of the practical insignificance of the δ_{RL} values. The statistical insignificance means that the values of a and $|b - 1|$ do not exceed the confidence intervals of their uncertainty. It is necessary to take into account that, in practice, the analysis is conducted with use of k replicate titrations. In particular, for the number of linear regression points of $n = 9$ and $k = 5$ we get:

Statistical insignificance:

$$a \le \frac{t(95\%, n-2) \cdot s_a}{\sqrt{k}} = \frac{1.89 \cdot s_a}{\sqrt{5}} = 0.85 \cdot s_a.$$
$$|b-1| \le 0.85 \cdot s_b. \quad (5.29)$$

5.9.5.2. Residual standard deviation (s_o)

In accordance with the approach of the Eur. Ph. (Table 5.7), the confidence interval of the random component of the uncertainty of the titrometric procedure is about a third of the total uncertainty $max\Delta_{As}$. The last in the case of a medicinal substance analysis is identical with symmetrical content tolerances, i.e.

$$\Delta_R\% \le \frac{1}{3} \cdot \max\Delta_{As}. \tag{5.30}$$

One of the main differences between the linearity verification of a titrometric procedure and a chromatographic procedure is that a titration regression point is a result of an analysis of a single sample weight under the specification. The conclusion about sample quality is based on the titration of a certain number (k) of replicate sample weights. Depending on the size of the k, the confidence interval of the mean result is modified, which characterizes the random component of the titration procedure uncertainty, i.e. given (5.29), we obtain [26]:

$$\Delta_R = \frac{t(95\%, n-2) \cdot s_o}{\sqrt{k}} \le \frac{1}{3} \cdot \max\Delta_{As}. \tag{5.31}$$

Since the value of s_o is obtained for n = 9 points of the linear relationship, the *t-value* is taken for the number of degrees of freedom n - 2 = 7 [11].

Given the ratios of (5.3-5.4), we can find the requirements to the residual standard deviation s_o, but we need to standardize the k value. Titration procedures are characterized by relatively low values of standard deviations of repeatability, which in many cases can get acceptable results even with titration of k = 2-3 sample weight replicates. However, it should be borne in mind that these procedures are typically validated for routine analysis of medicinal substances or drug products, and the conclusion about a large batch quality often depends on this analysis. Bearing in mind also the simplicity, speed and low cost of the titration procedures, to produce the statistically reliable results in serious analyses, the number of replicate titration cannot be less than 5, i.e. must be $k \ge 5$. Then the ratio (5.31) will get the requirements to the residual standard deviation s_o:

$$s_o \le \frac{\sqrt{5} \cdot \max\Delta_{As}}{3 \cdot t(95\%,7)} = \frac{2.24}{3 \cdot 1.89} \cdot \max\Delta_{As} = 0.39 \cdot \max\Delta_{As}. \tag{5.32}$$

Critical values of s_o are represented in the Table 5.8.

5.9.5.3. Correlation coefficient (*r*)

The correlation coefficient is calculated according to the formula [1-6, 9]:

$$R_c = r = \sqrt{1 - \frac{s_0^2}{s_Y^2}}. \qquad (5.33)$$

Knowing the value of s_o and given that s_Y = 13.69% for the range 80-120% (9 points) (see the Table 2.1), it is possible to calculate the critical values of the correlation coefficient *r*, which are represented in the Table. 5.8.

Given the high values of the correlation coefficient *r*, it is sometimes convenient to use their squares r^2, which are also represented in the Table 5.8.

Table 5.8

Critical values of the systematic (*max δ*) and total ($max\Delta_{As}$) uncertainties of the quantitative titration procedures and the parameters of a linear relationship $Y_i = b \bullet X_i + a$ for various tests, g = 9 points, s_Y = 13.69% and various content tolerances *B*

B, %	$max\Delta_{As}$%	*max δ* (is a greater value of $max\ \delta_{RL,80}$ and $max\ \delta_{RL,120}$) %	s_o , %	*min r*	$min\ r^2$
Medicinal substances					
0.5	0.5	0.33	0.20	0.99990	0.99979
1.0	1.0	0.67	0.39	0.99959	0.99917
1.5	1.5	1.00	0.59	0.99907	0.99814
2.0	2.0	1.33	0.79	0.99835	0.99670
Drug products					
5.0	1.6	1.07	0.63	0.99894	0.99789
7.5	2.4	1.60	0.94	0.99762	0.99524
10.0	3.2	2.13	1.26	0.99576	0.99154

5.9.5.4. Limit of detection (*DL*) and limit of quantitation (*QL*)

These values are not required when we carry out the validation of the assay procedures, but they are useful as information about how the procedure range exceeds its limits (procedure "safety margin").

DL and *QL* values are calculated on the base of the standard deviation of the extrapolated intercept s_a of the linear regression and its slope (*b*) in the same way as for spectrophotometric and chromatographic procedures (see the sections 1-4):

$$DL = 3.3 \cdot s_a / b \approx 3.3 \cdot s_a , \tag{5.34}$$

$$QL = 10 \cdot s_a / b \approx 10 \cdot s_a , \tag{5.35}$$

5.9.6. Accuracy and precision

They are evaluated in the same way as for spectrophotometric and chromatographic procedures on the base of data obtained in studying the linearity (see the sections 1-4).

5.10. Example. Validation of the titrometric assay procedure of the taurine substance

Experimental verification of the proposed approach is illustrated by the visual acid-base titration (the indicator is phenolphthalein) of the medicinal substance of taurine (titration with *0.1 M sodium hydroxide*).

Procedure. About m_T = 250 mg (the accurate weight) of the test substance is dissolved in 30 ml of water, add 5.0 ml of *Formaldehyde solution R* and titrate with *0.1 M sodium hydroxide* until slightly pink coloration (indicator is 0.1 ml of *Phenolphthalein solution R1*).

Simultaneously conduct the blank experiment.

1 ml of *0.1 M sodium hydroxide* is equivalent to 12.52 mg of $C_2H_7NO_3S$.

The content of $C_2H_7NO_3S$ (2-aminoethanesulphonic acid) in the test substance should be not less than 99.0% and not more than 101.0%, calculated with reference to the dried substance. Thus, considering the (5.3), $max\Delta_{As}$ = 1.0%.

The purity of substances used for validation (see the section 5.9.2). For the validation of the titration procedure we used the series No. 401107 of the taurine substance which met the requirements of the specification. The loss on drying was 0.043%.

Related substances are controlled by TLC. No additional spots were found (i.e. the content of impurities of less than 0.1%), so we can take the substance meets the requirement (5.23) to the purity of the substance used for the validation of titrometric procedures (i.e., $\Sigma Im_i \leq 0.32 \bullet max\Delta_{As}$ = 0.32%) Considering this and insignificant loss on drying (0.043%), in further calculations we considered the content of the base material was equal to 100.0%.

Standardization of titrant. In order to reduce the uncertainties, the titer of *0.1 M Sodium hydroxide* was determined with use of not *0.1 M Hydrochloric acid* (as in SPU [1]) but with use of the pharmacopoeial primary standard for volumetric solutions - *Potassium hydrogen phthalate RV* (M_r 204.2). With this, about 0.45 g (accurate weight) of *Potassium hydrogen phthalate RV*, dried to constant weight, was dissolved in 30 ml of *water R*

and titrated with *0.1 M Sodium hydroxide* until slightly pink coloration (indicator is 0.1 ml of *Phenolphthalein solution R1*, as for the taurine substance). The titer was calculated as a mean result of 5 replicate titrations. The correction factor to the nominal concentration of the volumetric solution was found as K_T = 1.0159 with a relative standard deviation *RSD* = 0.11% and a confidence interval Δ*(titr)* = 0.11%. As we can see, the requirements of the SPU-Eur. Ph. to the repeatability of the titer determination results (≤ 0.2%) are met (see the section 5.3.1).

Nominal titration volume (see the ratio (5.24)).

$$V_T = \frac{m_T}{K_T \cdot m_{1mL}} \cdot (1 - \frac{LD}{100}) = \frac{250}{1.0159 \cdot 12.52} \cdot (1 - \frac{0}{100}) = 19.66 ml. \quad (5.36)$$

The nominal titration volume is 78.6 % of the 25 ml burette capacity, i.e. it meets the SPU-Eur. Ph. requirements (about 80 %) [1-3].

Influence of indicator titration. *Phenolphthalein solution R1* is 10 mg/ml solution of *Phenolphthalein R* (M_r 318.3) in 95% alcohol [1]. 0.1 ml of this solution contains 0.1·10/318.3 = 0.00314 mg moles of phenolphthalein. Titration of it takes 0.00314/(1.0159·0.1) = 0.031 ml of 0.10159 M solution of sodium hydroxide. It is 100·0.031/19.7 = 0.16 % ≤ 0.32% of the nominal titration volume that is not significant compared with the target procedure uncertainty $max\Delta_{As}$ = 1.0%.

Blank experiment volume V_o = 0.37 ml, that is 1.9 % of the nominal titration volume.

So significant value of V_o is a cause of inevitable oxidation of formaldehyde to formic acid

Sample weights for the linearity study. Take the weights of taurine substance corresponding to the different regression line points (i) direct, which are 80, 85, 90, 95, 100, 105, 110, 115 and 120% of the nominal weight of 250 mg. To study reproducibility in different experiments of the linearity study, take 2 sample weights for each point (*i*), indicating them respectively *i_1* and *i_2* (see the Table 5.9). Respectively receive 2 sets of 9 points, which are separately processed with the least squares method in a straight line. For compare, a pooled set of 18 points is also processed. The criteria for it are calculated by the principles set out above.

Normalized coordinates. In the ratio of (5.25) we used m_T = 250 mg and V_T = 19.66 ml. Values of X_i, Y_i и Z_i are presented in the Table 5.9.

Table 5.9

The linearity study results in the normalized coordinates

Sample weight number	Taurine sample weight, m_i, mg	X%	V_i, ml	$V_i - V_0$	Y%	Z %
80_1	203.6	81.44	16.4	16.03	81.55	100.14
80_2	200.2	80.08	16.05	15.68	79.77	99.62
85_1	210.0	84.00	16.85	16.48	83.84	99.81
85_2	212.5	85.00	17.00	16.63	84.61	99.54
90_1	222.7	89.08	17.77	17.4	88.52	99.38
90_2	224.7	89.88	17.97	17.6	89.54	99.62
95_1	237.4	94.96	18.95	18.58	94.53	99.55
95_2	240.5	96.20	19.19	18.82	95.75	99.53
100_1	245.3	98.12	19.55	19.18	97.58	99.45
100_2	253.8	101.52	20.25	19.88	101.14	99.63
105_1	264.2	105.68	21.05	20.68	105.21	99.56
105_2	263.6	105.44	21.03	20.66	105.11	99.69
110_1	274.0	109.60	21.83	21.46	109.18	99.62
110_2	276.0	110.40	22.00	21.63	110.05	99.68
115_1	287.8	115.12	22.85	22.48	114.37	99.35
115_2	281.0	112.40	22.63	22.26	113.25	100.76
120_1	300.4	120.16	23.95	23.58	119.97	99.84
120_2	301.7	120.68	24.07	23.70	120.58	99.91

Linear regression. The results of processing results by the least squares method for each of two sets of points are presented in Table 5.10. The criteria are taken from the Table 5.8 (for medicinal substances) and correlations of (5.28, 5.29). For compare, a pooled set of 18 points is also processed. The criteria for it are calculated by the principles set out above.

Table 5.10

Characteristics of the linear regression $Y = a + b \cdot X$

Parameter	Value	Standard deviation (SD)	Criteria of statistical insignificance ($\leq 0.85 \cdot SD$)	Criteria of practical insignificance	Conclusion
Set *i*_1					
A	0.47	0.64	a ≤ 0.54		Conform

B	0.9915	0.0063			
$\lvert 1-b \rvert$	0.0085	0.0063	$\lvert 1-b \rvert \leq 0.0054$		*Not conform*
s_o	0.246			≤ 0.39	Conform
R	0.99986			≥ 0.99959	Conform
r^2	0.99973			≥ 0.99917	Conform
$\delta_{RL,80}$	0.26			≤ 0.67	Conform
$\delta_{RL,120}$	0.46			≤ 0.67	Conform
DL		2.1			
QL		6.4			
General conclusion about linearity for set *i*_1					**Conform**
Set *i*_2					
a	-1.59	1.02	$\lvert a \rvert \leq 0.87$		*Not conform*
b	1.0137	0.0101			
$\lvert 1-b \rvert$	0.0137	0.0101	$\lvert 1-b \rvert \leq 0.0086$		*Not conform*
s_o	0.386			≤ 0.39	Conform
r	0.99965			≥ 0.99959	Conform
r^2	0.99930			≥ 0.99917	Conform
$\delta_{RL,80}$	0.59			≤ 0.67	Conform
$\delta_{RL,120}$	0.059			≤ 0.67	Conform
DL		3.4			
QL		10.2			
General conclusion about linearity for set *i*_2					**Conform**
Pooled set of 18 points					
a	-0.54	0.65	$\lvert a \rvert \leq 0.51$*		*Not conform*
b	1.0025	0.0064			
$\lvert 1-b \rvert$	0.0025	0.0064	$\lvert 1-b \rvert \leq 0.0050$*		Conform
s_o	0.35			≤ 0.39	Conform
r	0.99967			≥ 0.99959	Conform
r^2	0.99934			≥ 0.99917	Conform
$\delta_{RL,80}$	0.43			≤ 0.67	Conform
$\delta_{RL,120}$	0.20			≤ 0.67	Conform
DL		2.1			
QL		6.5			
General conclusion about linearity for pooled set of 18 points					**Conform**

* $0.78 \cdot SD$

As can be seen from the Table 5.10, the requirement of simultaneous statistical insignificance of values $\lvert a \rvert$ and $\lvert 1-b \rvert$ fails for both sets of 9 points. It is not even for the

pooled set of 18 points. At the same time, both sets of 9 points and the pooled set of 18 points satisfy with the practical suitability of the linear relationship.

Note that the maximum value of the systematic error can be achieved as for 80% of the nominal content ($\delta_{RL,80}$) (the set of ***i*_2** and pooled set of 18 points) and for 120% ($\delta_{RL,120}$) (the set of ***i*_1**). This confirms the need for the use of the ratio (5.28) for practical insignificance of the systematic error.

Comparison of ***i*_1** and ***i*_2** sets shows that the parameters of the linear relationship may differ markedly from each other (especially vividly reflected by *a* values). However, the conclusion on the acceptability of a linear relationship is not changed. The conclusion is also not changed when we extend the set of points (from 9 to 18). This important result indicates the reproducibility of the validation studies of linearity.

Detection limit (*DL*) and Quantitation limit (*QL*)

In the Table 10 are also presented for information limits of detection (*DL)*) and limits of quantitation (*QL*) calculated from equations of (5.34-5.35). They all do not exceed 32%, i.e. significantly don't affect the assay (see the section 3.1.1).

Precision and accuracy.

Table 5.11

The study of precision and accuracy

X%	***Y%***	***Z* = 100·*Y/X***	***X%***	***Y%***	***Z* = 100·*Y/X***	***X%***	***Y%***	***Z* = 100·*Y/X***
Pooled set of 18 points			**Set *i*_1**			**Set *i*_2**		
81.44	81.55	100.14	81.44	81.55	100.14	80.08	79.77	99.62
80.08	79.77	99.62	84	83.84	99.81	85	84.61	99.54
84	83.84	99.81	89.08	88.52	99.38	89.88	89.54	99.62
85	84.61	99.54	94.96	94.53	99.55	96.2	95.75	99.53
89.08	88.52	99.38	98.12	97.58	99.45	101.52	101.14	99.63
89.88	89.54	99.62	105.68	105.21	99.56	105.44	105.11	99.69
94.96	94.53	99.55	109.6	109.18	99.62	110.4	110.05	99.68
96.2	95.75	99.53	115.12	114.37	99.35	112.4	113.25	100.76
98.12	97.58	99.45	120.16	119.97	99.84	120.68	120.58	99.91
101.52	101.14	99.63						
105.68	105.21	99.56						
105.44	105.11	99.69						
109.6	109.18	99.62						
110.4	110.05	99.68						
115.12	114.37	99.35						
112.4	113.25	100.76						
120.16	119.97	99.84						

120.68	120.58	99.91						
Mean ($\bar{x}$)		**99.70**			**99.63**			**99.78**
SD		0.33			0.26			0.38
$\Delta_R(0.95; 17)$		0.13	$\Delta_R(0.95; 8)$		0.16	$\Delta_R(0.95; 8)$		0.24
$\delta = \lvert\bar{x} - 100\rvert$		**0.30**			**0.37**			**0.22**
Statistical insignificance of the systematical error: $\delta \leq \Delta_R$								
Not conform: 0.30 > 0.13			*Not conform:* 0.37 > 0.16			Conform: 0.22 < 0.24		
Practical insignificance of the systematical error: $\delta \leq 0.67$								
Conform: $\Delta = 0.30 \leq 0.67$			Conform: $\delta = 0.37 \leq 0.67$			Conform: $\delta = 0.22 \leq 0.67$		
Practical acceptability of the precision on the base of 6 replicate titrations: $\Delta_R(0.95; 5) = t(0.95; f) \cdot SD/\sqrt{5} \leq 0.33$								
Conform: : **0.25** ≤ 0.33			Conform: : **0.21** ≤ 0.33			Conform: : **0.32** ≤ 0.33		
General conclusion about precision and accuracy:								
Conform			**Conform**			**Conform**		

As we can see, the statistical insignificance requirement to the systematical error may as hold (set ***i*_2**) and as not hold (set ***i*_1** and pooled set of 18 points). The practical insignificance of the systematical error holds for all sets under investigation. The requirement of the precision practical acceptability for 6 replicate titrations holds as well.

In whole, the procedure complies with the requirements for the precision and accuracy.

6. VALIDATION OF QUANTITATION PROCEDURES FOR *IN VITRO* BIOEQUIVALENCE STUDY [68]

The study of dissolution profiles is widely used in the *in vitro* confirmation of bioequivalence and bioavailability of generic drugs [63]. Methodology of studying the dissolution profiles (DP) is a generalization of the pharmacopoeial “Dissolution” test for solid dosage forms [33].

In accordance with the requirements of the State Pharmacopoeia of Ukraine (SPU) [11], which are harmonized with the relevant guidance of the European Pharmacopoeia [4], all analytical procedures must be validated. It should be noted that the validation of a quantitative procedure for a DP study makes sense only in the case when the quantitative procedures for “Assay” and “Dissolution” tests have been already validated. It means that the validation problems connected with the peculiarity of the method proper are resolved. There are unresolved problems connected mainly with the peculiarity of the DP study proper. In particular, procedure robustness has been already confirmed (for example, the impact of fluctuations in the composition of the mobile phase in liquid chromatography, the stability of absorbance in spectrophotometry, etc. have been studied). Therefore, these questions, associated with the method proper, are not discussed in this article.

Standardized validation schemes have been developed and documented for all principle pharmacopoeial analytical methods and quality tests [11], including the «Dissolution" test. However, the direct application of the standardized scheme, described for the “Dissolution” test, to the DP study has encountered significant difficulties connected with the difference between these two tests. The same problems arise in the validation of methods of analysis used to describe the profiles of release (see annex 2).

In particular, the DP study takes place in a much broader analytical range than the "Dissolution" and "Assay" tests. It complicates the application of the reference standard method [11] which is normally used for these analytical procedures. In addition, the determination of the dissolution degree in the “Dissolution” test is carried out in just one time point (usually in 45 minutes [33]) and under conditions close to equilibrium. In the DP study, the determination of the DP degrees is carried out in several time points and in non-equilibrium conditions that increases the uncertainty of the results. At the same time, the quantitative procedure for the DP study should be consistent with the quantitative procedures for “Assay” and “Dissolution” tests. Otherwise, the connection of the “Dissolution” test with bioequivalence becomes undefined.

Analysis of the critical factors, influencing the “Dissolution” test, is described, in particular, in the article [64]. There are proposals for a validation of quantitative procedures for the DP study in the article. However, the proposed criteria are preliminary and do not take into account the peculiarity of the DP studies, in particular, need to examine the linearity over a wide concentration range.

To study factors affecting reproducibility of the DP, we need a validated quantitative procedure.

This section gives a systematic study of the issues related to the validation of the quantitative procedures for the DP study in frames of *in vitro* bioequivalence confirming [63], and proposes a standardized validation scheme for such procedures, which are both suitable and for routine test «Dissolution". Next the application of the reference standard method is expected [15].

THEORY

6.1. SPU metrological requirements to the study of *in vitro* dissolution profiles and to the «Dissolution" test

In accordance with the section 6.2.3 of the general article 5.N.2 [63], 12 samples are studied for both investigated and reference drugs. The standard deviation from the mean, as a percentage of the nominal content of the analyte in the dosage form, for each of these drugs should be not more than 20% for the first time in control and not more than 10% for all other control points. Given the *t-criterion t(95%, 11)* = 1.80 [26],, this corresponds to a confidence intervals of 35.9% for the first point and 18.0% for the other points. In accordance with the *Insignificance principle* (see the section 2.3.1), the total quantitative procedure uncertainty (Δ_{As}) for the DP study should meet the following requirements (as a percentage of the nominal concentration):

DP: 1st point
$$\Delta_{As} \le \max \Delta_{As} = 0.32 \cdot 35.9 = 11.5\%. \quad (6.1)$$

DP: other points
$$\Delta_{As} \le \max \Delta_{As} = 0.32 \cdot 18.0 = 5.7\%. \quad (6.2)$$

Requirements of (6.2) are substantially stricter than the value (7.0%), proposed by the authors [64]. However, for correct conducting the “Dissolution” test, the total quantitative procedure uncertainty, according to the SPU, must meet the ratio [11]:

“Dissolution” test:
$$\Delta_{As} \le \max \Delta_{As} = 3.0\%. \quad (6.3)$$

As can be seen, the pharmacopeial requirements of (6.3) to the analytical procedure for the “Dissolution” test [11] are much stricter than the requirements of (6.1-6.2) for the DP study. This is due to the fact that the “Dissolution” test regulates only one point in a state of near-equilibrium, while the DP points are recorded mainly in a non-equilibrium state.

Thus, if we want to use the procedure both for the DP study and the “Dissolution” test, we must meet the requirements of (6.3), i.e.:

DP + “Dissolution” test:
$$\Delta_{As} \le \max \Delta_{As} = 3.0\%. \quad (6.4)$$

In this case, the maximum acceptable systematic error must meet the requirements (as a percentage of the nominal concentration) (see the section 2.3.3):

$$\delta \le \max \delta = 0.32 \cdot 3.0 = 1.0\%. \tag{6.5}$$

6.2. Analytical range and number of points

According to the SPU requirements [11], linear relationship of analytical response on concentration must be verified through the analytical range. In normalized coordinates the linear relationship is of the form (see the section 2.2):

$$Y = b \cdot X + a. \tag{6.6}$$

Normalized coordinates are calculated by the equations (see the section 2.2):

$$X_i = \frac{C_i}{C^{st}} \cdot 100\%, \quad Y_i = \frac{A_i}{A^{st}} \cdot 100\%. \tag{6.7}$$

Here: C_i , C_{st} are the concentration of the test and reference standard solutions respectively, A_i , A_{st} are the analytical responses of the test and reference standard solutions respectively.

The wider the analytical range, the harder it is to meet the linearity requirements, so too a broad analytical range use is inappropriate. Bearing in mind the requirements for uniformity of content and the fact that the first (lower) time point of measurement is typically above 20% of the release degree, for the DP study we can recommend the analytical range 20-120% of the nominal content.

The lower time point of the range (20%) can be justified as follows. As shown below (see the relation of (6.17)), the quantification limit (*QL*) should not be more than 9.3% of the nominal content. To conduct any quantitative estimates below *QL* (i.e. below the 9.3%) is metrological incorrect. To get the reliable results, the measured value should be at least twice the *QL*, which justifies the lower point of the range of 20% to the nominal content.

As a top of the range for the validation of the “Dissolution” test, it is recommended to take 130-135% of the nominal content of [11]. This is due to the requirements for the uniformity of content ($\pm$ 25% of the nominal content) and the "safety margin" of 5-10%. However, unlike the uniformity of content [32], to the "Dissolution" test the SPU [33] establishes requirements only to the bottom of the dissolution (usually Q = 80%) because it is only of interest (everything above it already meets the requirements). Formally, the high degree of dissolution (> 120%) may signal a meaningful contribution of background absorption or degradation products. However, to control this, the specificity and solution stability in time are checked. Therefore, strictly speaking, need to increase the ceiling to 130-135% is not, and we may well use the usual upper limit of 120% of the nominal content for validation of quantitative procedures [4, 11], as it is further proposed to make.

Because the range of 20-120% is much wider than commonly used in validation of quantitative procedures (80-120%) [4, 11], it would be useful to increase the number of calibration line (4.6) points with recommended 9 [11] to 11 that would alleviate the linearity criteria. Thus, the studied concentrations in normalized coordinates (see section 2.2) are of the form:

Range: X **= 20, 30, 40, 50, 60, 70, 80, 90, 100, 110 и 120%** of the nominal value (6.8)

The range is characterized by a nominal standard deviation

$$SD_{range} \le 33.2\%. \tag{6.9}$$

The value SD_{range} is used for the calculation of the critical correlation coefficient value (see the section 2.3.4.2 and ratio (6.11))

6.3. Problem of model solutions

One of the features of the validation of the quantitative procedures for the *in vitro* DP study is the inability to prepare model solutions in usually adopted analytical sense, i.e. that the ratio of active substances and excipients corresponds to the nominal composition of the test drug product. This is due to the fact that release speeds from a solid dosage form (tablets) of a target (active) substance and excipients, generally speaking, are different. Therefore, their ratio in a dissolution medium varies according to a time of release. Accordingly, influence of the excipients on the analysis changes. However, for the standardization of the subject, it is further proposed to consider the release of the target and auxiliary substances are at the same speed. Accordingly, when preparing the model solutions of (6.8), in each model solution at the last stage of preparation we add the placebo solution that corresponds to the nominal composition of the test drug product.

6.4. Linearity requirements

Given the *t-criterion t(95%, 9)* = 1.83 [26] (9 is the number of freedom degrees of the 11 points linear relationship (6.6)), we can get from the ratio (6.4) the requirements to residual standard deviation of the straight line:

$$SD_{rest} \le 3.0/1.83 = 1.6\%. \tag{6.10}$$

The correlation coefficient is calculated according to the formula (see the section 2.3.4.2):

$$R_c = \sqrt{1 - \frac{SD_{rest}^2}{SD_{range}^2}}. \tag{6.11}$$

Taking into account the ratios of (6.9-6.11) we can get the requirements to the correlation coefficient of the straight line (6.6):

$$R_c \le 0.99878. \qquad R_c^2 \le 0.99757. \tag{6.12}$$

Given the high values of the correlation coefficients, it is more convenient to use the R^2_c values.

To correctly use the reference standard method, the *Y*-intercept (*a*) of the linear relationship (4.6) must be insignificant - statistically or practically (see the section 2.3.3).

Statistical insignificance means that the *Y*-intercept (*a*) must be insignificantly different from zero, i.e. for the case of 11 points (9 degrees of freedom) the ratio should be performed (see the section 2.3.3):

Statistical insignificance:

$$a \le t(95\%, g-2) \cdot s_a = 1.83 \cdot s_a. \tag{6.13}$$

The equation (6.8) shows that X_{min} = 20%. Also bearing in mind the requirements of (6.5) to the systematical error, we'll get the requirements to practical insignificance of the *Y*-intercept of the straight line in the normalized coordinates (see section 2.3.3):

Practical insignificance:

$$a \le \left| \frac{\max \delta}{1 - (X_{\min} / 100)} \right| = 1.2\%. \tag{6.14}$$

The requirement (6.14) of practical insignificance is applied only in the case when the requirement (6.13) of statistical insignificance is not met (see the section 2.3.3).

6.5. Quantitation limit (*QL*)

The *QL* is calculated by the ration (see the section 2.3.5):

$$QL = 10 \cdot s_a / b \approx 10 \cdot s_a, \tag{6.15}$$

When we validate a quantitative procedure (range 80-120%), this characteristic has an informational meaning. However, in our case the lower bound of the range (20-120) is small. It is obvious that the *QL* value should be associated with the maximum acceptable uncertainty $max\Delta_{As}$. It is reasonable to assume that the $max\Delta_{As}$ value must be insignificant compared with the *QL*. In this case, *QL* did not significantly affect the results of the analysis. Thus, the maximum acceptable uncertainty of the ratio (6.4) must be insignificant (see the section 2.3.1) compared with the *QL*, i.e.:

$$\max \Delta_{As} = 0.32 \cdot QL. \tag{6.16}$$

Hence, given the (6.4), we obtain [5]:

$$QL \le 9.3\%. \tag{6.17}$$

6.6. Specificity

When validating the quantitative spectrophotometeric or chromatographic procedures, we generally use such requirement for the characteristic of procedure specificity: the total absorbance (or chromatographic response) of all impurities and excipients at the analytical wavelength (or in place of the peak of the target substance) should not exceed the maximum acceptable systematic error of the ratio (6.5).

When examining the DP and "Dissolution" test, sometimes we have to contend with a noticeable decomposition of a target substance (e.g., aspirin) in the process of conducting the study. But this decomposition does not indicate a poor quality product, and is the only feature of these tests. These tests are conducted for solid dosage forms which meet the other requirements of the specifications, in particular, for the content of permissible impurities. Therefore, the specificity of the procedure in relation to the further degradation (during test conducting) is not useful in this case and it can be harmful. A systematic error in the results of the quantitative determination is induced by a placebo.

Taking into account discussed in the previous sections, while studying the dissolution profiles (DP), as a measure of the procedure specificity it is appropriate to require that a proportion of the placebo analytical signal (optical density in spectrophotometry or height or peak area in chromatography - $S_{placebo}$) on the site of the peak (or wavelength) of the target substance in relation to the reference standard analytical signal of the target substances does not exceed the maximum acceptable value of the systematic error (6.5), i.e.:

$$100 \cdot \frac{S_{placebo}}{S_{st}} \le \delta = 1.0\%. \tag{6.18}$$

6.7. Metrological characteristics of results

To study the repeatability, we use the results obtained in the linearity study (see the section 2). Hereby we explore the uncertainty of $Z = 100 \cdot Y/X$, which is the concentration found as percentage of the concentration entered (see the section 2.2). However, the use of the Z value is correct only for the sufficiently narrow analytical range in which it can be assumed the approximate constancy of *relative* uncertainty. In the case of the DP study with the usual wide range of 20-120%, this assumption is incorrect. In this case it is more correct to assume the constancy of the *absolute* uncertainty. Note that this assumption is used when processing the line (6.6) by the least-squares method.

So, the validation of our procedure is formulated thus: in normalized coordinates the procedure must be characterized by the same uncertainty of no more than 3.0 percent through the whole range 20-120%.

To assess this uncertainty let's define the value:

$$\Delta Z_i = Y_i - X_i \,. \tag{6.19}$$

If the *Y*-intercept of the linear relationship (6.6) is insignificantly (statistically or practically) different from zero (i.e. the linear relationship (6.6) passes through the origin of coordinates), the *Y* value in normalized coordinates represents the found concentration value. Therefore, the ΔZ_i value in the equation (6.19) is the difference between the found (Y_i) and entered (X_i) concentrations as a percentage of the nominal concentration.

6.7.1. Accuracy

The mean value of ΔZ_i should be statistically or practically insignificantly different from zero, i.e. for $g = 11$ we can get:

Statistical insignificance:
$$\Delta Z = \left|\overline{\Delta Z}_i\right| \le \frac{t(95\%, g-1)}{\sqrt{g}} \cdot SD_{\Delta Zi} = 0.55 \cdot SD_{\Delta Zi}. \tag{6.20}$$

In case of failure of the ratio (20) for statistical insignificance, we can apply the requirement of practical insignificance: the mean value of ΔZ_i must not exceed the maximum acceptable value of the systematic error of the ratio (6.5), i.e.:

Statistical insignificance:
$$\Delta Z = \left|\overline{\Delta Z_i}\right| \le \delta = 1.0\%. \tag{6.21}$$

6.7.2. Repeatability

Given the ratios of (6.20-6.21), the uncertainty of the ΔZ_i should not exceed the maximum acceptable uncertainty of the analytical procedure in the ratio (6.4), i.e.:

$$\Delta_{\Delta Zi} = t(95\%, g-1) \cdot SD_{\Delta Zi} = 1.812 \cdot SD_{\Delta Zi} \le 3.0\%. \tag{6.22}$$

6.7.3. Intermediate precision

Another feature of validation of analytical procedures for the DP study is the inability to test the intermediate precision in its normal sense. In the normal case, to prove the intermediate precision we analyze several samples of the same drug product on different days, combine the results and calculate the relative standard deviation and confidence interval, which must not exceed $max\Delta_{As}$ (see the section 2.3.6). In our case, this approach is not applicable, since the various tablets of the same batch, which

meet the specification, may differ by up to 30% (because of content nonuniformity [32]) and may have, besides, the different dissolution speed. I.e., we'll check the quality of the technology but not the correctness of the analytical procedure. In addition, in this approach, we check the intermediate precision for only one concentration but not for the whole analytical range.

Therefore, as a proof of the intermediate precision we can offer to repeat the validation study the other day. The results must meet the above criteria and it is a proof of the intermediate precision. Note that in this way we check the precision not for a single concentration point but for the whole analytical range of concentrations.

6.8. Example. Validation of the quantitation procedure for the dissolution profile study of L-thyroxin tablets

The developed validation scheme is intended to validate quantitative procedures used to describe the dissolution (release) profiles (see the Addendum 2). An example is the description of dissolution profiles in an *in vitro* bioequivalence study of L-thyroxine tablets when optimizing their composition (see the Addendum 2. Chapter 3).

6.8.1. Object of study

As an object of study is the validation of the quantitative procedure for the dissolution profiles study of L-thyroxine-Farmak tablets 25-150 μg. It must again be emphasized that we validate not the release procedure but the quantitative analytical procedure for determining the concentrations in this test.

6.8.2. Description of the drug substance

Levothyroxine Sodium (LTS) is described in the SPU-Eur.Ph. [65], Levothyroxine Sodium tablets – in British [66] and United States [67] Pharmacopoeias.

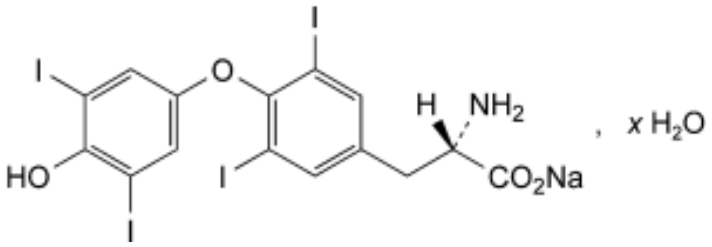


Рис. 6.1. Levothyroxine Sodium [65].

As can be seen from the Figure 6.1, LTS is a salt of strong base and relatively weak aminocarboxylic acid, so its saturated solution in water has a pH of 8.9 [67]. In line with this, LTS is very little soluble in water (due to hydrolysis of anion to the little soluble free acid) but soluble in alkalis [65, 67]. The NH_2 group enhances the solubility of LTS in strongly acidic media as well.

Note that the pharmacopoeial term "very little soluble" means the solubility in the range of 1: 1000 to 1: 10000, i.e. 1 mg of the LTS substance dissolves in the 1-10 ml

of water. Taking into account the dose (25 mg) and definition of a high soluble substance of high definition according to the *Biopharmaceutical classification system* (BCS) [63, section 6.1] (the highest dose should be dissolved in 250 ml of water with pH 1.2-6.8), we can assume that the LTS is the substance with high solubility by BCS. At the same time, it can be expected that the dissolution speed of LTS in an acid medium (pH 4.5) must be significantly lower than in a strongly acidic (pH 1.2) and more basic (pH 6.8) media.

6.8.3. Choice of dissolution media

In accordance with BCS [63, section 6.2.3], *in vitro* bioequivalence studies are carried out in these media: hydrochloric acid solution of pH 1.2, acetate buffer solution of pH 4.5, phosphate buffer solution of pH 6.8. In addition, such studies should be carried out in the dissolution medium used in the "Dissolution" test for quality control of the levothyroxine tablets, i.e. 0.01 M hydrochloric acid plus 0.2% Sodium Lauryl Sulfate [14]. This allows us to link the bioequivalence with the quality of the drug product according to its specification.

6.8.4. Analytical procedure

The test is carried out in accordance with the requirements of the SPU general chapters 5.N.2 [63] and 2.9.3 [33] using the Apparatus 2 (Paddle apparatus), rotation speed is 75 RPM. Volume of dissolution medium is 500 ml.

Dissolution media. In accordance with the requirements of the SPU general chapter 5.N.2 [63], the study is conducted in the three media, that are described in the SPU general chapter 2.9.3 [33], having pH 1.2, 4.5 and 6.8, as well as in 0.01 M HCl with adding of 0.2% Sodium Lauryl Sulfate.

The test solution. In our case of analysis of thyroxine tablets, place in the dissolution device the number of the tablets equivalent to 600 μg of LTS.

At appropriate intervals take 50 ml of the dissolution media from the vessel and filter through a paper filter "Blue Ribbon", discarding the first 30 ml of the filtrate.

Solution A. 400 mg of *sodium hydroxide R* dissolve in 500 ml of water and add 500 ml of *methanol R*.

Reference substance solution. 150 mg of levothyroxine sodium (USP RS) dissolve in 30 ml of solution A and bring up the volume to 100.0 ml with the same solution. 4.0 ml of the resulting solution then place in a measuring flask with a capacity of 50.0 ml and bring up to the mark with the solution A. 1 ml of the resulting solution then place in measuring flasks with a capacity of 100 ml and bring up to the mark with the buffer solutions of pH 1.2, 4.5 and 6.8 or *0.01 M hydrochloric acid* with the addition of 0.2% of *Sodium Lauryl Sulfate R*. The nominal concentration of levothyroxine sodium in the final reference substance solution is 1.2 μg/ml.

Model solutions (solutions to the linearity study). Prepare solutions with concentration of 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 110%, 120% of the nominal concentration of levothyroxine sodium (1.2 μg/ml). Initial dilutions of sodi-

um levothyroxine is prepared in solution A, in the final solutions add equivalent amounts of the levothyroxine tablet placebo solution and bring up to the mark with the corresponding buffer solution of pH: 1.2; 4.5, 6.8 or 0.01 M HCl with the addition of 0.2% Sodium Lauryl Sulfate.

Chromatographic conditions [67]:

Column (steel)*:*

- *size*: l = 0.250 m, θ = 4.6 mm;
- *stationary phase*: silica for chromatography, nitrile (5 μ).

Mobile phase: *acetonitrile R, phosphoric acid R, water R* (400 : 0.5 : 600 v/v/v).

Flow rate: 1.5 mL/min.

Detection: spectrophotometer at 225 nm.

Column temperature: (30 ± 1)°C.

Injection: 100 μL.

System suitability:

- *relative standard deviation* (with reference solution): meet the requirements of the SPU general chapter 2.2.46 [13];
- *column efficiency*: minimum 3000 theoretical plates calculated for the peak due to levothyroxine sodium in the chromatogram obtained with reference solution.

Part of levothyroxine sodium (X_2), which dissolves from the tablets into the solution, as a percentage of the nominal content in the tablet, calculate by the formula:

$$X_3 = \frac{S_1 \cdot m_0 \cdot 4 \cdot 1 \cdot 500 \cdot P_0 \cdot 100 \cdot (100 - W)}{S_0 \cdot 50 \cdot 100 \cdot 100 \cdot a \cdot n \cdot 100} = \frac{40 \cdot S_1 \cdot m_0}{S_0 \cdot a \cdot n} \cdot \frac{P_o}{100} \cdot \frac{100 - W}{100},$$

Here: S_1 is a mean area of levothyroxine sodium peaks, calculated from the chromatograms of the sample solution;

S_0 is a mean area of levothyroxine sodium peaks, calculated from the chromatograms of the reference solution;

a is a nominal content of levothyroxine sodium in one tablet, gram;

m_0 is a weight of levothyroxine sodium, taken for reference solution preparation, gram;

P_0 is a content of levothyroxine sodium in the reference substance taken for the reference solution preparation, per cent.

In accordance with the requirements of the specification, the tested tablets meet the requirements of the “Dissolution” test, if the dissolution degree of sodium levothyroxine for the tested tablets meets the requirements of the SPU general chapter 2.9.3

[33]. After 45 minutes it should be Q ≥ 70% of the nominal content of the levothyroxine in tablets.

6.8.5. Linearity study

From the text of the analytical procedure (see above) we can see that the injection size is a fairly large - 100 µl of the dissolution medium with dissolved tablet components. In addition, the dissolution media have a pH in the wide range of 1.2-6.8, so their influence on HPLC-analysis is different.

This means that validation should be conducted for all 4 dissolution media.

Conduct studies in the analytical range specified in the ratio (6.8). For this prepare the solutions of appropriate concentrations. The linearity study results are presented in the Tables 6.1-6.4.

Table 6.1

The linearity study results for the dissolution medium of pH = 1.2

Solution number	Peak area S_{ik}	Mean value S_i	$Y\% = 100 \cdot S_i/S_{st}$	Concentrations C_i, μ/mL	$X_i\% = 100 \cdot C_i/C_{st}$
St	238.8 238.3 237.6	238.2 = S_{st}	-	1.2100= C_{st}	-
1	47.4 47.3 47.6	47.4	19.9	0.2421	20.0
2	70.1 70.2 70.4	70.2	29.5	0.3631	30.0
3	94.0 94.1 93.3	93.8	39.4	0.4842	40.0
4	119.7 119.9 119.1	119.6	50.2	0.6052	50.0
5	142.5 142.8 142.4	142.6	59.8	0.7262	60.0
6	167.8 167.5 165.6	167.0	70.1	0.8473	70.0
7	189.8 187.0 188.5	188.4	79.1	0.9683	80.0
	214.6				

8	214.8 214.1	214.5	90.0	1.0894	90.0
9	238.8 238.3 237.6	238.2	100.0	1.2100	100.0
10	258.4 258.9 258.1	258.5	108.5	1.3314	110.0
11	288.5 290.2 289.0	289.2	121.4	1.4525	120.0

Table 6.2

The linearity study results for dissolution medium: 0.01 M hydrochloric acid + 0.2% Sodium Lauryl Sulfate

Solution number	Peak area S_{ik}	Mean value S_i	$Y\% = 100 \cdot S_i/S_{st}$	Concentrations C_i, μ/mL	$X_i\% = 100 \cdot C_i/C_{st}$
St	236.5 236.5 236.1	236.4 = S_{st}	-	1.2096= C_{st}	-
1	47.4 47.2 47.4	47.3	20.0	0.2419	20.0
2	71.8 71.5 71.6	71.6	30.3	0.3629	30.0
3	95.4 95.1 95.3	95.3	40.3	0.4838	40.0
4	113.8 113.5 113.5	113.6	48.1	0.6048	50.0
5	137.1 137.0 137.1	137.1	58.0	0.7258	60.0
6	170.1 169.9 169.4	169.8	71.8	0.8467	70.0
7	191.1 190.1 190.9	190.7	80.7	0.9677	80.0
	216.3				

8	216.0 216.1	216.1	91.4	1.0886	90.0
9	237.3 237.4 237.2	237.3	100.4	1.2096	100.0
10	263.4 263.2 263.6	263.4	111.4	1.3306	110.0
11	287.3 286.9 287.1	287.1	121.5	1.4515	120.0

Table 6.3

The linearity study results for the dissolution medium of pH = 4.5

Solution number	Peak area S_{ik}	Mean value S_i	$Y\% = 100 \cdot S_i/S_{st}$	Concentrations C_i, μ/mL	$X_i\% = 100 \cdot C_i/C_{st}$
St	221.9 219.6 220.2	220.6 = S_{st}	-	1.2100= C_{st}	-
1	45.2 45.0 45.0	45.1	20.4	0.2421	20.0
2	67.2 66.7 66.3	66.7	30.3	0.3631	30.0
3	88.1 89.9 88.1	88.7	40.2	0.4842	40.0
4	111.1 110.5 110.0	110.5	50.1	0.6052	50.0
5	132.8 132.5 132.3	132.5	60.1	0.7262	60.0
6	155.3 155.1 154.5	155.0	70.3	0.8473	70.0
7	180.4 180.9 180.1	180.5	81.8	0.9683	80.0
8	201.1 200.2	200.8	91.1	1.0894	90.0

	201.2				
9	221.9 219.6 220.2	220.6	100.0	1.2100	100.0
10	248.8 245.5 247.4	247.2	112.1	1.3314	110.0
11	265.4 266.3 264.6	265.4	120.3	1.4525	120.0

Table 6.4

The linearity study results for the dissolution medium of pH = 6.8

Solution number	Peak area S_{ik}	Mean value S_i	$Y\% = 100 \cdot S_i/S_{st}$	Concentrations C_i, μ/mL	$X_i\% = 100 \cdot C_i/C_{st}$
St	238.3 237.1 237.4	237.6 = S_{st}	-	1.2100= C_{st}	-
1	47.1 47.5 47.8	47.5	20.0	0.2421	20.0
2	70.8 71.3 70.3	70.8	29.8	0.3631	30.0
3	95.3 94.5 95.4	95.1	40.0	0.4842	40.0
4	119.4 119.2 119.2	119.3	50.2	0.6052	50.0
5	143.4 142.4 140.5	142.1	59.8	0.7262	60.0
6	167.4 167.3 168.0	167.6	70.5	0.8473	70.0
7	185.3 187.0 187.3	186.5	78.5	0.9683	80.0
8	219.0 218.0 219.2	218.7	92.1	1.0894	90.0

9	241.0 240.7 240.1	240.6	101.3	1.2100	100.0
10	261.7 261.7 260.4	261.3	110.0	1.3314	110.0
11	289.3 289.9 289.2	289.5	121.8	1.4525	120.0

The results were processed by the least squares method [26] for the line (6) and compared to developed above the acceptability criteria. Results of such calculations are presented in the Tables 2. 6.5-6.9. The typical regression line is shown in the Figure 6.2.

Table 6.5

Metrological performance of the regression lines $Y = a + b * X$ for different dissolution media (n = 11)

Parameter	Values found	Criteria	Conclusions
pH = 1.2			
a	-0.46	Statistical insignificance: $\lvert a \rvert \leq 1.83 \cdot 0.56 = 1.02$	Meet
		Practical insignificance: $\lvert a \rvert \leq 1.2$	Meet
SD_a	0.56		
b	1.0038		
SD_b	0.0072		
SD_{rest}	0.76	≤ 1.6	Meet.
R_c^2	0.99954	≥ 0.99757	Meet.
QL	5.5	$\leq$ 9.3	Meet.
General conclusion for pH = 1.2			**Meet**
0.01 M hydrochloric acid + 0.2% Sodium Lauryl Sulfate			
a	-1.01	Statistical insignificance: $\lvert a \rvert \leq 1.83 \cdot 0.85 = 1.57$	Meet
		Practical insignificance: $\lvert a \rvert \leq 1.2$	Meet.
SD_a	0.85		

b	1.020		
SD_b	0.011		
SD_{rest}	1.17	≤ 1.6	Meet
R_c^2	0.99892	≥ 0.99757	Meet
QL	8.4	≤ 9.3	Meet
General conclusion for 0.01 M HCl + 0.2% Sodium Lauryl Sulfate			**Meet**
pH = 4.5			
a	-0.015	Statistical insignificance: $\lvert a \rvert \leq 1.83 \cdot 0.51 = 0.94$	Meet
		Practical insignificance: $\lvert a \rvert \leq 1.2$	Meet
SD_a	0.51		
b	1.0089		
SD_b	0.0067		
SD_{rest}	0.70	≤ 1.6	Meet
R_c^2	0.99954	≥ 0.99757	Meet
QL	5.1	≤ 9.3	Meet
General conclusion pH = 4.5			**Meet**
pH = 6.8			
a	-0.67	Statistical insignificance: $\lvert a \rvert \leq 1.83 \cdot 0.70 = 1.28$	Meet
		Practical insignificance: $\lvert a \rvert \leq 1.2$	Meet
SD_a	0.70		
b	1.0147		
SD_b	0.0091		
SD_{rest}	0.95	≤ 1.6	Meet
R_c^2	0.99928	≥ 0.99757	Meet
QL	6.9	≤ 9.3	Meet
General conclusion for pH = 6.8			**Meet**

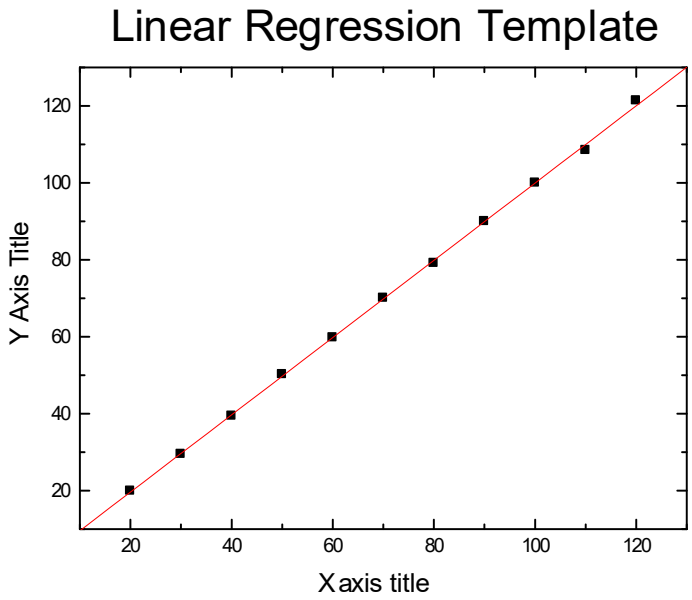


Figure 6.2. Typical regression line for the dissolution medium pH 1.2

Table 6.6

Results of accuracy and precision study for the dissolution medium pH = 1.2

№ solution	Y_i%	X_i%	$\Delta Z_i = Y_i - X_i$	Criteria	Conclusions
1	19.9	20.0	-0.10		
2	29.5	30.0	-0.53		
3	39.4	40.0	-0.64		
4	50.2	50.0	0.17		
5	59.8	60.0	-0.18		
6	70.1	70.0	0.06		
7	79.1	80.0	-0.93		
8	90.0	90.0	0.01		
9	100.0	100.0	0.00		
10	108.5	110.0	-1.54		
11	121.4	120.0	1.37		
		Mean ΔZ	-0.21		
		$SD_{\Delta Zi}$ =	0.73		
Statistical insignificance of ΔZ: $\Delta Z \leq 0.55 \cdot SD_{\Delta Z\,i}$				≤ 0.40	Meet

Practical insignificance of ΔZ:		≤ 1.0	Meet
Repeatability: 1.812· $SD_{\Delta Zi}$	1.33	≤ 3.0	Meet
General conclusion for pH = 1.2			**Meet**

Table 6.7

Results of accuracy and precision study for the dissolution medium: 0.01 M hydrochloric acid + 0.2% Sodium Lauryl Sulfate

№ solution	Y_i%	X_i%	$\Delta Z_i = Y_i - X_i$	Criteria	Conclusions
1	20.0	20.0	0.03		
2	30.3	30.0	0.31		
3	40.3	40.0	0.30		
4	48.1	50.0	-1.94		
5	58.0	60.0	-2.01		
6	71.8	70.0	1.84		
7	80.7	80.0	0.68		
8	91.4	90.0	1.44		
9	100.4	100.0	0.39		
10	111.4	110.0	1.44		
11	121.5	120.0	1.46		
		Mean ΔZ	0.36		
		$SD_{\Delta Zi}$ =	1.30		
Statistical insignificance of ΔZ: $\Delta Z \leq 0.55 \cdot SD_{\Delta Z\,i}$				≤ 0.71	Meet
Practical insignificance of ΔZ:				≤ 1.0	Meet
Repeatability: 1.812· $SD_{\Delta Zi}$			2.35	≤ 3.0	Meet
General conclusion for 0.01 M HCl + 0.2% Sodium Lauryl Sulfate					**Meet**

Table 6.8

Results of accuracy and precision study for the dissolution medium pH = 4.5

№ solution	Y_i%	X_i%	$\Delta Z_i = Y_i - X_i$	Criteria	Conclusions
1	20.4	20.0	0.43		
2	30.3	30.0	0.25		
3	40.2	40.0	0.20		
4	50.1	50.0	0.10		
5	60.1	60.0	0.07		
6	70.3	70.0	0.24		
7	81.8	80.0	1.79		
8	91.1	90.0	1.02		
9	100.0	100.0	0.00		
10	112.1	110.0	2.05		
11	120.3	120.0	0.30		
Mean ΔZ			0.59		
$SD_{\Delta Zi}$ =			0.72		
Statistical insignificance of ΔZ: $\Delta Z \leq 0.55 \cdot SD_{\Delta Z\, i}$				≤ 0.39	*not meet*
Practical insignificance of ΔZ:				≤ 1.0	Meet
Repeatability: $1.812 \cdot SD_{\Delta Zi}$			1.30	≤ 3.0	Meet
General conclusion for pH = 4.5					**Meet**

Table 6.9

Results of accuracy and precision study for the dissolution medium pH = 6.8

№ solution	Y_i%	X_i%	$\Delta Z_i = Y_i - X_i$	Criteria	Conclusions
1	20.0	20.0	-0.03		
2	29.8	30.0	-0.21		
3	40.0	40.0	0.00		
4	50.2	50.0	0.18		

5	59.8	60.0	-0.21		
6	70.5	70.0	0.50		
7	78.5	80.0	-1.52		
8	92.1	90.0	2.03		
9	101.3	100.0	1.26		
10	110.0	110.0	-0.08		
11	121.8	120.0	1.79		
		Mean ΔZ	0.34		
		$SD_{\Delta Zi}$ =	1.02		
Statistical insignificance of ΔZ: $\Delta Z \leq 0.55 \cdot SD_{\Delta Z\, i}$				≤ 0.56	Meet
Practical insignificance of ΔZ:				≤ 1.0	Meet
Repeatability: $1.812 \cdot SD_{\Delta Zi}$			1.84	≤ 3.0	Meet
General conclusion for pH = 6.8					**Meet**

6.8.6. Intermediate precision study

To check the intermediate precision, carry out the repeated linearity and precision studies the other day. The results of these studies are illustrated below in the Tables 6.10-6.12 for two of the most important dissolution media – of pH 1.2 and 6.8.

Table 6.10

Metrological performance of the regression lines $Y = a + b * X$ (n = 11) at the stage of the intermediate precision verification

Parameter	Values found	Criteria	Conclusion
pH = 1.2			
a	-1.17	Statistical insignificance: $\lvert a \rvert \leq 1.83 \cdot 0.64 = 1.16$	*not meet*
		Practical insignificance: $\lvert a \rvert \leq 1.2$	Meet
SD_a	0.64		
b	1.030		

SD_b	0.008		
SD_{rest}	0.87	≤ 1.6	Meet
R_c^2	0.99942	≥ 0.99757	Meet
QL	6.2	≤ 9.3	Meet
General conclusion for pH = 1.2			**Meet**
pH = 6.8			
a	-0.67	Statistical insignificance: $\lvert a \rvert \leq$ 1.83·0.69 = 1.26	Соотв.
		Practical insignificance: $\lvert a \rvert \leq 1.2$	Meet
SD_a	0.69		
b	0.9941		
SD_b	0.0089		
SD_{rest}	0.94	≤ 1.6	Meet
R_c^2	0.99928	≥ 0.99757	Meet
ПКО	6.9	≤ 9.3	Meet
General conclusion for pH = 6.8			**Meet.**

Table 6.11

Results of the accuracy and precision studies for the dissolution medium pH = 1.2 at the stage of the intermediate precision verification

Solution №	Y_i%	X_i%	$\Delta Z_i = Y_i - X_i$	Criteria	Conclusions
1	20.0	20.0	-0.02		
2	30.4	30.0	0.37		
3	39.8	40.0	-0.24		
4	49.7	50.0	-0.33		
5	60.4	60.0	0.42		
6	70.7	70.0	0.68		

7	80.5	80.0	0.51		
8	92.2	90.0	2.16		
9	100.1	100.0	0.10		
10	112.7	110.0	2.75		
11	123.5	120.0	3.49		
		Mean ΔZ	0.90		
		$SD_{\Delta Zi}$ =	1.29		
Statistical insignificance of ΔZ: $\Delta Z \leq 0.55 \cdot SD_{\Delta Z\,i}$				≤ 0.71	*not meet*
Practical insignificance of ΔZ:				≤ 1.0	Meet
Repeatability: $1.812 \cdot SD_{\Delta Zi}$			2.34	≤ 3.0	Meet
General conclusion for pH = 1.2					**Meet**

Table 6.12

Results of the accuracy and precision studies for the dissolution medium pH = 6.8 at the stage of the intermediate precision verification

Solution №	Y_i%	X_i%	$\Delta Z_i = Y_i - X_i$	Criteria	Conclusions
1	19.7	20.0	-0.34		
2	29.2	30.0	-0.79		
3	38.9	40.0	-1.12		
4	50.2	50.0	0.22		
5	58.3	60.0	-1.71		
6	68.1	70.0	-1.92		
7	78.1	80.0	-1.86		
8	89.3	90.0	-0.71		
9	100.8	100.0	0.84		
10	108.4	110.0	-1.60		
11	118.3	120.0	-1.70		
		Mean ΔZ	-0.97		
		$SD_{\Delta Zi}$ =	0.92		

Statistical insignificance of ΔZ: $\Delta Z \leq 0.55 \cdot SD_{\Delta Z\, i}$		≤ 0.50	*Not meet*
Practical insignificance of ΔZ:		≤ 1.0	Meet
Repeatability: $1.812 \cdot SD_{\Delta Zi}$	1.66	≤ 3.0	Meet
General conclusion for pH = 6.8			**Meet**

The Tables 6.10-6.12 show that the linearity and precision requirements are met when we repeat the analysis the other day. Thus, the intermediate precision is met.

The general conclusion: the procedure is validated for *in vitro* bioequivalence studies in accordance with the requirements of the SPU general chapter 5.N.2 [63] and the "Dissolution" test in accordance with the SPU general chapter 2.9.3 [33].

The validated procedure has been applied to the study of the dissolution profiles of various compositions of thyroxine tablets 25 μ at the stage of development of the technology for their production (see the Addendum 2, Chapter 3).

7. STANDARDIZED VALIDATION PROCEDURE FOR ATOMIC ABSORPTION ASSAYS OF MEDICINES, USING CALIBRATION GRAPH METHOD [69]

Atomic absorption spectrophotometry (AAS) is a pharmacopoeial method of analysis [2] and is widely used for the quantitative determination of metals in drug products (HP). Thus, the use of AAS is quite normal for the control of potassium and iron in the finished medicinal products – "Asparcam" [76], tablets of potassium gluconate, ferrous fumarate, iron gluconate, etc. [67]. In the case of vitamins and nutritional supplements the United States Pharmacopoeia (USP) uses AAS as the primary method for pharmacopoeial quantitative determination of metals [67].

Like any other pharmacopoeial procedure, method, an AAS procedure should be validated. Recommendations for validation of AAS procedures are in the State Pharmacopoeia of Ukraine (USP) [2] which is harmonized with the European Pharmacopoeia (Eur.Ph.) [3]. However, their implementation raises many questions. It should be noted that the SPU describes the standardized validation schemes for drug quality control procedures [11], which are being developed for all principal pharmacopoeial methods: spectrophotometry (SPh), HPLC and GC for assays, related substances control, residual solvents control, and titration (see chapters 2-6). Specificity of validation of assay procedures for compounded preparations are enough developed as well [77].

However, a direct application of the approaches, developed earlier in Chapters 1-6, to assay procedures using AAS is encountering significant difficulties connected with the specificity of the AAS. The main problem is the problem of linearity. AAC procedures often have to contend with the lack of direct proportionality of the dependence of the absorbance on the concentration in analytical range. This makes it necessary to use the calibration graph method (CGM) instead of the reference standard method (RSM)). At the same time, validation of the procedures that use the CGM, has its own specific character, which was not addressed in the previous chapters.

This chapter conducts the critical analysis of the SPU-Eur.Ph. approach to the AAS procedure validation and develops the standardized scheme for AAS procedure validation in the option of the CGM. At the same time we consider the criteria for applicability of the more simple reference standard method to the AAS-procedures with corresponding their validation on the previously developed schemes. The findings and results are applicable to any procedure that uses the calibration graph method.

7.1. SPU-Eur.Ph. approach to validation of AAS procedures

7.1.1. Method [2-3]

Ensure that the concentrations to be determined fall preferably within the linear part of the calibration curve. If this is not possible, the calibration plots may also be curved and are then to be applied with appropriate calibration software.

Determinations are made by comparison with reference solutions with known concentrations of the element to be determined either by the method of direct calibration (Method 1) or the method of standard additions (Method 2).

Method 1 – Direct calibration

For routine measurements 3 reference solutions and a blank solution are prepared and examined (test solution) as prescribed in the monograph. Prepare no fewer than 3 reference solutions of the element to be determined, the concentration of which span the expected value in the test solution. For assay purposes, optimal calibration levels are between 0.7 and 1.3 times the expected content of the element to be determined or the limit described in the monograph. For purity determination, calibration levels are the limit of detection and 1.2 times limit specified for the element to be determined. Any reagents used in the preparation of the test solution are added to the reference and blank solutions at the same concentration.

Introduce each of the solutions into the instrument using the same number of replicates for each of the solutions to obtain a steady reading.

Calculation. Prepare a calibration curve from the mean of the readings obtained with the reference solutions by plotting the means as a function of concentration. Determine the concentration of the element in the test solution from the curve obtained.

Method 2 - standard additions

Add to at least 3 similar volumetric flasks equal volumes of the solution of the substance to be examined (test solution) prepared as prescribed. Add to all but 1 of the flasks progressively larger volumes of a reference solution containing a known concentration of the element to be determined to produce a series of solutions containing steadily increasing concentrations of that element known to give responses in the linear part of the curve, if possible. Dilute the contents of each flask to volume with solvent.

Introduce each of the solutions into the instrument, using the same number of replicates for each of the solutions, to obtain a steady reading.

Calculation. Calculate the linear equation of the graph using a least-squares fit and derive from it the concentration of the element to be determined in the test solution.

7.1.2. Validation of the method [2-3]

Satisfactory performance of methods prescribed in monographs is verified at suitable time intervals.

LINEARITY

Prepare and analyse not fewer than 4 reference solutions over the calibration range and a blank solution. Perform not fewer than 5 replicates.

The calibration curve is calculated by least-square regression from all measured data. The regression curve, the means, the measured data and the confidence interval of the calibration curve are plotted. The operating method is valid when:

— the correlation coefficient is at least 0.99,

— the residuals of each calibration level are randomly distributed around the calibration curve.

Calculate the mean and relative standard deviation for the lowest and highest calibration level.

When the ratio of the estimated standard deviation of the lowest and the highest calibration level is less than 0.5 or greater than 2.0, a more precise estimation of the calibration curve may be obtained using weighted linear regression. Both linear and quadratic weighting functions are applied to the data to find the most appropriate weighting function to be employed. If the means compared to the calibration curve show a deviation from linearity, two-dimensional linear regression is used.

ACCURACY

Recovery. For assay determinations a recovery of 90 per cent to 110 per cent is to be obtained. For other determinations, for example, for trace element determination the test is not valid if recovery is outside of the range 80 per cent to 120 per cent at the theoretical value.

REPEATABILITY

The repeatability is not greater than 3 per cent for an assay and not greater than 5 per cent for an impurity test.

Validation of analytical procedures is described in the USP general chapter <1225> *Validation of compendial procedures* [67] which is harmonized with the corresponding ISH Guidance. So it differ little from the general chapter SPU-Eur.Ph. [11]. The USP don't give some special recommendations for validation of the AAS procedures.

7.2. Consideration of the SPU-Eur.Ph. approach

7.2.1. Method

7.2.1.1. Problem of linearity and choice of a procedure scheme

A characteristic feature of AAS is that, in contrast to absorption spectrophotometry, the linear dependence of absorbance on concentration has no theoretical background, but under reproducible conditions the linearity is usually holds [67 <851>]. In practice the linearity holds in a quite narrow range. The typical dependence of absorbance on concentration is shown in the Figure 7.1.

As can be seen, we can speak about the directly proportionality (i.e. the calibration line passes through the origin of coordinates) only for the absorbance range below approximately 0.25. In addition, for various elements to be determined and various

equipments this range, generally speaking, can be different. In other cases the linear relationship holds for a narrow range of concentrations, but the Y-intercept is not zero.

This leads to the fact that SPU-Eur.Ph., though recommends working in the linear range of the calibration curve, but permits the use of curved sections of the curve after its processing using appropriate mathematical software [2-3]. For other pharmacopoeial methods (HPLC, GC, SPh, titration) the SPU-Eur.Ph. gives no such recommendations.

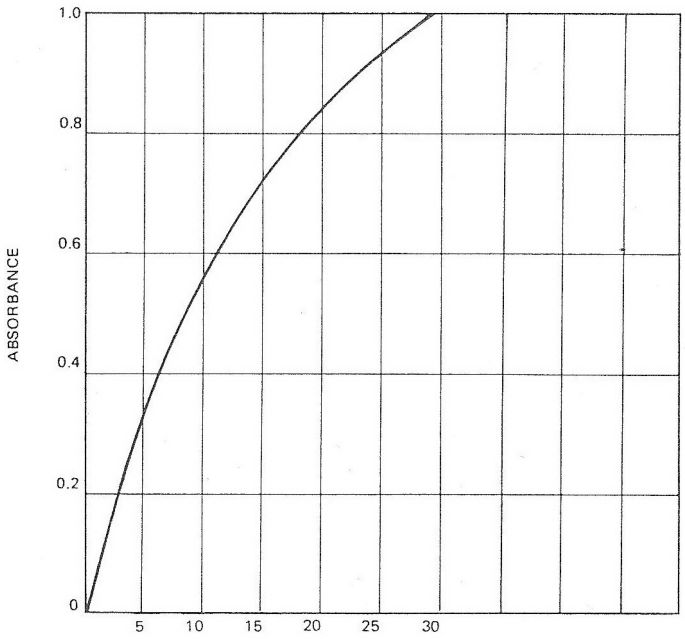


Figure 7.1. Nameplate dependence of absorbance on iron concentration (ppm) for Varian atomic absorption apparatus 220 FS Double Beam AA (USA)

This uncertainty with the linearity causes in the Eur. Ph. [2-3] allows the reference standard method for AAS, but not describes it. Eur.Ph. describes only the *Method 1 – direct calibration* and *Method 2 - standard additions*. This is because, apparently, that historically the first AAS method found application in the control of metals in alloys and other objects with a broad analytical range of concentrations. For these ranges the linearity of the AAS is generally performed badly, and if they are, then the Y-intercept of the calibration line is usually statistically and practically significant, which makes it impossible to use the reference standard method and, accordingly, the use of the standardized validation scheme (see chapters 1-6). Perhaps, therefore, the Eur.Ph. describes the *Methods 1* and *2* [2-3].

At the same time, the analytical ranges at the control of drug products are much narrower than for the control of alloys, and it may allow, in certain cases, application of the reference standard method (RSM). Therefore, the USP in the general chapter <851>*Spectrophotometry and light-scattering* [67] describes the RSM for the AAS as a primary, and in specific monographs on drug products uses both the RSM (Ferrous

Fumarate Tablets) and the calibration graph method (CGM) (*Ferrous Fumarate and Docusate Sodium Extended-Release Tablets, Oil-and Water-Soluble Vitamins with Minerals Oral Solution*) [67]. With this in mind, the SPU introduced the RSM in the national part of the general chapter on AAS [2].

Note, however, that there are no acceptance criteria of the RSM applicability to the AAS procedures in the SPU-Eur.Ph. (as well as in the USP).

In cases where the AAS procedure is included into the registration dossier (and therefore it is assumed its use in the state control laboratories which may have an equipment of lower level), use of the calibration graph method (CGM) for analysis of medicines is more general than the use of the reference standard method (RSM).

Given the narrow range of linearity in the AAS, the possibility of applying *Method 2 - standard additions* for quantitative analysis of medicines gives very great rise to doubt. Indeed, if the application of the RSM or SGM requires (according to the SPU-Eur.Ph.) the linearity in the range 70-130% of the nominal value, then the use of the standard additions method requires the linearity in the range 70-230%, which is hardly possible with good accuracy (see the Figure 1). Talking about the application of the standard additions method to the non-linear calibration curve is incorrect, since we don't know a priori, what range of this curve corresponds to the test solution. Therefore, in general, the use of the standard additions method in the AAS procedures is possible for control of impurities (where required accuracy is low), but not for quantitative determination of medicines.

7.2.1.2. Metrological correctness of SPU-Eur.Ph. AAS procedure schemes

The SPU-Eur.Ph. pointes out that a routine analysis is carried out with use of 3 reference solutions to generate the calibration graph. In this case, the number of degrees of freedom for the calibration line ($Y = a + b \cdot X$) is equal to $\nu = 3-2 = 1$. One-tailed *t*-criterion for probability of 0.95 is 6.31. Talking about the reliability of the calibration line is not necessary. In view of the uncertainty of measurements of a test solution, this procedure is hardly amenable to validation.

It is obvious that no three-point curvilinear calibration line may be even mentioned, since even for a quadratic function ($Y = a + b \cdot X + c \cdot X^2$) the number of degrees of freedom is equal to zero.

Note that the USP in the case of AAC procedures is much more correct. So, for example, to quantify iron the *Ferrous Fumarate and Docusate Sodium Extended-Release Tablets* monograph uses the AAS procedure with plotting a calibration graph by 5 points (instead of three, as recommended by the SPU-Eur.Ph.) [67].

7.2.2. Correctness of SPU-Eur.Ph. validation criteria

7.2.2.1. Limitation of a ratio of relative standard deviations (RSD) for points with the largest and smallest concentrations of a calibration line

This ratio should be in the range of 0.5-2.0. Otherwise, the calculation of the calibration line needs in using weighting factors for points, or using a weighted least-squares method.

Given that the *RSD* values for the largest and smallest concentrations have the equal number of degrees of freedom $v_1 = v_2 = 5\text{-}1 = 4$, and using the Fisher distribution [26], it can be shown that an interval of 0.5-2.0 corresponds to a confidence probability of 0.90. However, that probability of the *RSD* values difference is not statistically significant. In analytical practice, that distinction of *RSD* values is significant if the probability is above 0.95 and is highly significant with probability above 0.99 [26]. In our case (v1 = v2 = 5-1 = 4) it corresponds to the ratio ranges of 0.4-2.5 ($p = 0.95$) and 0.25-4.0 ($p = 0.99$). The actual *RSD* ratios are almost always met to such a wide tolerances.

The issue of limitation of *RSD* values ratio becomes even more confusing if we consider that plotting an unweighted calibration graph uses the assumption of constancy of the *absolute*, rather than relative, standard deviations in the analytical range (otherwise we must use the weighting factors). The analytical range covers 70-130% of nominal concentrations. So, considering the constancy of the absolute standard deviations, we get that the relative standard deviations *RSD* for the largest and smallest concentrations will differ in the 130/70 = 1.86 times, that is almost equal to the limits of the requirements (0.5-2.0). Given the inevitable statistical fluctuations of the *RSD* values of the 5 replicates, the *RSD* values ratio beyond the range of 0.5-2.0 is a fairly usual event.

As can be seen, limitation of the ratio of *RSD* values for the largest and smallest concentrations within 0.5-2.0 is very controversial.

7.2.2.2. Use of weight factors for obtaining the calibration line

In this case, we use a weighted least squares method (WLSM). The inverse values of the corresponding absolute dispersions of the points are usually used as weights [78]. The WMNK allows plotting the calibration straight line (in the general case, the curve line) more correct. However, this is only true if the variances are universe or are close to them (i.e., obtained for a large number of points). In the case of 5 measurements (such sample size is recommended by the SPU-Eur.Ph. to obtain points dispersions when plotting the calibration graph in AAS procedures), the points dispersions in another series of experiments may be different in 2 times (which is statistically insignificant by the Fisher criterion at the level of 95% [26]), and the "weighted" calibration line will be completely different, i.e. irreproducible.

Even more confusing is the question of the statistical evaluation of the "weighted" calibration straight line. Indeed, the residual standard deviation and correlation coefficient of such a "weighted" straight line does not have the meaning which they have for a common "unweighted" straight line. So, for example, an analytical procedure, based on a "weighted" calibration straight line with a correlation coefficient of 0.990, is not necessarily better than a procedure based on a usual “unweighted” calibration straight line with the correlation coefficient of 0.985.

As can be seen, the question of introducing weight factors is quite controversial and uncertain for validation.

7.2.2.3. Requirements to a correlation coefficient

From the text of the EPU-Eur.Ph. methodology for the validation (see the section 1.2) it is not clear how many points are processed by the least square method (LSM). Indeed, the expression "*the calibration curve is calculated by least-square regression from all measured data*" can be seen as 4-5 = 20 ordinate values are processed versus 4 abscissa values (concentrations). This processing option using the LSM is meaningless when checking the linearity, because it only masks the possible non-linearity and creates the appearance of increasing the number of degrees of freedom. Going this way, we can try (unsuccessfully) to process using the LSM the 20-multiple measurements of one concentration.

On the other hand, it read: "When the ratio of the estimated standard deviation of the lowest and the highest calibration level is less than 0.5 or greater than 2.0, a more precise estimation of the calibration curve may be obtained using weighted linear regression". Permission to use the weight factors for points indicates that we process with LSM only n = 4 ordinate values (mean of 5 measurements) versus 4 abscissa values (concentrations).

The correlation coefficient R_c can be calculated from the ratio [26]:

$$R_c^2 = 1 - \frac{SD_{rest}^2}{SD_{Co}^2}. \tag{7.1}$$

Here SD_{rest} is the residual absolute standard deviation around the points of the calibration line, SD_{Co} is the standard deviation of the calibration line points around the average (nominal value). Values of SD_{rest} and SD_{Co} in equation (7.1) can be taken in any of the same units. To evaluate the correctness of the correlation coefficient, these values are suitable to take as the percentage of the nominal value, and that is what we will do next.

It should be noted that for narrow tolerances and broad analytical ranges, the R_c values can exceed 0.999 (see, for example, the Table 7.1). So in many cases it is more convenient to use the square of the correlation coefficient (R_c^2) instead of the R_c, because the R_c^2 is smaller.

Since we have a range of 70-130% of a nominal value, and to plot a calibration graph 4 points are taken, it is advisable to take these values: 70, 90, 110 and 130% of a nominal value. It corresponds to SD_{Co} = 25.82%. Then, considering the R_c = 0.99, from the equation (7.1) we can get SD_{rest} = 3.64%.

Each point is the analysis of one concentration. Thus, the confidence interval of Δ_{As} for a single analysis is equal to [26]:

$$\Delta_{As} = t(95\%, n-2) \cdot SD_{rest} = 2.92 \cdot 3.64 = 10.6\%. \quad (7.2)$$

Here: n = 4 (according to the SPU-Eur.Ph,[2]).

As can be seen, the resulting confidence interval for points of the calibration graph is unacceptable for quantitative analysis of medicines, since corresponds to tolerances of (±) 10.6/0.32 = 33.2% of nominal value [11]. But it is still just the uncertainty of the calibration graph. Assuming that the uncertainty of the test solution should be, at least, not less, we'll get the total procedure uncertainty of $\sqrt{2} \cdot 33.2 = 47.0\%$.

Of course, this uncertainty is unacceptable for pharmaceutical analysis. As we can see, the SPU-Eur.Ph. requirements to the correlation coefficient are incorrect.

Note another aspect. If for obtaining the calibration curve during the validation 4 points are required, at that for routine analysis we may use only 3 points. There is a violation of the basic principle of validation: validation of a procedure must be carried out under the same conditions as the procedure itself.

7.2.2.4. Requirements to a residual standard deviation around a calibration line

In accordance with the SPU-Eur.Ph., these deviations must be "distributed around the calibration line randomly."

What this requirement means is unclear, since we have only 4 points. Therefore, options for distribution of points around the straight line only two: 1) three on one side and one on the other; 2), two on each side (it is clear that all four points in principle cannot be on one side of the straight line, obtained by means of the least squares method).

What does it mean in this case "random" and "nonrandom" distribution of points? As can be seen, this requirement is incorrect when using the method of least squares.

7.2.2.5. Accuracy and repeatability

An assay or trace analysis is characterized by a total procedure uncertainty which includes a systematic error (characterizing accuracy of results) and a random component of the uncertainty that characterizes repeatability.

The SPU - Eur. Ph. does not define any requirements for accuracy. It only states: "For assay determinations a recovery of 90 per cent to 110 per cent is to be obtained. For other determinations, for example, for trace element determination the test is not

valid if the recovery is outside of the range 80 per cent to 120 per cent at the theoretical value". It is presumed that those requirements should be developed for each particular case. However, it is unclear how they should be developed. In addition, it is unclear, why we explore the linearity in the range 70-130% if correctness is checked in a narrower range.

More specifically the SPU-Eur.Ph. expresses the requirements for repeatability of results: "The repeatability is not greater than 3 per cent for an assay and not greater than 5 per cent for an impurity test". Apparently, the confidence interval of the repeatability of the single sample definition is meant. However, since there are no requirements to the total procedure uncertainty (taking into account, inter alia, the uncertainty of calibration), the requirements to the repeatability of results do not allow to characterize the precision of the procedure.

Summing up, it can be concluded that the SPU-Eur.Ph. approach to validation of AAS procedures does not allow to confirm that the procedure has the necessary repeatability and accuracy, because the proposed criteria are incorrect and inadequate.

7.3. The proposed approach to validation of quantitative AAS procedures for quality control of medicinal products

The objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose [1, 3]. Therefore, validation must be carried out under the same conditions as the procedure.

The calibration graph method (CGM) is more general in nature than the reference standard method (RSM). So when obtaining the calibration graph, it is reasonable to verify the possibility of using the simpler RSM instead of the GSM. As shown in the Chapters 1-5, in the case where the Y-intercept (a) of the calibration line (7.3A) is not significantly different from zero (statistical and practical insignificance criteria are described earlier (see chapters 1-5)), the RSM is applicable. Validation of an AAS procedure in this case, formally, is not different than other methods such as spectrophotometry (see Chapter 2). The basic principles of validation of the AAS procedure using the RSM are described in chapters 1-5 and are outlined in the SPU [11].

As a result of validation we should also develop system suitability requirements which allow assessing at the routine analysis whether current conditions are met to those during the validation.

7.3.1. Specificity of validation of AAS procedures with use of a calibration line

For AAS procedures we have often to consider with a narrow linear range and that the calibration straight line can significantly (statistically or practically (see the Chapter 1) not pass through the origin. In this case the reference standard method (RSM) is not applied, and we have to apply the calibration graph method (CGM). The concentration of the analyte (C) in the test solution is calculated on the basis of the measured absorbance (A) using the equation of the calibration straight line. This equation can

be written using both true concentrations (C) and normalized coordinates - X (as a per cent of the nominal concentration) and Y (as a per cent of the nominal absorption):

$$X = b \cdot Y + a \,. \tag{7.3a}$$

$$X = b_1 \cdot A + a_1. \tag{7.3b}$$

$$C = b_2 \cdot A + a_2. \tag{7.3c}$$

As noted above, the standardized validation schemes for drug quality control procedures (see Chapters 1-6) has been developed for the RSM proper.

Thus, there is a general problem of developing the standardized validation scheme for procedures using the MCG.

The RSM uses simultaneous measurements of absorbances of a test and reference standard solutions. These solutions are completely equivalent, and the uncertainty of their absorbance measurement is assumed to be the same. Calculation of the concentration in the test solution is carried out using a simple proportion. Prognosis of a total procedure uncertainty is based on requirements to repeatability of a final analytical operation and metrological requirements to volumetric glassware and weights (uncertainty of sample preparation).

The CGM uses simultaneous absorbance measurements of a test solution and several standard solutions that are used to plot the calibration straight line (7.3). The uncertainty of determination of the test solution concentration using the equation (7.3) includes the uncertainty of absorbance measurement of the test solution and the uncertainty of the calibration line (7.3). The uncertainty of the calibration is not predicted (as for the RSM). It is calculated on the basis of metrological characteristics of the calibration straight line (7.3) and is associated both with the uncertainty of calibration solution absorbances (including the final analytical operation and sample preparation) and deviations from linearity.

Different values may be used as a measure of uncertainty evaluation of the calibration line (7.3). In particular, they are uncertainties of a and b coefficients. However, a more correct evaluation of the calibration uncertainty seems to be the residual standard deviation of SD_{rest}.. Indeed, if the calibration solutions are obtained and measured in the same conditions as the test solution, the point corresponding to the test solution is of the same parent population as the calibration solutions, and can therefore be described by SD_{rest}.

It should be noted that the same approach we applied in the development of the standardized validation scheme for procedures using the reference standard method (see Chapters 1-6).

7.3.2. The number of points of a calibration line

As noted above, a feature of the CGM is that a total uncertainty of results for determining a concentration of an analyte (Δ_{As}) depends not only on the uncertainty (including the sample preparation) of the analytical signal (in the case of AAS it is the absorbance), but also from the uncertainty of the calibration graph. This means that we must establish criteria for the correctness of the analytical signal measurements both to the analyte, and the parameters of the calibration line.

In a general case, a calibration graph must be plotted every time together with an analysis of a test solution (it is common for the AAS). Those cases where the same calibration graph is available in the same lab for a few days (weeks, months), must be justified in separately each time. Such justification, generally speaking, is beyond the validation of the procedures using the CGM.

Thus, CGM has to consider the need to plot every time a calibration graph, which significantly extends the analysis compared to the RSM. So checking linearity and plotting the calibration line using 9 points (plus 1 reference standard), as is customary when validating the RSM procedure (see Chapter 1-2), for the AAS procedures using the CGM is unrealistic in practice.

The SPU-Eur.Ph. recommends to use $n = 4$ points to plot the calibration line [2]. However, in this case, the calibration line has the $v = 4\text{-}2 = 2$ degrees of freedom and the *t*-criterion of 2.92 [26]. Increase of the number of points to $n = 5$ allows to reduce the *t*- criterion up to 2.35 [26], which significantly reduces the uncertainty of the calibration line, and, therefore, the total procedure uncertainty. It should be noted that this is the case of the USP AAS procedure of determination of iron in the *Ferrous Fumarate* and *Docusate Sodium Extended-Release Tablets* [67].

7.3.3. Normalized coordinates

Requirements to validation characteristics are easier developed in normalized coordinates, because they do not depend on the specifics of a particular object, but only from the content tolerances and range (see Chapter 2).

In the RSM (see section 2.2), the conversion to the normalized coordinates is done by dividing the concentration and the analytical signal of the sample solution by the concentration and the analytical signal of the reference solution. In the CGM, the calibration solution with a nominal concentration C_{nom} and corresponding nominal absorbance A_{nom} can be used instead of the reference standard solution. In this case, the expressions in the normalized coordinates would be the same as for RSM (see section 2.2):

$$X_i\,(\%) = 100 \cdot C_i\ / C_{st}. \quad C_{st} = C_{nom}.$$

$$Y_i\ (\%) = 100 \cdot A_i\ / A_{st}. \quad A_{st} = A_{nom}. \tag{7.4}$$

$$Z_i\ (\%) = 100 \cdot Y_i\ / X_i\ .$$

However, the use of the normalized coordinates in the CGM is not as efficient as in the RSM.

Note that the *Y* value is only need to construct a calibration graph in the visual normalized coordinates. To obtain the correlation coefficient, the range, the residual standard deviation and the requirements to accuracy and repeatability we use only the *X* values. The recovery factor (*RF*) is a far more important value than *Y* value to obtain validation characteristics:

RF(%) = (introduced concentration/found concentration)·100. (7.5)

In the RSM, the *RF* coincides with the $Z_i = 100 \cdot Y_i/X_i$. But it is easy to see that in the CGM we have $RF_i \neq Z_i$, as in this case, unlike the RSM, we have $A_i \cdot C_{nom}/A_{nom} \neq C_i$ (found). Thus:

Reference standard method: $Z = RF.$ (7.6)

Calibration graph method: $Z \neq RF.$ (7.7)

Given (7.7), in the CGM it is sufficient to use only the normalized *X* value calculated by the ratio (7.4). In this case the calibration line (7.3b) is plotted in the *X-A* coordinates, and the recovery ration *RF* is calculated by the ratio of (7.5). However, as it will be shown below, the use of the complete normalized coordinates, i.e. plotting the line (3a) in the *X-Y* coordinates allows also evaluating the applicability of the much simpler RSM for a particular AAS procedure on basis of the same experimental data. Therefore, further we'll use complete normalized coordinates (7.4) and the line (7.3a) as well.

7.3.4. Linearity range study

The SPU-Eur.Ph. [2] requires that the range for an assay should cover not narrow than 70-130% of the nominal value. In view of the fact that this procedure can be applied to the "Dissolution" test, the range can be extended to 50-150% of the nominal value. It should be noted that the USP for the control of vitamins and nutritional supplements uses close ranges of the calibration line. For example, in the case of *Oil-and Water-Soluble Vitamins with Minerals Oral Solution* [67] for the determination of iron the calibration line is plotted in the range of 2.0-8.0 μg/ml, with the mean value

5 μg/ml. It corresponds to the range of 40.0-160% of the nominal value, which is close to the 50-150%.

Note that in some cases (for narrow tolerances content) we can use the "common" [11] SPU-Eur.Ph. range of 80-120%.

Given the number of points of the calibration graph n = 5, the Table 7.1 shows the recommended concentrations of calibration solutions (as a per cent of the nominal concentration of 4 *ppm*) to plot the calibration line for the CGM with different analytical ranges, as well as their corresponding standard deviations SD_{Co}. The Table 7.1 shows the recommended concentrations (as a per cent of the nominal concentration of 4 *ppm*) for n = 9 model solutions, which are also used in the RSM to verify the linearity as well as their corresponding standard deviations SD_{Co}.

Table 7.1

The recommended concentrations of solutions (as a per cent of the nominal concentration of 4 *ppm*) to construct a calibration graph in the CGM, the number of points (n) of the calibration line and the corresponding number of degrees of freedom (v), the recommended concentrations of model mixtures (used also to check the linearity in the RSM) and their corresponding standard deviations SD_{Co}

№	CGM			RSM		
	range 80-120%	range 70-130%	range 50-150%	range 80-120%	range 70-130%	range 50-150%
1.	80.0	70.0	50.0	80.0	70.0	50.0
2.	90.0	85.0	75.0	85.0	77.5	62.5
3.	100.0	100.0	100.0	90.0	85.0	75.0
4.	110.0	115.0	125.0	95.0	92.5	87.5
5.	120.0	130.0	150.0	100.0	100.0	100.0
6.				105.0	107.5	112.5
7.				110.0	115.0	125.0
8.				115.0	122.5	137.5
9.				120.0	130.0	150.0
n	5			9		
v	3			7		
SD_{Co}	15.81	23.72	39.53	13.69	20.54	34.23

7.3.5. Total procedure uncertainty and its components

The AAS method is applied in the pharmacopoeial analysis to assays of drug products only. In the case of the impurities control, the AAS method is also applied to medicinal substances. However, this analysis does not formally different from the assay of drug products. Therefore, further consideration is only for the drug products.

As we have seen (see Chapter 2), the total relative uncertainty of the concentration determination Δ_{As} should not exceed the maximum value of $max\Delta_{As}$, which in the case of the drug product is calculated from the equation:

$$\Delta_{As} \le \max \Delta_{As} = 0.32 \cdot B. \tag{7.8}$$

Here: B is the tolerance (half-sum of upper and lower limits) of the analyzed component of the medicine, as a percentage of the nominal content.

Total uncertainty of the AAS procedure Δ_{As} includes a few constituents:

1. Systematic error (δ_1) is caused by matrix effects (i.e., the placebo effect). The δ_1 value characterizes the procedure specificity (see below) both for RSM and for CGM and should be insignificant compared with the maximum acceptable total uncertainty of the procedure ($max\Delta_{As}$), i.e., given (7.8) (see section 2.3.3):

$$\delta_1 \le \max \delta_1 = 0.32 \cdot \max \Delta_{As} = 0.1 \cdot B. \tag{7.9}$$

   The δ_1 value can be, in principle, is reduced to an acceptable level by changing the procedure proper, for example, by introducing a placebo into the reference standard and calibration solutions.

2. The uncertainty of calibration (Δ_{cal}). A characteristic feature of the uncertainty of calibration (Δ_{cal}) is that it cannot be reduced when we run the procedure without modifying the calibration scheme and/or analytical range. Sources of the calibration uncertainty (Δ_{cal}) vary considerably for the RSM and CGM. The Δ_{cal} can be evaluated and regulated at the linearity study in the RSM (see the Section 2.3.4) or when we plot the calibration line in the CGM.

3. The random component of the uncertainty related directly with the analysis of the test solution (CGM) or with the analysis of the test and reference standard solutions (RSM). This uncertainty includes both the uncertainty of the final analytical operation (absorbance measurement) and the uncertainty of sample preparation. The latter for the procedure with multiple dilutions of the analyzed solutions can greatly exceed the uncertainty of the final analytical operation [17]. It is easy to see [26] that in the RSM the random component of the uncertainty about √2 times greater than in the CGM or specific absorbance method. A random component can be reduced when we run the analysis proper by increasing the number of replicate definitions (including weighing).

7.3.6. Criteria of procedure specificity

The criteria of procedure specificity are the same for the RSM and CGM and are the same as for the spectrophotometry method (see Section 2.6.2). Absorbances of the blank (A_{blank}) and placebo ($A_{placebo}$) solutions should not significantly influence decisions about the quality of the sample, so their part in the total absorbance (A_{nom}) of the test sample of nominal composition should be insignificant compared with the maxi-

mum acceptable total procedure uncertainty ($max\Delta_{As}$), i.e., taking into account the (7.8-7.9):

$$\begin{aligned} 100 \cdot (A_{blank} / A_{nom}) &\le \max \delta_1 = 0.32 \cdot \max \Delta_{As} = 0.10 \cdot B. \\ 100 \cdot (A_{placebo} / A_{nom}) &\le \max \delta_1 = 0.10 \cdot B. \end{aligned} \tag{7.10}$$

7.3.7. Validation criteria for the reference standard method

This question is discussed in detail earlier (see Section 2.3). A specific feature of validation of procedures in the RSM is that all validation characteristics are obtained in the study of linearity using 9 model mixtures.

The uncertainty of calibration (Δ_{cal}) in the RSM is associated with significance (statistical or practical) of the *Y*-intercept of the calibration line (3a) and has a systematic character [2, 11, 26]. Given the general analytical requirements for maximum reducing the systematic error, it would be appropriate to require that the systematic error of the calibration (Δ_{cal}) in the RSM was not significant compared with the maximum acceptable total procedure uncertainty ($max\Delta_{As}$), i.e., given (7.8) (see Chapter 2):

Reference standard method:

$$\Delta_{cal} = \delta_2 \le \max \delta_2 = 0.32 \cdot \max \Delta_{As} = 0.1 \cdot B. \tag{7.11}$$

For the nominal concentration (i.e. X = 100 %) δ_2 = 0, reaching maximum values at the boundaries of the analytical range of concentrations (X_{min} and X_{max}). As it was shown for the RSM (see Chapter 2), the δ_2 value for all points of the analytical range does not exceed the maximum value ($max\delta_2$) of the ratio (7.10) if the *Y*-intercept (a) of the ratio (7.3a) meets the requirement for drug product procedures:

Reference standard method:

$$a \le \frac{0.1 \cdot B}{1 - (X_{\min} / 100)}. \tag{7.12}$$

A characteristic property of the systematic error (δ_2) associated with the ratio (7.12), is its predictability. It is entirely defined by the *Y*-intercept (a) of the ratio (7.3) and cannot be reduced within the RSM without reduction of the a value or without narrowing the analytical range (i.e., the X_{min} value). Therefore, the requirement (7.11) of insignificance of the $\delta_2$value is consistent with regular analytical practice requiring elimination of the systematic error.

It should be noted that the systematic error (δ_2) connected with the ratio (7.12) has a different sign (plus or minus) on the ends of the analytical range. Therefore, the average recovery factor *RF* (7.5) for the model mixtures scattered across the range, can be very close to 100% and does not have a significant systematic error. I.e. the mean of the (100-*RF*) values for the model mixtures cannot detect in the RSM the systematic error associated with inequality to zero of the *Y*-intercept (a). This systematic error is

regulated by the relationship (7.12). The average recovery factor (*RR*) for analysis of the model mixtures using the RSM should not have the significant systematic error, i.e. it must meet the ratio (7.9).

In the case of the RSM, the criterion (7.12) is checked for the analysis of 9 model mixtures (v = 7, Table 7.1) (see Chapter 2). Formally, in the case of the CGM, it can be tested at the stage of plotting the calibration graph using 5 points. However, due to the low number of degrees of freedom (v = 3, table 7.1), the uncertainty of the *Y*- intercept (a) is so great (see the experiment below) that testing the criterion (7.12) becomes impossible.

Because of the systematic error in the RSM is not significant, then the total procedure uncertainty (Δ_{As}) coincides with the random uncertainty of analysis of model mixtures (Δ_{sample}), i.e.:

(7.13)

RSM: $$\Delta_{sample} \le \max \Delta_{sample} \approx \max \Delta_{As} = 0.32 \cdot B.$$

Bearing also in mind that in the case of RSM v = 7 (Table 7.1), we can obtain [26]:

RSM: $$\Delta_{sample} = t(0.95;\ 7) \cdot SD_{rest} = 1.89 \cdot SD_{rest}. \quad (7.14)$$

From (7.13-7.14) we can get the requirement to the residual standard deviation SD_{rest}:

RSM: $$SD_{rest} \le \max \Delta_{As} / 1.89. \quad (7.15)$$

Substituting (7.15) in the expression (7.1), we can obtain the requirement to the minimum correlation coefficient of the line plotted on 9 model mixtures.

The total requirements for the validation characteristics of the RSM are presented in the Table 7.2.

Table 7.2

Validation criteria for the reference standard method (analysis of 9 model mixtures)

B%	$max\Delta_{As} = max\Delta_{sample}$%	$max\delta_1$ %	$max\delta_2$ %	SD_{rest} %	$minR_c^2$	*max a* %
Range 80-120%						
5.0	1.6	0.50	0.50	0.84	0.99620	2.50
7.5	2.4	0.75	0.75	1.27	0.99144	3.75
10.0	3.2	1.00	1.00	1.69	0.98478	5.00
12.2	3.9	1.22	1.22	2.06	0.97735	6.10
15.0	4.8	1.50	1.50	2.53	0.96577	7.50
20.0	6.4	2.00	2.00	3.38	0.93914	10.0
Range 70-130%						

5.0	1.6	0.50	0.50	0.84	0.99831	1.67
7.5	2.4	0.75	0.75	1.27	0.99620	2.50
10.0	3.2	1.00	1.00	1.69	0.99324	3.33
12.2	3.9	1.22	1.22	2.06	0.98994	4.07
15.0	4.8	1.50	1.50	2.53	0.98478	5.00
20.0	6.4	2.00	2.00	3.38	0.97295	6.67
Range 50-150%						
5.0	1.6	0.50	0.50	0.84	0.99939	1.00
7.5	2.4	0.75	0.75	1.27	0.99863	1.50
10.0	3.2	1.00	1.00	1.69	0.99757	2.00
12.2	3.9	1.22	1.22	2.06	0.99638	2.44
15.0	4.8	1.50	1.50	2.53	0.99452	3.00
20.0	6.4	2.00	2.00	3.38	0.99512	4.00

7.3.8. Validation criteria for the calibration line method

Unlike the RSM, in the CGM the linearity is checked not at the stage of analysis of 9 model mixtures, but already during the stage of plotting the calibration graph (by 5 points - see above). The linearity criteria for the calibration line are the criteria for the system suitability test as well. If these criteria fail, the analysis of the samples cannot be carried out.

Calibration uncertainty in the CGM (Δ_{cal}) is connected with uncertainty of the calibration graph parameters. The Δ_{cal} value is associated with both uncertainty of absorbance of calibration solutions (random component) and deviations from linearity. The last is systematic for each concentration, but random for the entire line (as it is obtained by the least squares method). Therefore, if the calibration line is plotted for each test (and this is the case we are considering), we can consider that the uncertainty of calibration in the CGM has a random character.

Using the parameters of the calibration graph in the CGM, we can evaluate the confidence interval Δ_{cal} with some probability, which formally does not differ from a confidence interval of repeatability for the tested solution. Therefore, the requirement of insignificance of the Δ_{cal} value as compared with the total procedure uncertainty Δ_{As} in the CGM may be too tough. With this in mind, the regulation of Δ_{cal} in CGM, unlike RSM, is not definite, and here different approaches may be offered, that will be considered below.

One of the features of procedure validation in the CGM is that here we cannot evaluate the procedure uncertainty on the basis of the linearity study only (as it is done during procedure validation in the RSM - see Chapters 1-5). The linearity study characterizes only the uncertainty of calibration Δ_{cal} and can be used in the formation of the system suitability criteria.

The total uncertainty of analysis results (in particular, analysis of model mixtures) (Δ_{As}) is determined by several factors, among which the main are:

- The uncertainty associated with the calibration line (Δ_{cal}); it is caused by the uncertainty of parameters *a* and *b* of the calibration line (7.3a). This uncertainty is characterized by the residual standard deviation SD_{rest}, which corresponds to a confidence interval, similar to (7.14) for RSM, but with the number of degrees of freedom, ν = 3 (see the Table 7.1):

$$CGM: \qquad \Delta_{cal} = t(0.95;\ 3) \cdot SD_{rest} = 2.35 \cdot SD_{rest}. \tag{7.16}$$

  The Δ_{cal} characterizes the uncertainty of calibration of the AAS procedure and must not exceed the maximum acceptable value of $max\Delta_{cal}$..

  From the equation (7.16) we can get an expression for SD_{rest} of 5-point line that is similar to the expression (7.15):

$$SD_{rest} = \Delta_{cal} / 2.35. \tag{7.17}$$

- The uncertainty associated with the test solution (Δ_{sample}), i.e. with the uncertainty of measuring its absorbance (*A*) and sample preparation. The Δ_{sample} can be found in the validation process by the analysis of model mixtures and calculation of their concentrations using the calibration line (7.3a).

  If the requirements of specificity (7.10) are met, the Δ_{sample} value describes the repeatability of results (i.e. the random component of uncertainty) of the AAS procedure.

With this in mind, the total uncertainty of the concentration determination (Δ_{As}) can be written as follows [26]:

$$\Delta_{As} = \sqrt{\Delta_{cal}^2 + \Delta_{sample}^2} \le \max \Delta_{As}. \tag{7.18}$$

The analysis of only one sample (according to the procedure) and calculation of its concentration on the calibration line (7.3a) can assesses only the uncertainty of calibration Δ_{cal} (using the metrological characteristics of the calibration line). The actual Δ_{sampl} value is unknown for us. It can be estimated from the results of the analysis of model mixtures in the process of validation.

Thus, for correct validation we need to bring some criteria of acceptability to the ratio of the Δ_{cal} and Δ_{sample} values.

The ratio (7.8) shows that there is a criterion for the total Δ_{As} only, so we need some assumptions (which must be confirmed experimentally) for one of the variables - Δ_{cal} or Δ_{sample}. It is reasonable to set these assumptions to the Δ_{cal} value, since this value will always be in calibration, and therefore the assumptions will always be checked during the system suitability test.

To develop criteria for acceptability of the Δ_{cal} value in the CGM, we can offer 2 approaches based on different assumptions.

7.3.8.1. Approach 1: assumption of insignificance of calibration uncertainty

The calibration line is plotted using more solutions than the analysis of the test sample. Therefore, similar to the RSM (see above), it can be assumed that the uncertainty of the calibration in the CGM should also be insignificant. This assumption of the insignificance of the calibration uncertainty is quite in line with the usual approach to procedure validation in the RSM, and the formulas for the criteria coincide with previously obtained expressions for the RSM (see Chapter 2).

Under this assumption, the Δ_{cal} value must be insignificant compared with maximum acceptable total uncertainty of the procedure $max\Delta_{As}$, i.e., given (7.8):

$$\textit{CGM. Approach 1}: \quad \Delta_{cal} \le \max \Delta_{cal} = 0.32 \cdot \max \Delta_{As} = 0.10 \cdot B. \quad (7.19)$$

This ratio is the same as the requirement (7.11) of the RSM. From the equations (7.17, 7.19) we can get requirements to the residual standard deviation SD_{rest} of the calibration line:

$$SD_{rest} \le \max SD_{rest} = \max \Delta_{cal} / 2.35 = 0.10 \cdot B / 2.35 = 0.0435 \cdot B. \quad (7.20)$$

From the ratios (7.1, 7.20) we can find the requirements *to* $minR^2$ or *minR* in the *Approach 1*.

An advantage of the *Approach 1* is that in this case we can ignore the calibration uncertainty in the total procedure uncertainty (Δ_{As}), which, in accordance with the equations (7.18- 7.19), comes to Δ_{sample} , i.e., given (7.8):

$$\Delta_{sample} \le \max \Delta_{sample} \approx \max \Delta_{As} = 0.32 \cdot B. \quad (7.21)$$

This considerably simplifies the evaluation of the validation results. In addition, the *Approach 1* transfers the total acceptable procedure uncertainty on a test specimen, which makes requirements to the procedure uncertainty more liberal.

A disadvantage of the *Approach 1* is that it imposes the strict requirements (7.20) to the calibration line, which not always can be implemented in practice. This is due, in particular, with a small number of degrees of freedom of the line ($\nu = 3$), which leads to a large *t*-criterion (2.35) in the equation (7.16).

7.3.8.2. Approach 2: calibration uncertainty is equal to a sample analysis uncertainty

When the calibration uncertainty is significant, i.e. ratios (7.19-7.20) are not (or can be not) completed, we must make some assumptions about the proportion between the uncertainties of calibration Δ_{cal} and repeatability of the results of the test sample Δ_{sample} in the total procedure uncertainty Δ_{As}. Because the precision of the analysis of

the calibration solutions and test solution is roughly the same, it can be assumed that their maximum acceptable uncertainties are equal, i.e.,

$$\max \Delta_{cal} = \max \Delta_{sample}. \tag{7.22}$$

In this case, the ratios (7.18) and (7.22) give:

$$\begin{aligned} &\max \Delta_{As} \le \sqrt{2} \cdot \max \Delta_{cal} = \sqrt{2} \cdot \max \Delta_{sample}. \\ &\max \Delta_{cal} = \max \Delta_{sample} \le 0.707 \cdot \max \Delta_{As} = 0.226 \cdot B. \end{aligned} \tag{7.23}$$

Then from the equations (7.17) and (7.23) we can get the requirements to the residual standard deviation of the calibration line SD_{rest}:

$$SD_{rest} \le \max SD_{rest} = \max \Delta_{cal} / 2.35 = 0.0961 \cdot B. \tag{7.24}$$

Inserting (7.24) into (7.1), we can obtain the requirements to the correlation coefficient in the *Approach 2*.

The *Approach 2* sets much more liberal requirements (7.24) to the residual standard deviation SD_{rest} and correlation coefficient R_c of the calibration line (7.3a) as compared with the *Approach 1* (7.20). Indeed, the comparison of ratios (7.18-7.19) and (7.23-7.24) shows that the in the *Approach 2* the allowable residual standard deviation SD_{rest} is 2.26 times more than in the *Approach 1*.

Critical values of repeatability of a test sample ($max\Delta_{sample}$), residual standard deviation ($maxSD_{rest}$) and correlation coefficient ($minR_c$) for different ranges, content tolerances and approaches are presented in the Table 7.3. They are based on the ratios (7.1), (7.8), (7.9), (7.19-7.24) and Table 7.1.

Table 7.3

Critical values of validation characteristics of linearity for different tolerances (*B*%), ranges and approaches in the calibration graph method (CGM) for the stage of calibration

B%	$max\Delta_{As}$	$max\delta_1$ %	**CGM: *Approach 1***			**CGM: *Approach 2***		
			$max\Delta_{sample}$	SD_{rest} %	$minR_c^2$	$max\Delta_{sample}$	SD_{rest} %	$minR_c^2$
Range 80-120%								
5	1.6	0.50	1.6	0.22	0.99981	1.13	0.48	0.99908
7.5	2.4	0.75	2.4	0.33	0.99957	1.70	0.72	0.99792
10	3.2	1.00	3.2	0.44	0.99924	2.26	0.96	0.99631
12.2	3.9	1.22	3.9	0.53	0.99887	2.76	1.17	0.99450

15	4.8	1.50	4.8	0.65	0.99830	3.39	1.44	0.99169
20	6.4	2.00	6.4	0.87	0.99697	4.53	1.92	0.98522
Range 70-130%								
5	1.6	0.50	1.6	0.22	0.99992	1.13	0.48	0.99959
7.5	2.4	0.75	2.4	0.33	0.99981	1.70	0.72	0.99908
10	3.2	1.00	3.2	0.44	0.99966	2.26	0.96	0.99836
12.2	3.9	1.22	3.9	0.53	0.99950	2.76	1.17	0.99756
15	4.8	1.50	4.8	0.65	0.99924	3.39	1.44	0.99631
20	6.4	2.00	6.4	0.87	0.99865	4.53	1.92	0.99343
Range 50-150%								
5	1.6	0.50	1.6	0.22	0.99997	1.13	0.48	0.99985
7.5	2.4	0.75	2.4	0.33	0.99993	1.70	0.72	0.99967
10	3.2	1.00	3.2	0.44	0.99988	2.26	0.96	0.99941
12.2	3.9	1.22	3.9	0.53	0.99982	2.76	1.17	0.99912
15	4.8	1.50	4.8	0.65	0.99973	3.39	1.44	0.99867
20	6.4	2.00	6.4	0.87	0.99952	4.53	1.92	0.99764

Table 7.3 shows that the requirements to the square of the correlation coefficient R_c^2 depend on the range and content tolerances and for the pharmacopoeial range 70-130% in all cases exceed 0.997. Therefore, the SPU-Eur.Ph. requirements to the correlation coefficient (> 0.99, i.e. $R_c^2 > 0.98$) [2] are clearly inadequate.

7.3.9. Validation criteria for the calibration graph method

7.3.9.1. Systematic error

As it was already noted above, systematic error (which characterizes accuracy) has two components.

Systematic error of the first type δ_1 is due to matrix effects (placebo effects) and is checked at the stage of verification of procedure specificity. This type of systematic error is roughly the same for whole concentration range and, in accordance with the ratios (7.9-7.10), should be insignificant. As noted in the section 3.5, the δ_1 value can be, in principle, reduced to an acceptable level when performing the analysis, for example, by introducing a placebo in the reference standard or calibration solutions.

If the requirement (7.10) is met when checking the procedure specificity, the systematic error for analysis of model mixtures for the average recovery factor *RF* should be also insignificant, i.e.:

$$\left| \overline{RF - 100} \right| \le \max \delta_1 = 0.1 \cdot B. \qquad (7.25)$$

Systematic error of the second type δ_2 is connected with the calibration uncertainty, can considerably vary for different concentrations and is irremovable on principle though is of different nature in the RSM and CGM.

In the RSM, the δ_2 value is associated with the significance of the *Y*-intercept (*a*) of the line (7.3a) (see the section 2.3.4.3). Criteria of insignificance of the δ_2 value are presented by the relations (7.11, 7.12).

In the CGM the δ_2 value is associated with deviations from linearity and is a part of the calibration uncertainty Δ_{cal}, which is characterized by the residual standard deviation SD_{rest} and corresponding confidence interval (7.16). Since the calibration line is calculated using the least squares method, the actual deviations from it have different value and sign for different concentrations. Therefore, generally speaking, an experimental systematic error δ_2 would be necessary to determine for each narrow range of concentrations that practically little doable. The model solutions cover the whole analytical concentration range, so the algebraic sum of systematic errors for them must be largely compensated. Therefore, the average value of the recovery factor *RF* (7.5) for these solutions must not have significant systematic error δ_2. The analysis of model mixtures using the RSM shall meet the requirements of the Table 7.2.

Bearing in mind also the insignificance of the systematic error δ_1, it is easily to see, that the requirement (7.25) of insignificance of systematic error δ for the average of all model mixtures recovery factor *RF* (7.5) must be met for both the SRM and CGM. This requirement is ensured by the requirement of specificity (7.10), the requirement (7.12) in RSM, requirements (7.20) (*Approach 1*) and (7.24) (*Approach 2*) at the CGM, which are checked by validation and shall comply with the Table 7.3.

7.3.9.2. Repeatability

In the RSM and *Approach 1*of the CGM, the calibration uncertainty (Δ_{cal}) is not significant, so the total uncertainty of model mixtures analysis (Δ_{As}) is defined by the uncertainty of analysis of the sample proper (Δ_{sample}).

In general case, the uncertainty (Δ_{sample}) of repeatability of a single recovery factor (*RF*) for $n = 9$ model solutions should not exceed the maximum acceptable procedure uncertainty $max\Delta_{As}$, i.e., taking into account the ratio (7.8):

Reference standard method and *Approach 1* of the CGM:

$$\Delta_{sample} = t(95\%, n-1) \cdot SD_{RR} = 1.86 \cdot SD_{RR} \le \max \Delta_{As} = 0.32 \cdot B. \quad (7.26)$$

Here SD_{RF} is a standard deviation of the recovery factor *RF* (7.5).

In the case of *Approach 2* of the CGM, we have a significant calibration uncertainty Δ_{cal}, and the requirements to repeatability of the recovery factor (*RF*), in accordance with the ratio (7.23), are more stringent:

Approach 2: $\Delta_{sample} = 1.86 \cdot SD_{RR} \leq 0.707 \cdot \max \Delta_{As} = 0.226 \cdot B.$ (7.27)

7.3.10. The criteria for system suitability

A task of a system suitability test is to ensure that metrological characteristics of the system meet those, which were obtained during its validation.

The suitability of a calibration graph. In the case of the CGM, this means that the metrological characteristics of the calibration graph (the residual standard deviation SD_{rest} and the correlation coefficient R_c) and repeatability of the replicate measurements of the analytical signal (absorbance) $\Delta_{A,r}$ must meet the requirements of the system suitability.

In the case of the RSM, repeatability $\Delta_{A,r}$ of the replicate absorbance measurements of the test and reference standard solutions is the only experimentally observable metrological value.

In the case of the CGM, the system suitability for the SD_{rest} and R_c values means that they must meet the requirements of correctness of linearity (Table 7.3).

A more complex situation is with repeatability of the replicate absorbance measurements ($\Delta_{A,r}$). In UV-VIS spectrophotometry, the most part of the procedure uncertainty is the uncertainty of sample preparation and calibration. The repeatability of the analytic signal $\Delta_{A,r}$ is usually insignificant compared to the total procedure uncertainty (see 2.2.25. Absorption spectrophotometry, ultraviolet and visible [1]). It can be assumed that the same situation is for measuring absorbance in the AAS. In the light of the *Insignificance principle* (see the Section 2.3.1) and the requirements (7.9) to the total procedure uncertainty, we can obtain the requirements to the relative uncertainty of repeatability of the replicate absorbance measurements $\Delta_{A,r}$:

$$\Delta_{A,r} \leq 0.32 \cdot \max \Delta_{As} = 0.10 \cdot B. \quad (7.28)$$

Given that [26]

$$\Delta_{A,r} = t(95\%, n-1) \cdot SD_{A,r} / \sqrt{n} \quad (7.29)$$

and the ratios (7.28-7.29), we can get the requirements to the relative standard deviation of the replicate absorbance measurements $SD_{A,r}$, depending on the number n of the replicate measurements:

$$SD_{A,r} \leq \max SD_{A,r} = 0.10 \cdot B \cdot \sqrt{n} / t(95\%, n-1). \quad (7.30)$$

Values of $maxSD_{A,r}$, depending on the number (n) of the replicate measurements and content tolerances B, calculated on the ratio (7.30), are presented in the Table 7.4.

Table 7.4

Dependence of $maxSD_{A,r}$ on number (n) of replicate absorbance measurements and content tolerances (B) for the CGM and RSM

n =	3	4	5	6	7	8	9
$t(95\%,n-1)$=	2.92	2.35	2.13	2.02	1.94	1.89	1.86
$B\% \downarrow$	Values of $maxSD_{A,r}$ %						
5.0	0.30	0.42	0.52	0.61	0.68	0.75	0.81
7.5	0.44	0.64	0.79	0.91	1.02	1.12	1.21
10.0	0.59	0.85	1.05	1.22	1.36	1.49	1.61
12.2	0.72	1.04	1.28	1.48	1.66	1.82	1.97
15.0	0.89	1.27	1.57	1.82	2.04	2.24	2.42
20.0	1.19	1.70	2.10	2.43	2.72	2.99	3.23

If the requirements of the Table 7.4 for the given number n are not met, it is necessary to increase the n.

7.3.11. Limit of detection (*DL*) and limit of quantitation (*QL)*

These values are not required when validate the assay procedures, but they are useful as information about how the procedure range surpasses its limit capabilities ("safety margin" of the procedure). In case of impurities control, obtaining the *DL* and *QL* values is required (see Chapter 1).

In accordance with the SPU [11], the *DL* and *QL* values can be calculated from the standard deviation s_a of the *Y*-intercept (a) of the linear relationship $A = b \cdot X + a$ and its slope (b). Division by the slope is necessary for transformation of the absorbance into the concentration. However, in the case of the ratio (7.3a) such transformation does not need. So it is possible here to calculate the *DL* and *QL* values from the following ratios with the criteria that were received earlier (see the section 2.3.5):

$$DL = 3.3 \cdot s_a \le 32\%. \tag{7.31}$$

$$QL = 10 \cdot s_a \le 32\%. \tag{7.32}$$

The *DL* value is calculated in this case as a percentage of the maximum acceptable content of impurities *ImL*, and *QL* - as a percentage of the nominal content

DL and *QL* values are calculated from the parameters of the line, based on 9 model mixtures (see the section 2.3.5). In principle, we can calculate the *DL* and *QL* values using the parameters of the line plotted on 5 points. However, due to the small number of degrees of freedom (n = 3), the *DL* and *QL* values in this case are too unreliable.

7.3.12. Intermediate precision

Intermediate precision characterizes influence of internal laboratory factors on the procedure: influence of different days, analysts, equipments and so on. When checking the intermediate precision, it is reasonable to verify:

1) reproducibility of developed parameters of system suitability, i.e. the acceptability of the residual standard deviation (SD_{rest}), correlation coefficient (R_c) (Table 7.3) and relative standard deviation of repeatability of the replicate absorbance measurements ($SD_{A,\,r}$) (Table 7.4);
2) reproducibility of found values of concentration.

In the case of a system suitability test, the situation is sufficiently unambiguous (the criteria for system suitability should be met). Unlike this, for checking the reproducibility of the found values of concentration it may be offered different approaches. For example, we can prepare three mixtures with composition, close to the nominal, and analyze them in two different days. The difference between the mean recovery factor (of three mixtures) in two different days (*RF(1)* and *RF(2)*) shall be practically (see the Section 2.3.3) insignificant, i.e. [26]:

$$\left|RF(2)-RF(1)\right| \le \sqrt{2}\cdot \max\Delta_{As}/\sqrt{3} = \sqrt{2/3}\cdot 0.32\cdot B = 0.26\cdot B. \quad (7.33)$$

In addition, for each day system suitability requirements must be met.

7.4. Example. Determination of iron in the drug product Gesticare by AAS with use of the calibration line method

Biphasic tablets Gesticare is a specialized multivitamin-mineral complex (MVMC) intended to improve the nutritional status of women during pregnancy and the postpartum period for all nursing and non-nursing mothers which improves feeling of pregnant. Particularly it reduces toxicity and protects against stresses, reduces the risk of premature birth, congenital deformities and coronary heart disease.

Gesticare contains (per one tablet) complex of vitamins: B1 (2.7-4.05 mg), B2 (2.7-3.9 mg), B3 (18-23.4 mg), B6 (42-62.5 mg), B9 – folic acid (0.9-1.35 mg), B12 (7.2-10.4 μg), vitamin C (108-144 mg), D3 (378-693 IU), E (27-36.6 IU), and microelements as well: calcium (180-230 mg), iron (25.2-32.2 mg), zinc (13.5-17.25 mg), йод (135-172.5 μg). Excipients are sodium carboxymethyl cellulose, magnesium stearate , silicon dioxide, SMCC, components for coating and polishing.

The task is to validate AAS assay procedure (using the CGM) for iron (as iron fumarate) in this drug product, which is acceptable for “Dissolution” test as well.

Equipment. Apparatus of atomic absorption Varian 220 FS Double Beam AA (USA). To standardize the method, we received and investigated an atomic absorption spectrum at a wavelength of 248.3 nm in air-acetylene flame. Passport dependence of absorption on iron concentration at 248.3 nm is presented in the Figure 7.1.

For each solution we made 5 measurements of absorption.

Used reagents and reference standards (solutions) met to the USP33-NF28 requirements [67].

Sample solution preparation (Gesticare tablets). Finely powder 10 tablets. About 5 g (accurate weight) of the sample place in a crucible, which is heated on a stove to charring. Then place the crucible in a preheating muffler and burn the sample at temperature (550 ±5)° C for 1 hour. Cool and quantitatively transfer the contents into a beaker with a capacity of 200 ml. For this, add drop by drop 20 ml of concentrated hydrochloric acid in the crucible, bring it to a boil, cool and transfer the liquid in a glass. Wash the crucible with water and place rinse waters in the same glass.

Boil the contents of the glass for 30 min. Cool and quantitatively transfer it to a measuring flask with a capacity of 200 ml. Glass washed with 6 M hydrochloric acid and place the rinse waters in the same measuring flask and dilute it to the mark with water. Mix and filter the solution in the flask, discarding the first 10 ml of the filtrate. Place 10 ml of the filtrate in a measuring flask with a capacity of 100 ml and dilute it to the mark with *0.1 M hydrochloric acid*.

The calibration range. Bearing in mind that the procedure should be appropriate for quantification of iron and for the "Dissolution" test, the calibration range was 50-150% of the nominal value, or 2-6 ppm. It corresponds to the absorbance range of 0.05-0.14 (see Table 7.3) that is located in the linearity range (see Figure 7.1.). Number of calibration solutions is $n = 5$.

Preparation of calibration solutions. In accordance with the Table 7.1, for the range of 50-150%, prepare the calibration solutions with concentrations of 50.0, 75.0, 100.0, 125.0 and 150.0% of the nominal content. This range corresponds to $SD_{Co} = 39.53\%$. For this, place 10.0 ml of the *1000 ppm iron standard solution* in a measuring flask with a capacity of 100 ml and dilute it to the mark with *0.1 M hydrochloric acid*. Place 2.0, 3.0, 4.0, 5.0, and 6.0 ml of the resulting solution in five measuring flasks with a capacity of 100 ml flasks and dilute them to the mark with *0.1 M hydrochloric acid*.

The calibration solutions have concentrations of $C_{st,i}$ = 2.0 ppm (50.0%), 3.0 ppm (75.0%), 4.0 ppm (100.0%), 5.0 ppm (125.0%) and 6.0 ppm (150.0%). The iron nominal concentration is $C_{nom} = 4$ ppm.

Preparation of model solutions. Weigh 404.3 mg of iron fumarate and 5.0018 g of the placebo (all components of the medicine, except iron) in a crucible and continue as described above for the sample solution preparation. Thus prepare 9 model mixtures with the target iron content of 50.0, 75.0, 62.5, 87.5, 100.0, 125.0, 112.5, 137.5 and 150.0% (see table 7.1) of the nominal content in the tablet (28.7 mg). The actual concentrations of iron in model solutions are presented in the Table 7.8.

7.4.1. Verifying specificity

Results of verifying specificity in accordance with the validation criteria (7.10) are presented in the Table 7.5. For transformation the data into normalized coordinates and evaluation of the specificity by the ratio (7.10), the solution with the nominal concentration C_{st} = 4 ppm and $A_{st} = A_{nom}$ = 0.0955 was considered as the standard (see the Table 7.6).

Table 7.5

Absorbances of the placebo and blank solutions

Solution	Absorbances						% of A_{nom} = 0.0955	Criterion %
	1	2	3	4	5	Среднее		
Placebo	0.0009	-0.0003	0.0003	0.0007	0.0012	0.0006	0.59	≤1.22
Blank	0.0006	-0.0005	-0.0001	-0.0005	0.0002	-0.0003	0.06	≤1.22

As we can see, the absorbances of the placebo and blank solutions meet the requirement of insignificance (≤1.22), i.e. the procedure can be considered to be specific.

7.4.2. Linearity verification

Results of analysis of the calibration solutions are presented in the Table 7.6. The normalized coordinates calculated by the ratios (7.4) are presented as well. As the standard we used the calibration solution with the nominal concentration of C_{st} = 4.0 ppm, A_{st} = 0.0955.

Table 7.6

Replicate absorbances of the calibration solutions

C ppm	X %	Absorbances, $A \cdot 10^4$						Y%	$SD_{A,r} \leq$ 1.28 %	Conclusion about $SD_{A,r}$
		1	2	3	4	5	mean			
2.0	50	499	493	502	501	498	499	52.2	0.70	Meet
3.0	75	729	731	723	737	722	728	76.2	0.84	Meet
4.0	100	962	959	951	956	949	955	100.0	0.57	Meet
5.0	125	1190	1194	1190	1195	1195	1193	124.8	0.22	Meet
6.0	150	1406	1396	1412	1403	1407	1405	147.0	0.42	Meet

As we can see, all the solutions meet the system suitability requirements to the standard deviation ($SD_{A,r} \leq$ 1.28%, see the Table 7.4).

It was built a linear relationship (7.3 a) for X versus Y using the least squares method [26]. The results are presented in the Table 7.7 (the criteria are taken from the Table 7.3) and the calibration graph is shown in the Figure 7.2.

Table 7.7

Metrological characteristics of the calibration line (3a) $X\% = b \cdot Y(\%) + a$

Parameter	Value	**Calibration graph method**			
		Approach 1		*Approach 2*	
		Criterion	Conclusion	Criterion	Conclusion
a	-4.95				
s_a	1.02				
b	1.049				
s_b	0.0096				
SD_{rest}	0.725	≤ 0.53	Fail	≤ 1.17	Meet
R_c^2	0.99966				
Range 80-120%		≥ 0.99887	Meet	≥ 0.99450	Meet
Range 70-130%		≥ 0.99950	Meet	≥ 0.99756	Meet
Range 50-150%		≥ 0.99982	Fail	≥ 0.99912	Meet

The Table 7.7 shows the requirements of the Table 7.3 to the square of the correlation coefficient R_c^2 and the residual standard deviation SD_{rest} for calibration graph method (CGM) are not met in the case of the *Approach 1* for the range 50-150%. For the *Approach 2*, the criteria of which are much softer, the requirements are met for all three ranges.

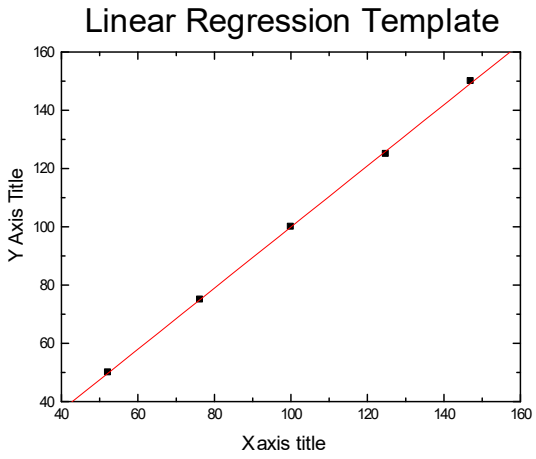


Figure 7.2. Calibration relationship *X* versus *Y*.

7.4.4. Analysis of model mixtures

Absorbances of the model mixtures and corresponding normalized coordinates are presented in table 2. 8. For the calculation of the normalized coordinates the calibration solution with the nominal concentration of C_{st} = 4 ppm and A_{st} = 0.0955 is used as a standard (see the Table 7.8).

Table 7.8

Concentrations and absorbances of the model solutions

C mg/L	X%	Replicate absorbances $A \cdot 10^4$						*Y*%	$SD_{A,r} \leq$ 1.28 %	Conclusion
		1	2	3	4	5	Mean			
2.038	50.95	502	500	496	497	498	499	52.19	0.48	Meet
2.521	63.03	609	613	610	610	611	611	63.91	0.25	Meet
3.031	75.78	720	733	728	734	729	729	76.28	0.76	Meet
3.565	89.13	848	851	861	844	859	853	89.24	0.85	Meet
4.058	101.45	969	962	971	966	965	967	101.17	0.36	Meet
4.516	112.90	1075	1080	1067	1069	1073	1073	112.29	0.48	Meet
5.016	125.40	1192	1189	1189	1190	1184	1189	124.43	0.25	Meet
5.434	135.85	1289	1288	1278	1282	1290	1286	134.54	0.40	Meet
5.875	146.88	1387	1396	1381	1382	1393	1388	145.26	0.48	Meet

As we can see, all the solutions meet the system suitability requirement to the standard deviation ($SD_{A,r} \leq$ 1.28%, see the Table 7.4).

In accordance with the standard validation scheme developed for validation of the reference standard method (RSM) (see Chapter 2), it was built the linear relationship

X versus *Y* (7.3a). Its metrological characteristics are presented in the Table 7.9. The criteria for the RSM are taken from the Table 7.2.

Table 7.9

Metrological characteristics of the line (7.3a) $X\% = b \cdot Y(\%) + a$ for the model solutions

Parameter	Value	Reference standard method	
		Criterion	Conclusion
a	-2.85		
Range 80-120%		$\lvert a \rvert \leq 6.1$	Meet
Range 70-130%		$\lvert a \rvert \leq 4.1$	Meet
Range 50-150%		$\lvert a \rvert \leq 2.4$	Fail
s_a	0.01		
b	1.031		
S_b	0.0001		
SD_{rest}	0.010	≤ 2.06	Meet
R_c^2	1.00000		
Range 80-120%		≥ 0.97735	Meet
Range 70-130%		≥ 0.98994	Meet
Range 50-150%		≥ 0.99638	Meet
DL%	0.33	32	Meet
QL%	1.15	32	Meet

The Table 7.9 shows the reference standard method (RSM) is applied to ranges of 80-120% and 70-130%. For the range of 50-150% RSM is not applicable because of significant systematic error due to the absolute term (*a*). Note that for AAS validation and calibration line plotting the SPU-Eur.Ph. recommends a range of 70-30% [2].

The *X* and *Y* values (Table 7.8) and the calibration line (Table 7.9) are used for calculations by the CGM and RSM. Also a deviation of each point from its true value is calculated as well. The results are presented in the Table 7.10.

Table 7.10

Results of analysis of the model mixtures with use of the reference standard method (RSM) and calibration graph method (CGM)

X%	*Y*%	RSM		CGM		
		$Z = 100 \cdot (Y/X) = RF\%$	*RF*-100%	X_{calc}%	*RF*%	*RF*-100%
50.95	52.19	102.43	2.43	49.79	97.72	-2.28
63.03	63.91	101.40	1.40	62.08	98.50	-1.50
75.78	76.28	100.67	0.67	75.06	99.05	-0.95
89.13	89.24	100.13	0.13	88.65	99.46	-0.54
101.45	101.17	99.73	-0.27	101.16	99.72	-0.28
112.90	112.29	99.46	-0.54	112.82	99.93	-0.07
125.40	124.43	99.23	-0.77	125.55	100.12	0.12
135.85	134.54	99.04	-0.96	136.16	100.23	0.23
146.88	145.26	98.90	-1.10	147.40	100.36	0.36
Mean		100.11			99.46	
$\delta_1 = \lvert RF\text{-}100 \rvert \leq 1.22\%$		0.11			0.54	
Conclusion for δ		Meet			Meet	
SD_{RF}		1.19			0.88	
$\Delta_{sample} = 1.86 \cdot SD_{RF}$		2.21			1.64	
Criteria for $\Delta_{RF} = \Delta_{sample}$:						
Approach 1: $\Delta_{sample} \leq 3.90$		-			Meet	
Approach 2: $\Delta_{sample} \leq 2.76$		-			Meet	
RSM: $\Delta_{sample} \leq 3.90$		Meet				

As can be seen from the Table 7.10, the requirements (7.25) to the systematic error (δ) and the random uncertainty of the test sample (Δ_{sample}) are met as for the reference standard method and the calibration graph method in options of the *Approach 1* and *Approach 2*. The insignificance of the systematic error δ is really related to the fact that the *RF*-100% values have different signs on the edges of the analytical range and are mutually compensated by summation. The actual systematic errors at the edges of the range are much higher and so the data of the Table 7.10 must be considered along with the results of the Table 7.9.

In view of the results of the Table 7.9 (compliance with the requirements to the absolute term *a* of the linear relationship (7.3a)), the RSM is applicable only for the ranges of 80-120% and 70-130%.

Because the requirements are not met 1 residual standard deviation SDrest (table 7.7) of the calibration line. Therefore 1 is not applicable for all three bands. At the same time, the Approach is applicable to 2 MG for all three bands.

The requirements to the residual standard deviation SD_{rest} of the calibration line for the *Approach 1* are not met (see the Table 7.7). Therefore the *Approach 1* is not applicable for all three ranges. At the same time, the *Approach 2* is applicable to CGM for all three ranges.

7.4.5. Intermediate precision

In two different days we prepared and analyzed by 3 model mixtures of composition close to the nominal. Every time we built the calibration line. Absorbances of the model and calibration solutions are presented in the Table 7.11. It contains also the normalized values of X and Y calculated by the ratio (7.4). For this we used the concentration C_{st} = 4 ppm and corresponding values of absorbances A = 0.0986 (1st day) and 0.0999 (2nd day).

Table 7.11

Concentrations and absorbances of the model solutions

C ppm	X%	Replicate aabsorbances A						Y%	$SD_{A,r}$ ≤ 1.28 %	Conclusion
		1	2	3	4	5	Mean			
1st day: calibration										
2.00	50	0.0497	0.0489	0.0493	0.0493	0.049	0.0492	49.94	0.64	Meet
3.00	75	0.0743	0.0747	0.076	0.0736	0.0739	0.0745	75.56	1.26	Meet
4.00	100	0.0987	0.0992	0.0982	0.0986	0.0983	0.0986	100.00	0.40	Meet
5.00	125	0.1221	0.1211	0.1202	0.1214	0.1214	0.1212	122.96	0.57	Meet
6.00	150	0.1444	0.1448	0.1448	0.1426	0.1432	0.1440	146.00	0.70	Meet
1st day: test sample analysis										
4.007	100.2	0.0971	0.0973	0.0967	0.0975	0.0977	0.0973	98.7	0.40	Meet
4.020	100.5	0.0977	0.0970	0.0974	0.0986	0.0971	0.0976	99.0	0.66	Meet
4.000	100.0	0.0974	0.0983	0.0976	0.0966	0.0956	0.0971	98.5	1.07	Meet
2nd day: calibration										
2.00	50	0.0516	0.0512	0.0514	0.0516	0.0517	0.0515	51.55	0.39	Meet
3.00	75	0.0772	0.0774	0.0772	0.0763	0.0758	0.0768	76.88	0.90	Meet
4.00	100	0.0993	0.1009	0.0991	0.1002	0.1001	0.0999	100.00	0.73	Meet
5.00	125	0.1263	0.1251	0.1237	0.1227	0.1243	0.1244	124.52	1.10	Meet
6.00	150	0.1460	0.1464	0.1458	0.1456	0.1457	0.1459	145.05	0.22	Meet
2nd day: test sample analysis										

4.027	100.7	0.0995	0.0996	0.0994	0.1004	0.1003	0.0998	99.90	0.47	Meet
4.004	100.1	0.0991	0.0994	0.0993	0.0997	0.0989	0.0993	99.40	0.31	Meet
4.027	100.7	0.0991	0.1004	0.1005	0.0995	0.0997	0.0998	99.90	0.60	Meet

As can be seen, the system suitability requirement in all cases is met i.e. the $SD_{A,r}$ values are below the critical value of 1.28%.

Metrological characteristics of the calibration lines are presented in the Table 7.12.

Table 7.12

Metrological characteristics of calibration lines (3a) $X\% = b \cdot Y(\%) + a$

Parameter	Value	**Calibration graph method**			
		Approach 1		*Approach 2*	
		Criterion	Conclusion	Criterion	Conclusion
1st day					
a	-3.16				
s_a	1.52				
b	1.043				
s_b	0.015				
SD_{rest}	1.10	≤ 0.53	Fail	≤ 1.17	Meet
R_c^2	0.99936				
Range 80-120%		≥ 0.99887	Meet	≥ 0.99450	Meet
Range 70-130%		≥ 0.99950	Fail	≥ 0.99756	Meet
Range 50-150%		≥ 0.99982	Fail	≥ 0.99912	Meet
2nd day					
a	-5.35				
s_a	1.06				
b	1.049				
s_b	0.016				
SD_{rest}	1.17	≤ 0.53	Fail	≤ 1.17	Meet
R_c^2	0.99913				
Range 80-120%		≥ 0.99887	Meet	≥ 0.99450	Meet

Range 70-130%		≥ 0.99950	Fail	≥ 0.99756	Meet
Range 50-150%		≥ 0.99982	Fail	≥ 0.99912	Meet

As we can see, in the *Approach 1* the criteria are met only for the range of 80-120%. In the *Approach 2* the criteria are met for all three ranges. Thus, the system suitability criteria are met for the *Approach 2*.

Results of calculation of model solution concentrations are presented in the Table 7.13.

Table 7.13

Results of analysis in 2 different days ofr 3 model mixtures with use of the reference standard method (RSM) and the calibration graph method (CGM)

1st day					2nd day				
X%	*Y%*	RSM	CGM		*X%*	*Y%*	RSM	CGM	
		Z= RF %	X_{calc}%	*RF%*			*Z=RF %*	X_{calc}%	*RF%*
100.18	98.6	98.47	99.74	99.56	100.68	99.92	99.25	100.21	99.54
100.50	98.9	98.45	100.06	99.56	100.10	99.36	99.26	99.62	99.52
100.00	98.5	98.48	99.57	99.57	100.68	99.92	99.25	100.21	99.54
		98.47		**99.56**			**99.25**		**99.53**
$\lvert RF(2)- RF(1) \rvert \le 0.26 \cdot 12.2 = 3.2$							0.78		0.03
							Meet		Meet

Table 7.13 shows that the requirements of (7.33) to the intermediate precision are met as for the CGM and RSM.

Summing up the results of the Tables 7.11-7.13, it can be concluded that the requirements to absorbance repeatability (Table 7.11) are met in all cases for all ranges as for the CGM and RSM. System suitability requirements (suitability of the calibration – see the Table 7.12) in the case of the CGM are met for all three ranges only for the *Approach* 2. With this, in the *Approach 1* the requirements are met only for the range of 80-120%. Requirements of reproducibility of certain concentrations (see the Table 7.13) are met as for the CGM and RSM.

In general, it can be concluded that use of the *Approach 2* in the AAS with broad ranges (70-130, 50-150%) is much more reliable than use of the *Approach 1*.

7.4.6. General conclusions of the validation of the AAS procedure

Summary results of the validation of the AAS-procedure of determination of iron in the *Gesticare* preparation are presented in the Table 7.14.

Table 7.14

Summary results of the validation of the AAS-procedure of determination of iron

Item	Reference standard method			Calibration graph method		
Range →	80-120	70-130	50-150	80-120	70-130	50-150
System suitability:						
1) Repeatability of the replicate absorbance measurements ($SD_{A,r}$)						
Requirement to $SD_{A,r}$ %	≤ 1.28					
Calibration solutions	Meet			Meet		
Model solutions	Meet			Meet		
Solutions for intermediate precision	Meet			Meet		
2) Requirements to the calibration line						
Requirements to SD_{rest}						
Approach 1				≤ 0.53		
Approach 2				≤ 1.17		
Experimental SD_{rest}				0.72, 1.10, 1.17		
Conclusion for SD_{rest}				*Approach 1* – **Fail** *Approach 2* – Meet		
Experimental R_c^2				0.99966, 0.99936, 0.99913		
Requirements to $minR_c^2$						
Approach 1				0.99944	0.99950	0.99982
Conclusion, *Approach 1*				**Fail**	**Fail**	**Fail**
Approach 2				0.99450	0.99756	0.99912
Conclusion, *Approach* 2				Meet	Meet	Meet
Analysis of 9 model mixtures						
Criterion for *max* $\lvert a \rvert$	≤ 6.1	≤ 4.1	≤ 2.4			
Experimental $\lvert a \rvert$	2.85					

	Meet	Meet	**Fail**			
Experimental SD_{rest}	0.010					
Criterion for $maxSD_{rest}$	≤ 2.06					
Conclusion for SD_{rest}	Соотв.					
Experimental R_c^2	1.00000					
Criterion for $minR_c^2$	0.97735	0.98994	0.99638			
Conclusion for R_c^2	Meet	Meet	Meet			
$\delta_1 = \left\| RF\text{-}100 \right\| \leq 1.22\%$	Meet			Meet		
Experimental Δ_{RF}						
$max\ \Delta_{RF} = max\Delta_{sample}$:	2.21			1.64		
Approach 1 and RSM: $\Delta_{sample} \leq 3.90$	Meet	Meet	Meet	Meet	Meet	Meet
Approach 2: $\Delta_{sample} \leq 2.76$	Meet	Meet	Meet	Meet	Meet	Meet
General conclusion on procedure validation						
RSM	**Meet**	**Meet**	**Fail**			
CGM – *Approach 1*				**Fail**	**Fail**	**Fail**
CGM – *Approach 2*				**Meet**	**Meet**	**Meet**

The Table 7.14 shows that in the analytical concentration range (50-150% of the nominal value of iron in the product) only the *Approach 2* is applied, which can be recommended for the use of CGM in the AAS. For the ranges of 80-120% and 70-130% (i.e. conventional pharmacopoeial ranges [2]) the RSM is applied as well.

Thus, in general, it is more reliable the *Approach 2* based on the assumption of parity of calibration and test sample uncertainties. The reference standard method in the AAS is typically applied for sufficiently narrow analytical ranges, i.e. no wider than 70-130% of the nominal value.

8. VALIDATION OF SPECTROPHOTOMETRIC QUANTITATION PROCEDURES OF MEDICINES USING THE SPECIFIC ABSORBANCE METHOD [72, 73]

Spectrophotometric assay of pharmaceuticals using the specific absorbance method (SAM) is a pharmacopoeial method of analysis [15]. The SAM is a direct method of analysis (such as, for example, titration), which does not require the use of certified reference standards. It is the main advantage of the SAM

Metrological aspects of the SAM in the quality control of medicines are covered in some detail earlier [81]. Professional testing results showed [81], that in Ukraine use of the SAM is metrological correctly only for assay of drug products with tolerances of $\pm$10% and wider. This is due, primarily, to poor reproducibility in Ukraine of specific absorbance values in different laboratories [81]. The reasons are several: poor equipment and volumetric glass qualification and lack of quality assurance systems for the analysis results, the human factor, etc.

Same problems with use of the SAM were until recently in EU countries as well. However, in recent years the situation has changed significantly. Spectrophotometers, providing high accuracy of absorbance (up to 0.003 absorbance unit), are routine, qualification of equipment and volumetric glassware are mandatory, as well as availability of quality assurance systems for analysis results. This has made it possible to introduce into the European Pharmacopoeia (Eur.Ph.) the requirements to control of absorbance accuracy using a potassium dichromate solution, as well as the requirements for control of the wavelength scale, stray light and cell uniformity [82]. These requirements are introduced into the State Pharmacopoeia of Ukraine (SPU), harmonized with the Eur.Ph. [15, 82].

This has largely standardized the specific absorbance values in different laboratories and made it possible to introduce the spectrophotometric assay procedures based on the SAM into pharmacopoeias. Here is particularly visible in the British Pharmacopoeia, where the SAM has been widely used to quantify substances and drug products. A textbook example is the assay of Paracetamol tablets using SAM at 257 nm [83]. The tolerances of the paracetamol content are $\pm$ 5%.

The Eur.Ph. also often uses the SAM for the quantification of substances and herbals. The SPU is harmonized with the Eur.Ph. so the corresponding monographs are introduced into the SPU. There are 10 medicinal substance monographs and 24 assay procedures of 21 herbals using the SAM are described in the 1st Edition SPU [15] (see the Table 8.1)

Table 8.1

SPU monographs describing the spectrophotometric assay with use of the specific absorption method (SAM)

№	Name	SPU, page	Tolerances* %	λ nm	$A_{1cm}^{1\%}$	C_{nom} mg/100 ml	A_{nom}	max δ_A%	Δ_{Dil} %

Субстанции									
1.	*Betamethasone dipropionate*	1.1, p.298	97-103	240	305	1.980	0.604	2.3	0.67
2.	*Hydrocortisone acetate*	1.1, p.313	97-103	241.5	395	1.990	0.786	1.8	0.56
3.	*Prednisolone*	1.2, p.531	97-103	243.5	415	1.980	0.822	1.7	0.56
4.	*Prednisolone sodium phosphate*	1.2, p.532	96-103	247	312	1.840	0.574	2.5	0.65
5.	*Riboflavine*	1.1, p.445	97-103	444	328	1.281	0.420	3.4	0.52
6.	*Rifampicin*	1.1, p.446	97-102	475	187	1.980	0.370	3.8	0.56
7.	*Testosterone propionate*	1.2, p. 559	97-103	240	490	0.995	0.488	2.9	0.95
8.	*Chloramphenicol*	1.2, p.573	98-102	278	297	1.990	0.591	2.4	0.56
9.	*Chloramphenicol sodium succinate*	1,4, p.457	98-102	276	220	1.960	0.431	3.3	0.62
10.	*Cyanocobalamin*	1.2, p.589	96-102	361	207	2.200	0.455	3.1	0.80
Herbals**									
11.	*Berch leaf* ΣF	1.4, p.295	≥ 1.5	425	500	0.540	0.270	5.2	0.78
12.	*Elder flower* ΣF	1.2, p.377	≥ 0.80	425	500	0.864	0.432	3.3	0.77
13.	*Hawthorn leaf and flower* ΣF	1.3, p.165	≥ 1.5	410	405	1.350	0.547	2.6	0.66
14.	*Hawthorn berries* ΣPr	1.2, p.414	≥ 1.0	545	75	5.375	0.403	3.5	0.43
	N: ΣF		≥ 0.05	425	500	0.344	0.172	8.2	0.77
15	*John's wort* Σ HP	1.2, p. 443	≥0.08	590	870	0.576	0.501	2.8	0.68
	N: ΣF		≥1.2	425	500	0.648	0.324	4.4	0.78
16.	*Calndula tincture*^{N} ΣF	1.4, p.332	≥ 0.04	425	500	0.800	0.400	3.5	0.78
17.	*Motherwort* ΣF	1.2, p.544	≥ 0.2	425	500	0.352	0.176	8.0	0.77
18.	*Motherwort tincture*^{N} ΣF	1.3, p.211	≥ 0.01	425	500	0.400	0.200	7.1	0.78
19.	*Knotgrass* ΣF	1.3, p.212	≥ 0.30	425	500	0.418	0.209	6.8	0.77
20.	*Equisetum stem* ΣF	1.3, p.215	≥ 0.3	425	500	0.432	0.216	6.5	0.77
21.	*Passion flower* ΣF	1.2, p.525	≥1.5	401	628	0.675	0.424	3.3	0.66
22.	*Yarrow* ΣPrA	1.2, p.421	≥ 0.02	608	23.8	8.800	0.209	6.8	0.17
23.	*Ribwort plantain* ΣDHA	1.3, p.204	≥ 1.5	525	185	1.688	0.312	4.5	0.79
24.	*Plantago major leaf*^{N}, ΣDHA	1.4, p.337	≥ 1.5	525	185	1.613	0.298	4.7	0.79
25.	*Senna pods, tinnevelly*	1.3, p.187	≥ 2.2	515	240	1.210	0.290	4.9	0.66

	ΣHAG								
26.	*Senna pods, Alexandrian* ΣHAG	1.3, p.188	≥ 3.4	515	240	1.870	0.449	3.2	0.66
27.	*Senna leaf* ΣHAG	1.3, p.190	≥ 2.5	515	240	1.375	0.330	4.3	0.66
28.	*Cascara* ΣHAG	1.4, p.313	≥ 8.0	515	240	2.880	0.518	2.7	1.03
	ΣCas		≥ 4.8	515	180	4.608	0.829	1.7	
29.	*Frangula bark* ΣGF	1.4, p.320	≥ 7.0	515	204	2.975	0.607	2.3	1.05
30.	*Turmeric Javanese* ΣDcm	1.4, p.322	≥ 1.0	530	2350	0.110	0.259	5.5	0.58
31.	*Greater cilandine* ΣAlk	1.2, p.592	≥ 0.6	570	933	0.243	0.227	6.2	0.90

*For herbal drugs the lower content limit $Cont_L$ % is used.

**For herbal drugs the nominal concentration is $Cont_L$ /0.80 .

As shown by us [80], in the case of spectrophotometric quantification of herbal drugs, the SAM is the main pharmacopoeial option of use of spectrophotometry (24 objects of 36) [80]. In view of improvement of the instrument base and widespread mandatory introduction of quality systems, it can be expected that in the future the SAM may replace the reference standard method and become the main approach in the pharmacopoeial quantification of medicines.

This is especially true for medicinal substances for which the status of the section "Assay" is significantly diminished with the emergence of objective and precise chromatographic methods of impurity control and significant lowering of tolerances of these impurities. Due to this, currently, medicinal substances in the Eur.Ph. are fully defined objects (the "transparency of the monograph" is a cornerstone of the Eur.Ph.). So it is not necessary to determine quantitatively the basic material, because its content is easily calculated by subtracting of impurities from 100%.

The assay for the medicinal substances in this case, in fact, plays the role of identification, and its task is to show that the content of the base material is not significantly different from 100% (in limits of acceptable uncertainty). Therefore, the tolerances of the base material content in the medicinal substances can be expanded, for example, to 97-103% (see the Table 8.1) that creates the preconditions for the metrologically correct use of the SAM for their assay.

In recent years, the situation began to change in Ukraine as well. Advanced manufacturers have been modernized and implemented the quality systems. The instrumental base of the regional state laboratories is significantly improved as well. Therefore, the use of the SAM for pharmacopoeial spectrophometric quantification in Ukraine becomes real. This raises a number of questions:

1. To what extent is metrological substantiated, from the viewpoint of the SPU-Eur.Ph. requirements, the use of the SAM for quantification of the pharmacopoeial medicines?
2. Under what conditions the use of the SAM is metrological correct?

3. How to carry out the validation of procedures using the SAM?

This section gives a systematic consideration of these questions. The findings are applicable primarily to synthetic substances and drug products based on them. Validation of summarized drugs (particularly herbal drugs) requires a separate additional consideration, since some validation characteristics (accuracy, specificity) for them are uncertain [70-80].

8.1. A general expression for a procedure uncertainty

A general expression for the total relative procedure uncertainty ($\Delta_{As}\%$) in the case of the specific absorbance method (SAM) has the form (see the Chapter 2):

$$\begin{aligned}\Delta^2_{As} &= [\delta^2_{noise} + \Delta^2_{FAO} + \Delta^2_{SP}] + \delta^2_{cal} = \\ &= \Delta^2_{sample} + \delta^2_{cal} \le \max \Delta^2_{As}.\end{aligned} \tag{8.1}$$

Here:

- Δ_{Sample} (expression in the square brackets) is the uncertainty associated directly with the test sample.

- δ_{noise} is the uncertainty introduced by the impurities and excipients (placebo). It has a systematic (albeit unknown) character for each individual analysis and describes the specificity of the procedure. It can be reduced by improving the sample preparation (for example, by extraction). Note that for different batches of the test sample it is random.

- Δ_{FAO} is the uncertainty of the final analytical operations, i.e. of the absorbance measurement. It depends on the spectrophotometer level, is random and can be reduced by increasing the number of replicates.

- Δ_{SP} is the uncertainty of the sample preparation. It has two components related to dilution (i.e. the uncertainty of weighing and volumetric glassware) and processing samples (extraction, chemical reactions, etc.). It is random and can be reduced by increasing the number of replicates and their care performance.

- δ_{cal} is the uncertainty of calibration associated with a difference of absorbance at different spectrophotometers and with a deviation from a direct proportionality of absorbance versus concentration. It is systematic for each spectrophotometer and cannot be reduced by increasing the number of replicates, care performance and improving the sample preparation.

- $max\Delta_{As}$ is a maximum acceptable total procedure uncertainty.

In the case of a medicinal substance assay, the value of $max\Delta_{As}$ is related to the upper content tolerance of $B_H(\%)$ by the ratio (see the Chapter 2):

Substance: $$\max \Delta_{As} = B = B_H - 100. \tag{8.2}$$

In the case of a drug product assay, the value of $max\Delta_{As}$ is related to the half-difference of the upper (B_H) and lower (B_L) content tolerances (as per cent of nominal) by the ration (see the Chapter 2):

Drug product:

$$B = (B_H - B_L)/2. \qquad \max \Delta_{As} = 0.32 \cdot B. \tag{8.3}$$

The ratios of (8.1-8.3) do not cause a problem for synthetic medicines, where there are upper and lower tolerances of content. However, in the case of herbal drugs only the lower content tolerance ($Cont_L$) is standardized. The question arises: must the herbal drugs be assumed as the substances or drug products and how to calculate $max\Delta_{As}$?

The herbal drugs are described in the SPU-Eur.Ph. as substances, because it is no dosage form. But their assay validation is closer to the drug products. This is because, unlike substances, the herbal drugs are mostly objects of unspecified composition and tolerance limits of active substance contents can vary widely in different herbal drug batches. In addition, even within the same batch, the actual contents of the active substances between small herbal drug samples can also vary considerably because the effects of heterogeneity.

Given that only the lower content limits ($Cont_L$) are usually standardized for herbal drugs (for example, not less than 1.5% of the flavonoid sum for *Birch leaf* - see the Table 8.1), it is necessary *to agree* on what are the nominal contents ($Cont_{nom}$) of active substances in the herbal drugs. These nominal concentrations are references for a nominal composition of a herbal drug product with double-sided standardization of active substances. Without knowledge of the nominal concentrations we cannot turn into normalized coordinates and formulate the invariant criteria of acceptability.

Given a sufficiently large permissible variation of a concentration of an active substance in a herbal drug, we can put that the lower permissible concentration limit in it ($Cont_L$) is at 20% below the nominal content ($Cont_{nom}$) of the active substance, that is 80% of the nominal concentration. In this case, the nominal content for the herbal drug is equal to:

$$Cont_{nom}(Herb) = Cont_L / 0.8. \tag{8.4}$$

Accordingly, the total maximum acceptable procedure uncertainty for the herbal drug is (see the Chapter 2):

Drug product:

$$B = 20\%. \qquad \max \Delta_{As} = 0.32 \cdot B = 6.4\%. \tag{8.5}$$

The actual content ($Cont\%$) of an active substance in a herbal drug can several times exceed the lower limit ($Cont_L$) due to the common problem of herbal drug

standardization. However, for quality control of the herbal drug this doesn't mean: it is important that $Cont \geq Cont_L$.

The question is why we choose just B = 20% for herbal drugs? Why not more? As shown [85], the criteria of uniformity of dosage units are based on the assumption that the maximum relative standard deviation of population in different units does not exceed 10%. This is consistent with the double-sided confidence interval of 20% [26], which can be considered the maximum acceptable tolerance of content for drug products, within which there is no significant difference in pharmacological action.

8.2. Normalized coordinates

It is easier to obtain requirements to validation characteristics in normalized coordinates, because in this case they depend not on specificity of a particular object, but only from the content and range of tolerance (see the Section 2.2).

In the case of the SAM, the definition of the normalized coordinates is identical to the definition for the calibration graph method (CGM) (see the Chapter 7):

$$\begin{aligned} X_i\,(\%) &= 100 \cdot C_i\ / C_{st}. \quad C_{st} = C_{nom}. \\ Y_i\ (\%) &= 100 \cdot A_i\ / A_{st}. \quad A_{st} = A_{nom}. \\ Z_i\ (\%) &= 100 \cdot Y_i\ / X_i\ . \end{aligned} \tag{8.6}$$

Here C_{nom} is a concentration of the analyte (g/100 ml), which is the nominal concentration of the test solution by the specification. C_{nom} is calculated on the base of the nominal sample weight (m_{nom}, g), nominal analyte content in the test sample ($Cont_{nom}$,%), loss on drying or water content in per cent (LOD) and dilution Dil.

$$\begin{aligned} C_{nom} &= [m_{nom} \cdot (100 - LOD)] \cdot Dil \cdot (Cont_{nom}/100). \\ A_{nom} &= A_{1cm}^{1\%} \cdot C_{nom}. \end{aligned} \tag{8.7}$$

In the case of herbal drugs the $Cont_{nom}$ value is calculated by the ratio (8.4) on the base of the lower tolerance ($Cont_L$). For medicinal substances $Cont_{nom}$ = 100%. The values of C_{nom} and A_{nom} , calculated by the ratio (8.7), are presented in the Table 8.1.

8.3. Specificity

The question arises: should be always specificity required for a spectrophotometric assay using the SAM?

In line with the general ratio (8.1), verifying the specificity of the spectrophotometric assay is the proof of insignificance (practical or statistical) of the uncertainty associated with the background absorption (δ_{noise}%) at the analytical wavelength (see the Chapter 2), as compared with maximum acceptable procedure uncertainty

($max\Delta_{As}$). It is easy to show that this means the insignificance of the sum of information coefficients (r) of all impurities (including δ_{imp} of degradation products and δ_{exc} of excipients) at their maximum acceptable (by the specification) concentrations compared with the maximum acceptable (by the specification) uncertainty $max\Delta_{As}$, i.e. (see the Chapter 2):

Drug product:

$$\delta_{noise}(\%) = \delta_{exc} + \delta_{imp} = 100 \cdot \sum_{i=1}^{k} r_{imp.i} \approx$$
$$\approx \frac{100 \cdot \sum_{i=1}^{k} A_{\mathrm{Im}\,p,i}}{A_{nom}} \le 0.32 \cdot \max \Delta_{As}. \qquad (8.8)$$

The expression (8.8) for the SAM is the same expression for the reference standard method (see the Chapter 2), except it includes the rated nominal absorbance (A_{nom}) instead of the reference standard solution absorbance (A_{st}).

In the case of a drug product the ratio (8.8) is no doubt. The quantitative composition (and for an external control, often, the qualitative composition as well) of a drug product is a priori unknown. Active component content, unlike a medicinal substance, cannot be calculated using the impurities contents. An objective of a drug product assay, unlike a medicinal substance, is really a determination of the active substance content. Therefore an assay procedure for a drug product should be specific. Conditions of the spectrophotometric assay of the drug product should be chosen such that the ratio of (8.8) is met. The spectrophotometric assay of ambroxol tablets and its validation is an example of such an analysis using the reference standard method (see the section 2.6).

For medicinal substances, the situation is significantly different. As discussed above, the task of assay for them is not a determination of the active component content (this can be done much better by subtracting of the total impurities content and water from 100%). The task of a substance assay is a proof that the content, found within acceptable statistical uncertainty, is not significantly different from 100%, that is, another characteristic of identification. Therefore, if we have a tight control of impurities (usually using chromatographic methods), the assay of the substance can be carried out (and commonly is used) with a nonselective method. In particular, for assay of medicinal substances the SPU-Eur.Ph. traditionally widely uses non-specific titrimetric methods. Application of the spectrophotometric SAM for quality control of substances, from this point of view, is no different from the titrimetry.

Therefore, evidence of specificity of the SAM spectrophotometric substance assay for the validation stage, in general, is not required.

The question arises: maybe the requirement of specificity (8.8) for substance assay procedures, included into the SPU-Eur.Ph., is met in fact? Let's conduct relevant assessments.

In the case of medicinal substances, the impurities sum content is usually determined by HPLC using internal normalization [1]. Therefore, we can use the maximum tolerated impurities sum ($\sum imp\%$) (according to the specification) as the δ_{imp} estimation. Considering that in the case of medicinal substances δ_{exc} = 0, the ratio (8.8) gives for the SAM:

Substance, SAM:

$$\delta_{noise} = \delta_{imp} \approx \Sigma imp\% \leq \max \Sigma imp = 0.32 \cdot \max \Delta_{As}. \quad (8.9)$$

From the Table 8.4 we can see that the requirement (8.9) of insignificant influence of impurities (the requirement of specificity) is only met for two of ten medicinal substances - for riboflavine (0.025% under the criterion of 0.96%) and chloramphenicol (0.5% under the criterion of 0.64%). For the remaining eight substances the ratio (8.9) is not met. With this, the impurities sum ($\sum imp$) in some cases by more than 6 times is higher than the critical value (chloramphenicol sodium succinate: 4.0% under the criterion of 0.64%). As we can see, the requirement of specificity (8.9) for the SAM spectrophotometric assays of the medicinal substances, included in the SPU-Eur.Ph., in general, is not met in fact.

The question arises, and what about herbal drugs? As shown previously [80], for herbal drugs the concepts of "specificity" and "accuracy" are generally indefinite, so for them, this issue requires a special consideration.

8.4. Range

When the assay procedures are validated, the SPU requires to study linearity within the concentration range of not closer 80-120% of the nominal value [11]. This range can be extended up to 55-135% depending on the analytical task (dissolution study, content uniformity) (see the Chapter 2). In the case of a herbal drug analysis the range can be even wider. The common number of points is 9, distributed evenly across the range. Normalized concentration values, RSD_{range} and corresponding criteria for different ranges and analytical tasks are described earlier (see Chapter 2).

It should be noted that if the procedure is intended for medicinal substance assays only (content tolerances for them, as a rule, not exceed 97-103%), the range of 90-110% is quite enough for the linearity study. Given that the narrower the range of the study, the easier to achieve the necessary linearity, narrowing the study range for the analysis of the medicinal substances can be critical in some cases.

8.5. Uncertainty of a final analytical operation

The SPU recommends carrying out three replicate absorbance measurements with cell pulling out [15]. For such a procedure the SPU recommends to use the value Δ_{FAO} = 0.49% for prognosis of the uncertainty of the final analytical operation. This value is based on the relative standard deviation RSD_A= 0.52% of replicate absorbance measurements with cell pulling, resulting in the great inter-laboratory experiment [17]. The actual RSD_A values for modern spectrophotometers, as well as the

recommendations of the SPU for qualification of spectrophotometers (≤ 0.25% [15]), are at least 2 times less.. Note that the value Δ_{FAO} = 0.49% is not significant compared with the maximum acceptable total procedure uncertainty $max\Delta_{As}$ for all drug products, for the assay of which the spectrophotometry is used [81], and compared with the uncertainty of calibration as well (see the Table 8.1).

Thus, for the modern spectrophotometers, the uncertainty of the absorbance measurement Δ_{FAO} does not play a significant role in the mono-component spectrophotometric analysis using the SAM. That brings it with the titrimetry (see the Chapter 2).

8.6. Uncertainty of sample preparation

The uncertainty of sample preparation (Δ_{SP}), generally has two components: the uncer*t*ainty Δ_{Dil} related to the dilution of the sample (which includes the uncertainties of weighing, pipettes and flasks) and the uncertainty Δ_{Handle} related to the sample processing (extraction, evaporation, chemical reactions etc), i.e.

$$\Delta_{SP}^2 = \Delta_{Dil}^2 + \Delta_{Handle}^2. \tag{8.10}$$

The uncertainty of dilution Δ_{Dil} can be prognosed, based on the SPU requirements to volumetric glassware [11]. Results of such calculations of the Δ_{Dil} values are presented in the Table 8.1.

Prognosis of the sample processing uncertainty Δ_{handle} , in general case, is not possible. When analyzing the synthetic drug products and the lack of interaction effects (acid-base reaction, color, etc.), the total sample preparation uncertainty is the same as the uncertainty of dilution, i.e. $\Delta_{SP} = \Delta_{Dil}$. In particular, this case has a place for all medicinal substances in the Table 8.1. The dilution uncertainty can be reduced by changing the volumetric glassware and analytical procedures. So it is logical to require that, in the case of the SAM spectrophotometric analysis by characteristic absorption of the synthetic medicines, the sample preparation uncertainty must be not significant compared to the total procedure uncertainty, i.e.

Synthetic drug products, characteristic absorption

$$\Delta_{SP} \approx \Delta_{Dil} \le 0.32 \cdot \max \Delta_{As}. \tag{8.11}$$

It should be noted that the actual dilution uncertainty is usually much higher, due to the human factor (qualification of personnel) [81].

In the case of herbal drugs, the assay typically involves multiple extractions, evaporations, reagent processing, etc. [80]. So for them the ratio (8.11) fails and the issue requires a special consideration.

8.7. Uncertainty of calibration

The equation (8.1) shows that the systematic error has two components: the uncertainty δ_{noise} caused by background absorption, and the uncertainty of calibration

δ_{cal}. The uncertainty δ_{noise} can be evaluated when checking the specificity (see the ratio (8.8)). The situation with the uncertainty of calibration δ_{cal} is much more complicated.

Validation of procesures using the SAM is in many ways similar to the validation of procedures using the calibration graph method (CGM), which was considered by us earlier [69]. Similar to the CGM, the uncertainty of calibration δ_{cal} in the SAM has two main components:

$$\delta_{cal}^2 = \delta_A^2 + \delta_{line}^2. \tag{8.12}$$

Here δ_A is the uncertainty associated with the irreproducibility of the specific absorbance (or the same as the irreproducibility of the absorbance of the same solution) at the different spectrophotometers; δ_{line} is the uncertainty associated with the deviation of the a calibration graph from direct proportionality (i.e. the absolute term of the line is not zero) [69]. The contribution of these values in the total δ_{cal} value varies considerably.

8.7.1. Uncertainty associated with a deviation of a calibration line from a direct proportionality (δ_{line})

The general equation of the line in the normalized coordinates is of the form (see the section 2.3.4):

$$Y = b \cdot X + a\ . \tag{8.13}$$

Application of the reference standard method and the SAM is based on the assumption of insignificance (statistical or practical) of the absolute term (a) of the line (see the Chapter 2).

The specific absorbance (or any value that is proportional to it), included in the particular specification, must be defined for the nominal concentration. In normalized coordinates the nominal values are: X_{nom}= Y_{nom}=100%. Accordingly, the specific absorbance (AC) in the normalized coordinates for the nominal concentration is AC_{nom} = (100·b+ a)/100 = b + (a/100). For the lower concentration range limit (X_L), AC_L= b + (a/X_L). The relative change (in %) of the specific absorbance (δ_{line}) should be insignificant compared with the maximum acceptable procedure uncertainty $max\Delta_{As}$ (see the Chapter 2):

$$\delta_{line} = \left|\frac{AC_{nom} - AC_L}{AC_{nom}}\right| \cdot 100 = \left|\frac{a \cdot [(1/X_L) - (1/100)]}{b + (a/100)}\right| \cdot 100 =$$
$$= \left|\frac{a \cdot (100 - X_L)}{X_L}\right| \le 0.32 \cdot \max \Delta_{As}. \tag{8.14}$$

From here we can obtain the requirement to the absolute term of the linear relationship (8.13):

$$\left|a_{line}\right| \le \frac{0.32 \cdot \max \Delta_{As} \cdot X_L}{100 - X_L}. \qquad (8.15)$$

This requirement to the absolute term in the SAM is some tougher than the requirement for the reference standard method (see the Chapter 2). Using the ratios (8.2, 8.3, 8.5) and setting the lower concentration range limit X_L, we can get the requirements to the a_{line} value for different ranges and tolerances of medicinal substances and drug products. Results of such calculations are presented in the Table 8.3.

However, it should be noted that the requirements (8.14, 8.15) refer only to the stage of the SAM procedure development when there is no uncertainty of the specific absorbance associated with transfer to another spectrophotometer. For the validation of the SAM procedure on another spectrophotometer (that is the case we are considering), the ratios (8.14, 8.15) in their pure form are not applied, since the systematic error δ_{line} interferes with the systematic error δ_A (see below), and they cannot be separated.

8.7.2. Uncertainty of a specific absorbance (δ_A)

The δ_A value has a systematic character for the particular spectrophotometer but has a random character for different spectrophotometers. It cannot be reduced by careful experiment. It can be assumed that this value is the same for different wavelengths, but, accordingly to the SPU-Eur.Ph. requirements, it strongly depends on the absorbance value.

In accordance with the SPU-Eur.Ph. requirements [15, 82], absorbance of a potassium dichromate solution with an accurate concentration of 0.060 mg/ml at the different spectrophotometers must differ from the nominal value (A_0) not more than $\Delta A_0 = \pm 0.01$ absorbance units, regardless of the A_0 value. This makes it possible to calculate the maximum permissible relative uncertainty of the absorbance (or the same as the specific absorbance) of the potassium dichromate solution of the same concentration at any spectrophotometer. Results of such calculations are presented in the Table 8.2.

The specific absorbance uncertainty δ_A % is a maximum permissible statistically insignificant (from the point of view of the SPU) the relative difference between the absorbance values of the potassium dichromate solution of the same concentration at different spectrophotometers.

Statistically insignificant the relative difference of two absorbance values of the same concentration at the different spectrophotometers, which can be considered as the uncertainty of the specific absorbance ($max\delta_A$), is $\sqrt{2}$ times more than the permissible absorbance deviation (ΔA) from the nominal value of the A_{nom} [26], i.e.:

$$\max\delta_A = \sqrt{2}\cdot\frac{100\cdot\Delta A}{A_{nom}};\quad \Delta A = 0.01. \tag{8.16}$$

Results of $max\delta_A$ value calculations for various nominal absorbance values A_{nom} are presented in the Table 8.2.

Table 8.2

SPU-Eur.Ph. permissible deviation limits [81, 82] of the specific absorbance of potassium dichromate from the nominal values at different spectrophotometers at different wavelengths (λ); A_{nom} is the nominal absorbance of the pharmacopoeial solution of potassium dichromate (60 mg/ml); $max\delta_A$ is the maximum permissible relative uncertainty of the specific absorbance

λ, nm	$nom\ A_{1см}^{1\%}$	A_{nom}	ΔA	$100\cdot\Delta A/A_{nom}$	$max\ \delta_A$ %
SPU-Eur.Ph. requirements					
235	124.5	0.75	0.01	1.34	1.9
257	144.5	0.87	0.01	1.15	1.6
313	48.6	0.29	0.01	3.43	4.8
350	107.3	0.64	0.01	1.55	2.2
430	15.9	0.95	0.01	1.05	1.5
Estimated values					
		0.15	0.01	6.67	9.4
		0.20	0.01	5.00	7.1
		0.30	0.01	3.33	4.7
		0.40	0.01	2.50	3.5
		0.50	0.01	2.00	2.8
		0.60	0.01	1.67	2.4
		0.70	0.01	1.43	2.0
		0.80	0.01	1.25	1.8
		0.90	0.01	1.11	1.6
		1.00	0.01	1.00	1.4

As can be seen, the SPU-Eur.Ph. maximum permissible relative difference of the specific absorbance at the different spectrophotometers $max\,\delta A$ depends on the

absorbance value. As optimal absorbance range for the SAM we can consider the range of A_{nom} = 0.5-1.0, i.e.:

***SAM**:* $$Optimum\ A_{nom} = 0.5 - 1.0. \tag{8.17}$$

The Table 8.2 shows, for this absorbance range the calibration error is between 1.4 to 2.8%, which corresponds to maximum tolerances for substances 97-103%. It is also consistent with the results of professional testing [81], which showed that in Ukraine the use of the SAM is correct only for assay of drug products with tolerances of $\pm$ 10% and wider (i.e. $max\Delta_{As} \geq 3.2\%$) [81].

It should be noted that the further A_{nom} increase is meaningless, since the absorbance range $A_{nom} \geq 1.0$ is not covered by the SPU-Eur.Ph. requirements (see the Table 8.2).

The main conclusion of the Table 8.2 is that the specific absorbance uncertainty δ_A, and with it the total systematic procedure error δ_{tot} cannot be made insignificant compared to the total procedure uncertainty $max\Delta_{As}$. It is therefore necessary, as in the case of the calibration graph method (see the Chapter 7), to make any assumptions about its relationship with $max\Delta_{As}$.

8.8. The accuracy and precision of SAM

The total procedure uncertainty Δ_{As} can be represented as the sum of two summands: the total systematic δ_{tot} and random Δ_{prec} components, i.e.:

$$\Delta_{As}^2 = \delta_{tot}^2 + \Delta_{prec}^2 \leq \max \Delta_{As}^2. \tag{8.18}$$

The total systematic error (δ_{tot}) describes the procedure accuracy. It includes several factors (see above). They are: the specific absorbance uncertainty (δ_A), deviations from linearity (δ_{line}) and the impurities influence (δ_{imp}). It should be noted that although the specificity for SAM analysis of substances is not required (see above), but the presence of impurities can cause significant deviations of the obtained content values from 100%. If the specific absorbance values of the impurities are higher than that of the basic component, they systematically overestimate results compared to 100%, and if lower, then underestimate.

These summands cannot be separated, but to estimate their total contribution at the stage of validation is required.

The random summand Δ_{prec} describes the procedure precision and includes as a final analytical operation and sample preparation.

We can, like for the calibration graph method (CGM) (see the Chapter 7), suppose that the maximum acceptable contributions of systematic (δ_{tot}) and random (Δ_{prec}) summands of the total procedure uncertainty ($max\Delta_{As}$) are about the same, i.e.:

$$\delta_{tot} \le \max \delta_{tot} = \max \Delta_{prec} \ge \Delta_{prec}. \tag{8.19}$$

In this case, as shown for the CGM, we can get from the ratio (8.18) (see Chapter 7):

$$\max \delta_{tot} = \max \Delta_{prec} = (\sqrt{2}/2) \cdot \max \Delta_{As} = 0.71 \cdot \max \Delta_{As}. \tag{8.20}$$

Given the ratios of (8.2, 8.3, 8.5), we can get:

Substances: $$\max \delta_{tot} = \max \Delta_{prec} = 0.71 \cdot B. \tag{8.21}$$

Drug products: $$\max \delta_{tot} = \max \Delta_{prec} = 0.23 \cdot B \ . \tag{8.22}$$

Herbal drugs: $$\max \delta_{tot} = \max \Delta_{prec} = 0.23 \cdot B. \tag{8.23}$$

The total systematic error (δ_{tot}) includes also the specific absorbance uncertainty (δ_A) from the ratios (8.16) and (8.20). So we can get the requirements to the minimum value of the nominal absorbance:

$$A_{nom} \ge \frac{2}{\max \Delta_{As}}. \tag{8.24}$$

The minimum A_{nom} values calculated by the ratio (8.24) are presented in the Table 8.3.

Comparison of the ratio (8.23) with the Table 8.1 shows that, despite the very wide limits for the herbal drugs, the requirements of the ratio (8.23) are not met for the majority of the herbal drugs and the main reason for this is the low values of the nominal absorbance (A_{nom}), i.e. the failure of the ratio (8.24).

The requirement (8.21) fails also for a number of substances of the Table 8.1: the specific absorbance uncertainty (δ_A) exceeds in some cases the $max\Delta_{As}$ values of the ratio (8.2) for substances. The comparison of the Tables 8.1-8.3 shows that the $max\Delta_{As}$ values must be at least 2.5-3.0%. In this case, the ratio (8.21) is met.

The results of $max\delta_{tot}$ calculations by the ratios of (8.19-8.23) are presented in the Table 8.3.

Table 8.3

Critical values of systematic ($max\ \delta_{tot}$) and total procedure uncertainty ($max\Delta_{As}$) and parameters of the linear relationship $Y_i = b \bullet X_i + a$ for various tests, g = 9 points and various tolerances of content (B)

Критические значения систематической ($max\ \delta_{tot}$) и полной неопределенности ($max\Delta_{As}$) методики анализа и параметров линейной зависимости $Y_i = b \bullet X_i + a$ для различных испытаний, g = 9 точек и различных допусков содержания B

Test*	Range, step, RSD_{range} %	B%	$max\Delta_{As}$ %	$max\delta_{tot} = max\Delta_{prec}$ %	RSD_o %	$min\ R^2_c$	$maxa$ %	$min\ A_{nom}$
Substances								
Assay	Range =80-120 step = 5 RSD_{range}=13.69	1.0	1.0	0.7	0.37	0.9993	1.6	2.00
		1.5	1.5	1.1	0.56	0.9983	2.2	1.33
		2.0	2.0	1.4	0.75	0.9970	2.9	1.00
		2.5	2.5	1.8	0.93	0.9954	3.7	0.80
		3.0	3.0	2.1	1.1	0.9933	4.4	0.67
Drug products								
Assay	Range =80-120 step = 5 RSD_{range}=13.69	5.0	1.6	1.1	0.60	0.9981	2.3	1.25
		7.5	2.4	1.7	0.90	0.9957	3.5	0.83
		10.0	3.2	2.3	1.2	0.9924	4.7	0.63
		15.0	4.8	3.4	1.8	0.9829	7.0	0.42
Assay Herb	Range =50-150 step = 12.5 RSD_{range}=34.23	20.	6.4	4.5	2.4	0.9952	5.0	0.31
ConU	Range =70-130 step = 7.5 RSD_{range}=20.54		3.0	2.1	1.1	0.9934	3.1	0.67
Dis	Range =50-130 step = 10 RSD_{range}=30.43		3.0	2.1	1.1	0.9934	2.3	0.67
	Range =55-135 step = 10 RSD_{range}=27.39		3.0	2.1	1.1	0.9934	2.4	0.67
Assay ConU	Range =55-135 step = 10	5.0	1.6	1.1	0.60	0.9981	1.3	1.25
		7.5	2.4	1.7	0.90	0.9957	1.9	0.83
		10.0	3.2	2.3	1.2	0.9924	2.6	0.63

Dis	RSD_{range}=27.39	15.0	4.8	3.4	1.8	0.9829	3.9	0.42
		20.0	6.4	4.5	2.4	0.9696	5.2	0.31
Assay ConU Dis	Range =60-135 step = 9.4 RSD_{range}=25.67	5.0	1.6	1.1	0.60	0.9981	1.4	1.25
		7.5	2.4	1.7	0.90	0.9957	2.1	0.83
		10.0	3.2	2.3	1.2	0.9924	2.7	0.63
		15.0	4.8	3.4	1.8	0.9829	4.1	0.42
		20.0	6.4	4.5	2.4	0.9696	5.5	0.31

* ConU – content uniformity, Dis – dissolution, Herb – herbal drugs

8.9. The criteria of linearity

It is reasonable to carry out the linearity study using$g$ = 9 points as usual (see the Chapter 2).

8.9.1. A residual standard deviation RSD_o

A confidence interval of points variation around the line $Y_i = b \bullet X_i + a$ is equal to $t(95\%,g\text{-}2)\bullet RSD_o$ and is a confidence interval of the test sample analysis uncertainty (Δ_{prec}), which should satisfy the inequalities of (8.19-8.23). With this in mind, as well as [26], we can get:

Substance: $$\Delta_{prec} = t(95\%, g-2)\cdot RSD_o = 1.89\cdot RSD_o \le 0.71\cdot B. \quad (8.25)$$

Drug product, herbal drug: $$\Delta_{prec} = 1.89\cdot RSD_o \le 0.23\cdot B. \quad (8.26)$$

From here we can obtain the requirements to the RSD_0 value (g = 9):

Substance: $$RSD_o \le 0.37\cdot B. \quad (8.27)$$

Drug product, herbal drug: $$RSD_o \le 0.12\cdot B. \quad (8.28)$$

In the case of tests “Content uniformity” and ”Dissolution” the maximum analysis uncertainty is $max\Delta_{As}$ = 3.0%, which corresponds to the formal tolerances of B = 9.3% (see Chapter 2). This value should be put for these tests data into the ratios of (8.27-8.28).

8.9.2. Correlation coefficient

The correlation coefficient is calculated from the formula [26]:

$$R_c = \sqrt{1 - \frac{RSD_0^2}{RSD_{range}^2}}. \qquad (8.29)$$

The use of normalized coordinates, ratios (8.27-8.28) and RSD_{range} (the standard deviation of all the model solution concentrations in the normalized coordinates (see the section 2.2)) allows us to obtain the acceptability criteria for the R_c values. Given the high values of R_c, it is advisable to regulate the R^2_c values instead of the R_c values. These calculations are presented in the Table 8.3.

8.9.3. *Y*-intercept

The absolute term (a) of the line (8.13) characterizes a systematic error. In accordance with the section 2.3.4, the requirements to it can be of two types:

1. A *statistically* insignificant difference from zero: the value (a) must be less than the confidence interval of its uncertainty, i.e. (g = 9):

Statistical insignificance: $$a \le t(95\%, g-2) \cdot s_a = 1.89 \cdot s_a. \qquad (8.30)$$

Here s_a is the standard deviation of the absolute term of the line (a) obtained by the least squares method.

2. *An acceptable value* of the absolute term (*Y-intercept*). This concept in the case of the SAM replaces the concept of the "*Practical insignificance* of the absolute term" which is applied to the reference standard method (see the Chapter 2).

In the case of the reference standard method the absolute term is insignificant if the systematic error, caused by it, is not significant compared with the maximum acceptable analysis uncertainty $max\Delta_{As}$. In the case of the SAM, at the stage of the procedure development, this requirement is also applied, and it is the ratio (8.15).

However, at the stage of the SAM procedure validation for external analysis (i.e. for another spectrophotometer) we have to consider, as it is illustrated in the section 8.7.2, with the systematic error (δ_A) which can reach, in accordance with the Table 8.2, very large values.

The systematic error δ_A (which is the main part of the total systematic error δ_{tot}) corresponds to an absolute term $a_{\delta A}$, the absolute value of which, taking into account the ratio (8.18), corresponds to the ratio:

$$\left|a_{\delta A}\right| \le \max \delta_A \le \max \delta_{tot} = 0.71 \cdot \max \Delta_{As}. \qquad (8.31)$$

Comparison of ratios (8.15) and (8.31) shows that $a_{\delta A}$ depends entirely on $max\Delta_{As}$, while a_{line} is also dependent on the range, i.e. X_L. Values of a_{line} and $a_{\delta A}$ are random in relation to each other, so the requirements to the resulting absolute term (a) are of the form [26]:

Acceptability requirement:

$$|a| \le \sqrt{\max a_{line}^2 + \max a_{\delta A}^2}. \qquad (8.32)$$

We cannot talk about the practical insignificance of the absolute term (a) in the case of the SAM (ratio (8.32)). A similar situation is in the calibration graph method (see the Chapter 7). It is rather to talk about *acceptability* of the a value.

With this in mind, in the case of the SAM, the absolute term (a) can be considered acceptable for solving the task, if a systematic error, contributed by it, does not exceed the requirements of the ratio (8.32), where the values of a_{line} and $a_{\delta A}$ meet the requirements of (8.15) and (8.31). The results of a value calculations by the ratios of (8.15, 8.31, 8.32) are presented in the Table 8.3.

Like for the reference standard method, the requirement of acceptability (8.32) is applied only if the criterion (8.30) of statistical insignificance fails.

8.9.4. Limit of detection (*DL*) and limit of quantitation (*QL*)

These values are not required when the assay validation is carried out, but they are useful as information about how a range of application surpasses its limit capabilities ("safety margin" of the procedure). In case of impurities control, acquisition of the *DL* and *QL* values is required (see the Chapter 1).

In accordance with the SPU-Eur.Ph. requirements [11], *DL* and *QL* values can be calculated on the base of the absolute term standard deviation (s_a) of the line and its slope (b):

SAM:

$$DL = 3.3 \cdot s_a / b. \qquad (8.33)$$

SAM:

$$QL = 10 \cdot s_a / b. \qquad (8.34)$$

8.10. Solution stability study

Checking the stability of the test and reference solutions is one of the elements of the procedure robustness study (see the section 2.3.7) and must be carried out before all other validation studies. Usually we need to show that solutions are stable for at least 1 hour (see the section 2.3.7). This means that the systematic error, contributed by their instability (δ_t), must be insignificant compared with the maximum acceptable total uncertainty of analysis ($max\Delta_{As}$), i.e. (see the section 2.3.1):

$$\delta_t \le 0.32 \cdot \max \Delta_{As}. \qquad (8.35)$$

In the case of SAM spectrophotometric analysis, we must show that the change in the absorbance of the test sample solution during 1 hour in normalized coordinates (i.e. the change of the Y value of the equation (8.6)) meets the requirements of the ratio (8.35). To do this, we carry out the replicate absorbance measurements after t = 0, 15, 30, 45 and 60 minutes, then calculate by the equation (8.6) the Y_t values, their

standard deviation (RSD_t %) and the confidence interval Δ_t % (one-tailed *t*-criterion for 4 degrees of freedom and the probability of 0.95 is 2.13 [26]), which must conform to the requirements of the ratio (8.35), i.e.:

$$\Delta_t(\%) = 2.13 \cdot RSD_t \le 032 \cdot \max \Delta_{As}.. \quad (8.36)$$

The $max\Delta_{As}$ value is found from the equations $max\Delta_{As}$ (8.2-8.3).

8.11. Prognosis of the total procedure uncertainty

Prognosis of the total analysis uncertainty can be carried out in the common way (see the section 2.4) by the equation (8.1) using the ratios (8.10-8.12, 8.14, 8.16) and considering Δ_{FAO} = 0.49% (see the section 8.5). Such a prognosis is only possible for synthetic medicinal substances and drug products, where sample preparation uncertainty is the uncertainty of sample dilution, i.e. the ratio (8.11) is met.

It should be noted, however, that the δ_{line} value is a priori unknown. It makes difficult to use this approach. More correct is to apply the ratio (8.18), in which the limit value $max\delta_{tot}$ should be taken for δ_{tot} (as it is unknown). With this, the $max\delta_{tot}$ value is taken from the Table 8.3 and Δ_{prec} is calculated by the ratio:

$$\Delta^2_{prec} = \Delta^2_{SP} + \Delta^2_{FAO}. \quad (8.37)$$

The uncertainty of calibration Δ_{SP} is calculated in the common way (see the section 2.4), and Δ_{FAO} is considered to be Δ_{FAO} = 0.49% (see the section 8.5). In this case the ratio (8.18) takes the form:

$$\Delta^2_{As} \le \max \delta^2_{tot} + \Delta^2_{SP} + \Delta^2_{FAO} \le \max \Delta^2_{As}. \quad (8.38)$$

For herbal drugs, the uncertainty of Δ_{Handle} related to the sample processing (extraction, evaporation, chemical reactions etc) is very important. It cannot be predicted in general case. Therefore, the question of the uncertainty prognosis for herbal drugs requires of separate consideration.

The predicted total analysis uncertainty should not exceed the maximum acceptable uncertainty of analysis $max\Delta_{As}$ (Table 8.3).

8.12. Intermediate precision

The intermediate precision study for procedures using the reference standard method is carried out with use of the *Confirming approach* (see the section 1.7.2), i.e. the confidence interval of the normalized values of Z (see the ratio of (8.6)), received under different conditions, must not exceed the maximum acceptable procedure uncertainty ($max\Delta_{As}$). To do this, examine under the specification procedure n = 5 samples (sample weights) of the same batch of the drug product under investigation in the m = 3 different days. Studies are carried out different analysts using different equipment (spectrophotometers, cells, volumetric glassware). All the results obtained

(Z_i) must belong to the same population. So calculate for them the pooled mean (Z_{intra}), standard deviation ($SD_{Z\text{-}intra}$%) and the relative confidence interval (Δ_{intra} %) (see the section 2.6.1). The Δ_{intra} value should not exceed the $max\Delta_{As}$ value of the equations (8.2-8.3) and Table 8.3, i.e.:

$$\Delta_{intra} = t[95\%,(n\cdot m-1)]\cdot SD_{Z-intra} \le \max\Delta_{As}. \qquad (8.39)$$

This approach has worked well for drug products (see the Chapter 2). However, it is uncertain for a medicinal substance assay. The reason is the indefiniteness of the concept “5 samples” for the medicinal substance. Are there different batches of the same substance? But they can have different impurities content that could affect the results of the assay and does not allow us to get a sample from the same population. In addition, in the case of a drug product analysis, the different samples (model mixtures) have different concentrations within the analytical range. In the case of the medicinal substances we check only one some point of the range, since the concept of a "model mixture" for them is absent. It is obvious that to confirm the intermediate precision we have to analyze different dilutions of the same substance in different days.

Therefore, it is advisable to use an approach that we proposed for the validation of analytical procedures for dissolution profile studies [71]. It is that validation studies are repeated in the other day at the same spectrophotometer. The results must meet the above mentioned criteria for linearity, accuracy and precision. In addition, the pooled sample of 18 points should meet the requirements for precision of (8.21), i.e.

$$\Delta_{intra} = t[95\%,17)]\cdot SD_{Z-intra} = 1.76\cdot SD_{Z-intra} \le \max\Delta_{prec} = 2.1\%.. \qquad (8.40)$$

The advantage of this approach is that the intermediate precision is confirmed for the whole analytical range in two independent experiments, as well as for the pooled (two times larger) sample.

The question is why the intermediate precision study should be carried out at the same spectrophotometer? The fact of the matter is that the absorbance at different spectrophotometers can vary quite significantly (see the Table 8.1) by the calibration error of δ_A. For randomization of this error use of two spectrophotometers is too little, they must be at least five. However, this is already the inter-laboratory experiment (for it we must take in the ratio of (8.40) the $max\Delta_{As}$ value instead of the $max\delta_{prec}$ value). Therefore, to prove the intermediate precision this approach is more appropriate (in fact in the laboratory the analysis is carried out at the same spectrophotometer). It should be noted that such a problem is absent in the reference standard method - due to the lack of the calibration error of δ_A.

It should also be noted that, in connection with the mandatory GMP requirements in Ukraine, quality control of medicinal substances is carried out almost exclusively by manufacturers, i.e. within one laboratory.

8.13. Example. Validation of an assay of the prednisolone substance by SAM

8.13.1. Choice of the object of study

The Table 8.4 shows that only 2 medicinal substances (*Hydrocortisone acetate* and *Prednisolone*) meet the requirements of the Table 8.3 to the minimum nominal absorbance value ($minA_{nom}$). We can perform the validation for them without correction of the nominal absorbance or content tolerances. In all other cases the systematic error of the specific absorbance ($max\delta_A$) is too large for the correct external spectrophotometric analysis with use of the SAM.

Table 8.4

The metrological characteristics for the medicinal substances described in the SPU, assay of which is carried out by the SAM

№	Наименование	Допуски	$max\Delta_{As}$ %	A_{nom}	$max\delta_A$ %	$minA_{nom}$	$\sum imp$ %	$max \sum imp$ %
1.	*Betamethasone dipropionate*	97.0-103.0	3.0	0.604	2.3	0.67	2.5	0.96
2.	*Hydrocortisone acetate*	97.0-103.0	3.0	0.786	1.8	0.67	1.5	0.96
3.	*Prednisolone*	97.0-103.0	3.0	0.822	1.7	0.67	2.0	0.96
4.	*Prednisolone sodium phosphate*	96.0-103.0	3.0	0.574	2.5	0.67	3.0	0.96
5.	*Riboflavine*	97.0-103.0	3.0	0.420	3.4	0.67	*0.025*	0.96
6.	*Rifampicin*	97.0-102.0	2.0	0.370	3.8	1.00	3.5	0.64
7.	*Testosterone propionate*	97.0-103.0	3.0	0.488	2.9	0.67	1.0	0.96
8.	*Chloramphenicol*	98.0-102.0	2.0	0.591	2.4	1.00	*0.5*	0.64
9.	*Chloramphenicol sodium succinate*	98.0-102.0	2.0	0.431	3.3	1.00	4.0	0.64
10.	*Cyanocobalamin*	96.0-102.0	2.0	0.455	3.1	1.00	3.0	0.96

Given these data, the *Prednisolone* substance was chosen as an object of study. To neutralize the influence of the substance impurities, validation studies were conducted with use of the Prednisolone SPU CRS, certificate № 1/11/2143 with the certified value of 99.8%. According to the certificate, this CRS is intended for one-wave spectrophotometric assays with $max\Delta_{As} \geq 1.6\%$ that is quite suitable for assay of *Prednisolone* substance ($max\Delta As = 3.0\%$).

8.13.2. Equipment qualification [81]

8.13.2.1. Cells

Estimation the cell difference between a pair of standard cells (A:B) were conducted by measuring the absorbance of a compensation solution (according to the procedure it is *96% alcohol R*) and then cell turn on 180° and the second measurement. The difference between the absorbance means of the three replicate measurements of the compensation solution in the original cell position and turned on 180° was $\Delta A = A_{cp.1} - A_{cp.2} = 0.0949\text{-}0.0933 = 0.0016$, i.e. met the requirement [81]:

$$\delta_{dif} \leq 0.002. \tag{8.41}$$

8.13.2.2. Accuracy of absorbance

Before starting the experiment we conducted testing of the absorbance accuracy for the spectrophotometer (SPECORD-200) using the pharmacopoeial potassium dichromate solution in 0.005 M sulphuric acid ($m_n = 0.060$ g) in accordance with the SPU methodology [15, 81]. The results are presented in the Table 8.5.

Table 8.5

Verifying the absorbance accuracy

Wavelength λ, нм	Absorbance		Specific absorbance		Absorbance deviation				Specific absorbance uncertainty, %	
					absolute		relative			
	A^*	A_{nom}	$A_{1\text{см}}^{1\%}$	$nomA_{1\text{см}}^{1\%}$	ΔA	ΔA_0	ΔA,%	$\frac{\Delta A}{A_{nom}} \times 100$	δ_A	$max\delta_A$
235	0.7493	0.747	124.88	124.5	0.0023	0.01	0.31	1.34	0.31	1.9
257	0.8662	0.867	144.37	144.5	0.0008	0.01	0.09	1.15	0.09	1.6
313	0.2918	0.292	48.63	48.6	0.0002	0.01	0.07	3.43	0.07	4.8
350	0.6461	0.646	107.68	107.3	0.0001	0.01	0.02	1.55	0.36	2.2
430	0.9285	0.954	15.90	15.9	0.0255	0.01	2.67	1.05	0.01	1.5

* The mean of the three absorbance measurements

As can be seen from the Table 8.5, the absorbance accuracy meet the SPU requirements [15].

8.13.2.3. Repeatability of absorbance with the cell withdrawal

Qualification of the spectrophotometer was carried out, getting thirty replicate absorbance values of the tested prednisolone substance solution against the compensation solution using random cell positions. Calculated on the basis of

experimental data (A_{cp} = 0.8240, SD = 0.0021) the relative standard deviation of absorbance RSD = 0.25% complies with the SPU requirements HFCS [15, 81]:

$$S_{Ac,r} = 0.25 \leq 0.25\%. \quad (8.42)$$

8.13.2.4. Limit of stray light

Stray light limits the maximum absorbance that can be achieved at the spectrophotometer at a given wavelength. The level of stray light, in general, is inversely proportional to the fourth power of the wavelength [81], therefore the stray light level control is relevant for the ultraviolet region.

Determination of the stray light level was carried out in accordance with the SPU-Eur.Ph. requirements [15, 82], under specified wavelengths with appropriate solutions. The mean absorbance value of the three measurements of the test solution (12 g/l *potassium chloride R*) in a cell with a thickness of 1 cm is dramatically increased in the wavelength range of 220, 200 and 198 nm using *water R* as a compensation solution and was equal to A_{mean} = 2.554. The result met to the SPU requirements (2.554 > 2.0) [15].

8.13.2.5. Requirements to solvents

96% alcohol R is used for the prednisolone assay as a solvent. Its absorbance measured against the air at the analytical wavelength λ = 243 nm was equal to 0.1665 ≤ 0.2, i.e. met the SPU requirements [15, 82].

8.13.3. Validation of the assay of the prednisolone substance

8.13.3.1. Requirements to the maximum acceptable total procedure uncertainty ($max\Delta_{As}$).

According to the relation (8.2) and the Table 8.4, for prednisolone, $max\Delta_{As}$ = 3.0%.

8.13.3.2. Prognosis of the sample preparation uncertainty (Δ_{SP}).

Prognosis of the sample preparation uncertainty Δ_{SP} (see the section 2.6.6.6) is shown in the Table. 8.6.

Table 8.6

Prognosis of the sample preparation uncertainty for the prednisolone substance assay procedure

Sample preparation stage	Formula parameter	Uncertainty,%
Test solution		
Weighing	m_0	0.2 mg/100 mg · 100 % = 0.2 %

Diluting to the volume in a 100 ml volumetric flask	100	0.12 %
Aliquot sampling with a 2 ml pipette	2	0.5 %
Diluting to the volume in a 100 ml volumetric flask	100	0.12 %
	$\Delta_{SP} = \sqrt{0.2^2 + 0.12^2 + 0.5^2 + 0.12^2} = 0.56 \leq 0.96\%$	

According to the relation (8.11), the requirement of insignificance of the Δ_{SP} value compared with the maximum acceptable analysis uncertainty (Δ_{As}) must be satisfied:

$$\Delta_{SP} \leq 0.32 \cdot \max \Delta_{As} = 0.32 \cdot 3.0 = 0.96\% .$$

As can be seen from the Table 8.6, this requirement is met.

8.13.3.3. Prognosis of the total procedure uncertainty (Δ_{As})

Such a prognosis was carried out by the ratio (8.39). The predicted total analysis uncertainty should not exceed the maximum acceptable analysis uncertainty ($\Delta_{As} \leq 3.0\%$). The total predicted assay uncertainty for the prednisolone substance we calculated on the basis of the ratio (8.39) and the Table 8.3 (from which we found $max\Delta_{As} = 3.0\%$, $max\delta_{to} = 2.1\%$) and assuming $\Delta_{FAO} = 0.49\%$ (see section 8.5):

$$\Delta_{As} \leq \sqrt{\max\delta_{tot}^2 + \Delta_{SP}^2 + \Delta_{FAO}^2} = \sqrt{2.1^2 + 0.56^2 + 0.49^2} = 2.2 \leq 3.0\%.$$

As we can see, the predicted total uncertainty of the prednisolone substance assay complies with the SPU requirements.

8.13.3.4. Assessment of the procedure specificity

As discussed above (see the section 8.3), a spectrophotometric medicinal substance assay using the SAM does not require proving specificity. However, assessment of the procedure specificity is useful. It can be obtained by the ratio (8.9), from which it follows that the maximum permissible sum of all impurities should be insignificant compared to the total maximum acceptable procedure uncertainty, i.e. the next ratio must met (see the Table 8.3):

$$\Sigma imp \leq 0.32 \cdot \max \Delta_{As} = 0.96\%. \qquad (8.43)$$

The Table 8.4 shows, for the prednisolone substance we have $\sum imp = 2.0\%$. As we can see, the requirement of specificity fails, i.e. impurities have a significant influence on the spectrophotometric analysis.

8.13.3.5. Robustness

Checking the stability of the solutions was carried out within an hour (every 15 minutes), measuring the absorbance values of the test solution with the nominal according to the procedure. Results of the study are presented in the Table 8.7.

Table 8.7

Results of the test solution stability study

Test solution	Time of the study n_t, min					$C_{r,t}$	$S_{r,t}$	RSD_t, %	Δ_t, %	$0.32 \cdot max\Delta_{As}$ %
	0	15	30	45	60					
A_i	0.8152	0.8209	0.8230	0.8216	0.8218	99.78	0.2558	0.26	**0.55**	**0.96**
	0.8191	0.8166	0.8212	0.8223	0.8215					
	0.8171	0.8202	0.8170	0.8230	0.8224					
$A_{cp.}$	0.8171	0.8192	0.8204	0.8223	0.8219					
$Y_i=100*A_t/A_{nom}$, % (A_{nom}=0.822 нм)	99.41	99.66	99.81	100.04	99.99					

The stability of the absorbance of the test solution within one hour is characterized by the confidence interval of ± 0.55% and is insignificant compared with the maximum acceptable total procedure uncertainty $max\Delta_{As}$ ($0.55 \leq 0.96$).

An effect of minor fluctuations in *pH* on the test solution absorbance was studied adding by one drop of the 0.01 M hydrochloric acid or 0.01 M sodium hydroxide to get *pH* fluctuations within ± 10%. The absorbance values of the obtained model solutions were measured at 243.5 nm. The results of influence of *pH* fluctuations on analysis results are presented in the Table 8.8.

Table 8.8

The study of influence of pH on the absorbance of model solutions

Test solution	Absorbance A_t^* (λ = 243.5 nm)			$C_{r,pH}$	$S_{r,pH}$	RSD_{pH}, %	ΔpH, %	$0.32*max\Delta_{As}$, %
	Solution 1: + *X* drops of 0.01 M HCl	Solution 2: without changes	Solution 3: + *X* drops of 0.01M NaOH					
Test solution + 1 drop of the reagent								
1	0.8165	0.8152	0.8176	99.59	0.11	0.11	0.33	0.96
2	0.8179	0.8191	0.8204					
3	0.8191	0.8216	0.8210					
A_{mean}	0.8178	0.8186	0.8197					
$Y_i=100 \cdot A_t/A_{nom}$, % ($A_{nom}$=0.822)	99.49	99.59	99.72					
Test solution + 2 drops of the reagent								
1	0.8166	0.8152	0.8194	99.74	0.21	0.21	0.62	0.96
2	0.8197	0.8191	0.8226					

3	0.8209	0.8216	0.8235					
A_{mean}	0.8191	0.8186	0.8218					
Y_i=100·A_i/A_{nom}, % (A_{nom}=0.822)	99.64	99.59	99.98					
Test solution + 3 drops of the reagent								
1	0.8187	0.8152	0.8205	99.96	0.33	0.33	0.96	0.96
2	0.8232	0.8191	0.8246					
3	0.8258	0.8216	0.8263					
A_{mean}	0.8226	0.8186	0.8238					
Y_i=100·A_i/A_{nom}, % (A_{nom}=0.822)	100.07	99.59	100.22					

* Mean of three absorbance measurements

Study on the robustness of the prednisolone substance assay procedure showed that pH fluctuations of the final solutions within ± 10% does not significantly affect the reproducibility of the absorbance: $\Delta_{pH} = 0.96 \leq 0.96$.

8.13.3.6. Linearity

Linearity of the procedure were studied for 9 model solution concentrations that cover the range from 80% to 120% of the nominal content. Calculations were carried out in normalized coordinates. Using all 9 solutions, we calculated by the least squares method [26] the dependence of the absorbance ratios $Y_i = (A_i/A_{nom})\cdot 100$ on the concentration ratios $X_i = (C_i/C_{nom})\cdot 100$, i.e. the dependence:

$$Y_i = b \cdot X_i + a\,.$$

The obtained linear relationships for the prednisolone substance are:

$$1 \text{ day: } Y_i = 0.9655 \cdot X_i + 1.3.$$

$$2 \text{ day: } Y_i = 0.9877 \cdot X_i + 1.2.$$

Metrological characteristics of these relationships are presented in the Table 8.9,

Table 8.9

The metrological characteristics of the linear relationships for prednisolone on different days. The range 80-120%, the number of points 9

Parameter	Value		Criteria (for tolerances 97-103%)	Conclusion (meet or fail)
	1 day	2 day		
b	0.9655	0.9877	-	-
s_b	0.0067	0.0012	-	-
a	1.3		1) statistical insignificance $\leq 1.89 \cdot s_a = 1.3$;	Meet

		1.2	$\leq 1.89 \cdot s_a = 2.3$; 2) acceptable value $a \leq 4.39$	Meet Meet
s_a	0.68	1.2	-	-
RSD_o	0.26	0.45	≤ 1.1	Meet
r^2	0.9996	0.9991	≥ 0.9933	Meet

Example of graphical data presentation - see the Figure. 8.1.

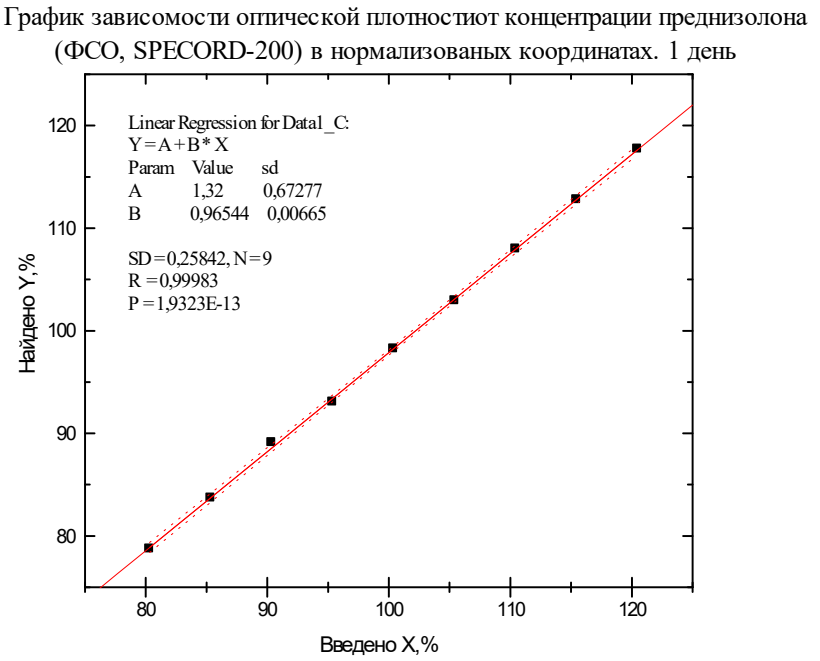


Рис. 8.1. The linear relationship of the absorbance on the prednisolone concentration in normalized coordinates: 1 day

As can be seen, the linearity requirements for the prednisolone substance assay procedure are met for two different days.

8.13.3.7. Accuracy and precision

The procedure precision and accuracy were evaluated on the base of the linearity study data, obtained during analysis of the same sample on different days in the same laboratory at the same device. Results of the precision and accuracy calculations are presented in the Table 8.10.

Table 8.10

The results of analysis of the model mixtures on different days and their statistical processing

Solution number	Entered concentration as % of reference standard solution concentration $X_i = (C_i / C_{nom}) \cdot 100\%$		Found concentration as % of reference standard solution concentration $Y_i = (A_i^* / A_{nom}) \cdot 100\%$		Found as % of entered $Z = (Y_i / X_i) \cdot 100\%$	
	Day 1	*Day 2*	*Day 1*	*Day 2*	*Day 1*	*Day 2*
1	80.32	80.16	78.75	80.31	98.04	100.18
2	85.34	85.17	83.69	85.63	98.06	100.54
3	90.36	90.18	89.10	89.99	98.61	99.79
4	95.38	95.19	93.05	95.14	97.55	99.95
5	100.40	100.20	98.24	100.80	97.85	100.60
6	105.42	105.21	102.94	104.93	97.65	99.74
7	110.44	110.22	107.99	109.61	97.78	99.44
8	115.46	115.23	112.77	115.73	97.67	100.43
9	120.48	120.24	117.72	119.70	97.71	99.55
Mean, *X%*					**97.9**	**100.0**
Relative standard deviaton, $RSD_x\%$					0.32	0.43
Relative confidence interval $\Delta_{prec}\% = t(95\%,8) \cdot RSD_x = 1.86 \cdot RSD_x$					**0.60**	**0.80**
Critical value of results repeatability $\Delta_{prec} \leq 2.1\%$					Соотв.	Соотв.
Systematic error $\delta = \lvert X - 100 \rvert$					**2.1**	**0.0**
Criterion of systematic error acceptability (1) $\delta \leq \Delta_{prec}/3 = 0.60/3 = 0.20$; $\delta \leq \Delta_{prec}/3 = 0.80/3 = 0.27$ If (1) fails then must be (2) $\delta \leq max\delta_{tot} = 2.1$					*Fail* Meet	 Meet Meet
Conclusion about the procedure for every day:					**Correct**	**Correct**
Intermediate procedure						
Pooled mean $Z_{intra}\% =$					**99.0**	

Pooled standard deviation $SD_{z\text{-}intra}\%=$	**1.2**
Pooled confidence interval $\Delta_{intra}\%= t(95\%,17)\cdot SD_{z\text{-}intra}\% = 1.74\ SD_{z\text{-}intra}\%$	**2.0**
Critical value of the results repeatability $\Delta_{intra} \leq 2.1\%$	Meet
Intermediate systematic error $\delta=$	**1.0**
Criterion of systematic error acceptability (1) $\delta \leq \Delta_{intra} / \sqrt{18} =$ 1.2/4.2 = 0.27% If (1) fails then must be (2) $\delta \leq max\delta_{tot}$ =2.1%	 *Fail* Meet
Intermediate procedure precision:	**Correct**
Общий вывод о методике:	**Correct**

***** Mean of three absorbance measurements

8.13.3.8. Intermediate precision

Calculations were carried out by the ratio (8.40), the results are presented in the Table 8.10. As can be seen, the requirements of the intermediate precision are met. The whole procedure is correct in general, as well.

Summarizing the researches on validation of the prednisolone substance assay procedure using the SAM, it can be concluded that the use of the SAM for medicinal substance assays requires careful analysis and consideration of many factors, among which the first is the qualification of the spectrophotometer. The use of the SAM for medicinal substance analysis is promoted by the obligatoriness of the GMP for the drug manufacturers. Thanks to this, the analysis of the medicinal substances is carried out almost entirely by the manufacturers.

9. STANDARDIZED VALIDATION PROCEDURE FOR ASSAYS OF SUMMARIZED DRUGS, USING THE CALIBRATION GRAPH METHOD [70]

Biologicals is now rapidly gaining market and are one of the main directions of development of pharmacy. A large part of the biological drugs are “summarized”, i.e. such medicines, biological activity of which is associated with a large number of compounds, many of which may be unknown (or known only as classes of chemical compounds) and the concentrations of which (absolute and relative) can vary widely. These concentrations are determined by the properties of the raw materials and manufacturing technology and cannot be changed at will [80]. The typical summarized drugs are, for example, the herbal drugs and various extracts of them [80].

A large part of the biologicals is also the summarized drugs. As compared to the herbal drugs, they have some specific features.

Pharmacological effect of the biologicals is associated with many different factors, and its standardization is ensured by the strict compliance with the technological process. Quality of the summarized biologicals is characterized by a set of quality indicators, each of which individually may not be directly associated with an activity, but out of limits of this parameter indicates the violation of manufacturing technology, storage conditions, or decomposition, and thus the violation of quality. These indicators can be therefore considered as signal [80].

This situation, in varying degrees, is typical to all summarized drugs [80] but for the summarized biological especially. This results in fact that these (signal) characteristics may be just the characteristics of quality, even when they are the quantitative contents of some groups of compounds. It has also influence on the validation of procedures of their determination.

A typical example of such procedures is an assay of a carbohydrate sum in a biological substance *Concentrate of deproteinized dermal layer of pigs* (CDDL).

The CDDL is a main active ingredient of the liposome drug product "Èfial′" [87]. One of the quantitative characteristics of the CDDL quality is a carbohydrate content (not less than 0.35 mg/ml). Determination of carbohydrates is carried out using a spectrophotometric procedure according to their color reaction with anthrone (Dreywood method [86]) in the option of the calibration graph method. It is fairly typical for biological drugs.

In accordance with the SPU requirements, all the quality control procedures of medicinal products, introduced into the specification, must be validated. The issue rises about validation of the procedure of carbohydrates determination in the CDDL.

Standardized validation schemes for quantitative procedures in the option of the reference standard method are described for most pharmacopoeial methods (see chapters 1-8). A standardized validation scheme for quantitative procedures in the option of the calibration graph method is described for atomic absorption procedures (see the Chapter 7). However this scheme for absorption spectrophotometry in the visible and ultraviolet regions is not yet discussed. The situation is further complicated by the fact that the CDDL is a summarized drug. Assays of these drugs have a number of features [80] which do not allow applying to them approaches to validation of analytical methods, previously developed in the chapters 1-7.

This chapter develops the standardized validation scheme for spectrophotometric assays of summarized biologicals in the option of the calibration graph method (CGM) and verifying it for the carbohydrate sum assay in the biological substance *Concentrate of deproteinized dermal layer of pigs* (CDDL).

As in the case of validation of the AAS-procedures in the option of the calibration graph method (see the Chapter 7), at the same time we also conduct an assessment of applicability of the more simple reference standard method. The findings are applicable to validation of any other assay procedure of a summarized drug using the methods of calibration graph or reference standard.

The objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose [1, 3].The most important stages of validation are: getting validation characteristics, comparing them with critical values and conclusions about the procedure correctness (see the Chapters 1-2). One of the most important validation characteristics are *Specificity* and *Accuracy*, which in turn are based on a maximum acceptable uncertainty of an assay procedure $max\Delta_{As}$.

9.1. Maximum acceptable uncertainty of an assay procedure

A characteristic feature of the summarized drug assays is that they are usually standardized only by the lower content limit $Cont_L$ (i.e. $\leq Cont_L$) (see the Chapter 8). Therefore there is no nominal content ($Cont_{nom}$) for them. It makes difficult to establish the maximum acceptable procedure uncertainty ($max\Delta_{As}$), as well as the analytical range in which a validation is carried out (as the range is defined as a percentage of the nominal concentration - see the Chapter 2).

It would seem we can consider the lower content limit $Cont_L$ as a nominal concentration $Cont_{nom}$. However, this is contrary to the general principles of standardization of medicines. In particular, in this case the medicines with the nominal content could not be released, because the probability of their failing (i.e. the probability of the concentration obtained in the control laboratory to be less than $Cont_{nom}$) is 50%.

One possible way out of this situation is the introduction of the formal nominal concentration and formal two-sided content tolerances.

When one-sided tolerances for a target compound group content are developed, variation of this content in the summarized drug can be very significant. Accordingly, the requirements to the maximum acceptable procedure uncertainty ($max\Delta_{As}$) are low. In this case the maximum acceptable procedure uncertainty of any medicine can be considered as $max\Delta^0_{As}$. This $max\Delta^0_{As}$ value can be obtained from the pharmacopoeial requirements to the content uniformity [81].

The requirements to the relative standard deviation, when performing the test for the content uniformity, are developed on a base of an assumption that the universe relative standard deviation must not exceed 10% [28, 85]. For the *t*-distribution, two-sided criteria and probability of 95% this corresponds to tolerances of $\pm$ 20% [26]. These tolerances can be thought as maximum tolerances for medicines. They can be recommended for establishing formal two-sided tolerances in the case of the one-sided standardization. Then the nominal content ($Cont_{nom}$) is associated with a lower tolerance limit $Cont_L$ by the ratio:

$$Cont_{nom} = 100 \cdot Cont_L / (100 - 20) = Cont_L / 0.8. \quad (9.1)$$

Accordingly, the maximum acceptable assay uncertainty ($max\Delta_{As}$) in this case is equal to (see the Chapter 2):

$$\begin{aligned} B &= 20\%. \\ \max \Delta_{As} &= \max \Delta^0_{As} = 0.32 \cdot B = 6.4\%. \end{aligned} \quad (9.2)$$

Here B is the half-sum of the upper and lower tolerances as a per cent of the nominal content.

9.2. Accuracy

Accuracy of an analytical procedure expresses the closeness of agreement between the value which accepted either as conventional true value or an accepted reference value and the value found. (see the section 1.8).

This definition, which is unambiguous for synthetic drugs, is meaningless for summarized drugs [80].

In the case of a spectrophotometric assay of a summarized drug we find a sum of conditional concentrations in terms of some single component [80] (in the case of CDDL, it is a sum of carbohydrates in terms of glucose). Depending on the choice of the component, wavelength, analysis conditions, etc., the results will be different. Therefore, the concepts of a "true value" or "reference value" for a sum of conditional concentrations in a summarized drug do not exist. Also we cannot conduct a comparative analysis by another method (for example, chromatography), since the results of this analysis will be significantly different.

Bearing in mind also that biological activity of compounds within the same target group can vary to a great extent (for example, in our case of the sum carbohydrates assay - between mono- and polysaccharides), the sum of the conditional concentrations in the summarized drugs is simply a signal characteristic of their quality. To speak about “accuracy” of this value is incorrect. We can only talk about its reproducibility in different laboratories that is a prerequisite for the application of this value as a quality characteristic. The extent to which this sum of the conditional concentrations reflects correctly the quality of the summarized drug is decided not at the stage of validation, but under procedure choice substantiation and development. In addition, as mentioned above, these characteristics are often a signal for the summarized drugs and out of their specification limits indicates a violation of the technology, storage conditions, or about degradation.

Thus, the validation characteristic "*Accuarcy*" is absent for summarized drug.

9.3. Specificity

Specificity for assay provides an exact result which allows an accurate statement on the content or potency of the analyte in a sample (see the section 1.8).

This definition was not in doubt for a synthetic drug of an exactly known composition. In this case the specificity is assessed and quantified: a total contribution of all non-target components to an analytical signal (absorbance, peak area, and so on) must be insignificant compared with the maximum acceptable procedure uncertainty ($max\Delta_{As}$) (see the Chapter 2).

In the case of a summarized drug, the concept of specificity is often rather vague [80]. A control of a target group of chemical compounds in a summarized drug by the spectrophotometry is usually carried out with use of color reactions (rarely own absorption) of these compounds with a group reagent followed by a measurement of an absorbance at an analytical wavelength [80]. It would seem, for the quantification of specificity of analysis of summarized drugs we can apply the same approach as for synthetic drugs. However, in the case of the summarized drugs, it is usually not possible to extract the target compound group from the drug (to get “pure placebo”), and therefore it is not possible to assess the non-target component contribution. In addition, the group reagent (not to mention procedures based on own absorption) may provide the absorbance at the analytical wavelength not only with the declared target group of chemical compounds [80].

In particular, in our case of the carbohydrates sum assay, the Dreywood method is based on dehydration of monosaccharides to hydroxylmethylfurfural which then reacts with anthrone [86]. As we can see, the hydroxylmethylfurfural derivatives will also produce coloring with anthrone. Therefore, strictly speaking, it is correct to say that we determine not the “sum of carbohydrates” but the “sum of compounds producing the absorbance with anthrone at 620 nm". The same situation generally is for other spectrophometric procedures of determination of target compound group sum in the summarized drugs [80].

In view of the above, the absorbance of the CDDL proper (without the reagent) can be considered as the characteristic of specificity of the carbohydrates sum assay in the CDDL by the reaction with anthrone. The ratio of the absorbance of the CDDL without reagent at λ = 620 nm (A_λ) to the absorbance of the same sample after the reaction with anthrone at 620 nm, less the blank absorbance (A_o), should be not significant [11] compared with the maximum acceptable procedure uncertainty ($max\Delta_{As}$), i.e., taking into account the (9.2):

Specificity:
$$\frac{A_\lambda}{A_o} \cdot 100 \le \max \delta = 0.32 \cdot \max \Delta_{As} = 2.0\%. \qquad (9.3)$$

9.4. Linearity

9.4.1. Problem of linearity study for an assay of summarized drugs

A characteristic of the summarized drug analysis (in options as the reference standard and calibration graph method) is that in this analysis two different substances take part

– a test sample and a reference standard. The analytical characteristics (in particular, the linearity in the validated range) of the reference standard, generally speaking, can differ from the test sample. Therefore, in general, we must check the linearity as for the test sample and reference standard.

The situation is further complicated by the fact that if the reference standard concentrations to build a calibration line are known, then the initial concentration of the test summarized drug sample (in the case of spectrophotometric procedures it is a conditional concentration [69]) is not known in principle. In order to determine it, we are in need of a validated procedure and to validate this procedure we are in need of known summarized sample concentrations. It is a vicious circle.

9.4.2. Normalized coordinates

As we have shown earlier [69], in the case of the calibration graph method (CGM), the normalized coordinates can be used only for the concentration axis. However, the use of the normalized coordinates for the ordinate axis allows determining the possibility of using the more simple reference standard method instead of the CGM on the base of the linearity study data [69].

In the reference standard method (see the section 2.2), conversion to the normalized coordinates is done by dividing the concentration and analytical signal of the sample solution by the concentration and analytical signal of the reference standard solution. In the CGM instead of the standard solution we can use the calibration solution with the nominal concentration C_{nom} and the corresponding absorbance A_{nom}. In this case, the expression for the normalized coordinates would be the same as that for the reference standard method (see the section 2.2):

$$\begin{aligned} X_i\,(\%) &= 100 \cdot C_i\ / C_{st}. \quad C_{st} = C_{nom}. \\ Y_i\ (\%) &= 100 \cdot A_i\ / A_{st}. \quad A_{st} = A_{nom}. \\ Z_i\ (\%) &= 100 \cdot Y_i\ / X_i\ . \end{aligned} \tag{9.4}$$

The linear relationship in the normalized coordinates is of the form (see the section 2.3.4):

$$X\ = b\ \cdot Y\ + a\ . \tag{9.5}$$

9.4.3. Range and number of points for obtaining the calibration line

The range of the linearity and metrological characteristics study for the assay procedure validation is usually at least twice exceeds the content tolerances. In particular, the common pharmacopoeial range (80-120) [11] is twice wider than the maximum content tolerances for the majority of drug products ($\pm$ 10%). Therefore the range of 50-150% of the nominal value $Cont_{nom}$, proposed earlier (see the Chapter 7) for the calibration graph method (CGM) seems quite justified. The number of points to build the calibration line (9.5) should not be less than n = 5 (see the Chapter 7). It corre-

sponds to 50, 75, 100, 125 and 150% of the nominal content $Cont_{nom}$. With this SD_{Co} = 39.53% (see the Chapter 7). It is desirable that the nominal solution was as close as possible to 100%.

Note that solution A volumes, pipetted for preparation of last dilutions of calibration solutions, do not cover the entire volume of a pipette. Therefore, in general, it is advisable to use weight dilutions. If $m_o(cal)$ is a weight of the solution A volume of the nominal concentration placed for the last dilution in a measuring flask of V_{cal} capacity, $m_i(cal)$ is a weight of a volume placed in another measuring flask of V_{cal} capacity for another dilution, then, given (9.4), the normalized concentrations of the different dilutions of the calibration solutions will be as follows:

$$X_i\,(cal) = 100 \cdot C_i\,(cal) / C_{st}(cal) = 100 \cdot m_i\,(cal) / m_o(cal). \qquad (9.6)$$

Concentrations of model solutions in mg/ml are equal to:

$$C_i\,(cal) = C_A \cdot m_i\,(cal) / (V_{cal} \cdot \rho_A). \qquad (9.7)$$

Here: C_A is a concentration (mg/ml) of the calibration solution before the last weight dilutions in a measuring flask of V_{cal} capacity, ρ_A is a specific density of this solution (g/ml).

Given (9.4), absorbance values of the calibration solutions ($A_i(cal)$) are conversed to the normalized values ($Y_i(cal)$) using the formula:

$$Y_i\,(cal) = 100 \cdot A_i\,(cal) / A_{st}(cal). \qquad (9.8)$$

Here $A_{st}(cal)$ is the absorbance of a model solution adopted as a reference standard.

9.4.4. Model mixtures, their analysis and processing

In the case of synthetic drugs they use n = 9 model solutions with such concentrations (see the Chapter 7): 50.0, 75.0, 62.5, 87.5, 100.0, 125.0, 112.5, 137.5 and 150% of the nominal concentration, which are characterized by the standard deviation of SD_{Co} = 34.23%.

However, in the case of a summarized drug it is usually impossible to prepare a model mixture with exactly known composition. It would seem, as the model mixtures we can use the different concentrations of the reference standard. However, in this case, we would have studied the assay of the reference standard instead of the test sample assay. It is more correct to study the different dilutions of the test sample. In particular, this approach is applied to the spectrophotometric assay (in the option of the specific absorbance method) of flavonoids and procyanidins sums in the complex tincture "Aterofit-norma" [88]. However, it is necessary to formulate the task, what the results we want to get from the studies of different dilutions of the test sample and develop the appropriate criteria.

As mentioned above, a true concentration (content) of an analyzed group of compounds in a summarized drug is not known, so the validation characteristic "Accura-

cy" is absent here. Accordingly, there is also absent such an important analytical characteristic as "recovery," which, in particular, we widely used in the validation of the AAS-procedures in the option of the calibration graph method (see the Chapter 7). With this in mind, during the validation of an assay procedure of a summarized drug we can experimentally verify only the necessary precision of the procedure.

The task of the study of different dilutions of the test sample can be formulated as follows: the uncertainty (in the form of a confidence interval) of variation of concentration values, calculated for $n = 9$ dilutions of the same drug sample in the analytical range of the conditional concentrations, must meet the specified criterion.

In a preliminary experiment, it is chosen the dilution of the drug sample, which approximately corresponds to the absorbance $A_{st}(cal)$ of the calibration solution adopted as a standard (see above). Assuming approximately proportionality of the absorbance on the concentration, we can get n dilutions of the drug sample in the range of 50-150% of this value.

In accordance with the SPU-Eur.Ph. requirements number of such dilutions should be not less than $n = 9$ [11], so pipetted volumes of the drug would not cover the entire volume of a pipette. Therefore, in general, like preparation of the calibration solutions (see above), it is advisable to use weight dilutions. If m_o is a weight of the drug sample volume (corresponding to its nominal concentration) placed in a measuring flask of V_b capacity, m_i is a weight of a volume placed in another measuring flask of V_{cal} capacity for another dilution, then the normalized concentrations of the different dilutions of the drug sample will be as follows (see (9.7)):

$$X_i\,(in) = 100 \cdot C_i\,(in) / C_o\,(in) = 100 \cdot (m_i\ / m_o). \qquad (9.9)$$

Values of X_i are chosen close to 50, 62.5, 87.5, 75, 100, 125, 112.5, 137.5 and 150%. Preparation of such model solutions of a summarized drug is not much different in the main from preparation of common model solutions for a synthetic drug.

Dilutions (Dil_i) of the drug model samples will be equal to:

$$Dil_i\ = V_b\ \cdot \rho_s / m_i\ . \qquad (9.10)$$

Here ρ_s is a specific density of the drug (g/ml), which must be defined in the preliminary experiment.

For each concentration obtained ($X_i(in)$), the absorbance (A_i) is measured, which is conversed by the equation (9.4) into the normalized value Y_i:

$$Y_i\ = 100 \cdot A_i\ / A_{st}(cal). \qquad (9.11)$$

Here $A_{st}(cal)$ is the absorbance of the calibration solution, adopted as the reference standard. The calculated values $X_i(out)$ are found using the calibration line (9.5) and Y_i values.

The concentrations $C_i(sample)$ (mg/ml) in the drug sample taken for preparing the model solutions are calculated as follows:

$$C_i\,(sample) = X_i\,(out) \cdot C_{st}(cal) \cdot Dil_i\ /100. \tag{9.12}$$

Here the $C_{st}(cal)$ value is calculated by the ratio (9.7) for the calibration solution adopted as the reference standard, and Dil_i is calculated by the ratio (9.10). For the $C_i(sample)$ values obtained, the mean concentration (C_{sample}), relative standard deviation ($RSD_{sample}\%$) and relative confidence interval (Δ_{sample}) are calculated. The Δ_{sample} value must meet the relevant criteria (see below).

9.4.4.1. Calculations for the reference standard method

The use of the normalized coordinates also allows us to assess the possibility of application of the more simple reference standard method (see the Chapter 7). For this, the parameters of the calibration line (9.5) are compared with the criteria obtained previously in the Chapter 7.

9.5. Validation criteria

Total uncertainty of an assay in the CGM option includes uncertainty of a calibration graph and uncertainty of a test sample analysis. The latter is a systematic error in the sample analysis. As shown in the Chapter 7, different approaches can be applied to choose the ratio between these two uncertainties. For the range of 50-150% of the nominal concentration, the most common one is the *Approach 2*: *maximum acceptable uncertainties of calibration and the test sample analysis are equal*, i.e., taking into account the ratio (9.2), we obtain (see the Chapter 7):

$$\max \Delta_{cal} = \max \Delta_{sample} \le 0.71 \cdot \max \Delta_{As} = 4.5\%. \tag{9.13}$$

This approach we'll apply further.

9.5.1. Uncertainty of a calibration line

As shown in the Chapter 7, in the case of the *Approach 2*, the residual standard deviation SD_{rest} of the calibration line (9.5) of 5 points, subject to the equation of (9.2), must meet the requirements:

$$SD_{rest} \le \max SD_{rest} = 0.096 \cdot B = 0.096 \cdot 20 = 1.9\%. \tag{9.14}$$

The square of the correlation coefficient (R_c^2) for the range of 50-150% must meet the requirements (see the Chapter 7):

$$R_c^2 \ge 0.99764. \tag{9.15}$$

If the claims of (9.14-9.15) are not met, then this calibration line is unusable for the correct analysis using the CGM within the *Approach 2* (see the Chapter 7).

9.5.2. Uncertainty of a test sample in the calibration line method

As mentioned above, the concept "Accuracy" in the analysis of summary drugs is absent. So we can only check reproducibility of quantitative determination of the concentrations for $n = 9$ various dilutions of the same drug sample ($C_i(sample)$) in the analytical concentration range (see (9.12)).

Thus, the relative confidence interval of variations of the concentrations, obtained for $n = 9$ various dilutions of the same drug sample ($C_i(sample)$), subject to the ratio (9.13), must meet the ratio (see Chapter 7):

$$\Delta_{sample} = 1.86 \cdot RSD_{sample} \le 0.707 \cdot \max \Delta_{As} = 4.5\%. \tag{9.16}$$

9.5.3. Uncertainty of a test sample in the reference standard method

The use of the normalized coordinates in the CGM allows evaluating the applicability for analysis of the more simple reference standard method (RSM) (see the Chapter 7).

To do this, let's calculate the linear relationship (9.5) by using the least squares method, in which Y_i and X_i are calculated on the ratios of (9.4). An absolute term of this linear relationship should, taking into account the ratio (9.2), meet the ratio (see the Chapter 7):

Reference standard method:
$$a \le \frac{0.1 \cdot B}{1 - (X_{\min}/100)} = 4.0\%. \tag{9.17}$$

The residual standard deviation SD_{rest} and the square of the correlation coefficient R_c^2 of the linear relationship must meet the requirements (see the Chapter 7):

Reference standard method:
$$SD_{rest} \le 3.4\%. \tag{9.18}$$

Reference standard method:
$$R_c^2 \ge 0.99512. \tag{9.19}$$

The confidence interval for the found concentration C_{sample}, given (9.2), must meet the ratio (see the Chapter 7):

Reference standard method:
$$\Delta_{sample} = 1.86 \cdot SD_Z \le 0.32 \cdot B = 6.4\%. \tag{9.20}$$

9.5.4. Intermediate precision

It is advisable to use the approach described in the Chapter 7. Carry out on three replicate drug assays in two different days. The difference between found in different days C_{sample} values should, taking into account the ratio (9.2), meet the relationship:

$$\left|\frac{2\cdot 100\cdot[C_{sample}(2)-C_{sample}(1)]}{C_{sample}(2)+C_{sample}(1)}\right| \le \sqrt{2}\cdot \max\Delta_{As}/\sqrt{3} = 0.26\cdot B = 5.2\%. \quad (9.21)$$

9.6. Example. Validation of the assay of the carbohydrates content in CDDL

All used reagents and volumetric solutions, volumetric glassware and equipment were met the SPU requirements [1]. Qualification of the spectrophotometer was conducted as recommended by the reference [81].

The specification on the substance *Concentrate of deproteinized dermal layer of pigs* (CDDL) standardizes the carbohydrates content in terms of glucose at least 0.35 mg/ml. Then the nominal content of carbohydrates in CDDL can be calculated by the ratio (9.1):

$$Cont_{nom} = 0.35/0.8 = 0.4375 mg/ml. \quad (9.22)$$

9.6.1. Carbohydrates determination procedure to be validated

Test solution: Place V ml of CDDL in a volumetric flack with capacity of V_b = 5 ml and dilute with *water R* to the mark.

Calibration solutions: Place 275.0 mg of glucose anhydrous (Fluka # 49152) in a volumetric flack with capacity of 100 ml and dilute with *water R* to the mark (solution A). The glucose concentration in the solution A is C_A = 2.75 мг/мл. Given the small concentration of glucose in the solution A (C_A), its specific density is equal to the specific density of water, i.e. ρ_A = 1.000 g/ml.

Place 1.25, 1.75, 2.25 (accepted as nominal), 2.75 и 3.25 ml of the solution A in the weighted volumetric flasks with capacity of V_{cal} = 100 ml, weigh (getting the weights of m_{sti}) and dilute with *water R* to the mark (calibration solutions).

Place on 1 ml of the test solution, calibration solutions and *water R* in test tubes and transfer for 5 minutes in an ice bath. Then add into each tube 3 ml of 0.1% solution of anthrone (100 mg of anthrone Fluka # 10740 place in a volumetric flask with capacity of 100 ml and dilute with *sulphuric acid R* to the mark. Use the solution freshly made). Mix well the contents of the tubes and place them in a water bath for 10 minutes. Then cool the tubes to room temperature and measure the absorbance values $A_i(cal)$ at 620 nm. As a control solution, use the solution based on the *water R*, prepared as described above.

9.6.2. Obtaining the calibration line

Values $X_i(cal)$ were calculated by the ratio (9.6); $C_i(cal)$ – by the ratio (9.7), i.e. $C_i(cal) = 2.75\cdot m_i(cal)/(100\cdot 1.000) = 0.0275\cdot m_i(cal)$; $Y_i(cal)$ – by the ratio (9.8).

Table 9.1

Analysis of calibration solutions

Volumes of solution A, ml	Weights of solution A, $m_i(cal)$, g	$C_i(cal)$	$X_i(cal)$	$A_i(cal)$	$Y_i(cal)$
1.25	1.2272	0.03375	54.97	0.315	48.99
1.75	1.7217	0.04735	77.12	0.478	74.34
2.25	2.2324 = $m_i(cal)$	0.06139 = $C_{st}(cal)$	100.00	0.643 = $A_{st}(cal)$	100.00
2.75	2.7291	0.07505	122.25	0.772	120.06
3.25	3.2218	0.08860	144.32	0.925	143.86

Build a linear relationship (9.5) as $X_i(cal)$ versus $Y_i(cal)$. Results of processing by the least squares method [26] and the above obtained criteria are presented in the Table 9.2. The calibration line is illustrated in the Figure 9.1.

Table 9.2

The metrological characteristics of the calibration line (9.5)

$X_i(cal) = b \cdot Y_i(cal) + a$

Parameter	Value	Calibration graph method (CGM)		Reference standard method (RSM)	
		Criterion	Conclusion	Criterion	Conclusion
a	7.25	-	-	4.0	**Fail**
s_a	2.26	-	-		
b	0.949	-	-		
s_b	0.022	-	-		
SD_{rest}	1.64	< 1.9	Meet	< 3.4	Meet
R_c^2	0.99787	> 0.99764	Meet	> 0.99512	Meet

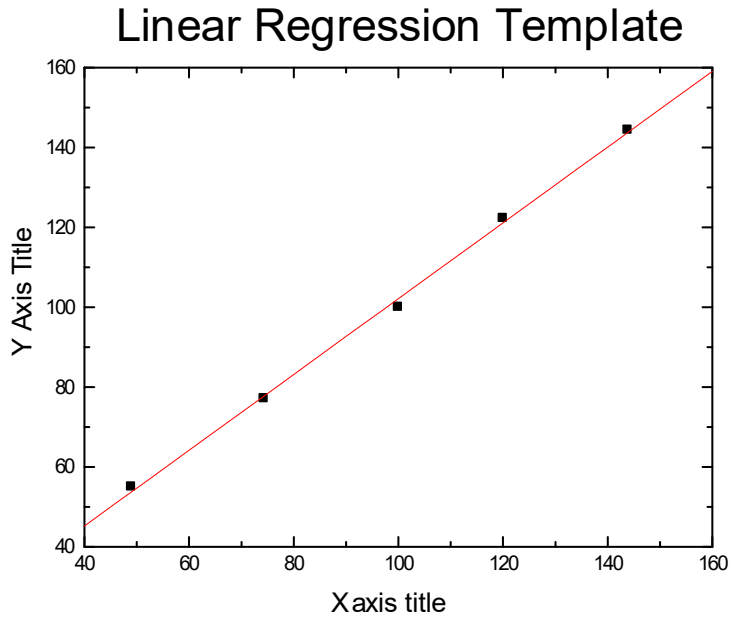


Figure 9.1. Calibration line of dependence of glucose anhydrous concentration on absorbance in normalized coordinates.

As can be seen, the linearity holds for the CGM, but not for the RSM - because of the practically significant absolute term (a) of the (9.5). I.e. in this case the **RSM is not applied**.

9.6.3. Analysis of model mixtures

9.6.3.1. Specificity

The absorbance of the original drug at 620 nm is 0.043. The nominal dilution of the drug under conducting the reaction equals to 6.73 (see below: the solution T5). Therefore, the absorbance of the original drug under the nominal dilution is A_λ = 0.043/6.74 = 0.0064. The absorbance of the drug solution T5 at 620 nm, processed under procedure conditions, equals to A_o = 0.643. Then 100 · A_λ/A_o = 1.0 ≤ 2.0%, i.e. the ratio (9.3) requirements are met. Therefore, the specificity holds.

9.6.3.2. Preparation and analysis of model solutions

In preliminary studies, it was found that the V_o = 0.75 ml of CDDL (see section 9.6.1, "Test solution") produces the absorbance which was approximately equal to the absorbance of the A_{st} = 0.6139 from the Table 9.1. 9 dilutions of the drug were prepared around this mean volume V_0 (see the Table 9.3). In the preliminary studies we have also found the drug specific density ρ_s = 1.012 g/ml, which we used to find the dilutions by the ratio (9.10), that in our case has a form of $Dil_i = 1.012 \cdot 5.0/m_i = 5.060/m_i$.

The results of preparing and analysis of the drug model mixtures are presented in the Table 9.3. The Y_i values were calculated by the ratio of (9.11) using $A_{st}(cal)$ = 0.643 from the Table 9.1, i.e. $Y_i = 100 \cdot A_i /0.643$. The $X_i(out)$ values were calculated by the ratio of (9.5) using the parameters from the Table 9.2, i.e. $X_i(out) = 0.949 \cdot Y_i + 7.25$. The carbohydrates concentration $C_i(sample)$ in the CDDL in terms of glucose (mg/ml) were calculated by the ratio (9.12), i.e. $C_i(sample) = X_i(out) \cdot 0.06139 \cdot Dil_i/100 = X_i(out) \cdot 0.0006139 \cdot Dil_i$. For this we used the $C_{st}(cal)$ = 0.06139 mg/ml from the Table 9.1. The Δ_{sample} value was calculated by the ratio (9.16).

Table 9.3

The analysis of model solutions

Solution N	Drug volume, V_i ml	Weight m_i, g	Dilution Dil_i	A_i	Y_i%	$X_i(out)$ %	$C_i(sample)$ mg/ml
T_1	0.98	0.9912	5.1052	0.871	135.46	135.80	0.4256
T_2	0.92	0.9299	5.4417	0.812	126.28	127.10	0.4246
T_3	0.86	0.8697	5.8184	0.759	118.04	119.27	0.4260
T_4	0.80	0.8046	6.2891	0.697	108.40	110.12	0.4252
T_5	0.74	0.7514	6.7344	0.648	100.78	102.89	0.4254
T_6	0.68	0.6912	7.3209	0.593	92.22	94.77	0.4259
T_7	0.62	0.6264	8.0783	0.532	82.74	85.77	0.4254
T_8	0.56	0.5703	8.8729	0.481	74.81	78.24	0.4262
T_9	0.50	0.5043	10.034	0.42	65.32	69.24	0.4265
T_1	0.98	0.9912	5.1052	0.871	135.46	135.80	0.4256

T_2	0.92	0.9299	5.4417	0.812	126.28	127.10	0.4246
						Mean C_{sample}	**0.4256**
						SD mg/ml	0.0006
						RSD_{sample} %	0.14
						$\Delta_{sample} = 1.86 \cdot RSD_{sample}$	**0.26** < 4.5%

As can be seen, the procedure is characterized by very good repeatability, and the requirements of (9.16) to Δ_{sample} are met with a very large margin. It also confirms the good linearity for the drug sample in the analytical range of concentrations of total carbohydrates content.

9.6.3.3. Intermediate precision

We conducted on three replicate drug determinations in two different days. Results were: $C_{sample}(1)$ =0.4256, $C_{sample}(2)$ = 0.4069, $\left|200 \cdot (0.4069-0.4256)/(0.4056+0.4269)\right| = 4.5 < 5.2\%$. As can be seen, the ratio of (9.21) is met, i.e. intermediate precision holds.

The total conclusion on the validation: the **procedure is correct.**

Addendum 1

Performing tests "Uniformity of dosage units" and "Dissolution test for solid dosage forms" by liquid chromatographic procedures under the production quality control of drug products

Issues related to requirements to metrological characteristics of the analytical procedures used for such pharmaceutical technical procedures as "Content uniformity" and "Dissolution", are very little discussed in the scientific press and practically unresolved. Arising from this, practical problems sometimes call into question the very possibility of correct conducting such tests, especially in the case of chromatographic methods that require a large number of replicate measurements and testing the system suitabilty.

The "Content uniformity test" is a two-level [31, 32]. At the *Level 1,* analyze 10 units of a dosage drug product (for example, tablets) are analyzed and check the pharmacopoeial criteria compliance. If they are not met, then under certain conditions, go to the *Level 2*. For that, analyze extra 20 units and check the pharmacopoeial criteria compliance for all 30 units. The decision based on the analysis of all 30 units is final.

The " Dissolution" test is a three-level [33]. At each level, check the pharmacopoeia criteria. The conclusion based on an analysis of all three stages is final. At the *Level 1,* control 6 units, at the *Level 2* - 6 more units (a total of 12 units), at the *Level 3* – 12 more units (for a total of 24 units).

As can be seen, testing content uniformity for tablets, we must analyze 10 tablets at the *Level 1*l [31, 32]. If we use directly the specification procedure described in the section "Assay", then the test performing is impracticable. Indeed, according to the SPU [13, 53], to analyze each tablet we must get (by alternate injections) at least 5 chromatograms of test and reference standard solutions for each. I.e., to analyze 10 tablets, we must get 100 chromatograms + additional 5 chromatograms to check suitability of chromatographic system. If we take the average run time of a 20 min for each chromatogram (rather common case), it can be can seen that already in the *Level 1* of the test for content uniformity we must spend about 35 hours of chromatographic time, without regard the time for sample preparation. If we'll go to the *Level 2* (additional analysis of 20 tablets more), the total time grows up to 100 hours. It should be noted that the same problem occurs and when testing the tablet blend uniformity [58].

Similar problem is with the "Dissolution" test [33]. At the *Level 1* we analyze 6 tablets, at the *Level 2* - additional 6 tablets and the *Level 3* - 12 tablets more. It is easy to estimate that only the chromatographic analysis at the *Level 1* is about 22 hours, with the *Level 2* it grows up to 42 hours and with *Level 3* is up to 82 hours.

Of course, it's unrealistic time for analysis of batch production. In practice, therefore, an analyst gets so many replicate chromatograms as sees fit. The metrological accuracy of the results is simply ignored. To talk in this case about the validation of "Content uniformity" and "Dissolution" test (all the analytical procedures should be validated [11]) and ensure the quality of the results is no sense. As a result, if a manufac-

ture control laboratory obtained positive results in carrying out these tests, this still does not mean that positive results will be obtained in a test laboratory. But the “Content uniformity” is a critical test for low dosage drug products, gradually replacing the section "Assay". The "Dissolution" test is also necessary for all solid dosage forms, in many cases already replacing the "Disintegration" test. It turns out that the most important pharmaceutical technical procedures for drug products cannot give reliable and metrological substantiated results.

Thus, correct application of "Content uniformity” and “Dissolution" tests needs to develop requirements to metrological characteristics of the analytical procedures used for their conduct, and, based on them, to substantially decrease the number of replicate chromatograms. Note that this is not contrary to articles “Content uniformity" [31] and" Dissolution "[33], as they indicate that the analytical procedure is provided by a specification. The purpose of this chapter is an attempt to develop the metrological substantiated scheme for such analytical procedure performing.

1. Requirements to the uncertainty of the analytical results obtained in “Content uniformity” and “Dissolution” tests"

1.1. Content uniformity

When setting requirements to the uncertainty of analytical results obtained in “Content uniformity” and “Dissolution” tests, it is logical to make use of the *Insignificance principle* (see the section 2.3.1 of the chapter entitled “Standardized validation schemes for drug quality control procedures”, hereinafter named as “Validation”): the results of content determination in individual dosage units must not significantly distort the real picture of the content heterogeneity caused by the technological reasons. In analytical practice, the use of a confidence interval for probability of 0.95 is accepted, so we will assume it further. Everywhere further we will also assume that the average content is 100%.

Using the *Insignificance principle* (see the section 2.3.1, "Validation") and "guaranteeing" tolerances, we have shown [28] that the confidence interval Δ_{As} (probability of 0.95) of the analytical procedure results for each dosage unit when conducting the "Content uniformity" test must meet the requirements:

$$\Delta_{As} \leq \max \Delta_{As} = 3.0\% \,. \qquad (1)$$

1.2. Dissolution

The difference in degree of dissolution between various units of a drug product is affected by two factors:

1) heterogeneity of an active substance content in individual dosage units of this drug product (this heterogeneity is characterized by the “Content uniformity” test);

2) the difference in a dissolution degree between various units of the drug product with the same active substance content, caused by different technological characteristics of these units (e.g., different degree of compression for different tabs).

So the difference in the dissolution degree between the various units of the drug product is always above the difference in the active substance content of these units. It resulted in the requirements for the "Dissolution" test are less stringent by reason of additional (to the heterogeneity of content) heterogeneity of the dissolution.

If the dissolution degree equals to 100%, the requirements to "Dissolution" test should automatically turn into the requirements to “Content uniformity” test. Therefore, given this limit case, it is advisable to set the requirements to the uncertainty of the analytical procedure for the "Dissolution" test the same as for “Content uniformity" test, i.e. requirements of the ratios of (1).

2. Calculation of a sufficient number of replicate chromatograms when performing the "Content uniformity” and “Dissolution" tests

The ratio of (1) shows that the requirements to the uncertainty of the analytical procedure for the “Content uniformity” and “Dissolution” test are fairly liberal - they are generally less stringent than for the assay procedures. Thus, if the test material tolerances are equal to $\pm$ 5%, the uncertainty of the analytical procedure should be not more than 1.6% (see Table 2.1, "Validation"). In addition, as will be shown below, it is possible to significantly increase the number of degrees of freedom that can substantially reduce the *t-value* in the calculation of a confidence interval. All this makes it possible to significantly reduce the number of replicate chromatograms when we perform the “Content uniformity” and “Dissolution" tests.

Further we'll use the following assumptions.

2.1. Basic assumptions

1. In all cases, the uncertainty of the results will be the relative confidence interval with a factor of confidence 0.95.
2. *RSD* of the repeatability of the chromatographic response (peak or height area) for the test and reference standard solutions are sample estimates of the same universe *RSD*. This is due to the fact that we analyze the same substance in close concentrations (usually not more than $\pm$ 15 per cent).
3. In the calculation of a confidence interval of analytical procedure for "Content uniformity” and “Dissolution” tests, as well as for the assay procedures, the one-tailed Student's distribution is used. This is because, when an individual dosage unit or a particular drug substance batch are analyzed, we, in fact, are concerned only with the received values are not exceeded or were below some value (for example, when conducting the "Content uniformity" test, we are only interested in whether or not the value of 112% with a confidence interval is less than 115% since 112%

more than 85%). In this case the two-tailed *t-value* corresponds to a probability of 95% at one-tailed distribution.

4. The sample preparation error (Δ_{SP}) is quite easily adjusted by correct sample weights and volumetric flasks and can therefore be made insignificant compared with maximum permissible (target) uncertainty of the final analytical operation ($max\Delta_{FAO}$) (in this case, chromatography), i.e., taking into account the ratio of (1) and Table 2.1, "Validation", we get:

$$\Delta_{SP} \le 0.32 \cdot \max \Delta_{FAO} \approx 0.32 \cdot \max \Delta_{As} = 0.96\%. \qquad (2)$$

2.2. Role of the chromatographic system suitability test

The system suitability tests represent an integral part of the method and are used to ensure adequate performance of the chromatographic system. Compliance with the system suitability criteria is required throughout the chromatographic procedure (European Pharmacopoeia, 2.2.46 [63]). One of the most important parts of the system suitability test is the maximum permitted relative standard deviation (*RSD*) of the replicate injections [63].

However, this is not entirely correct, because in fact the metrological characterization of the procedure is not this *RSD* but the total uncertainty of the analytical procedure Δ_{As} . It must meet the ratio of (1).

If the obtained *RSD* meets the requirements of "Chromatographic system suitability test" (i.e. $RSD \le RSD_{max}$), then there's a high probability that the ratio of (1) will meet. But this test is conducted with use of a reference standard solution (see SPU, 2.2.29 [53]) and the *RSD* values of a test sample, due to the influence of other concomitant substances, may not belong to the same population. Therefore, the ratio of (1) for the resulting uncertainty Δ_{As} do need to check.

On the other hand, failure to comply with the “System suitability” test (i.e. $RSD \ge RSD_{max}$) still does not necessarily mean that the resulting uncertainty Δ_{As} would not meet the ratio of (1). Incorporating other chromatograms obtained in the analysis process may well reduce Δ_{As} to an acceptable value.

Thus, failure to comply with the “System suitability” test for the *RSD* value is informative-warning nature, indicating that this system may not provide an acceptable uncertainty of results. The principal requirement is compliance with the ratio of (1) for the resulting Δ_{As}.

In view of the above, we propose the following scheme of analysis.

2.3. The experiment scheme for conducting the “Content uniformity” and “Dissolution" tests (hereinafter referred to as *Experiment scheme*)

1st stage. System suitability verification. At this stage, obtain n_o = 3-6 replicate chromatograms for the reference standard solution, calculate the relative standard deviation and compare it with the maximum acceptable value of RSD_{max}.

Given the existence of the second stage (see below), a difficult question is what are the requirements to the RSD_{max} value in the first stage. It is reasonable to set such RSD_{max} value that run of n_o replicate chromatograms for each of a test and reference standard solutions would provide uncertainty of results (Δ_{As}) for the test material not more the requirements of the ratio of (1). Because really we conduct only n_o replicate chromatograms of the reference standard solution, the number of degrees of freedom in this case is n_o - 1. Same number of degrees of freedom is *t-criterion*. Considering the *Basic assumptions 1, 3, 4*, the confidence interval of the results uncertainty in this case is:

$$\Delta_{As} \le \max \Delta_{As} = \sqrt{2} \cdot \frac{RSD_{\max} \cdot t_{90\%, n_o - 1}}{\sqrt{n_o}}. \qquad (3)$$

Hence, given the ratio of (1), we can get the requirements to the RSD_{max} value:

System suitability verification:

$$RSD \le RSD_{\max} = \frac{2.12 \cdot \sqrt{n_o}}{t_{90\%, n_o - 1}}. \qquad (4)$$

The RSD_{max} values for different n_o at the *1 stage* (verification of the chromatographic system suitability) are shown in the Table 1.

Table 1

The requirements to the RSD_{max} values for the "Content uniformity" and "Dissolution" test at the stage of the verification of the chromatographic system suitability

n_o	2	3	4	5	6	7	8	9	10
RSD_{max}%	0.48	1.26	1.80	2.23	2.58	2.89	3.17	3.42	3.66

The Table 1 shows that the requirements to the RSD_{max} values at the stage of the system suitability verification for the "Content uniformity" and "Dissolution" tests are rather liberal, that allows conducting these tests at any modern chromatograph. The RSD_{max} value (1.26%) for three replicate chromatograms (n_o = 3) is easily achieved in practice and this number of replicates we can be limited in most cases.

2nd stage. Analysis proper. Obtain chromatograms in the following order:

*n c*hromatograms of the 1st test solution - reference standard solution – *n* chromatograms of the 2nd test solution – reference standard solution ... – *n* chromatograms of the i^{th} test solution – reference standard solution ... – *n* chromatograms of the N^{th} test solution – reference standard solution.

As can be seen, we get a total of (n_o + N) chromatograms of the reference standard solution and (N-n) chromatograms of the test solutions 1...N. Calculate the relative standard deviation for the standard solution (S_{ro}) and for each of the test solutions (S_{ri}). According to the paragraph 2 of *Basic assumptions*, all of them are sample estimates of the universe *RSD*. Therefore, it is possible to calculate the pooled relative standard deviation by the ratio:

$$RSD^2_{\max} \geq RSD^2 = \frac{(n_o + N - 1) \cdot S^2_{ro} + (n-1) \cdot \sum_i^N S^2_{ri}}{n_o + N \cdot n - 1}. \quad (5)$$

As can be seen, RSD_{max} has the degrees of freedom number equal to $f = n_o + N \cdot n - 1$.

Find the requirements to the RSD_{max} value on the basis of the maximum acceptable (target) procedure uncertainty for the "Content uniformity" and "Dissolution" tests (see the ratio of (1)).

Considering the *Basic assumptions 1-4*, the procedure uncertainty of the mean result for each analyzed drug product unit can be found from the equation:

"Content uniformity" and "Dissolution" tests

$$\Delta_{As} = RSD \cdot t_{90\%, f} \cdot \sqrt{\frac{1}{n_o + N} + \frac{1}{n}}, \quad (6)$$

$$f = n_o + n \cdot N - 1,$$

$$RSD \leq RSD_{\max}.$$

The Δ_{As} value consists of two components: relative confidence intervals of the reference standard (first member below the root) and the test (second member below the root) solutions. The relative standard deviation (RSD_{max}) and *t*-criterion ($t_{90\%,f}$) for both the solutions are the same, but the number of replicates for calculation of the uncertainty of the mean result are different. They are (n_o + N) and n respectively.

Given that the Δ_{As} values for "Content uniformity" and "Dissolution" tests must meet the ratio of (1), from the equation of (6) we can calculate RSD_{max} values for different n_o, N and n (Tables 2-3).

2.3.1. Content uniformity

Table 2

Requirements to the RSD_{max} values when conducting the "Content uniformity" test for different n_o , N, n

n_o	Values of RSD_{max} % for the n values equal to:							
	1	2	3	4	5	6	7	8

$N = 10$								
1	1.58	2.26	2.71	3.05	3.32	3.54	3.72	3.88
2	1.60	2.28	2.74	3.09	3.36	3.59	3.78	3.95
3	1.62	2.30	2.77	3.12	3.40	3.64	3.84	4.01
4	1.64	2.32	2.79	3.15	3.44	3.68	3.89	4.07
5	1.65	2.33	2.81	3.17	3.47	3.72	3.93	4.12
6	1.66	2.34	2.82	3.20	3.50	3.76	3.97	4.17
$N = 30$								
1	1.74	2.46	2.99	3.41	3.76	4.07	4.34	4.58
2	1.74	2.46	2.99	3.41	3.77	4.08	4.35	4.60
3	1.74	2.47	2.99	3.42	3.78	4.09	4.36	4.61
4	1.75	2.47	3.00	3.42	3.78	4.10	4.38	4.62
5	1.75	2.47	3.00	3.43	3.79	4.11	4.39	4.64
6	1.75	2.47	3.01	3.43	3.80	4.11	4.40	4.65

From the Table 2 we can draw the following conclusions:

1. The RSD_{max} values depend little on the *1st stage* data (n_o) (verification of the chromatographic system suitability).
2. To obtain reliable information it is enough to obtain one replicate chromatogram ($n = 1$) for each unit of the analyzed drug product, since in this case the RSD_{max} value is perfectly acceptable both for $N = 10$ (1.58%) and $N = 30$ (1.74%). The real *RSD* values for modern chromatographs are usually at least three times less. However, when conducting the “Content uniformity" test we must check not only the *RSD* value of the individual unit contents (for this it is enough $n = 1$) but we must determine the contents itself which must be within appropriate limits. Therefore, for the calculation of these contents we should take at least two replicate chromatograms ($n = 2$).
3. The requirements to the RSD_{max} values differ little for $N = 10$ and $N = 30$, so as a requirements to the RSD_{max} values we can take the requirements for $N = 10$.

2.3.2. Dissolution

Table 3

Requirements to the RSD_{max} values when conducting the “Dissolution” test for different n_o , N, n

n_o	Values of RSD_{max} % for the n values equal to:

	1	2	3	4	5	6	7	8
				***N* = 6**				
1	1.44	2.10	2.51	2.80	3.02	3.19	1.32	3.46
2	1.49	2.14	2.56	2.87	3.10	3.29	3.45	3.58
3	1.53	2.18	2.61	2.93	3.18	3.38	3.54	3.68
4	1.56	2.21	2.65	2.98	3.24	3.45	3.62	3.78
5	1.58	2.24	2.68	3.02	3.29	3.51	3.70	3.86
6	1.60	2.26	2.71	3.06	3.34	3.57	3.76	3.93
				***N* = 12**				
1	1.62	2.31	2.77	3.13	3.41	3.65	3.85	4.02
2	1.64	2.32	2.79	3.16	3.45	3.69	3.90	4.08
3	1.65	2.34	2.81	3.18	3.48	3.73	3.94	4.13
4	1.66	2.35	2.83	3.20	3.51	3.76	3.98	4.17
5	1.67	2.36	2.84	3.22	3.53	3.79	4.02	4.21
6	1.68	2.37	2.86	3.24	3.56	3.82	4.05	4.25

As can be seen from the comparison of the Tables 2-3, the requirements to RSD_{max} values for the "Dissolution" test is little different from the requirements to the "Content uniformity" test, although are a little tighter because of lesser *N* value. Therefore, the conditions of the two tests conducting can be combined and we may offer the following recommendations.

2.3.3. Recommendations for conducting the "Content uniformity" and "Dissolution" tests (hereinafter referred to as *Recommendations*)

1. *1st stage. System suitability verification.* Obtain sequentially n_o = 2, 3, 4, 5 etc. replicate chromatograms for the reference standard solution and calculate the relative standard (*RSD*). Stop obtaining the replicate chromatograms after conformation of the *RSD* to the requirements of the Table 1.
2. *2nd stage. Analysis proper*. Carry out the *Level* 1 in accordance with the *2nd stage* of the *Experiment scheme,* obtaining 2 chromatograms for the every test solution of the every test drug product unit (n = 2). Using the pooled results of the *System suitability verification* and the *Analysis proper,* calculate the mean value of the chromatographic response (peak height or area) and the relative standard deviation for the reference standard solution (S_{ro}). Then calculate a mean value of the chromatographic response for the each test drug product unit, and using it and the mean value of the chromatographic response of the reference standard solution, calculate

the mean value and the relative standard deviation (S_{ri}). Using the S_{ro} and S_{ri} values, calculate the *RSD* value according to the ratio of (5). It must meet the requirements of the Table 1 and 2. For the obtained contents, calculate the mean value and the *RSD* value for it. The content values for the test drug product units and this *RSD* value around the pooled mean should be within the pharmacopoeial requirements [31-33].

3. If the *Level 1* requirements are not met, then hold the *Level 2* in accordance with the 2nd stage of the *Experiment scheme*, repeating all that is described above in the section 2.
4. Calculate (if necessary) the actual uncertainty of content for each test drug product unit at each level for each test using the ratios (5-6). It must meet the ratio of (1).

Gain in number of chromatograms (and the run time) compared to the conventional (pharmacopoeial) analysis (for $n_o = 5$):

1. *"Content uniformity" test*:

1.1. *Level 1*:

1.1.1. Number of chromatograms for the proposed scheme: 5 + 10 + 2·10 = 35.

1.1.2. Number of chromatograms for the conventional scheme (5 replicate chromatograms for both test reference standard solutions for each drug product unit + 5 replicates for the chromatographic system suitability verification): 5 + 10·5 + 10·5 = 105 chromatograms.

1.1.3. Gain in the number of chromatograms (and run time): 105/35 = 3 times.

1.2. *Level 2*:

1.2.1. Number of chromatograms for the proposed scheme: 5 + 30 + 2·30 = 95.
1.2.2. Number of chromatograms for the conventional scheme: 5 + 30·5 + 30·5 = 305 chromatograms.
1.2.3. Gain in the number of chromatograms (and run time): 305/95 = 3.2 times.

2. *"Dissolution" test:*

2.1. *Level 1*:

2.1.1. Number of chromatograms for the proposed scheme: 5 + 6 + 2·6 = 23.
2.1.2. Number of chromatograms for the conventional scheme: 5 + 6·5 + 6·5 = 65 chromatograms.
2.1.3. Gain in the number of chromatograms (and run time): 65/23 = 2.8 times.

2.2. *Level 2*:

2.2.1. Number of chromatograms for the proposed scheme: 5 + 12 + 2·12 = 41.
2.2.2. Number of chromatograms for the conventional scheme: 5 + 12·5 + 12*5 = 125 chromatograms.
2.2.3. Gain in the number of chromatograms (and run time): 125/41 = 3.0 times.

For $n_o < 5$ gain in number of chromatograms is still more.

As can be seen, the application of the proposed scheme allows reducing the analysis time, on average, three times. A similar scheme can be used for the simultaneous assay of multiple batches of a drug product.

3. Example. Content uniformity control of the “Renalgan” tablets with use of liquid chromatography

As mentioned above, the ideology of standardization in the “Content uniformity” and “Dissolution" multilevel tests is the same. These tests are different, in fact, only the number of drug product units at each level. This number is strictly fixed and cannot be changed: 10 and 30 units for the “Content uniformity” test and 6, 12 and 24 units for the "Dissolution" test. Therefore, the experimental demonstration of the above developed approaches we conducted on the example of the “Content uniformity” test (as more general test compared to the "Dissolution" test). In this case we did not attempt to evaluate the quality of the specific industrial drug product batches (it is the task of regulatory authorities). Purpose was to demonstrate the use of the developed approaches to real objects. Therefore we selected a laboratory batch of a drug product.

3.1. Test subject

For study we selected the laboratory tablet batch of the following composition:

Metamizole sodium (Analgin) – 0.5 g,
Pitophenone hydrochloride – 0.005 g,
Fenpiverinium bromide – 0.0001 g,
excipients – up to the tablet weight of 0.77 g.

In accordance with the requirements of the SPU general article "Tablets" [1], the Metamizole sodium content must be within the range 95-105% of the nominal (i.e. B = 5%), Pitophenone hydrochloride content must be within 90-110% of the nominal (B = 10%), Fenpiverinium bromide content must be within 85-115% (B = 15%).

Tablets of such composition are widely presented in the market of Ukraine («Baralgin», «Renalgan», «Spasgin», and so on), so reducing the number of replicate chromatograms when conducting their tests "Dissolution" and "Content uniformity" is quite relevant.

In accordance with the SPU requirements [30-31], the “Content uniformity” test must be conducted for Pitophenone hydrochloride and Fenpiverinium bromide.

As can be seen, this drug composition is characterized by very large differences in concentrations of active ingredients: Analgin: Pitophenon hydrochloride: Fenpiverinium bromide = 5000: 50: 1.

In Analgin substance, in accordance with the European Pharmacopoeia requirements, up to 0.5% of impurities are allowed, i.e. up to 0.0025 g to one tablet. It is 25 times higher than the concentration of Fenpiverinium bromide in one tablet. Therefore,

HPLC of this drug composition (other methods here are hardly applied) is of considerable complexity.

Significant differences in the concentrations of Pitophenon hydrochloride and Fenpiverinium bromide cause big differences in repeatability of replicate chromatograms for these compounds (see below) that allows us to test the proposed scheme of the analysis in a wide range of the RSD values. In addition, the presence of large amounts of impurities of Analgin makes for Fenpiverinium bromide more reliable application of the peak height as a chromatographic response but not the peak area (as for Pitophenon hydrochloride). This allows us to test the scheme for different chromatographic response range.

An important advantage of this composition batch as the object for the demonstration of the proposed analysis scheme, according to a preliminary research, is that the content uniformity of Pitophenon hydrochloride and Fenpiverinium bromide is in the critical zone. This allows us to check the proposed scheme for the most difficult cases, when it is necessary to distinguish between standard and substandard products.

3.2. Content uniformity procedure

Reference standard solution. Freshly prepared solution containing 5% of Analgin, 0.05022% of Pitophenon hydrochloride and 0.001046% of Fenpiverinium bromide in the mobile phase.

Procedure. Place each tablet in a conical flask with a capacity of 50 ml, add 10 ml of the mobile phase with a pipette, shake until tablet disintegration and filter through a glass filter POR 16, discarding the first portion of the filtrate.

It should be noted that the sample preparation uncertainty in this case meets the ratio of (2), i.e. it is not taken into account in further calculations.

Chromatographic conditions:

liquid chromatograph: “Waters Alliance” with UV detector;
steel column, size l = 0.25 m, Ø = 4.6 mm,
stationary phase: octylsilyl silica gel (C18);
mobile phase: acetonitrile – ion-pair reagent (40:60);
flow rate: 1.0 ml/min;
column temperature: 50 ºC;
detection: spectrophotometer at 200 nm;
injection: 20 µl.

Conduct the analysis of 10 tablets accordingly to the *Experiment scheme* taking into account the *Recommendations*.

A typical chromatogram is presented in the Fig 1.

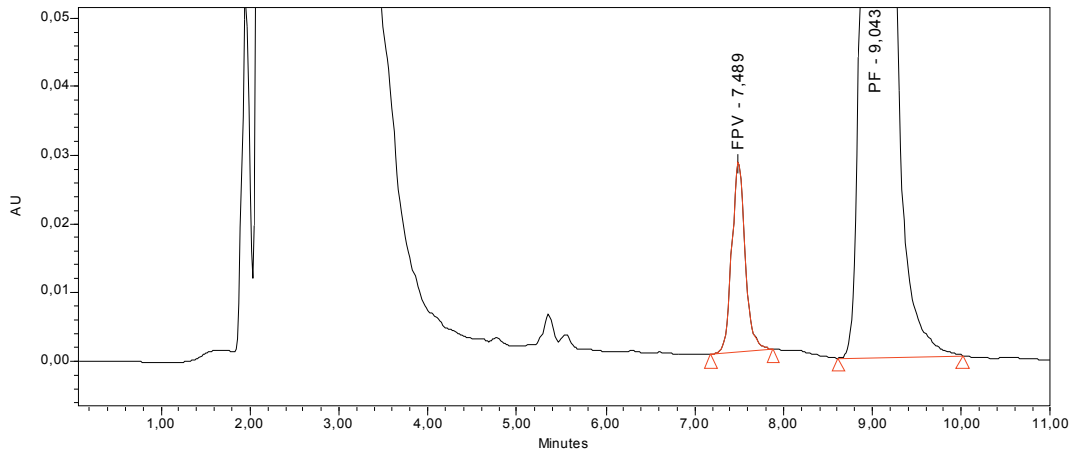


Рис. 1. Typical chromatogram of the drug composition analysis. From right to left: Pitophenon hydrochloride, Fenpiverinium bromide.

Calculate the Pitophenon hydrochloride content (X_{PH}) in one tablet, as a percentage of the nominal, by the equation:

$$X_{PH} = \frac{S_{PH} \cdot 100}{S^{st}_{PH}} \cdot \frac{C^{st}_{PH}}{0.05}.$$

Here: S_{PH} is the mean peak area of Pitophenon hydrochloride, calculated on the basis of chromatograms of the test solution;

S^{st}_{PH} is the mean peak area of Pitophenon hydrochloride, calculated on the basis of chromatograms of the reference standard solution;

C^{st}_{PH} is the concentration of Pitophenon hydrochloride in the reference standard solution.

Calculate the Fenpiverinium bromide content (X_{PH}) in one tablet, as a percentage of the nominal, by the equation:

$$X_{FB} = \frac{h_{FB} \cdot 100}{h^{st}_{FB}} \cdot \frac{C^{st}_{FB}}{0.001},$$

Here: h_{FB} is the mean peak area of Fenpiverinium bromide, calculated on the basis of chromatograms of the test solution;

h^{st}_{FB} is the mean peak area of Fenpiverinium bromide, calculated on the basis of chromatograms of the reference standard solution;

C^{st}_{FB} is the concentration of Fenpiverinium bromide in the reference standard solution.

Contents of Pitophenon hydrochloride and Fenpiverinium bromide in one tablet should conform to the requirements of the SPU general article 2.9.6 [30].

The average full time of one tablet analysis, including sample preparation, is about 20 minutes. Thus, the analysis of 10 tablets in accordance with the SPU requirements [53] will take about 35 hours. Really the analysis takes much more time: as the column is being poisoned by products of analgin decomposition (dozens of times greater than the content of Fenpiverinium bromide), the column must be periodically cleaned.

To reduce the analysis time, we used the described above *Experiment scheme* and *Recommendations*.

3.3. Results and their discussion

1st stage. System suitability verification.

Table 4

The results of the system suitability verification for different n_o

Chromatogram number	Pitophenon hydrochloride			Fenpiverinium bromide			n_o	Requirement of Table 1 to *RSD*
	S^{st}_{PHi}	Mean S^{st}_{PH}	*RSD* %	h^{st}_{FBi}	Mean h^{st}_{FB}	*RSD* %		
1.	*14202521*	-	-	*27459*	-	-	-	-
2.	*14149303*	14169442	0.27	*27129*	27294	0.85	2	0.48
3.	*14154777*	14168867	0.21	*28288*	27625	1.90	3	1.26
4.	*14171165*	14169442	0.17	*28036*	27728	1.91	4	1.80
5.	*14157743*	14167102	0.15	*28602*	27903	2.16	5	2.23

As can be seen, the requirements of Table 1 for limit *RSD* values are met for both substances, but at different n_o: for Pitophenon hydrochloride they are already met when n_o = 2, and for Fenpiverinium bromide when n_o = 5. Given that both substances are determined at the same chromatogram, it should be assumed for them both n_o = 5. In accordance with the Table 1, there was no point in getting further additional chromatograms (with n_o > 5) for the reference standard solution.

2nd stage. Analysis proper (N = 10).

Table 5

Assay of Pitophenon hydrochloride in 10 tablets

№ таблетки	Reference standard solution			Test solution				
	Peak areas			Peak areas				X_{PH} %
	S^{st}_{PHi}	S^{st}_{PH}*	S_{ro}% *	1	2	Среднее (S_{PH})	S_{ri} %	
1.	14240777			12466530	12460606	12463568	0.03	87.64
2.	14252192			13188619	13222866	13205743	0.18	92.86

3.	14316388	14282585	0.68	13977556	13981547	13979552	0.02	98.30
4.	14345217			13426522	13413966	13420244	0.07	94.37
5.	14399585			13957605	13996672	13977139	0.20	98.29
6.	14412474			13866804	13899359	13883082	0.17	97.62
7.	14370673			13143989	13183413	13163701	0.21	92.57
8.	14351441			13874245	13956640	13915443	0.42	97.85
9.	14399036			13775195	13776381	13775788	0.01	96.87
10.	14315488			13659478	13722502	13690990	0.33	96.27
Mean content $\overline{X_{PH}}$								***95.26***
RSD_{PH} around the mean content $\overline{X_{PH}}$								***3.61***

* Calculated on the basis of Tables 4-5 (total 15 chromatograms)

Substituting the S_{ro} and S_{ri} values in the equation of (5), for $n_o = 5$, $n = 2$, $N = 10$ we get $RSD = 0.54\%$, that is well below the critical value (2.33%) given in Table 1 for $n_o = 5$, $n = 2$, $N = 10$. I.e. the requirements of the Table 2 are met.

As provided by the requirements of the SPU-Eur.Ph. general article 2.9.40 [32], in our case ($\overline{X_{PH}} < 98.5\%$) the acceptance value AV at the *Level 1* (10 tablets) must meet the relationship:

$$AV = 98.5 - \overline{X} + 2.4 \cdot RSD \leq 15.0. \qquad (7)$$

In our case for Pitophenon hydrochloride we have AV_{PH} = 98.5 - 95.26 + 2.4·3.61 = 12.0 < 15.0, i.e. the relationship of (7) is met at the *Level 1*. Correspondingly the content uniformity requirements of the SPU-Eur.Ph. general article 2.9.40 are met.

On the basis of the $RSD = 0.54\%$, calculate the real relative uncertainty of Pitophenon hydrochloride content in each tablet (Δ_{As}) by the equation of (6). The number of degrees of freedom is $f = n_o + N$·n - 1 = 5 + 10·2 - 1 = 24. One-tailed *t-criterion* for f = 24 and 95% probability is equal to 1.7109 [26]. Then the ratio of (9) gives Δ_{As} = 0.70%, while the equation of (1) requires $\Delta_{As} \leq 3.0\%$, i.e. much more.

Table 6

Assay of Fenpiverinium bromide in 10 tablets

№ табл ет	Reference standard solution	Test solution	
	Peak heights	Peak heights	X_{FB}

ки	h^{st}_{FBi}	h^{st}_{FB}*	S_{ro}% *	1	2	Mean (h_{FB})	S_{ri} %	%
1.	27988			25446	25397	25421.5	0.14	95.17
2.	27872			23721	23772	23746.5	0.15	85.16
3.	27642			25616	25634	25625	0.05	95.93
4.	27474			24766	24614	24690	0.44	92.43
5.	27747			25062	25146	25104	0.24	93.98
6.	27529	27937	1.91	24285	23624	23954.5	1.95	89.68
7.	29198			25030	24674	24852	1.01	93.04
8.	27557			24437	23937	24187	1.46	90.55
9.	28116			26896	25382	26139	4.10	97.86
10.	28424			24078	23484	23781	1.77	89.03
Mean content								***92.28***
RSD around the mean content								***4.08***

* Calculated on the basis of Tables 4-5 (total 15 chromatograms)

Substituting the S_{ro} and S_{ri} values in the equation of (5), for $n_o = 5$, $n = 2$, $N = 10$ we get $RSD = 1.80\%$, which is less than the critical value (2.33%) given in the Table 2 for $n_o = 5$, $n = 2$, $N = 10$. I.e. the requirements of the Table 2 are met.

As provided by the requirements of the SPU-Eur.Ph. general article 2.9.40 [32], in our case ($\overline{X_{FB}} < 98.5\%$) the acceptance value AV at the *Level 1* (10 tablets) must meet the relationship (7).

In our case for Fenpiverinium bromide we have $AV_{FB} = 98.5 - 92.28 + 2.4 \cdot 4.08 = 16.0 > 15.0$, i.e. the relationship (7) is not met at the *Level 1*. Correspondingly the content uniformity requirements of the SPU-Eur.Ph. general article 2.9.40 are not met. We must hold the *Level 2*.

On the basis of $RSD = 1.80\%$, calculate the real relative uncertainty of Fenpiverinium bromide content in each tablet (Δ_{As}) by the equation (6). The number of degrees of freedom is $f = 24$ (see above). The one-tailed *t-criterion* for $f = 24$ and 95% probability is equal to 1.7109 [26]. Then the ratio of (6) gives $\Delta_{As} = 2.32\%$, while equation (1) requires $\Delta_{As} \leq 3.0\%$, i.e. the ratio of (1) is met. Thus the received negative results at the *Level 1* is not related to the lack of procedure precision, and are associated with inadequate technology, which leads to a very large decrease of the Fenpiverinium bromide content in tablets.

Note that this scheme is applied mutatis mutandis to the simultaneous chromatographic assay of several batches of a drug product [62].

4. Using two reference standard solutions when conducting the "Dissolution" and "Content uniformity" tests by chromatographic methods

Above was shown the effectiveness of metrological based scheme of simultaneous chromatographic analysis of several samples of a drug product, which was, in particular, applied for conducting the pharmacopoeial "Content uniformity" and "Dissolution" tests. This scheme enables us to significantly reduce the chromatographic experiment while maintaining the necessary precision of the analysis.

The usual scheme of the chromatographic experiment use one reference standard solution and one solution of a test sample. These solutions then are chromatographed as needed. This scheme is usually meant in a specification. It was usually clear in the analysis of 1-2 test samples, however, becomes more vulnerable with a substantial increase in the number of tested samples, for example, when we conduct the "Content uniformity" (10 or 30 samples) or "Dissolution" tests (6, 12 or 24 samples).

The feature of quantitative chromatographic procedures is that the uncertainty of the final analytical operation (chromatography proper) is controlled by the chromatographic system suitability test. At the same time, the sample preparation uncertainty is ensured only by the validated environment that is vulnerable to gross carelessness.

It should be noted the difference between the routine quality control under the drug production and analysis of drugs under the State control or arbitration. In the first case, the analysis is carried out continuously by the validated procedure, and the manufacturer control laboratory accumulates information on the chromatographic responses, which is the controlling factor for possible gross blunders in the sample preparation. In addition, the results are substantially expected. In the second case, the analysis is usually a one-time, data on procedure validation and historical data of the chromatographic responses are absent. In these circumstances, the gross error (mistake) with the sample preparation may affect the decisions about drug product quality.

An impact of a gross error in a sample preparation is particularly high in the case of a reference standard solution used for analysis of large quantity of samples, for example, when we run the "Content uniformity" or "Dissolution" tests. In this case, the gross error in the preparation of the reference standard solution makes incorrect an entire big experiment and conclusion about quality.

Effective way to control gross errors in the sample preparation is the simultaneous chromatographic analysis of two independently prepared reference standard solutions. However, the question arises as to what should be a scheme of analysis, how to calculate and how to control the correctness of the results.

Below is the modernization of the metrological grounded scheme (proposed earlier [28, 62] and described above) by means of inclusion of two reference standard solutions in order to control their sample preparation.

4.1. Experiment scheme

Upgrade the scheme proposed above with one reference standard solution (*ST*) as follows. Instead of a single *ST* alternately chromatograph the *ST1* and *ST2*.

1st stage. System suitability verification. At this stage obtain n_{0st1} chromatograms of the *ST1* and n_{0st2} chromatograms of the *ST2*. When this:

$$n_{0st} = n_{0st1} + n_{0st2}.$$

In the case of an even n_{0st} we have $n_{0st1} = n_{0st2} = 0.5 \cdot n_{0st}$. Verification of the chromatographic system suitability is carried out on the base of the total chromatogram number n_{0st}.

2nd stage. Analysis proper. Get the chromatograms in the following sequence, alternately chromatographing the *ST1* and *ST2* in every *2n* chromatograms of the test solution:

n chromatograms of the sample solution 1 → *n* chromatograms of the sample solution 2 → *ST1* → *n* chromatograms of the sample solution 3 → *n* chromatograms of the sample solution 4 → *ST2* →…. *n* chromatograms of the sample solution *N*-2 → *ST1* → *n* chromatograms of the sample solution *N*-1 → *n* chromatograms of the sample solution *N* → *ST2*.

At this stage we get *n* chromatograms of every test solution, n_{st1} chromatograms of the *ST1*and n_{st2} chromatograms of the st2. The total number of *ST1* and *ST2* chromatograms at the stage of analysis of the test sample is $n_{st} = n_{st1} + n_{st2}$. The total number of *ST1* chromatograms is equal $N_{st1} = n_{0st1} + n_{st1}$, the total number of *ST2* chromatograms is equal to $N_{st1} = n_{0st2} + n_{st2}$ and the total number of *ST1* and *ST2* chromatograms is equal to $N_{st} = n_{0st} + n_{st} = n_{0st} + 0.5 \cdot N$. Finally, we get:

$$\begin{aligned}
&n_{0st} = n_{0st1} + n_{0st2},\\
&n_{st} = n_{st1} + n_{st2} = 0.5 \cdot N,\\
&N_{st1} = n_{0st1} + n_{st1}, \quad N_{st2} = n_{0st2} + n_{st2},\\
&N_{st} = N_{st1} + N_{st2} = n_{0st} + n_{st} = n_{0st} + 0.5 \cdot N,\\
&N_{tot} = N \cdot n + N_{st} = N \cdot (n + 0.5) + n_{0st}.
\end{aligned} \tag{8}$$

At the same time, as shown above, the one-sided confidence interval (probability 0.95) Δ_{As} for the results of analysis of each drug product unit under the pharmacopoeial tests for the content uniformity or dissolution must meet the ratio of (1).

The uncertainty of sample preparation (Δ_V%) is quite easily adjusted by proper sample weights and volumetric flasks and can therefore be made insignificant compared with the maximum acceptable uncertainty of the final analytical operation ($max\Delta_{FAO}$%) (in our case, chromatography), i.e., the ratio of (2) must be met.

As mentioned above, the relative standard deviations (*RSD*) of repeatability of chromatographic responses (peak areas or heights) for the test and reference standard solutions are sample estimates of the universe *RSD*. This is due to the fact that we analyze the same substance in different close concentrations (usually not more than ± 15%) Therefore, in view of the relationship (2), the difference between the peak area values of two different reference standard solutions (*ST1* and *ST2*) is only determined by the chemical reference substance (CRS) weights (m_{st1} and m_{st2}) taken for their preparation.

4.2. Standardized *ST1* and *ST2* peak areas

Standardized ST1 and ST2 peak areas. In a specification indicate the nominal CRS weight (m_{st0}) taken for the reference substance solution preparation. Let's introduce standardized peak areas in two different reference substance solutions *ST1* and *ST2* in the following way:

$$S^0_{stij} = \frac{m_{st0}}{m_{sti}} \cdot S_{stij} \tag{9}$$

Here: i = 1 or 2 (respectively for *ST1* and *ST2*),

j = replicate chromatogram number for the *ST1* and *ST2*.

In view of the relation (8), the S^o_{stij} values for the *ST1* and *ST2* are samples from the parent population with the same mean and we can combine them. So for this pooled sample let's calculate the standardized value of the peak area of the reference standard solution S^o_{st}:

$$S^0_{st} = \frac{1}{n_{0st} + 0.5 \cdot N} \cdot \sum_{i,j} S^0_{stij} \,. \tag{10}$$

The content of the test material in each studied drug product unit calculate from the following equation (S_k is the mean peak area corresponding to the *k-th* unit under study, P – dilution factor):

$$X_k\% = 100 \cdot P \cdot \frac{S_k}{S^0_{st}} \cdot m_{st0} \,. \tag{11}$$

The confidence interval (probability 0.95) Δ_{As} for the results of analysis of each drug product unit under the "Content uniformity" or "Dissolution" tests must meet the requirements of the relationship (2).

4.3. Requirements to *RSD* values

1st stage. System suitability verification. At this stage, get n_{ost} = 2 – 6 (total) replicate *ST1* and *ST2* chromatograms (chromatograph alternately), calculate the relative standard deviation and compare it with the critical RSD_{max} value [13].

Given the existence of the *2nd stage* (see below), a difficult question is what are the requirements to RSD_{max} values at the *1st stage*. It is reasonable to set such the RSD_{max} values that n_{ost} replicate chromatograms of test and reference standard solutions should provide the results uncertainty of Δ_{As} for the test material not worse than requirements of the ratio (1). This is consistent with the above approach for one reference standard solution. Since at the *1st stage* (verification of the chromatographic system suitability) we perform n_{ost} (total) replicate *ST1* and *ST2* chromatograms, then the number of degrees of freedom in this case is n_o - 1. Same number of degrees of freedom is the *t-criterion*. Given this uncertainty, the confidence interval is the result:

$$\Delta_{As} \le \max\Delta_{As} = \sqrt{2} \cdot \frac{RSD_{\max} \cdot t_{90\%,n_o-1}}{\sqrt{n_o}}. \qquad (12)$$

Hence, given the relationship (2), we can get the requirements to RSD_{max} values:

System suitability verification:

$$RSD_{0st} \le RSD_{\max} = \frac{2.12 \cdot \sqrt{n_o}}{t_{90\%,n_o-1}}. \qquad (13)$$

RSD_{max} values for various n_{ost} at the *1st stage* (verification of chromatographic system suitability) are shown in the Table 7. Note that the RSD_{0st} values are applied to the standardized values of the *ST1* and *ST2* peak areas.

Table 7

Requirement to the RSD_{max} values under the "Content uniformity" and "Dissolution" tests performing at the 1st stage (verification of chromatographic system suitability)

n_{ost}	2	3	4	5	6	7	8	9	10
RSD_{max}%	0.48	1.26	1.80	2.23	2.58	2.89	3.17	3.42	3.66

Table 7 shows that the requirements to the RSD_{max} values at the 1st stage (verification of chromatographic system suitability) under the "Content uniformity" and "Dissolution" test performing are rather liberal, that allows conducting these tests on each modern chromatograph. The RSD_{max} value (1.26%) for three chromatograms (n_{ost} = 3) is easily achieved in practice and this chromatogram number is enough in the most cases. However, taking into account the presence of two reference standard solution

(*ST1* and *ST2*), it is recommended to obtain at least 4 chromatograms, i.e. $n_{ost} \geq 4$. The RSD_{max} values for $n_{ost} > 6$ are presented just for comparison.

2nd stage. Analysis proper. At this stage, we get a total of $n_o + 0.5 \cdot N$ chromatograms of the *ST1* and *ST2* and $N - n$ chromatograms of the test solutions 1 to N. Calculate the relative standard deviations for the *ST1* and *ST2* (RSD_{st}) and for every test solution (RSD_k). All of them (and the RSD_{ost} value) are sample estimates of the universe *RSD*. Given the (8), we can calculate the pooled (stages 1 and 2) relative standard deviation from the ratio:

$$RSD^2_{\max} \geq RSD^2_{tot} = \frac{[n_{0st} + 0.5 \cdot N - 1] \cdot RSD^2_{0st} + (n-1) \cdot \sum_k^N RSD^2_k}{n_{ost} - 1 + N \cdot (n - 0.5)}. \qquad (14)$$

As can be seen, RSD_{tot} has number of degrees of freedom equal to $f = n_o - 1 + N \cdot (n - 0.5)$.

Find the requirements to the RSD_{max} values, on the basis of the maximum acceptable procedure error under the “Content uniformity” and “Dissolution" tests (ratios (1-2)).

Given the ratios (1-2), (8) and (14), the uncertainty of the mean content in the each drug product unit under analysis can be found from the equation:

“Content uniformity” and “Dissolution” tests performing

$$\Delta_{As} = RSD_{tot} \cdot t_{90\%,f} \cdot \sqrt{\frac{1}{n_{0st} + 0.5 \cdot N} + \frac{1}{n}},$$
$$f = n_0 - 1 + N \cdot (n - 0.5), \qquad (15)$$
$$RSD_{tot} \leq RSD_{\max}.$$

The Δ_{As} value consists of two components: the relative confidence intervals of the reference standard (first member below the root) and the test (second member below the root) solutions. The relative standard deviation (RSD_{tot}) and *t-criterion* ($t_{90\%,f}$) for both the solutions are the same, but the number of replicates to account for the uncertainty of the mean value are different ($n_o + 0.5 \cdot N$) and n respectively.

Given that the Δ_{As} value under the “Content uniformity” and “Dissolution" testsperforming must meet the ratio of (1), we can calculate the RSD_{max} values for different n_{ost} , N and n from the equation of (15) (see the Tables 8-9).

4.4. Content uniformity

Table 8

Requirements to the RSD_{max} values under the “Content uniformity” test performing for various n_{ost} , N and n

n_{ost}	RSD_{max} % values for n values equal to:							
	1	2	3	4	5	6	7	8
Level 1: ***N* = 10**								
2	1.44	2.14	2.55	2.84	3.05	3.22	3.36	3.48
3	1.49	2.18	2.60	2.90	3.14	3.32	3.48	3.60
4	1.53	2.21	2.65	2.96	3.21	3.41	3.57	3.71
5	1.56	2.24	2.68	3.01	3.27	3.48	3.65	3.80
6	1.58	2.26	2.71	3.05	3.32	3.54	3.72	3.88
Level 2: **N = 30**								
2	1.67	2.39	2.88	3.25	3.56	3.82	4.04	4.24
3	1.68	2.40	2.89	3.27	3.58	3.85	4.08	4.27
4	1.69	2.41	2.90	3.29	3.60	3.87	4.11	4.31
5	1.69	2.41	2.91	3.30	3.62	3.90	4.13	4.34
6	1.70	2.42	2.92	3.32	3.64	3.92	4.16	4.37

From the Table 8 the following conclusions can be drawn:

1. RSD_{max} values little depend on the data of the 1st stage (n_o) - verification of the chromatographic system suitability.
2. To obtain reliable information, in theory it is enough of one replicate chromatogram (n = 1) for each unit of the drug product under study, since the RSD_{max} values are a perfectly acceptable for N = 10 (1.44%) and for N = 30 (1.67%) because the actual *RSD* values for modern chromatographs are usually at least three times less. However, under the "Content uniformity" test performing we check not only the *RSD* value of contents of individual drug product units (for this it is enough n = 1) but the contents themselves: they must be within appropriate limits. It is therefore advisable to obtain at least two replicate chromatograms (n = 2).
3. Requirements to the RSD_{max} values for N = 10 and N = 30 little differ, so as the requirements to the RSD_{max} values we can take more stringent requirements for N = 10.

4.5. Dissolution

Table 9

Requirements to the RSD_{max} values under the "Dissolution" test performing for various n_{ost} , N and n

n_{ost}	RSD_{max} % values for n values equal to:							
	1	2	3	4	5	6	7	8
Level 1: $\boldsymbol{N = 6}$								
2	1.28	1.98	2.10	2.60	2.79	2.93	3.04	3.13
3	1.38	2.05	2.11	2.71	2.92	3.08	3.20	3.31
4	1.44	2.10	2.12	2.80	3.02	3.19	3.34	3.46
5	1.49	2.14	2.12	2.87	3.10	3.29	3.45	3.58
6	1.53	2.18	2.13	2.93	3.18	3.38	3.54	3.68
Level 2: $\boldsymbol{N = 12}$								
1	1.44	2.16	2.56	2.85	3.06	3.23	3.37	3.49
2	1.49	2.19	2.61	2.91	3.15	3.33	3.48	3.61
3	1.53	2.23	2.66	2.97	3.22	3.41	3.58	3.72
4	1.56	0.18	2.69	3.02	3.28	3.48	3.66	3.81
5	1.58	0.18	2.72	3.06	3.33	3.55	3.73	3.89
6	1.60	0.18	2.75	3.10	3.37	3.60	3.79	3.96
Level 3: $\boldsymbol{N = 24}$								
1	1.62	2.34	2.80	3.15	3.44	3.67	3.87	4.04
2	1.64	2.35	2.82	3.18	3.47	3.71	3.92	4.09
3	1.65	2.36	2.84	3.21	3.50	3.75	3.96	4.14
4	1.66	2.37	2.86	3.23	3.53	3.78	4.00	4.19
5	1.67	2.38	2.87	3.25	3.56	3.81	4.04	4.23
6	1.68	2.39	2.88	3.27	3.58	3.84	4.07	4.27

As can be seen from the comparison of the Tables 8 and 9, the requirements to the RSD_{max} values under the "Dissolution" test performing are little different from the requirements for “Content uniformity” test, although are a little tighter by reason of the lesser N value. Therefore, the conditions of the two tests can be combined and offer the following recommendations.

4.6. Recommendations for conducting the “Content uniformity” and “Dissolution” tests

1. *1st stage*. *System suitability verification.* Get sequentially n_o = 2, 3, 4, 5, etc. replicate chromatograms of *ST1* and *ST2*, calculate the standardized area values by the ratio of (9) and the relative standard deviation RSD_{0st} for these standardized areas.

Cease getting the replicate chromatograms when the RSD_{0st} value meets the requirements of the Table 7.

2. *2^{nd} stage. Analysis proper*. Carry out the *Level* 1 of the Table 8 or 9 in accordance with the *2^{nd} stage* of the *Experiment scheme,* obtaining 2 chromatograms for the every test solution of the every *k-th* test drug product unit (n = 2). Using the pooled results of the *System suitability verification* and the *Analysis proper,* calculate by the ratio of (9) the standardized area values S^0_{stij} for *ST1* and *ST2*, their pooled mean S^0_{st} by the equation of (10) and relative standard deviation (RSD_{st}). For each *k-th* drug product unit calculate the mean peak area S_k and its relative standard deviation RSD_k. On the basis of the S_k and S^0_{st} values calculate the mean content value X_k. On the basis of the S_{ro} and S_{ri} values calculate the RSD_{tot} value by the ratio of (14). It must meet the requirements of the Table 8 or 9. For the obtained content values, calculate the mean value and *RSD* for it. The content values for the test drug product units and this *RSD* value should be within the requirements of the SPU general articles for the content uniformity (2.9.40) [32] or dissolution (2.9.3) [33].
3. If the *Level 1* requirements of the Table 8 or 9 are not met, then hold the *Level 2* of the Table 8 or 9 in accordance with the 2nd stage of the *Experiment scheme*, repeating all that is described above in the section 2. Similarly for the *Level 3* of the Table 9 for the dissolution.
4. The real uncertainty of content for each analyzed drug product unit at each stage for each test calculate (if necessary) by the ratio of (14-15). It meets the requirements of (1).

Gain in number of chromatograms (and the run time) compared to the conventional (pharmacopoeial) analysis (for n_o = 5):

1. *"Content uniformity" test* of the Table 8:

1.1. *Level 1*:

1.1.1. Number of chromatograms for the proposed scheme (equation (8)): 10·(2+0.5)+5 = 30.

1.1.2. Number of chromatograms for the conventional scheme (5 replicate chromatograms for both test reference standard solutions for each drug product unit + 5 replicates for the chromatographic system suitability verification): 5 + 10•5 + 10•5 = 105 chromatograms.

1.1.3. Gain in the number of chromatograms (and run time): 105/30 = 3.5 times.

1.2. *Level 2*:

1.2.1. Number of chromatograms for the proposed scheme: 30·(2+0.5)+5 = 80.

1.2.2. Number of chromatograms for the conventional scheme: 5 + 30•5 + 30•5 = 305 chromatograms.

1.2.3. Gain in the number of chromatograms (and run time): 305/80 = 3.8 times.

2. *"Dissolution" test* of the Table 9*:*

2.1. *Level 1*:

2.1.1. Number of chromatograms for the proposed scheme: 6·(2+0.5)+5 = 20.
2.1.2. Number of chromatograms for the conventional scheme: 5 + 6•5 + 6•5 = 65 chromatograms.
2.1.3. Gain in the number of chromatograms (and run time):: 65/20 = 3.3 times.

2.2. *Level 2*:

2.2.1. Number of chromatograms for the proposed scheme: 12·(2+0.5)+5 = 30.
2.2.2. Number of chromatograms for the conventional scheme: 5 + 12•5 + 12*5 = 125 chromatograms.
2.2.3. Gain in the number of chromatograms (and run time): 125/30 = 4.2 times.

For $n_o < 5$ gain in number of chromatograms is still more.

As can be seen, the application of the proposed scheme allows reducing the analysis time, on average, 3.5 times. A similar scheme can be used for the simultaneous assay of multiple batches of a drug product.

4.7. Supplementary control of the reference standard solutions

The above scheme ensures a correct conclusion about the quality of the samples. If the *ST1* and *ST2* peak areas differ greatly (a gross error in the sample preparation), the requirements to chromatographic system suitability (Table 7) and to the pooled RSD_{tot} value (ratio of (14)) will not hold. However, the differences between the *ST1* and *ST2* may be insignificant in terms of getting a correct conclusion about the quality, but may be significant in terms of a regular analytical practice. To determine this, we should calculate separately the mean standardized peak area values for the *ST1* and *ST2* by the equation:

$$\begin{aligned} S^0_{st1} &= \frac{1}{N_{st1}} \cdot \sum_j S^0_{st1j} = \frac{1}{n_{0st1} + n_{st1}} \cdot \sum_j S^0_{st1j}, \\ S^0_{st2} &= \frac{1}{N_{st2}} \cdot \sum_j S^0_{st2j} = \frac{1}{n_{0st1} + n_{st1}} \cdot \sum_j S^0_{st2j}. \end{aligned} \qquad (16)$$

The difference between the means shall not exceed the value of the pooled relative confidence interval:

$$100 \cdot \left| \frac{2 \cdot (S^0_{st1} - S^0_{st2})}{S^0_{st1} + S^0_{st2}} \right| \le RSD_{tot} \cdot t_{90\%,f} \cdot \sqrt{\frac{n_1 + n_2}{n_1 \cdot n_2}},$$
$$f = n_{0st} - 1 + N \cdot (n - 0.5), \qquad (17)$$
$$n_{0st} = n_{0st1} + n_{0st2},$$
$$n_{st1} = n_{0st1} + n_{st1}, \quad n_{st2} = n_{0st2} + n_{st2}.$$

If the ratio of (17) is not met, the requirement (2) of the insignificance of the sample preparation error for *ST1* and *ST2* does not hold. Therefore, there are problems with the preparation of the solutions.

In conclusion, it should be noted that it is reasonable to apply the schemes (as with the sections 1-3 and the section 4 for double reference standards) with use of the proper software.

Addendum 2

DESCRIPTION OF ACTIVE SUBSTANCE RELEASE PROFILES FOR VARIOUS DOSAGE FORMS

The SPU-Eur.Ph. [1] describes the tests for dissolution (release) of various dosage forms and substances: *2.9.3. Dissolution test for solid dosage forms*, *2.9.4. Dissolution test for transdermal patches, 2.9.25. Dissolution test for medicated chewing gums", 2.9.42. Dissolution test for lipophilic solid dosage forms, 2.9.43. Apparent dissolution.* These tests, in addition to quality control, are widely used to study release (dissolution) profiles, which is required at the stage of development of pharmaceutical drugs and evidence of bioequivalence. Therefore, the mathematical description of these curves is both an academic and practical interest.

Despite the differences in these tests, the curves of the dissolution (release) for them are similar and are determined mainly by two factors: the diffusion of the target component to the surface of contact with the liquid and dissolving into the liquid. In the most general case, these factors occur when describing the release curves for ointments and suppositories, so we consider them in the beginning. This allows us to obtain general equations, which can then be used in the simpler cases.

1. DESCRIPTION OF *IN VITRO* RELEASE PROFILES OF BIOLOGICALLY ACTIVE SUBSTANCES FOR SUPPOSITORIES AND OINTMENTS [89-92]

This section considers the description of *in vitro* profile (curve) release of biologically active ingredients from suppositories and ointments. It aims to:

- explore the type of the release profiles, obtained under different conditions at different devices;
- get general mathematical expressions for dissolution (release) curves and their special cases;
- explore factors affecting reproducibility of these curves;
- offer the standardized release procedure for ointments and suppositories, allowing to carry out comparative studies.

As a method of quantitative determination we use in all cases the UV spectrophotometry by absorption of the investigated compounds. All the ointment and suppository bases have some residual absorption, which prevents quantification. So to reduce it, we use in proper cases multi wave spectrophotometry [81].

1.1. Description of release profiles at a constant layer thickness

This section discusses the type of release profiles (curves), resulting under different conditions and on different devices.

1.1.1. Experiment

Objects of research. Suspension suppositories "Germicid", "Gibernal", "Pipolfen", "Diafilin", ointment with lidocaine ("Egis", Hungary), suppositories with metamizole sodium (Ukraine) with different bases and metamizole sodium (analgin) powder. The composition of these products is shown in the Table 1.1. The use of various objects and bases is intended to get the general conclusions regarding the nature of the release curves, independent of these factors.

Equipment and materials:

1. "Sartorius" absorption simulator (type SM 16750) with an ointment chamber (type SM 16754). Dissolution medium volume is 100 ml.
2. “Erweka” dissolution tester (Germany). Dissolution medium volume is 500 ml ("rotating basket") and 100 ml (directly from the suppositories).
3. As a semi-permeable membrane is used the dialyzing membrane of "Hoechst" with a thickness of 5.5 mm (Germany, Nadir-Dialysierschlauch). The membrane is previously soaked in 0.1 M solution of hydrochloric acid for 1 hour.
4. Dissolution media: 0.1 M hydrochloric acid; water for "Germicid-2" and "Diafillin" when released directly from the suppositories.

Table 1.1

Objects of study

N	Объекты	Composition, grams	Analytical wavelengths , nm			m^*
			1	2	3	
1.	Suppositories “Gibernal”	Aminazin base 0.1, white wax 0.065, cocoa butter 2.215	258	300	-	2.90
2.	Suppositories “Germicid-1”	Amidazophen 0.1, cocoa butter 1.35	255	280	-	2.38
3.	Suppositories “Pipolfen-1”	Promethazine base 0.025, white wax 0.045, cocoa butter 1.38	249	-	-	2.90
4.	Suppositories Analgin-1	Analgin 0.1, hard fat 1.3	258	290	-	2.80
5.	Suppositories Analgin-2	Analgin 0.1, Witepsol 1.3	258	290	-	2.80
6.	Analgin-powder	-	258	290	-	0.20

7.	Ointment with lidocaine	Lidocaine base 1.0, PEO-4000 9.0, PEO-300 5.0	269	271	282	8.50
8.	Suppositories "Germicid-2"	Amidazophen 0.1, Witepsol 1.35	255	280	-	1.45
9.	Suppositories "Pipolfen-2	Promethazine base 0.025, Witepsol 1.425	249	-	-	1.45
10.	Suppositories "Diafillin"	Aminophylline 0.36, Witepsol 2.04	267	-	-	2.40

* sample weight, grams

Release procedure. Conduct the release at the temperature $(37 \pm 0.5)^0$C with magnetic ("Sartorius", 4 stage) or glass ("Erweka") stirring at the constant speed of 100 rpm. Every hour (the "Sartorius") or in 5, 10, 15, 30 and 60 minutes ("Erweka") sample 30 ml ("Sartorius") or 20 ml (the "Erweka", "rotating basket") or 5 ml (directly from the suppositories) of the dialyzate for quantitative determination. Then add the same volume of the initial dissolution medium to the rest of the dializate.

Carry out not less than 5 replicates for each object (suppository or ointment).

In the case of "Sartorius", where there is the special ointment chamber, conduct the release through the semi-permeable membrane.

In the case of "Erweka" conduct the released in the "rotating basket" with a suppository, wrapped in the membrane, and directly from a suppository without the use of a membrane (place a suppository on the bottom of the dissolution vessel).

Such the different conditions of the experiment aimed to get some general conclusions independent of these conditions.

Final analytical operation. As a final analytical operation in all cases we used the UV spectrophotometry by absorption of the investigated compounds. For this we dilute the test dialyzate with 0.1 M solution of hydrochloric acid to a proper absorbance. To reduce the influence of ointment and suppository base, we use in proper cases multi wave spectrophotometry, whose questions have been discussed in detail in [81].

An important issue is the choice between single, double or triple wavelength spectrohotometry. The fact of the matter is that, for obtaining the practically important conclusions, we studied the industrial batches of the drugs. Therefore, we could not reliably predict the influence of a suppository or ointment base on the analysis. This situation is typical for the analytical work related to the release. Criterion for the choice between single, double and triple spectrophotometry is the calculated value of the concentration of the active ingredient. The additional base absorption overstates the analysis results. Use of the derivative (differential) spectrophotometry reduces this overstatement. Therefore, if the analysis result is not diminished when moving from the one-wave to two-wave spectrophotometry, we can use the one-wave spec-

trophotometry. If not, then you need to go next to a three-wave one, etc. In this way we had a choice of wavelengths number.

Conditions of quantitative determination are presented in the Table 1.1.

The degree of release (G) as a per cent of the total content of biologically active ingredient in the test sample, calculated by the equations:

$$G(t) = X(t) + (\frac{f}{V}) \cdot \sum_{i=1}^{t-1} X(i) \qquad (1.1)$$

$$X(t) = P \cdot C(t) \cdot V \cdot m_o /(y_o \cdot m) \qquad (1.2)$$

Here: t is a time in hours,

f = dialyzate volume, sampled for the analysis (30 mL for "Sartorius" and 20 or 5 mL for "Erweka"),

V = dialyzate volume (100 mL for "Sartorius", 500 or 100 mL for "Erweka"),

P = dialyzate dilution before the final analytical operation (spectrophotometry),

m_o = average weight of one suppository, grams (for ointments m_o = 1 g),

m = weight of a suppository or ointment, sampled for the release study, gram,

y_o = average mass of a biologically active ingredient in a suppository, gram.

$C(t)$ is equal to:

one-wave analysis: $C(t) = A(t)/E(t)$,

two-wave analysis: $C(t) = [A_1(t)-A_2(t)]/(E_1-E_2)$,

three-wave analysis: $C(t) = [A_1(t)-2A_2(t)+A_3(t)]/(E_1-2E_2+E_3)$,

where:

$A_j(t)$ = absorbance at j-th analytical wavelength in time t,

E_j = specific absorbance of an analyzed ingredient at j-th analytical wavelength.

Also we processed the results with use of the linear (LSM) and nonlinear (NLSM) weighted least squares method.

1.1.2. Results and discussion

The Table 1.2 illustrates the results obtained under the release procedure described above. Reproducibility of release for each object and time is characterized by an absolute standard deviation $S(t)$. The error of spectrophotometry proper does not usually exceed 1.0% relative, i.e., the $S(t)$ values are almost entirely associated with irreproducibility of the release conditions.

Table 1.2

The dependence of release degree *G(t)* (as a per cent of the initial content) on release time *t*; (*S(i)* values are in per cent)

Objects	*G(1)*	*S(1)*	*G(2)*	*S(2)*	*G(3)*	*S(3)*	*G(4)*	*S(4)*	*G(5)*	*S(5)*
Sartorius*										
Suppositories:										
Gibernal	15.9	1.5	28.1	1.7	37.4	1.9	44.7	1.2	51.1	1.5
Germicid-1	19.2	3.1	28.2	3.1	34.5	3.9	40.0	4.4	43.7	2.5
Pipolfen-1	16.4	0.6	25.5	0.5	32.3	0.7	37.8	0.9	42.7	1.0
Analgin-1	0.18	0.04	0.32	0.03	0.40	0.03	0.52	0.05	0.63	0.1
Analgin-2	2.6	0.6	3.3	0.6	4.0	0.6	4.8	1.0	5.4	1.0
Analgin powder	47.2	7.1	54.2	4.3	55.9	6.1	58.9	9.0	59.4	9.4
Ointment with lidocaine	6.8	1.2	8.6	1.5	11.2	1.7	13.7	2.0	16.2	2.4
Erweka**: a) rotating basket										
Suppositories:										
Germicid-2	16.3	2.4	23.7	3.0	31.6	4.2	58.1	4.2	68.9	4.5
Pipolfen-2	6.6	1.0	9.6	1.6	11.7	1.6	15.5	1.8	20.6	2.0
Diafillin	7.8	1.1	12.3	1.9	16.2	2.7	26.2	5.7	45.6	12
b) directly from a suppository										
Suppositories:										
Germicid-2	53.9	12.6	70.4	14.4	77.9	11.5	90.0	8.0	90.6	6.2
Pipolfen-2	75.3	10.9	90.9	8.2	95.4	5.4	98.8	2.7	100.7	1.2
Diafillin	41.9	6.4	62.2	5.5	75.3	3.5	91.6	1.6	98.0	1.0

* t = 1, 2, 3, 4, 5 hours

** t = 5, 10, 15, 30 60 minutes

As can be seen from the Table 1.2, the "Sartorius" has quite reproducible results on release, which are usually much better than using the "rotating baskets" and especially directly from a suppository ("Erweka"). This is because of much more standardized experiment conditions for "Sartorius".

To describe *in vitro* release curves *G(t)* different models are used [93-97], among which the most interesting is the one-compartment exponential model [99]:

$$G(t) = G_o \cdot [1 - \exp(-kt)], \tag{1.3}$$

as she describes the release throughout the whole time interval. The exponential model is usually used in the linear form:

$$\lg[1 - (G / G_o)] = -0.434 \cdot kt. \tag{1.4}$$

It is natural to assume the maximum release degree of G_o = 100% [93-97]. This assumption, however, in the case of suspension suppositories and ointments is not quite correct, since the release process is complicated by the dissolution of the suspended substance into the ointment or suppository base and its diffusion to the membrane surface. The diffusion process can be a determining factor [95]. When we use the semi-permeable membrane, the inverse diffusion of the dissolution medium into the ointment chamber in some cases (for example, for analgin powder and hydrophilic bases) is substantial. Therefore, although the release degree (*G)* tends to 100% with an infinite time of the release, but in the real experiment (time is within 8-10 hours) it does not exceed a certain unknown G_o <. 100%. In this case, the equation (1.3) cannot be transformed into the linear form about the parameters *k* and G_o like the equation (1.4). Bearing in mind also the different precision of *G(t)* values for various *t*, we must use the non-linear weighted least squares method (NWLSM) for finding these parameter [98]. As an optimization criterion, the residual standard deviation is used:

$$S_r = \{[n/(n-2)] \cdot \sum_{t=1}^{t=n} [G_{ex}(t) - G_{cal}(t)]^2 \cdot W(t)\}^{1/2} \tag{1.5}$$

Here the weights *W(t)* calculate with the ratio:

$$W(t) = [1/S(t)]^2 \cdot \sum_{t=1}^{t=n} [S(t))]^2. \tag{1.6}$$

Here:

$G_{ex}(t)$ and $G_{cal}(t)$ are the experimental and calculated *G* values for time *t,*

S(t) are the standard deviations of the experimental *G* values from their mean values for time *t* of the Table 1.2.

Results of use of the NWLSM for the description of the experimental data and obtaining the parameters G_o and *k* are presented in the Table 1.3.

It should be noted that the finding of the parameters of equation (1.3) by the NWLSM has no visibility. At the same time, we can get an approximate form of the equation (1.3), which is easily transformed to the linear form. To do this, present it as a form:

$$G(t) = G_o \cdot [\exp(kt) - 1] / \exp(kt) \,. \tag{1.7}$$

Expanding the exponent in the Maclaurin series to the second member and substituting it into the equation (1.7), we obtain the well-known Langmuir isotherm:

$$G(t) = G_o \cdot kt / [1 + kt] \,. \tag{1.8}$$

This equation is easily transformed to the linear form:

$$\frac{1}{G(t)} = \frac{1}{G_0} + \left(\frac{1}{G_0 \cdot k} \right) \cdot \frac{1}{t} \tag{1.9}$$

It is simpler and more practical than the exponential form (1.6), as the factors G_o and k can be assessed graphically or using the linear weighted least squares method (WLSM), obtaining the weights $w(t)$ on the basis of $S(t)$ values of the Table 1.3 by conventional formulas of propagation of uncertainties [98]. It gives the same results as the direct application of the NWLSM to the equation (1.8). Results of such calculations are presented in the Table 1.3.

As can be seen from the Table 1.3, for both models (the exponential (1.3) and Langmuir (1.8)) the residual standard deviations S_r do not exceed, by the Fisher criterion, the standard deviations of reproducibility $S(t)$. It indicates an adequate description of the experiment by these models [98]. This is also supported by the high values of correlation coefficients R. This description well holds for various objects, bases, for different devices ("Sartorius" and "Erweka") and the way of release (ointment chamber, "rotating basket", directly from suppositories, various dissolution media, different ratios of dissolution medium and sample dialyzate volumes, etc.), reflecting the general mechanism of the release and the nature of the curve. Comparison of S_r and R shows that the Langmuir model (1.8), at least not worse describes the experiment than exponential model (1.3), and can therefore, as more simple, be used in practice in the linear form (1.9). With this, the G and k values for both models can vary significantly. This is due to the fact that in the case of the Langmuir model (1.8) these values are simply the parameters of the equation.

Table 1.3

The results of description of the release process using the exponential (1.3) and Langmuir (1.8) models

	Exponential model (1.3)						Langmuir model (1.9)					
Objects	G_o	S_{Go}	k^* 100	S_k^* 100	S_r^* 100	R	G_o	S_{Go}	k^* 100	S_k^* 100	S_r^* 100	R
Sartorius												
Suppositories												
Gibernal	71.5	1.6	24.8	0.9	0.3	1.000	112.4	1.6	16.6	0.3	0.2	1.000
Germicid-1	48.0	2.0	46.0	4.6	1.3	0.993	65.5	2.5	39.2	2.5	0.8	0.997
Pipolfen-1	49.0	3.0	37.4	4.0	1.0	0.994	71.3	3.9	28.4	2.0	0.6	0.998
Analgin-1	0.95	0.2	19.6	6.6	0.02	0.985	1.56	0.4	12.4	3.3	0.02	0.987
Analgin-2	5.36	0.7	53.1	14.5	0.4	0.925	7.18	0.9	48.8	10.3	0.3	0.954
Analgin powder	57.1	1.0	166	18.6	1.5	0.925	63.1	0.8	295	23.4	0.8	0.980
Ointment with lidocaine	17.0	3.0	41.2	14.0	1.2	0.935	23.1	4.0	36.9	9.4	1.1	0.946
Erweka: a) rotating basket												
Germicid-2	76.7	7.0	245	42	3.3	0.987	108.5	14.9	188	47	3.6	0.985
Pipolfen-2	19.5	1.7	420	79	1.3	0.967	24.6	1.8	400	65	0.8	0.988
Diafillin-1	43.3	11.5	209	71	1.4	0.963	63.3	16.8	152	54	1.2	0.973

b) directly from a suppository												
Germicid-2	90.1	1.5	966	88	2.7	0.980	98.0	2.1	1557	222	2.5	0.983
Pipolfen-2	100.3	0.5	1492	173	1.2	0.929	103.3	0.5	4000	526	2.5	0.983
Diafillin-1	97.9	0.6	581	26	1.1	0.996	109.5	2.2	899	124	1.8	0.986

As we can see from the k values of the rate constants (Table 1.3), the release speed significantly increases when switching from the ointment chamber with the semi-permeable membrane ("Sartorius") to the "rotating basket" and especially to the release directly from suppositories, that is obvious. It should be noted that the release degree $G(t)$ is not usually determined by the rate constant k and limit value G_o. This is seen, for example, from a comparison of the values of $G(t)$ for the suppositories of "Gibernal" and Analgin-2 (Table 1.2). The $G(t)$ values for the "Gibernal" suppositories are much higher than for the Analgin-2 suppositories, although the k values for the latter are significantly higher. This is due to the considerably larger G_o for the "Gibernal" suppositories.

The main result of the description of the experimental data with the help of both models is the fact that the limit of G_o values, reached theoretically in infinite release time, in many cases, are considerably less than 100%. This illustrates the decisive role of diffusion of an active ingredient of a drug to the membrane surface under real experimental conditions (time of not more than 8-10 hours). This is particularly evident for the hydrophobic bases (Analgin-1suppositories), where the G_o = 1-1.5%. The value of G_o = 57-63% < 100%, even in the case of well soluble in water analgin powder, where the inverse diffusion of the dissolution medium into the ointment chamber is significant (during the experiment water entered the chamber). This process can be significant for hydrophilic ointment bases (ointment with lidocaine). The G_o value is, apparently, connected also with solubility of an active ingredient in a dissolution medium and with a dispersion degree of active substances of suspension suppositories.

Naturally assume that the G_o value is the closer to 100%, the smaller the thickness of the drug layer is adjacent to the membrane, since this reduces the influence of all above mentioned factors on the release process. This matter would be considered in the chapter 2 of this Addendum.

1.2. Dependence of release degree on drug layer thickness

In the section 1.1, it was shown that the *in vitro* release of suppositories and ointments, regardless of the conditions of the experiment, the equipment and the type of a drug, is well described by two-parametric exponential (1.3) and Langmuir (1.8) models. The equation (1.8) is preferred in practice because it is easy transformed to the linear form. The extrapolation limit value of the release degree G_0 under actual experiment conditions (5 hours) is close to 100% only for release directly from a suppository. When using semi-permeable membranes, the release degree depends on the type of a drug and is usually significantly lower (in our experiments the G_o values varied from 1% for analgin suppositories with hard fat base to 77% for the "Germicid" suppositories with Witepsol base).

Naturally assume that the G_o value is strongly dependent on the thickness of the layer adjacent to the membrane, increasing with decreasing this layer. So for the standardi-

zation of *in vitro* suppositories and ointments release, it is necessary to study the influence of this factor.

1.2.1. Theory

Despite a good description of the release process in the *in vitro* experiment (in our case during 5 hours), it is obvious that the expressions of (1.3) and (1.8) don't describe the release in the whole possible time interval, since the degree of release for an infinite time t (G_o) must be equal to 100%, but do not Go < 100% . This means that, after reaching under experimental conditions (5 hours) of a some limit value according to the equations (1.3) or (1.8), then the *release* degree (G) tends slow (much slower than according to the equations (1.3) or (1.8)) to 100% with the increase of time t.

The release process can be divided into 2 parallel running processes:

1) the dissolution of the suspended substance (if it's a suspension drug) into the base and its diffusion to the surface of the membrane;
2) the release proper, i.e. the transition of the substance through the membrane into the dissolution medium.

A good description of the experiment by equations (1.3) and (1.8) means that the first stage is limiting. In the general case, the two-stage release process is described by two-compartment exponential model [99], which in our case can be represented as:

$$G = G_o \cdot [1 - \exp(-k_2 t)] + (100 - G_o) \cdot [1 - \exp(-k_3 t)] \qquad (1.10)$$

The Langmuir version of this exponential model is the equation

$$G = [G_o \cdot k_2 t / (1 + k_2 t)] + [(100 - G_o) \cdot k_3 t / (1 + k_3 t)]\,. \qquad (1.11)$$

Since the first stage (dissolution and diffusion to the surface of the membrane) is limiting, then

$$k_2 >> k_3\,. \qquad (1.12)$$

In this case the equations (1.10-1.11) transform into the ratios (1.3-1.4), correctness of which is confirmed by large experiment in the section 1.1.

Thus, the release process consists of two stages: the fast stage characterized by the first members and the slow stage, characterized by the second members of the equations (1.10-1.11). The ratio between these two stages is defined by the G_o value: the smaller the value, the greater the impact of the first stage. Naturally to assume that the G_0 value grows with a reduction in a layer thickness in the ointment chamber and is equal to 100% at a zero layer thickness, when there is no diffusion process. At an infinitely large layer thickness, the G_o value is equal to 0. Therefore, for the descrip-

tion of the dependence of G_0 on layer thickness L we can propose the exponential model as well:

$$G_o = 100 \cdot \exp(-k_1 L) \tag{1.13}$$

and the corresponding Langmuir form:

$$G_o = 100/(1 + k_1 L)\,. \tag{1.14}$$

The equations (1.10, 1.13) and (1.11, 1.14) are the common expressions for the dependence of release degree on time and layer thickness. Given (1.12) (i.e. diffusion is a limiting stage), the second member of the equations (1.10-1.11) can be neglected. In this case we obtain the simple forms describing the release process in actual experiment (5 hours, sufficient layer thickness):

Simplified exponential model:

$$G = 100 \cdot \{\exp(-k_1 L)\} \cdot \{1 - \exp(-k_2 t)\}\,. \tag{1.15}$$

Simplified Langmuir model:

$$G = 100 \cdot k_2 t / \{(1 + k_1 L) \cdot (1 + k_2 t)\}\,. \tag{1.16}$$

1.2.2. Experiment

As shown above in the section 1.1, the nature of the release curves does not depend on the type of appliance, drug product and equipment. Therefore, release process studies have been conducted for pipolfen suppositories using "Sartorius" (ointment chamber of 45 mm diameter and of 5 mm height) using dialyzing membrane "Hoechst" with the thickness of 5.5 microns (Germany, Nadir-Dialysiersch-lauch). The conditions of the experiment are described above.

As an object of study there were selected "Pipolfen-1" suppositories (base: cocoa butter) with weight of 1.45 g, containing promethazine-base of 0.025. To study the influence of the layer thickness on the release process, placed 0.5, 1, 1.5 and 2 suppositories into the ointment chamber and carried out the experiment as described previously in the section 1.1,1, making no fewer than 5 replicate determinations. Under the experiment, the preparation melted, forming a layer with an average thickness of about 0.5, 1.0, 1.5, and 2.0 mm respectively (calculated on the basis of the density, suppository mass and geometry of the ointment chamber).

Calculations were carried out using the nonlinear weighted least squares method (NWLSM) (see section 1.1) on general equations (1.10, 1.13), (1.11, 1.14), as well as approximate ones (1.15) and (1.16). Compliance of these equations are largely con-

nected with the compliance of the ratios of (1.3, 1.8), so conducted the calculations with the NWLSM on equations (1.3, 1.8) for an each fixed layer thickness as well.

1.2.3. Results and discussion

The results, obtained for *in vitro* release of "Pipolfen" suppositories with a different layer thickness in ointment chamber, are presented in the Table 1.4. The release, as expected, heavily depends on the layer thickness, decreasing with growth of the latter.

Table 1.4

The dependence of release degree *G%* of "Pipolfen" suppositories on release time and layer thickness (*S* is an absolute standard deviation of reproducibility)

Layer thickness *L*, mm	Replicates number	Mean release degree *G* % in time *t* (hours)									
		1	*S*	2	*S*	3	*S*	4	*S*	5	*S*
0.5	9	45.2	4.3	59.0	5.2	66.5	6.3	70.6	6.9	73.1	7.1
1.0	5	32.8	1.7	46.3	0.7	56.3	2.0	62.2	2.7	67.5	4.1
1.5	5	21.7	3.0	31.8	1.6	39.2	1.0	45.3	0.9	50.8	0.8
2.0	5	16.4	0.6	25.5	0.5	32.3	0.7	37.8	0.9	42.7	1.0

The Table 1.5 shows the results of calculations using the NWLSM on the equations (1.3) and (1.8). As can be seen, in all cases there is good agreement with experiment that confirms the findings of the section 1.1. Herewith the Langmuir model (1.8) holds some better than of the exponential one (1.3).

Table 1.5

The results of description of the dependence of the release degree on time with the exponential (1.3) and Langmuir (1.8) models for the fixed layer thickness *L*

L, mm	Exponential model						Langmuir model					
	G_o %	S_{Go}	k	S_k	S_r	R	G_o %	S_{Go}	k	S_k	S_r	R
0.5	71.7	1.7	0.95	0.07	1.9	0.988	86.7	0.4	1.08	0.02	0.3	1.000
1.0	68.2	4.0	0.58	0.07	1.5	0.983	91.2	4.4	0.52	0.05	0.9	0.995
1.5	60.8	3.1	0.35	0.04	1.0	0.991	85.6	4.7	0.28	0.03	0.8	0.996
2.0	49.0	3.0	0.37	0.04	1.0	0.994	71.6	3.8	0.28	0.03	0.6	0.998

Processing of the Table 1.4 data using the NWLSM on the simplified equations (1.15) and (1.16), connecting the release degree with the layer thickness (L) and time, gave the following results (S is the corresponding standard deviation):

Simplified exponential model:

$$k_1 = 0.45,\ S_{k1}=0.02;\ k_2 = 0.57,\ S_{k2} = 0.05;\ S_r = 2.6\%;\ R = 0.977. \quad (1.17)$$

Simplified Langmuir model:

$$k_1 = 0.47,\ S_{k1} = 0.10;\ k_2 = 0.70,\ S_{k2} = 0.18;\ S_r = 4.9\%;\ R = 0.916. \quad (1.18)$$

As can be seen, both models are characterized by quite low residual standard deviations S_r and high correlation coefficients (R). The latters are much higher than the critical value 0.444 (for the probability of 0.95 and degrees of freedom 4 * 5-2 = 18) below which a correlation is insignificant. Note that the exponential model (1.15) holds better of the Langmuir one (1.16), while for the fixed thickness (equation (1.3, 1.8), the situation is usually reversed. Using the NWLSM, we also received the general expressions (1.10, 1.13), (1.11, 1.14) for the dependence of the release degree for the "Pipolfen" suppositories on the layer thickness and the time. These two-compartment models are characterized by the following metrological characteristics:

Two-compartment exponential model:

$$k_1=0.89,\ S_{k1}=0.09;\ k_2=1.11,\ S_{k2}=0.21;$$
$$k_3=0.075,\ S_{k3}=0.010;\ S_r=1.5\%,\ R=0.992. \quad (1.19)$$

Two-compartment Langmuir model:

$$k_1=0.95,\ S_{k1}=1.24;\ k_2=1.09,\ S_{k2}=1.31;$$
$$k_3=0.049,\ S_{k3}=0.077;\ S_r=4.7\%,\ R=0.922. \quad (1.20)$$

As can be seen, both models have high correlation coefficients (R) and low residual standard deviations S_r. Comparison with the results of (1.17-1.18) shows that the exponential two-compartment model (1.10, 1.13) statistically significantly better describes the experiment than its simplified form (1.15) (the experimental value of the Fisher criterion for S_r is 3.00, while the critical value for 0.95 probability and degrees of freedom 18 and 17 is 2.23). At the same time, the Langmuir two-compartment model (1.11, 1.14) has no advantages over its simplified form (1.16) and is characterized, moreover, by unacceptably large standard deviations of parameters of k_i. As the simplified Langmuir model (1.16) also describes worse the experiment than the simplified exponential model (1.15), then it can be concluded that the Langmuir model is applicable only to a fixed layer thickness where it more simple form (1.8), trans-

formed to the linear form (1.9) (see section 1.1), has an advantage over the more grounded exponential model (1.3).

It should be noted that although the two-compartment exponential model (1.10, 1.13) better (in the whole possible time interval) describes the experiment than its simplified form (1.15), but for the real time release (about 5 hours) the simplified form (1.15) is acceptable and can be used to describe the dependence of release degree on the time and layer thickness (or the drug amount) for suppositories and ointments.

Thus, the dependence of the release degree on the time and layer thickness is well described by equations (1.10, 1.13) for suppositories and ointments, and in a limited time interval – by the equation (1.15).

An important result of the Table 1.4 is that the standard deviations *S(t)* of release degree for every hour, in general, significantly increasing with decreasing in the layer thickness, that is, above all, connected with the simultaneous growth of irregularity (and, as a result, repeatability) of the layer thickness. When we reduce the layer thickness, it is also becoming increasingly apparent an influence of nonuniformity of the active substance content in the sample weight of the test ointment or suppository (especially for suspension drug products). This effect is the most noticeable for the lower layer thickness (0.5 mm), where the irregularity is visible to the naked eye and is apparently caused by incomplete wetting of the membrane with the test preparation. Thus, the layer thickness of the test preparation needs to be standardized and ought to be, apparently, not lower than 2 mm. A further increase in the layer thickness of the test preparation also is not desirable, since the release degree is significantly reduced. So, the calculations carried out using the equation (1.15) show that increasing the layer thickness to 1 mm reduces the release degree of the "Pipolfen" suppository roughly 1.5 times. Of course, for each drug product and release conditions these values are individual, but, to approximate the release *in vivo* by the *in vitro* study, the layer thickness of the drug product should not exceed 5 mm, because the radius of suppositories does not typically exceed this value.

As can be seen, the degree and reproducibility of release for suppositories and ointments are heavily dependent on the layer thickness of the preparation adjacent to the membrane. To obtain reproducible and comparable results, the layer thickness needs to be standardized and must be between 2-5 mm.

1.3. Standardized release procedure for ointments and suppositories

In previous sections we have shown that one of the most important factors, influencing the degree and reproducibility of the release, is the layer thickness of suppositories or ointments, adjacent to the membrane. It must be within 2-5 mm. The layer thickness lower 2 mm increases the uncertainty of release results and above 5 mm significantly reduces the release degree.

An *in vitro* release study is one of the most important stages of a pharmaceutical development of a drug product and in many cases allows us to optimize its composition.

However, the above mentioned results show that without standardization of a layer thickness we cannot properly compare different compositions on a degree and nature of release profiles.

Given that the radius of suppositories is typically 5 mm, optimal thickness of the layer, which has a certain correlation with *in vivo*, can be considered to be of 3 mm. This value we accepted in further study.

In previous sections we have shown that the results of the release on the "Sartorius" absorption simulator (Germany) are fairly well reproduced with a layer thickness of 2 mm and above. So this device may be recommended for release researches. However, it is clear that dissolution (release) of the suppositories and ointments with semi-permeable membrane is almost similar in form to release of transdermal patches described in the SPU-Eur.Ph. [1] (*2.9.4. Dissolution test for transdermal patches*). This general chapter uses for dissolution test the device of "Erweka" (Germany) type [1]. Therefore, with the view of standardization of *in vitro* release of ointments and suppositories, it would be desirable to offer for them the procedure which would minimally differ from the SPU-Eur.Ph. Developing such a methodology is facilitated by the fact that the mathematical form of a release curve does not depend on a type of a drug product and release conditions (see section 1.1), i.e. all previously received us in sections 1.1-1.2 conclusions remain valid.

To this end, we have developed a simple chamber for the release study of suppositories and ointments, which is similar to the extraction cell described in the Eur.Ph. general chapter 2.9.4. Layer thickness is 3 mm (see above). Further studies were conducted on this chamber.

1.3.1. Experiment

Object of study. “Pipolfen-1” suppositories (see the Table 1.1).

Equipment and materials. The “Erweka” dissolution tester "(Germany), fitted with our camera, which is placed at the bottom of the vessel with the membrane down. Dissolution medium is 0.1 M aqueous hydrochloric acid, warmed up to (37 $\pm$ 0.5)°C, volume is 900 ml. Conduct the release at different rotation speeds of a glass stirrer (50, 100, 200 and 300 rpm), which submerge at 2 cm below the surface of the dialyzate. There is a risk of chamber overturn at a high speed of stirrer for the lower immersion.

As a semi-permeable membrane it is used the dialyzing membrane of "Hoechst" with a thickness of 5.5 mm (Germany, Nadir-Dialysierschlauch), control number 896 899/1-5 . The membrane is previously soaked in 0.1 M solution of hydrochloric acid for 1 hour.

Release procedure. Place about 2.9 g (two suppositories) of the molten suppository mass into the weighed chamber, cool down to 33^0C and remove carefully the mass excess with a glass slide. Weigh the chamber again; determine the sample weight as the chamber weight difference (this sample weight is used further in release degrees

calculations). Then cover the chamber with the membrane, close and place with a glass stick on the bottom of the dissolution vessel with the membrane down.

This procedure is characterized by good enough reproducibility of the preparation sample weight (for 25 experiments the sample weight was equal to 2.300 g with the relative standard deviation of S = 3.6%), which provides good reproducibility of the preparation layer thickness. As shown earlier in the section 1.2, it is important to obtain the reproducible results of release. Putting the chamber with the membrane down creates a permanent contact of the preparation with the entire membrane surface.

Every hour, sample 10 ml of the dialyzate for analysis, and add to the rest of the dializate the same volume of the 0.1 M hydrochloric acid warmed up to (37 $\pm$ 0.5)°C. Measure the absorbance of the sampled dialyzate at the wavelength of 249 nm and calculate the degree release in per cent as previously described in the section 1.1. Carry out at least 5 replicates for each release time and the stirrer rotation speed.

To determine the reproducibility of the release in different laboratories, experiment was conducted in parallel in the State Center for Drug Science (SCDS) (Kharkov, Ukraine) and "Egis" (Budapest, Hungary).

To compare the reproducibility of the entire release curve in different laboratories we also conducted the calculations on the two-parametric exponential (1.3) and Langmuir (1.8) models, using Nonlinear weighted least squares method (NWLSM), as described above in the section 1.4.1.

1.3.2. Results and discussion

As can be seen from the Table 1.6, release results, obtained in the different laboratories, are not statistically different from each other and are characterized by fairly low standard deviations *S(i)*, which confirms the good reproducibility of the procedure. With this, the release results are not dependent on the stirrer rotary speed that allows us to recommend the rotary speed of about 100 rpm.

Table 1.6

The mean release degrees (*G%*) for the "Pipolfen" suppositories at different stirrer rotary speeds (number of replicates is 5)

Laboratory	φ^*	Release degree G% with the standard deviation *S%* abs. and release time *t* (hours)									
	rpm	t = 1		t = 2		t = 3		t = 4		t = 5	
		G	S_G	G	S_G	G	S_G	G	S_G	G	S_G
SCDS	50	13.8	0.4	19.9	0.3	24.4	0.5	28.3	0.2	31.8	0.4
Egis	50	12.8	3.8	18.2	5.3	22.4	7.1	27.1	7.1	33.0	6.5
SCDS	100	13.5	0.6	19.3	1.0	23.9	1.4	27.9	1.8	31.6	2.2

Egis	90	14.2	0.6	20.0	1.0	22.8	1.2	26.8	0.9	30.4	1.5
SCDS	200	13.9	1.5	19.3	1.4	23.9	1.5	27.5	1.7	30.7	2.1
SCDS	300	14.0	1.0	20.2	1.3	25.0	1.2	29.5	1.5	33.8	1.8

*rotary speed of the stirrer

Table 1.7

Results of description of the dependence of release degree on time with the exponential (1.3) and Langmuir (1.8) models (S_r is a residual standard deviation, R is a correlation coefficient)

Laboratory	φ* rpm	Exponential model						Langmuir model					
		G_o %	S_{Go}	k	S_k	S_r	R	G_o %	S_{Go}	k	S_k	S_r	R
SCDS	50	34.4	2.0	0.44	0.06	0.9	0.988	48.0	3.0	0.36	0.05	0.7	0.994
Egis	50	36.7	5.2	0.37	0.10	2.0	0.969	52.8	8.6	0.29	0.09	1.7	0.977
SCDS	100	31.4	2.6	0.53	0.08	1.2	0.979	43.6	3.6	0.43	0.07	0.9	0.990
Egis	90	29.2	1.9	0.63	0.10	1.4	0.977	38.9	2.6	0.55	0.09	1.0	0.988
SCDS	200	32.3	2.4	0.48	0.08	1.3	0.979	44.1	3.5	0.41	0.07	0.9	0.989
SCDS	300	35.9	3.3	0.44	0.08	1.5	0.980	50.6	5.0	0.35	0.07	1.1	0.989

*rotary speed of the stirrer

As can be seen from the Table 1.7, results of release are well described with the exponential and Langmuir models, which confirm the applicability of the earlier findings to the developed procedure. Coefficients of equations within the statistical uncertainties, are fairly well reproduced in different laboratories (and at different stirrer rotary speeds). This confirms the reproducibility in total of the release curves obtained by the proposed procedure in different laboratories, and allow to recommend this procedure for comparative studies of the *in vitro* release of ointments and suppositories at the stage of their pharmaceutical development.

2. DEPENDENCE OF SUBSTANCE SOLUBILITY ON ITS PARTICLE SIZE [109]

A study of a dependence of solubility of a substance on its particle size and dissolution time, as well as a study of a fractional composition of the substance, are a necessary part of a pharmaceutical development of any dosage form, but particularly important for solid dosage forms, including tablets. This raises the following questions:

1. What equations describe the dependence of solubility on dissolution time? This issue is important for both solid (tablets) and soft (suspension ointments and suppositories) dosage forms and liquids (eye drops, syrups, injections). In the first case it is directly connected with the "Dissolution" test prognosis [33], and the last - with validation of the technological process of dissolving substances during the drug manufacture.
2. What is the effect of the particle size of the poorly soluble substances on their rate of dissolving? This issue is closely linked to the first issue and is important for the technological requirements to quality of ingredients to assure manufacture of the drug product with sustained quality.
3. Is there a threshold of the particle size, below which this size does not affect the dissolution rate? This issue is important to standardize the quality of substances.
4. If there is a marked dependence of the dissolution rate on particle size, then how to determine the real (equilibrium) solubility?

As for suspension ointments and suppositories [100-101], the degree of dissolution increases with the decrease in the mean particle size only up to a certain point. Starting from the threshold of 65-90 μm (0.090-0.065 mm), a further reduction in the average size of particles does not increase the degree of dissolution. This conclusion was obtained for both easily soluble in water ethambutol hydrochloride, and very little soluble in water of rifampicin [101], i.e., it has a quite general nature. In the case of ointments and suppositories, this threshold is, apparently, so that from a certain particle size, dissolution of active substances from a particle into the ointment base is not longer the limiting stage of the process. This limiting stage becomes the diffusion of the active substances from the base into the water phase (medium).

The question, to what extent the findings of a threshold value of particle size and the magnitude of the threshold (0.065-0.090 mm) obtained for suppositories [100-101], may be applied to tablets?

Study of the influence of time and particle size on kinetics of dissolution is especially important for poorly water soluble substances, because these parameters can have a crucial effect on the processes of dissolving during the manufacture of liquid dosage forms (where quite often we have to prepare solutions of substances at the limit of their solubility [102]) and on the compliance with the pharmacopoeia requirements to the dissolution of solid dosage forms [33].

Currently, standardization of substances on the solubility becomes particularly relevant for domestic drug manufacturers due to the widespread use of dissolution profiles in *in vitro* bioequivalence studies of generics [103]. Comparison of dissolution profiles of a generic drug and the reference drug product without standardization of the generic substances on the solubility is incorrect, since the different series of solid dosage forms (such as tablets) resulting from substances with different solubility (meaning the solubility in the "pharmacopoeial range", i.e. up to 60 min [33]) can vary significantly by dissolution profiles and bioavailability.

The above issues were encountered in the development of dosage forms based on a new original oral antidiabetic fensuccinal substance, which is β-phenilethylamide of 2-oxysuccinanile acid [104].

Fensuccinal substance is soluble in dimethylformamide, little soluble in 96% alcohol, very little soluble in chloroform, and practically insoluble in water.

Since the main dosage form of fensuccinal is 0.25 g tablets for oral administration of a traditional release, and then its poor solubility in water causes difficulties in developing the "Dissolution" test. In this case, the size of the crystals may significantly affect the solubility of a substance [100-101] and therefore affect the reproducibility of the "Dissolution" test for tablets in accordance with the requirements of the SPU [33]. In particular, the dissolution degree of fensuccinal tablets can greatly vary for different batches of the substance. Accordingly, the bioavailability and pharmacological action of the drug can vary as well. Similar problems often arise for other poor water soluble substances.

Thus, the fensuccinal substance is a convenient target to examine the above matters. Therefore, the study of the kinetics of dissolution of fensuccinal substance of varying fractional compositions in dissolution pharmacopoeial media [33] is of interest.

Given the considerable influence of the particle size of the substances on the dissolution of solid dosage forms [100-101], the study of actual fractional composition of different series of fensuccinal substance is also of interest for purpose of its standardization.

2.1. Experiment

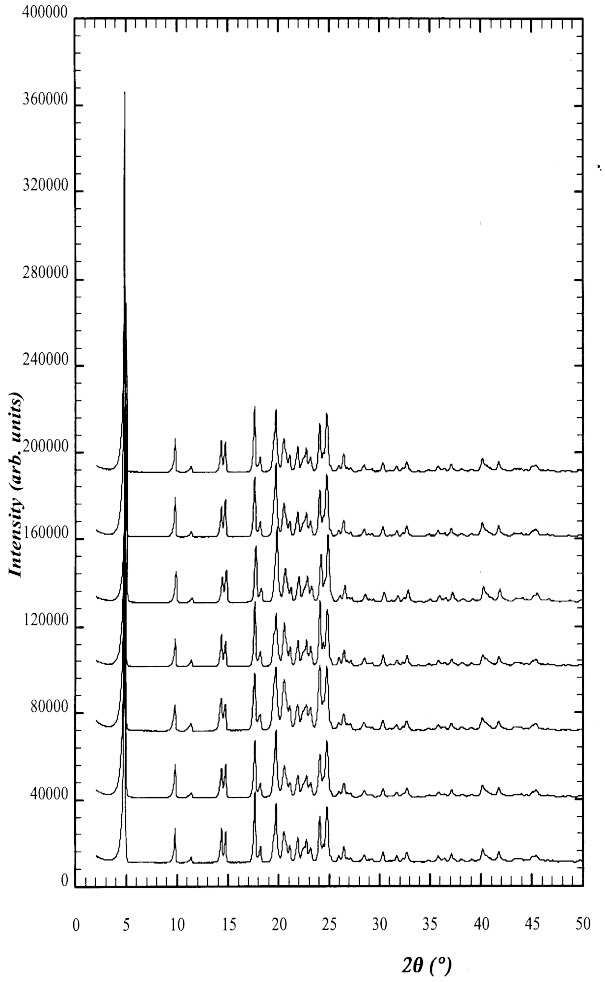
2.1.1. Substance purity

Fensuccinal substance batches used conform to the requirements of the specification. The actual content of the related impurities sum (by HPLC): not more than 0.6%, the sulfated ash: not more than 0.04%, loss on drying is less than 0.1%, heavy metals is less than 0.001%. Thus, impurities do not have a significant impact on the dissolution profile of the substance samples.

2.1.2. X-ray phase analysis of different samples of fensuccinal substance

The study of solubility is correct only for samples which have the same crystalline modification, since different crystalline modifications can have different solubility.

Crystallographic evidence for the sample homogeneity we conducted by their X-ray phase analysis using the powder diffractometer Siemens D500 in monochromatic Cu radiation (monochromator of pyrolytic graphite in the secondary beam). Analysis of diffraction patterns were carried out PDF-4 files [105] as well as by the Rietveld method [106] under the "FullProf" program [107]. A typical diffraction pattern is presented in Figure 2.1.



Fifure 2.1. Typical diffraction patterns of various fractions of fensuccinal substances

2.1.3. Production of fractions. The industrial series 010504 were sifted through the corresponding sieves, getting fractions with the sizes: 0.05-0.10 mm, 0.1-0.16 mm, 0.16-0.315, 0.315 mm-0.5 mm.

2.1.4. Dissolution media

Water, 0.1 M hydrochloric acid and phosphate buffer pH 6.8, conforming to the SPU [33].

2.1.5. Solubility study

Study of solubility of different fractions of fensuccinal were conducted with use a Paddle Apparatus for “Dissolution” test [33]. The temperature of the experiment 37^0C; the initial volume of dissolution medium 1000 ml; sample weight of fensucci-

nal substance was 300 mg; samples volume of dissolution medium was 25 ml. Samples were selected through time t_i = 5, 15, 25, 35, 45, 60, 120 and 180 minutes.

In the process of stirring, unsolved crystals are distributed throughout the volume of the liquid and some of them reach the sampled volume. So the sample was filtrated through the paper filter of "Blue Ribbon". In this procedure it is necessary to keep up the air temperature of 37^0C (dry-air thermostat was used). To prevent a possible precipitation of the substance in the transition to a room temperature (the solution was saturated at temperature of 37^0C), we used adding of alcohol, where the solubility of fensuccinal substance is better than in water.

At first, 10 ml of 96% alcohol was placed in a measuring flask with a capacity of 25 ml and 10 ml of the filtrated sample was added. Then the flask was diluted to the mark with 96% alcohol. The concentration of the resulting solution was determined spectrophotometrically by measuring the absorbance at wavelength of 244 nm (absorption maximum of fensuccinal) and using the specific absorbance. Three replicates were carried out for each fensuccinal fraction and each dissolution medium. The mean value of solubility and the standard deviation (*SD*) were calculated. For each fraction and dissolution medium the pooled SD_{pool} were calculated, as well as the total pooled $SD_{pool,\ tot}$ for the whole fraction [26]. The research results are presented in the Table 2.1 and Figure. 2.2.

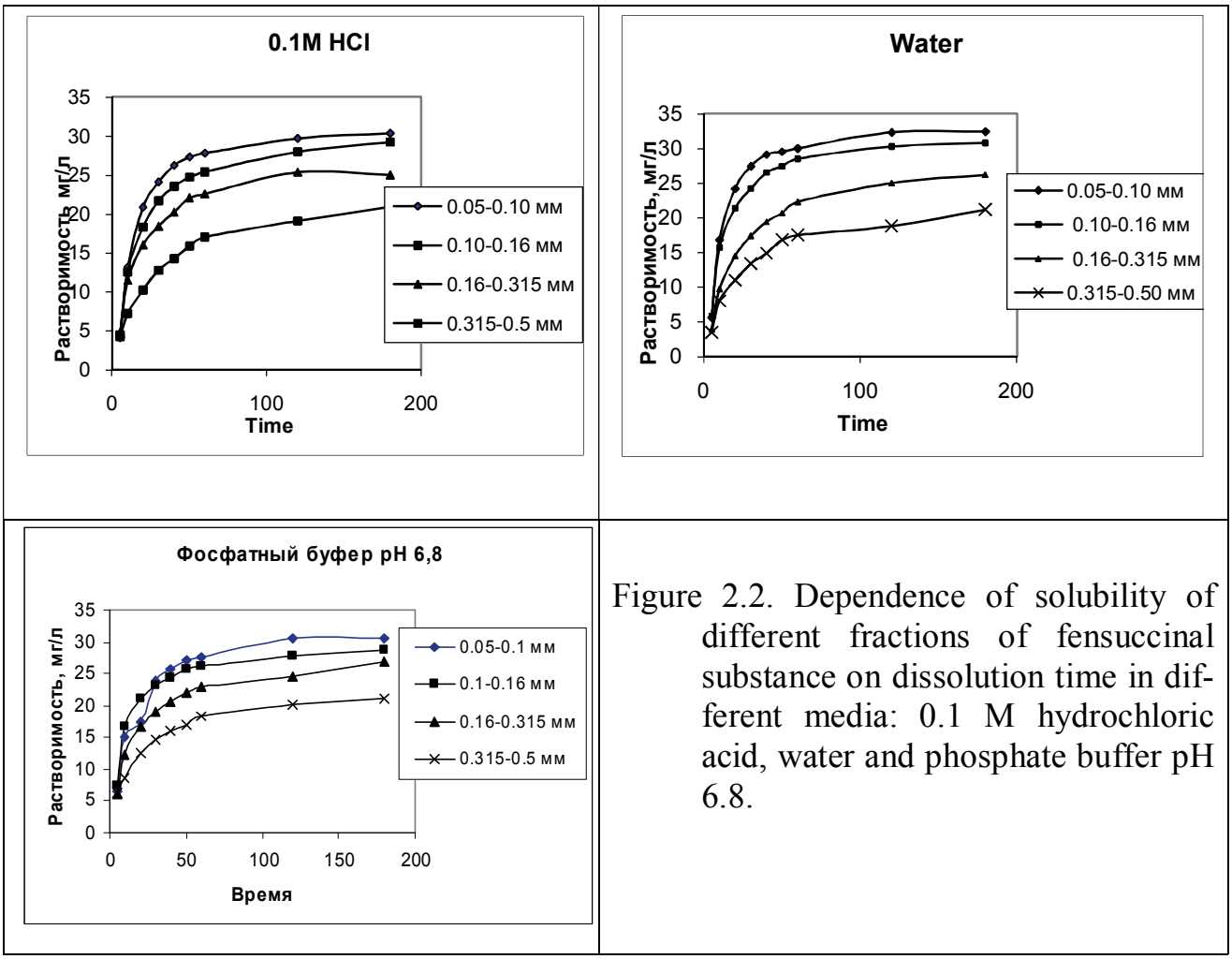


Figure 2.2. Dependence of solubility of different fractions of fensuccinal substance on dissolution time in different media: 0.1 M hydrochloric acid, water and phosphate buffer pH 6.8.

2.1.6. Actual distribution of various fensuccinal substance batches (samples) on particle size

Given the importance of the distribution of the substance on particle size, we studied it for the industrial series 010504 020504 030504 of fensuccinal substance, using the laser diffraction analyzer Fritsch Particle Sizer ‘analysette 22' (company Fritsch).

Fritsch Particle Sizer 'analysette 22'

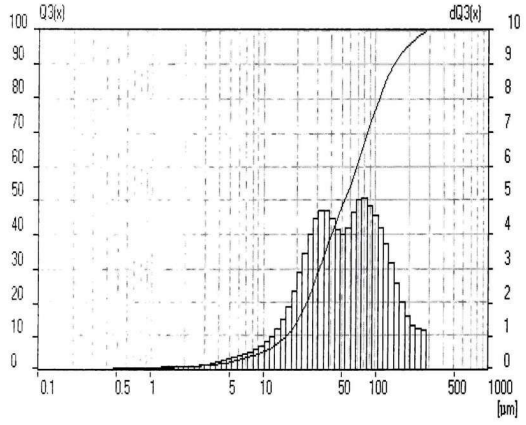
Meas.No. 39 | Date 05-31-2007 | Time 15:29 | Operator Akichev Andrey | ID 7645 | Serial No. 123456

Фенилсукцинал с 010504 проба 1

Measuring Range	0.31 [µm] - 300.74 [µm]	Pump	100 [%]
Resolution	62 Channels (17 mm / 114 mm)	Stirrer	3
Absorption	7.00 [%]	Ultrasonic	On
Measurement Duration	5 [Scans]		

Modell Independant

Fraunhofer Calculation selected.



Fritsch Particle Sizer 'analysette 22'

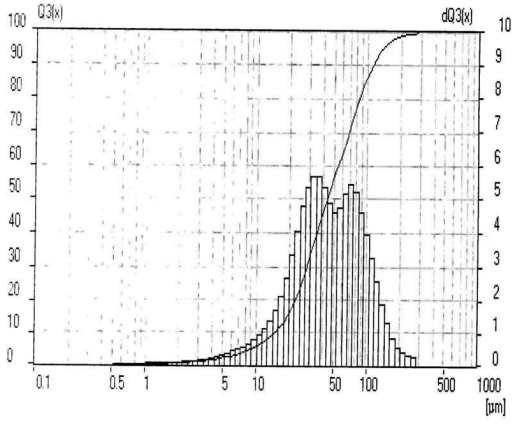
Meas.No. 42 | Date 06-01-2007 | Time 13:59 | Operator Akichev Andrey | ID 7645 | Serial No. 123456

Фенилсукцинал с 020504 проба 1

Measuring Range	0.31 [µm] - 300.74 [µm]	Pump	100 [%]
Resolution	62 Channels (17 mm / 114 mm)	Stirrer	3
Absorption	6.00 [%]	Ultrasonic	On
Measurement Duration	5 [Scans]		

Modell Independant

Fraunhofer Calculation selected.



Fritsch Particle Sizer 'analysette 22'

Meas.No. 45 | Date 06-01-2007 | Time 15:07 | Operator Akichev Andrey | ID 7645 | Serial No. 123456

Фенилсукцинал с.030504 проба 1

Measuring Range	0.31 [µm] - 300.74 [µm]	Pump	100 [%]
Resolution	62 Channels (17 mm / 114 mm)	Stirrer	3
Absorption	7.00 [%]	Ultrasonic	On
Measurement Duration	5 [Scans]		

Modell Independant

Fraunhofer Calculation selected.

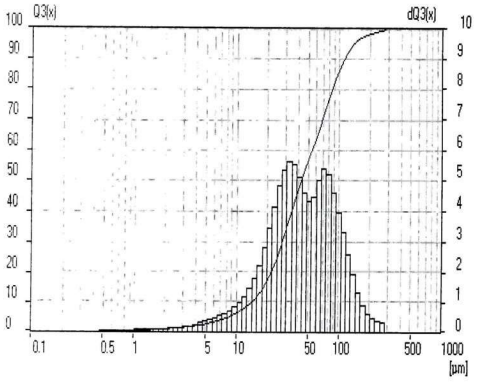


Рис. 2.3. Typical distribution on particle size for series 010504, 020504 and 030504.

As a media for dispersing substances we selected the purified water, in which fensuccinal is poorly soluble. For one test (at least 3 tests were conducted for each series) used a sample unit sufficient to saturate the suspension up to 7-8% (70-80 g/l). Given that the solubility of fensuccinal in water is about 30-40 mg/l, it can be seen that it does not affect the results.

Typical particle size distribution curves for different series are presented in Figure 2.3.

2.2. Theory

2.2.1. Dependence of fensuccinal substance solubility on dissolution time

Under standardized dissolution conditions and medium, amount (*S*) of a substance dissolved for the time *t* is a function of dissolution time (*t*) and the mean particle size (*L*), i.e. *S = S (L, t)*. Since the volume of dissolution medium is fixed (1000 mL), then instead of amount (*S*) of a substance dissolved is easy to use its concentration *C*(mg/ml).

In this case, the dependence of the concentration (*C*) ondissolution time (*t*) may be described by the one-compartment exponential model (see Chapter 1 of Add 2):

$$C(L,t) = C(L;t=\infty)\cdot[1-\exp(-k_t\cdot t)], \tag{2.1}$$

which is a function of two parameters: $C(L;\ t=\infty)$ and k_t .

The exponential equation (2.1) cannot be transformed to the linear frm that deprives it of visibility and makes it difficult to use in practice. To describe the experimental curves by the equation (2.1), we must use a non-linear least squares (NLSM) (see above Chapter 1 of Add 2).

As shown in Chapter 1 of Add 2, for practical purposes, the equation (2.1) may be approximated by a simpler Langmuir form:

$$C(L,t) = \frac{C_\infty(L,t=\infty)\cdot k_t\cdot t}{1+k_t\cdot t}, \tag{2.2}$$

Which is easy transformed to the linear form:

$$Y = \frac{1}{C(L,t)} = \frac{1}{C_\infty(L,t=\infty)} + \frac{1}{C_\infty(L,t=\infty)\cdot k_t}\cdot\frac{1}{t} = a + b\cdot X. \tag{2.3}$$

As shown in Chapter 1 of Add 2, the linear Langmuir form (2.3) often describes the experimental data not worse than the original exponential model (2.1).

2.2.2. Problem of uniform precision for ordinate values

It should be noted one important fact (which has a general nature for all descriptions of experimental data on theoretical equations), which becomes relevant when we are interested in not only the conformity of experimental data with the theoretical equations, but in parameters of these equations, as well. The matter is the weighting factors for ordinates, used in calculations on the theoretical equations.

In our case the values of particular interest are C (L; $t = \infty$) and k_t of equations (2.1-2.3) with quite specific physical meaning. So, C (L; $t = \infty$) value is the extrapolation limit of solubility (at infinite time t) for the given particle size L and dissolution medium, and the k_t value characterizes the dissolution rate.

If the experimental C values are obtained with uniform precision, the direct calculation on the equations (2.1-2.2) by using nonlinear least squares method (NLSM) will give us some values of the C (L; $t = \infty$) and k_t parameters and the linear least squares method (LSM) by using the equation (2.3) will give us, generally speaking, other values. This is because the direct calculation by using the NLSM on the equations (2.1-2.2) implies the uniform precision of the concentrations C, and the LSM using equation (2.3) involves the uniform precision of the $1/C$ values. In our case the LSM does not correspond to the actual situation and artificially overstates the contributions of points with small concentrations C. Hence, as is noted in Chapter 1 of Add. 2, the $C(L; t = \infty)$ and k_t values in the equation (2.3) have no physical meaning and are simply the model parameters. The situation can be changed by introduction of appropriate weights for the $1/C$ values (based on the rules of uncertainties propagation (see Chapter 1 of Add 2 and [26]), but this approach is slightly easier than the NLSM. Moreover, it is unclear how to assess the metrological characteristics obtained.

In general, for having a physical sense of the $C(L; t = \infty)$ and k_t values, it is better use the direct calculation on the equation (2.1) NLSM (see Chapter 1 of Add 2). However, this equation is obtained without visibility.

Given this factor, we carried out direct the calculations on the equation (2.1) using the NLSM, and the LSM used for the equation (2.3). For comparison, in the latter case, we also defined a real deviation from calculated values from the experimental values of concentrations on the basis of which assessed the residual standard deviations in terms of C values (mg/ml) and the corresponding general indices (coefficients) of correlation [26].

A typical linear relationship (2.3) is given in Fig. 2.4, the metrological characteristics of the line (2.3) are presented in the Table. 2.2-LL. For each dissolution media and particle size we calculated also the extrapolation limit concentration value $C(L; t = \infty) = 1/a$ and the value of $k_t = a/b$.

Direct calculation on the equation (2.1) with use of the NLSM are presented in the Table 2.2-EN. In this case we used the assumption of uniform precision of the concentrations (confirmed by experiment - see below). For comparison in the Table 2.2-EN also provides the values of $C(L, t = \infty)$ and k_t from the Table. 2.2-LL.

It should be noted that the use of the NLSM for processing of the equations (2.1), (2.4), (2.7) causes problems with convergence of the iterative procedure (so-called "ill-posed problems" [108]). The convergence of the procedure is significantly improved if to take for the calculation the dissolution time *t* in minutes and not in hours (it drastically reduces the derivative values of the parameters). The LSM does not requir it. However, since this approach is applied to the NLSM, in common with all calculations we took the time in hours.

2.2.3. Dependence of solubility on mean particle size

As was showed in the Chapter 1 of Add 2, for ointments and suppositories the release degree exponentially decreases with increasing thickness of layer in the releasing chamber. Influence of particle size on a dissolution process in many aspects is similar to an impact of the layer thickness of the ointments or suppositories in the releasing chamber on the dissolution degree. It can therefore be assumed that the concentration *C*s for each dissolution time *t* from the equation (2.1) also exponentially decreases with increasing the average particle size (*L*), i.e.:

$$C(L,t) = C(L=0,t) \cdot \exp(-k_L \cdot L) \tag{2.4}$$

or in the linear form:

$$\ln C(L,t) = \ln[C(L=0,t)] - k_L \cdot L. \tag{2.5}$$

Here *L* is an average particle size, *C(L=0, t)* is a concentration value for time *t* at the infinitesimal particle size *L*.

As the average particle size (*L*) we assumed half-widths of the corresponding fractions, i.e.:

$$\begin{aligned} &0.05 - 0.10 \text{ mm:} \quad L = 0.075 \text{ mm} = 75 \text{ μm;} \\ &0.10 - 0.16 \text{ mm:} \quad L = 0.13 \text{ mm} = 130 \text{ μm;} \\ &0.16 - 0.315 \text{ mm:} \quad L = 0.238 \text{ mm} = 238 \text{ μm;} \\ &0.315 - 0.50 \text{ mm:} \quad L = 0.408 \text{ mm} = 408 \text{ μm.} \end{aligned} \tag{2.6}$$

It is interesting to verify the suitability of the equation (2.5) for the description of the experimental data at different values of dissolution time *t*.

Metrological characteristics of obtained linear relationships are presented in the Table 2.3-EL. A typical line (5.5) is shown in Figure 2.5.

To test the adequacy of the parameters obtained using the linear least squares method (LSM) on the equation (2.5), we also held direct calculations on the equation (2.4)

using non-linear least squares method (NLSM), assuming the uniform precision of the concentrations (which is confirmed by the experiment - see below). Results of such calculations are presented in the Table 2.3-EN. For comparison purposes, the Table 2.3-EN shows also the values of $C(L=0, t)$ and k_L from the Table 2.3-EL (obtained by LSM).

2.2.4. General equation for dependence of solubility on particle size and time and its applications

A good description of the experimental data by using the equation (2.1) and (2.4) for different dissolution time t and of different average particle size L (see below) allows us to offer the general equation for dependence of the solubility on particle size and time. By analogy with the release of ointments and suppositories (see Chapter 1 of Add 2), this equation can be represented as (t - time in hours, L – particle size in mm):

$$C = C(L = 0, t = \infty) \cdot \exp(-k_L \cdot L) \cdot [1 - \exp(-k_t \cdot t)]. \qquad (2.7)$$

Here $C(L = 0, t = \infty)$ is a solubility at infinite time and infinite small particle size (actually this is the equilibrium solubility). Values of k_L and k_t have the same meaning as in the equations (2.1) and (2.4).

Calculations on this equation are carried out by using NLSM assuming uniform precision for solubility values C (which is confirmed by the experiment - see below).

This equation allows the most accurately to describe the experimental data and assess the values of $C(L = 0, t = \infty)$, k_L and k_t, as it covers the whole experiment and has a fairly large number of degrees of freedom (4·9 -3 = 33).

Calculations on this equation are presented in the Table 2.4.

2.2.4.1. Calculation of the critical value of particle size

Equation (2.7) allows us to calculate the critical average value L_{crit} of the average particle size, below which further powdering is impractical in terms of improving the solubility.

The use of substances with a lower average particle size improves their solubility (i.e. solubility in technologically reasonable time, because the equilibrium solubility doesn't depend on the particle size) and, accordingly, pharmaco-technological characteristics. However, substances with a smaller average size of particles are usually more expensive. In addition, there are often problems with their stability. Therefore, it is important for manufacture of a drug product to identify the critical average particle size L_{crit} below which there is no sense to crush further the substance because its solubility does not increase significantly.

The L_{crit} value can be defined in different ways, for example, on the basis of the insignificance principle (see the section 2.3.1).

Let's define the critical average particle size L_{crit} as the size, with a decrease of which twice the solubility increases only at 5% (adopted in analytical practice significance level). Then from the equation (2.7) we get:

$$1.05 = \frac{C_{0.5Lcrit}}{C_{Lcrit}} = \exp(0.5 \cdot k_L \cdot L_{crit}) \quad (2.8)$$

From this equation we get:

$$L_{crit} = \frac{\ln(1.05)}{0.5 \cdot k_L} = \frac{0.98}{k_L}. \quad (2.9)$$

The ratios (2.8-2.9) show that the L_{crit} value depends only on the constant of k_L, so for the calculation we could use not the equation (2.7), but the simpler equations (2.4-2.5). However, the k_L values of these equations are very much dependent on time, due to the small number of degrees of freedom (4-2 = 2). Therefore the L_{crit} values thus obtained for different time may differ by several times and wonder how it is interpreted. Therefore to calculate the L_{crit} value we must use equation (2.7), which describes the solubility at any time and particle size and covers the entire experiment with a large number of degrees of freedom (4·9-3 = 33).

2.2.4.2. Substance dissolution degree G% for specified time t

For prediction of solubility of solid and suspension drug products in accordance with the requirements of the SPU [33] and during its pharmaceutical development, it is informative a fraction (*G*%) of a substance dissolved as a percentage of the maximum concentration (the equilibrium solubility) $C(L = 0, t = \infty)$. From equation (2.7) we get:

$$G\% = 100 \cdot \exp(-k_L \cdot L) \cdot [1 - \exp(-k_t \cdot t)]. \quad (2.10)$$

It should be noted that the value of G% can be found for each particle size *L* from a simpler equation (2.1) - as $100 \cdot C(L; t)/ C(L; t=\infty)$. However, calculations using equation (2.10) are more accurate, encompassing the entire experimental material.

The "pharmacopoeial area» of time is *t* = 15, 30, 45 and 60 min [33, 103]. Examples of such calculations using the equation (2.10) are presented in the Table 2.5.

2.2.4.3. Dissolution time t required for achievement of specified dissolution degree G%

For technological purposes (for example, when preparing solutions for injections, eye drops or syrups), it is often important to know the time *t* of the dissolution of a frac-

tion *G%* of the substance as a percentage of the equilibrium solubility. This dependence can be obtained from the equation (2.10):

$$t = \frac{1}{k_t} \cdot \ln\left[1 - \frac{G\%}{100 \cdot \exp(-k_L \cdot L)}\right]. \tag{2.11}$$

Dissolving time *t* for the dissolution degree G% and particle size *L* can be found and more simple way - from the equation (2.1). However, the equation (2.11) is much more precise because of the greater number of degrees of freedom in the calculation of its parameters. Examples of calculations on the equation (2.11) are presented in the Table 2.6.

2.2.5. Study of actual distribution of industrial fensuccinal substance batches on particle size

The laser diffraction analyzer Fritsch Particle Sizer 'analysette 22' allows us to measure the fractional distribution as a percentage of the total number of particles, i.e. the dependence of the numerical proportion of particles *Fr(N)* on their size *L*. For technological purposes it is of interest the dependence of mass proportion of particles *Fr(m)* on their size *L*. The particle mass is proportional to the third degree of its size. Since we are interested in relative values, the relationship between values *Fr(m)* and *Fr(N)* is:

$$Fr(m)_i = \frac{Fr(N)_i \cdot L_i^3}{\sum_{i=1}^{i=n} Fr(N)_i \cdot L_i^3}. \tag{2.12}$$

In addition, it is of interest the average particle size – both on the number of particles and mass of particles, which are calculated, respectively, by the equations:

$$\overline{L(N)} = \sum_{i=1}^{i=n} Fr(N)_i \cdot L_i. \tag{2.13}$$

$$\overline{L(m)} = \sum_{i=1}^{i=n} Fr(m)_i \cdot L_i. \tag{2.14}$$

We also determined the proportions of particles with a size greater than 0.1 mm (100 μm) and 0.16 mm (160 μm), as well as the maximums on the curves *Fr(m)* and *Fr(N)*. Results of such calculations are presented in the Table 2.6.

2.3. Results and discussion

2.3.1. Crystallographic uniformity of samples

As can be seen from the Figure 2.1, radiographs of samples are almost identical. There are only small differences in the ratio of heights of some of the peaks. This is due to differences in the size of the crystals in different samples, although all samples before radiography were ground to powder in the mortar. As demonstrated by the Rietveld method calculation [105], all lines, observed in the diffraction patterns, were the lines of one crystalline form of fensuccinal, the impurity lines were found. This means that the investigated samples do not contain crystalline impurities within the method sensitivity (97-99%). Amorphous admixtures cannot be detected with such X-ray phase analysis. Thus, we may speak about a crystallographic homogeneity of the samples.

2.3.2. Solubility of different fractions of fensuccinal substance in different pharmacopoeial dissolution media

As can be seen from the Table 2.1, water solubility of the fensuccinal substance does not depend on the pH of the medium for any fraction. The standard deviation (*SD*) for solubility results, generally, does not depend on dissolution time (and hence the concentration *C*). Using limit (equilibrium) values of $C(L = 0, t = \infty)$ from the Table 2.4, it is possible to calculate the standard deviation as a percentage of this maximum value. As can be seen from the Table 2.1, the pooled on a fraction standard deviation $SD_{pool,\text{tot}}$ does not exceed 10% (the recommended the Guidance [103] for *in vitro* bioequivalence study). However, for individual media and especially for different dissolution times, the $SD_{pool,tot}$ values, as the percentages of the $C(L = 0, t = \infty)$, considerably exceed 10% (for example, in the phosphate buffer pH 6.8 for the fraction of 0.05-0.10 mm, t = 10 minutes we have SD = 6.02 mg or 6.02·100/32.8 = 18.4%). It indicates the heterogeneity of the fractions.

It can be shown that the *SD* sample for each fraction and dissolution medium, as well as for the whole *SD* population, are uniform by the Cochran criterion [26]. This means that when processing the results on the equations (2.1, 2.4, 2.7), we can accept the hypothesis of uniform precision of the concentrations (*C*). i.e.:

$$SD(C) = const \tag{2.15}$$

and , unlike Chapter 1 of Add 2, can use in equations (2.1, 2.4, 2.7) unweighted nonlinear MNCS.

The Table 2.1 shows that the solubility decreases with the increase of the average particle size. Particularly it is evident from the Figures 2.1-2.3. Differences are particularly large for the "pharmacopeial range" of dissolution time of dissolution: up to 60 min [33]. In particular, for fractions of 0.05-0.1 mm and 0.315-0.5 mm in this field the differences in solubility of fensuccinal are 1.5-2 times.

An interesting fact is that the solubility curves of various fractions of the fensuccinal (Figures 2.1-2.3) do not converge even after 3 hours (180 min) of dissolution, although in theory it should be, because thermodynamic (equilibrium) solubility of the fensuccinal does not depend on the size of the particles. Thus the equilibrium solubility of the fensuccinal is not achieved even through 3 hours. As we can see, the concept of solubility for the fensuccinal is not quite certain. The question arises: in what way we must determine it, in particular, in accordance with the requirements of the SPU [1]? This question seems to arise for other poor water soluble substances.

As expected, reducing in the particle size increases the solubility of the fensuccinal only up to some limit. The Figures 2.1-2.3 show that the difference in solubility between the fractions of 0.05-0.10 mm and 0.10-0.16 mm is very small and it can be expected that the further decrease in the particle size will not lead to the increase in the solubility of the fensuccinal. This conclusion confirms the result obtained by authors of [2.2-2.3] for dependence of the dissolution degree of suppositories on particle size: the decrease in the particle size below 0.065-0.090 mm does not increase the dissolution degree. Thus, the particle size < 0.10 mm is critical not only for suppositories, but for solid dosage forms as well.

The dependence of the solubility of the fensuccinal on particle size it is similar to the situation with the dependence of dissolution degree of ointments and suppositories on the thickness of the layer in the releasing chamber (see Chapter 1 of Add 2). This indicates the similarity of the kinetics of dissolution in both cases.

Table 2.1

The dependence of solubility of different fractions of fensuccinal on time

Time, minute (hour)	Concentration (*C*) mg/L, Mean of 3 replicates			Standard deviation *SD*, mg/L		
	0.1 M HCl	Water	phosphate buffer pH 6.8	0.1 M HCl	Water	phosphate buffer pH 6.8
Fraction 0.05 - 0.10 mm						
5 (0.0833 hour)	4.07	5.6	6.4	1.16	0.74	1.73
10 (0.167 hour)	13.2	16.6	15.0	3.06	2.19	6.02
20 (0.333 hour)	20.9	24.2	17.4	2.65	2.30	5.99
30 (0.50 hour)	24.1	27.4	23.9	1.73	2.33	5.35
40 (0.667 hour)	26.2	29.0	25.8	1.21	2.15	5.07
50 (0.833 hour)	27.3	29.4	27.2	1.08	1.97	3.87
60 (1 hour)	27.8	30.0	27.7	0.85	2.20	3.00
120 (2 hour)	29.7	32.3	30.7	0.85	0.00	0.28

180 (3 hour)	30.4	32.4	30.6	0.78	0.07	0.42
Pooled SD_{pool}				*1.76*	*1.92*	*4.38*
Pooled SD_{pool} , per cent of *$C(L=0, t = \infty)$*				*5.2*	*5.6*	*12.9*
Pooled, total on the fraction $SD_{pool,tot}$				*2.94*		
$SD_{pool,tot}$, per cent of *$C(L=0, t = \infty)$*				*8.6*		
Fraction 0.10 - 0.16 mm						
5 (0.0833 hour)	5.8	4.46	7.5	1.50	0.59	0.20
10 (0.167 hour)	15.7	12.6	16.7	1.86	1.72	1.88
20 (0.333 hour)	21.5	18.3	21.1	1.54	1.78	1.31
30 (0.50 hour)	24.2	21.7	23.2	1.64	1.50	1.65
40 (0.667 hour)	26.5	23.5	24.3	2.05	1.18	1.50
50 (0.833 hour)	27.4	24.7	25.8	2.19	1.23	1.12
60 (1 hour)	28.6	25.4	26.3	1.73	1.10	1.04
120 (2 hour)	30.3	28.0	27.9	2.26	1.41	1.91
180 (3 hour)	30.8	29.3	28.7	1.48	1.27	1.06
Pooled SD_{pool}				*1.82*	*1.35*	*1.37*
Pooled SD_{pool} , per cent of *$C(L=0, t = \infty)$*				*5.3*	*4.0*	*4.0*
Pooled, total on the fraction $SD_{pool,tot}$				*1.53*		
$SD_{pool,tot}$, per cent of *$C(L=0, t = \infty)$*				*4.5*		
Fraction 0.16 – 0.315 mm						
5 (0.0833 hour)	4.80	3.9	6.1	0.87	0.53	0.90
10 (0.167 hour)	11.5	9.7	12.3	1.75	1.54	0.72
20 (0.333 hour)	16.0	14.5	16.8	2.31	0.70	1.44
30 (0.50 hour)	18.4	17.4	18.9	1.78	0.78	0.61
40 (0.667 hour)	20.2	19.4	20.7	1.70	0.59	2.80
50 (0.833 hour)	22.1	20.7	22.1	1.60	0.78	1.17
60 (1 hour)	22.6	22.3	22.9	1.12	0.42	1.60
120 (2 hour)	25.4	25	24.7	1.77	0.71	0.78
180 (3 hour)	25.0	26.2	26.9	0.00	0.42	0.78
Pooled SD_{pool}				*1.60*	*0.81*	*1.42*

Pooled SD_{pool}, per cent of *$C(L=0, t = \infty)$*				*4.7*	*2.4*	*4.2*
Pooled, total on the fraction $SD_{pool,tot}$				*1.32*		
$SD_{pool,tot}$, per cent of *$C(L=0, t = \infty)$*				*3.9*		
Fraction 0.315 - 0.5 mm						
5 (0.0833 hour)	4.2	3.5	6.2	0.71	1.27	0.42
10 (0.167 hour)	7.2	8.0	8.6	0.36	2.68	1.38
20 (0.333 hour)	10.2	11.0	12.6	0.58	3.62	3.27
30 (0.50 hour)	12.8	13.4	14.7	1.66	4.34	2.47
40 (0.667 hour)	14.3	14.8	15.9	2.11	4.60	2.23
50 (0.833 hour)	15.9	16.8	17.0	2.63	4.60	2.18
60 (1 hour)	17.0	17.5	18.2	2.75	4.87	2.72
120 (2 hour)	19.1	18.8	20.1	0.14	1.70	1.27
180 (3 hour)	21.0	21.2	21.2	0.07	1.06	1.63
Pooled SD_{pool}				*1.68*	*3.69*	*2.18*
Pooled SD_{pool}, per cent of *$C(L=0, t = \infty)$*				*4.9*	*10.8*	*6.4*
Pooled, total on the fraction $SD_{pool,tot}$				*2.66*		
$SD_{pool,tot}$, per cent of *$C(L=0, t = \infty)$*				*7.8*		

One of the obvious conclusions of the Table 2.1 is the need to standardize the poorly soluble substances on particle size. Otherwise, we can get solid dosage forms (such as tablets) which are non-reproducible by the "Dissolution" test [33]. Consequently, without such standardization it is incorrect to compare the dissolution profiles to demonstrate bioequivalence according to the Guidelines [103].

2.3.3. Description of dependence of concentration on time

2.3.3.1. Verifying the adequacy of the linearized Langmuir equation (2.3), using linear least-squares method

As we can see from the Figure 2.4 and Table 2.2-LL, the linear dependences (2.3) are good enough for all the studied dissolution media. It confirms the correctness of application of the Langmuir model to describe the kinetics of dissolution (see Chapter 1 of Add 2).

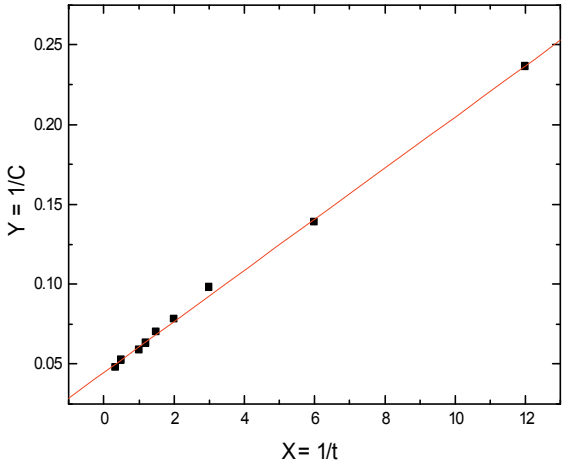


Figure. 2.4. Dissolution medium: 0.1 M HCl, fraction 0.315-0.5 mm. Typical regression of

$$\frac{1}{C} = \frac{1}{C(L, t=\infty)} + \frac{1}{C(L, t=\infty)\cdot k_t}\cdot\frac{1}{t}$$

.

The Table 2.2-LL shows that the maximum extrapolation concentration of the fensuccinal *C(L, t = ∞)* very much depends on the size of its particles, decreasing in 2-4 times with increase in the average particle size from 0.05-0.10 mm to 0.315-0.50 mm. Such a large difference raises doubts as to the correctness of the obtained estimates of the *C(L, t = ∞)* values and is associated with the ununiform precision of ordinate values of (1/*C*).

Table 2.2-LL

Metrological characteristics of the linear regressions (2.3)

$$Y = \frac{1}{C} = \frac{1}{C(L,t=\infty)} + \frac{1}{C(L,t=\infty)\cdot k_t}\cdot\frac{1}{t} = a + b\cdot X.$$

for different dissolution media and particle sizes (*SD* – standard deviation, SD_r – residual *SD*, *r* – correlation coefficient, *n* = 9), *t* - time (hour)

Particle size, mm	***a* ·100**	***SD*$_a$ ·100**	***b* ·100**	***SD*$_b$ ·100**	***SD*$_r$* ·100**	***r****	***Significance level of r* [10], %**	***C(L, t = ∞) = 1/a***	***k*$_t$ = *a/b***
0.1 M HCl									
0.05-0.10	1.17	0.91	1.75	0.20	2.08 (13.0)	0.959 (0)	100.00	85.4	0.67
0.10-0.16	1.86	0.69	1.56	0.15	1.58 (6.64)	0.970 (0.553)	100.00	53.9	1.19
0.16-0.315	2.72	0.50	1.40	0.11	1.14	0.980	100.00	36.8	1.94

					(3.11)	(0.887)			
0.315-0.50	4.47	0.11	1.60	0.02	0.25 (0.50)	0.999 (0.996)	100.00	22.4	2.79
Water									
0.05-0.10	1.61	0.65	1.22	0.14	1.47 (8.99)	0.958 (0)	100.00	62.3	1.31
0.10-0.16	2.03	0.53	1.15	0.11	1.21 (5.66)	0.967 (0.717)	100.00	49.3	1.77
0.16-0.315	2.33	0.60	1.81	0.13	1.37 (3.95)	0.983 (0.840)	100.00	42.8	1.29
0.315-0.50	3.52	0.58	1.99	0.12	1.32 (1.80)	0.987 (0.947)	100.00	28.4	1.77
Phosphate buffer pH 6.8									
0.05-0.10	2.40	0.37	1.03	0.08	0.85 (3.01)	0.980 (0.930)	100.00	41.6	2.34
0.10-0.16	2.78	0.34	0.81	0.07	0.77 (2.69)	0.973 (0.915)	100.00	36.0	3.45
0.16-0.315	3.18	0.26	1.04	0.06	0.59 (1.37)	0.990 (0.977)	100.00	31.5	3.05
0.315-0.50	4.72	0.20	1.00	0.04	0.46 (0.76)	0.994 (0.989)	100.00	21.2	4.72

* *SD* and *r* values in parentheses are calculated on the base of the deviations of calculated regression points from the experimental *C* values in mg/ml.

At the same time, if you calculate the residual standard deviations (SD_r) and correlation coefficients (r) in terms of concentration (C), the actual description of the experiment looks significantly worse (the values in parentheses). This is because, as mentioned above, of the ununiform precision of the concentrations C, rather than values of $1/C$. Hence it is of interest a direct description of the experimental data on the equation (2.1) using the non-linear least squares method (NLSM).

2.3.3.2. Verifying the adequacy of the exponential model (2.1), using non-linear least-squares method

As can be seen from a comparison of the SD_r and r values in parentheses in the Table 2.2-LL and the SD_r and r values in the Table 2.2-EN, the NLSM for the exponential model (2.1) better describes the experiment than the linear LSM for the linear Langmuir (2.3). Consequently, a much more accurate are the extrapolation $C(L, t = \infty)$ values: the differences between them in the Table 2.2-EN are much smaller and more real than in the Table. 2.2-LL.

Table 2.2-EN

Metrological characteristics of the equations (2.1)

$$C(L,t) = C(L,t=\infty)\cdot[1-\exp(-k_t \cdot t)]$$

for different dissolution media and particle sizes (SD – standard deviation, SD_r – residual SD, r – correlation coefficient, n = 9), t - time (hour)

Particle size, mm	***C(L, t = ∞)***	***SD_C***	***k_t***	***SD_k***	***SD_r***	***R***	***Significance level of r* [10], %**	**Data of Table 2.2-LL**	
								C(L, t = ∞)	***k_t***
0.1 M HCl									
0.05-0.10	29.8	0.77	3.22	0.29	1.32	0.989	100.00	85.4	0.67
0.10-0.16	27.9	0.72	2.97	0.25	1.19	0.989	100.00	53.9	1.19
0.16-0.315	24.5	0.61	3.00	0.25	1.00	0.989	100.00	36.8	1.94
0.315-0.50	19.8	0.62	2.11	0.18	0.87	0.987	100.00	22.4	2.79
Mean	***25.5***		***2.82***					***49.6***	***1.65***
Water									
0.05-0.10	31.7	0.83	3.88	0.39	1.56	0.984	100.00	62.3	1.31
0.10-0.16	29.8	0.72	3.61	0.32	1.30	0.987	100.00	49.3	1.77
0.16-0.315	25.2	0.61	2.35	0.17	0.91	0.992	100.00	42.8	1.29
0.315-0.50	19.7	0.63	2.35	0.22	0.94	0.986	100.00	28.4	1.77
Mean	***26.6***		***3.05***					***45.7***	***1.54***
Phosphate buffer pH 6.8									
0.05-0.10	30.00	0.87	3.07	0.30	1.45	0.984	100.00	41.6	2.34
0.10-0.16	27.0	0.69	4.59	0.49	1.39	0.978	100.00	36.0	3.45
0.16-0.315	24.7	0.78	3.26	0.36	1.34	0.978	100.00	31.5	3.05

0.315-0.50	19.8	0.71	2.92	0.34	1.16	0.973	100.00	21.2	4.72
Mean	***25.4***		***3.46***					***32.6***	***3.39***

As can be seen from comparing the coefficients $C(L,\ t = \infty)$ and k_t received by the equations (2.3) (Table 2.2-LL) and (2.1) (Table 2.2-EN), they are very different. This is especially clear from the mean values, which usually differ in 1.5-2 times.

Thus, if we are interested not only in the fact of observance of the ratio (2.3), but in the coefficients $C(L,\ t = \infty)$ and k_t as well, we must carry out the direct calculation on the exponent (2.1) using the NLSM.

2.3.4. Description of dependence of concentration on particle size

2.3.4.1. Verifying the adequacy of linearized exponential model (2.5), using linear least-squares method

As can be seen from the Table 2.3-EL and Figure 2.5, despite a big approximation of the relationships (2.6), the equation (2.5) is well met for all the studied dissolution media. The exception is the results obtained for the dissolution time $t = 5$ min. This is due to the fact that in this case, the range of ordinate variation is often less of uncertainty of these ordinate values.

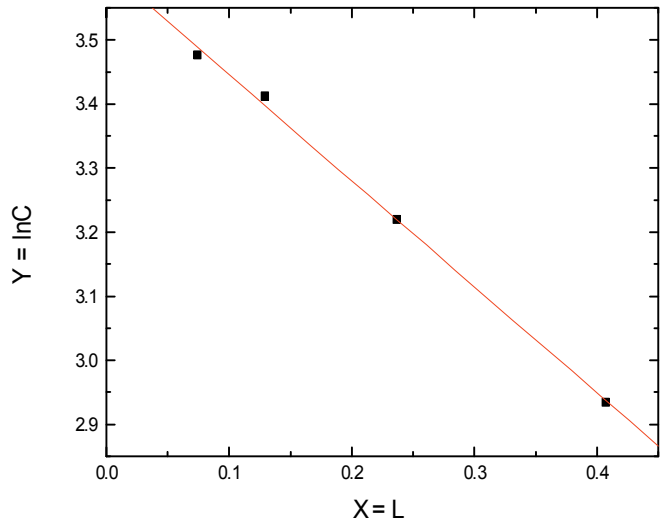


Figure 2.5. Dissolution medium: phosphate buffer pH 6.8. Time – 40 min. The typical linear regression

$$Y = \ln C(L,t) =$$
$$= \ln[C(L=0,t)] - k_L \cdot L =$$
$$= a_1 + b_1 \cdot X$$

Thus, the dependence of solubility of the fensuccinal substance on its particle size is exponential in nature and similar to the dependence of the dissolution degree of the ointments and suppositories on the layer thickness in the releasing chamber, that we obtained in Chapter 1 of Addendum 2.

Table 2.3-EL

Metrological characteristics of the linear dependences (2.5)

$$Y = \ln C = \ln[C(L=0,t)] - k_L \cdot L = a_1 + b_1 \cdot X.$$

for different dissolution time and media (*SD* – standard deviation, SD_r – residual *SD*, *r* – correlation coefficient, *n* = 4, *L* - mm). Linear least squares method.

Dissolution time, min	a_1	SD_a	$-b_1 = k_L$	SD_b	SD_r	*-r*	*Significance level of r* [10], %	$C(L=0,t) = exp(a_1)$, *mg/mL*
0.1 M HCl								
5	1.46	0.08	-0.07	0.34	0.09 (0.39)	*-0.134* (0)	*13.4*	4.3
10	2.77	0.09	1.83	0.35	0.09 (0.98)	0.965 (0.932)	96.5	16.0
20	3.21	0.05	2.09	0.22	0.05 (0.85)	0.989 (0.982)	98.9	24.7
30	3.30	0.03	1.78	0.14	0.04 (0.43)	0.994 (0.996)	99.4	28.0
40	3.37	0.03	1.70	0.13	0.04 (0.50)	0.994 (0.995)	99.4	30.1
50	3.40	0.04	1.51	0.15	0.04 (0.73)	0.990 (0.989)	99.0	31.0
60	3.41	0.03	1.37	0.10	0.03 (0.47)	0.994 (0.995)	99.4	31.1
120	3.48	0.04	1.25	0.16	0.04 (0.83)	0.984 (0.984)	98.4	33.4
180	3.49	0.02	1.09	0.10	0.03 (0.50)	0.992 (0.993)	99.2	33.3
Water								
5	1.87	0.12	1.63	0.47	0.12 (0.57)	*0.926* (0.877)	*92.6*	6.5
10	2.97	0.13	2.33	0.51	0.13 (1.45)	0.956 (0.941)	95.6	19.6

20	3.35	0.08	2.45	0.30	0.08 (1.22)	0.985 (0.980)	98.5	28.5
30	3.45	0.06	2.18	0.25	0.06 (1.17)	0.987 (0.983)	98.7	31.5
40	3.52	0.05	2.07	0.20	0.05 (1.07)	0.991 (0.986)	99.1	33.7
50	3.51	0.05	1.74	0.21	0.05 (1.15)	0.986 (0.980)	98.6	33.3
60	3.54	0.03	1.70	0.14	0.03 (0.90)	0.994 (0.988)	99.4	34.5
120	3.61	0.01	1.66	0.05	0.01 (0.43)	0.999 (0.998)	99.9	37.0
180	3.58	0.01	1.31	0.05	0.01 (0.39)	0.999 (0.997)	99.9	36.0
Phosphate buffer pH 6.8								
5	1.95	0.10	0.33	0.39	0.10 (0.68)	*0.515* (0)	*51.5*	7.0
10	2.95	0.11	1.92	0.43	0.11 (1.69)	0.954 (0.880)	95.4	19.2
20	3.08	0.13	1.23	0.54	0.14 (2.59)	*0.849* (0.667)	*84.9*	21.7
30	3.31	0.03	1.53	0.11	0.03 (0.65)	0.995 (0.988)	99.5	27.4
40	3.37	0.01	1.48	0.05	0.01 (0.29)	0.999 (0.998)	99.9	29.2
50	3.43	0.02	1.44	0.07	0.02 (0.42)	0.998 (0.996)	99.8	30.7
60	3.43	0.01	1.28	0.04	0.01 (0.29)	0.999 (0.998)	99.9	30.8
120	3.50	0.01	1.24	0.06	0.01 (0.43)	0.998 (0.995)	99.8	33.2

180	3.51	0.03	1.09	0.12	0.03 (0.82)	0.987 (0.980)	98.7	33.5

The calculations of the residual standard deviations (SD_r) and correlation coefficients (r) in terms of concentrations (C, mg/l) (values in parentheses) do not affect the visible picture.

2.3.4.2. Verifying the adequacy of the exponential model (2.4), using a non-linear least-squares method

As can be seen from a comparison of the SD_r and r values in parentheses in the Table 2.3-EL and the SD_r and r values in the Table 2.3-EN, in this case there is not much difference between linear (2.5) and non-linear (2.4) LSM.

This is due to the fact that the equation (2.5) considers the uniform precision of the lnC values. Taking into account formulas of uncertainties propagation [26], it means the assumption of constancy of relative standard deviations (*RSD*). The assumption of *RSD(C)* = const, given the considerable variation in *SD* values (see the Table 2.1), is performed slightly worse than assumption (2.15) *SD (C)* = const, which results in the good performance of the equation (2.5) in terms of concentrations C.

Values of k_L and $C(L = 0, t)$ in the Table 2.3-EL and 2.3-EN also differ little. Particularly evident is clear from mean values, which do not practically differ for the linear (Table 2.3-EL) and non-linear (Table 2.3-EN) LSM.

Thus, in this case, to calculate k_L and $C(L = 0, t)$ values, it is quite possible to use the simple linear relationship (2.4).

Table 2.3-EN

Metrological characteristics of the dependences (2.4)

$$C(L,t) = C(L=0,t) \cdot \exp(-k_L \cdot L)$$

for different dissolution time and media (*SD* – standard deviation, SD_r – residual *SD*, r – correlation coefficient, n = 4, L - mm). Nonlinear least squares method.

Dissolution time. min	***C(L, t=0)***	***SD_C***	***k_L***	***SD_k***	***SD_r***	***r***	***Significance level of r* [10], %**	**Data of Table 2.3-EL**	
								C(L=0,t) mg/mL	***k_L***
0.1 M HCl									
5	4.44	0.42	0.00	0.38	0.43	*0*	*0*	4.3	-0.07
10	15.5	1.18	1.65	0.38	0.93	0.938	93.8	16.0	1.83
20	24.3	1.09	2.00	0.24	0.82	0.984	98.4	24.7	2.09

30	27.8	0.54	1.85	0.10	0.42	0.996	99.6	28.0	1.78
40	29.9	0.62	1.77	0.11	0.48	0.996	99.6	30.1	1.70
50	30.7	0.89	1.56	0.14	0.71	0.989	98.9	31.0	1.51
60	30.9	0.57	1.43	0.09	0.47	0.995	99.5	31.1	1.37
120	33.1	0.95	1.27	0.14	0.80	0.985	98.5	33.4	1.25
180	33.4	0.58	1.15	0.08	0.50	0.993	99.3	33.3	1.09
Mean	***25.6***		***1.41***					***25.8***	***1.39***
Water									
5	6.59	0.71	1.70	0.55	0.56	*0.879*	*87.9*	6.5	1.63
10	20.4	2.03	2.53	0.57	1.40	*0.945*	*94.5*	19.6	2.33
20	29.4	1.68	2.61	0.33	1.14	0.982	98.2	28.5	2.45
30	32.3	1.54	2.31	0.26	1.10	0.985	98.5	31.5	2.18
40	34.2	1.41	2.15	0.22	1.04	0.987	98.7	33.7	2.07
50	33.7	1.45	1.81	0.22	1.13	0.981	98.1	33.3	1.74
60	34.6	1.13	1.71	0.17	0.89	0.988	98.8	34.5	1.70
120	36.9	0.53	1.64	0.07	0.42	0.998	99.8	37.0	1.66
180	36.0	0.46	1.30	0.06	0.39	0.997	99.7	36.0	1.31
Mean	***29.3***		***1.97***					***29.0***	***1.90***
Phosphate buffer pH 6.8									
5	7.03	0.70	0.33	0.42	0.68	*0*	*0*	7.0	0.33
10	18.8	2.15	1.78	0.59	1.67	*0.883*	*88.3*	19.2	1.92
20	21.4	2.98	1.13	0.66	2.57	*0.673*	*67.3*	21.7	1.23
30	27.3	0.79	1.51	0.14	0.65	0.988	98.8	27.4	1.53
40	29.1	0.35	1.46	0.06	0.29	0.998	99.8	29.2	1.48
50	30.6	0.50	1.41	0.08	0.41	0.996	99.6	30.7	1.44
60	30.7	0.34	1.26	0.05	0.29	0.998	99.8	30.8	1.28
120	33.3	0.51	1.26	0.07	0.43	0.996	99.6	33.2	1.24
180	33.3	0.92	1.06	0.13	0.81	0.980	98.0	33.5	1.09
Mean	***25.7***		***1.24***					***25.9***	***1.28***

2.3.5. Verifying the adequacy of the general equation (2.7), using non-linear least-squares method

As can be seen from the Table 2.4, the general equation (2.7) of the dependence of the solubility on dissolution time and particle size is well performed.

This equation allows to get the equilibrium concentrations ($C(L = 0, t = \infty)$ values) in all three studied dissolution media. They are respectively: 34.1 mg/l (0.1 M hydrochloric acid), 37.8 mg/ml (water), 32.8 mg/ml (phosphate buffer pH 6.8), that is a little different. These values are close to the maximum solubility values obtained in fact (Table 2.1, 0.05-0.1 mm): 30.4 mg (0.1 M hydrochloric acid), 32.4 mg/ml (water), and 30.6 mg/ml (phosphate buffer pH 6.8). As can be seen, these differences do not exceed, in total, 15%. This once again confirms the conclusion that reducing the particle size below 0.10 mm does not lead to a significant increase in the solubility of the substance.

Table 2.4

Metrological characteristics of the dependences (2.7)

$$C = C(L = 0, t = \infty) \cdot \exp(-k_L \cdot L) \cdot [1 - \exp(-k_t \cdot t)]$$

C – mg/L, t – hour*, L* - mm

C(L=0, t = ∞), mg/L	***SD [C(L=0,t=∞)]***	k_L	$SD(k_L)$	k_t	$SD(k_t)$	SD_{rest}	***r****
0.1 M HCl							
34.1	0.73	1.48	0.091	2.94	0.14	1.22	0.988
L_{crit} = 0.066 mm = 66 μm							
Water							
37.8	0.96	1.84	0.12	3.26	0.19	1.59	0.983
L_{crit} = 0.053 mm = 53 μm							
Phosphate byffer pH 6.8							
32.8	0.81	1.30	0.10	3.46	0.20	1.48	0.978
L_{crit} = 0.075 mm = 75 μm							
Average value on all three dissolution media L_{crit} **= 0.065 мм = 65 мкм**							

* All correlation coefficients are significant at a level upper 99.99% [26].

2.3.5.1. Critical value of particle size

Coefficient of k_L of the equation (2.7) allows finding a critical value of particle size L_{crit} on the equation (2.9). The L_{crit} values for each dissolution media are also presented in the Table 2.4 and the average value for all dissolution media as well.

As we can see, the calculated $\boldsymbol{L_{crit}}$ values for different dissolution media vary in a fairly narrow range (0.053-0.075 mm) with an average value of 0.065 mm (65 μm). Using the fraction width of 0.025 mm for the range of 0.05 – 0.10 mm (see the ratios (2.6)), the average value of 0.065 mm corresponds to the fraction of 0.040-0.090 mm = 40-90 μm. Given sufficient approximation of the relationship (2.6), this range is the same as found for suppositories critical fraction of 65-90 μm [100-101], and the investigated us fraction 0.050-0.10 mm = 50-100 microns.

It should be noted that the found assessments of the critical average particle size are identical with the requirements to the maximum size of the particles for suspension liquid and soft eye drugs (not more than 90 μm [1]). This confirms the correctness of the assessments found.

Thus, the value of 0.10 mm, as in the case of the suppositories [100-101], is critical for the average particle size of the substance. When the particle size is less than 0.10 m, the fraction composition of the substance does not need further standardization (in terms of solubility). For larger fractions, the solubility of the substance can substantially depend on the size of the particles, so the particle size distribution of such fractions (more than 0.10 mm) needs further standardization.

So the proportion of the fraction with particle size of more than 0.10 mm is a characteristic of a substance quality: the less this proportion, the better. From this point of view, it is of interest the actual particle size distribution of industrial fensuccinal batches and the proportion in these batches of the fraction with the particle size of more than 0.10 mm.

2.3.5.2. Substance dissolution degree G% for specified time t

The Table 2.5 illustrates the calculated by the equation (2.10) the dissolution degree (G%) of the fensuccinal as percentage of the equilibrium (maximum) concentration of $C(L = 0, t = \infty)$ for different average particle sizes (L) in 15, 30, 45 and 60 minutes ("pharmacopoeial region" [33, 103]).

Table 2.5

Dissolution degrees of different fractions of fensuccinal in different media

Fraction, mm	**Dissolution degree *G%* for the dissolution medium**		
	0.1 M HCL	**Water**	**Phosphate buffer pH 6.8**
t = 15 min			
0.05-0.10	46.6	48.6	52.5

0.10-0.16	42.9	43.9	48.9
0.16-0.315	36.6	36.0	42.5
0.315-0.50	28.5	26.3	34.1
t = 30 min			
0.05-0.10	68.9	70.0	74.6
0.10-0.16	63.5	63.3	69.5
0.16-0.315	54.2	51.9	60.4
0.315-0.50	42.1	38.0	48.4
t = 45 min			
0.05-0.10	79.6	79.6	83.9
0.10-0.16	73.4	71.9	78.1
0.16-0.315	62.6	59.0	68.0
0.315-0.50	48.7	43.1	54.5
t = 60 min			
0.05-0.10	84.8	83.8	87.9
0.10-0.16	78.1	75.7	81.8
0.16-0.315	66.6	62.1	71.1
0.315-0.50	51.8	45.4	57.0

As we can see, for all dissolution times, the difference in the dissolution degree between the smallest (0.05-0.10 mm) and the largest (0.315-0.50 mm) fractions is 1.5-2 times. Such differences can make it difficult to standardize the technological process of preparation of the solution.

2.3.5.3. Dissolution time t required for achievement of specified dissolution degree G%

The Table 2.6 presents the results of a calculation, using the equation (2.11), of the time required for obtaining the dissolution degree of G%.

Table 2.6

Time *t*, required for achieving the dissolution degree of *G%*

G%	**Dissolution time *t* for the average particle size of *L*(mm) =**			
	0.075	**0.13**	**0.238**	**0.408**

0.1 M HCL				
80	45.8	71.4	Not achieved	Not achieved.
70	31.1	38.5	107.5	Not achieved
60	22.7	26.5	39.1	Not achieved
50	16.7	19.0	25.3	50.0
Water				
80	46.1	Not achieved.	Not achieved	Not achieved
70	30.0	40.5	Not achieved	Not achieved
60	21.5	26.4	48.6	Not achieved
50	15.7	18.6	27.4	Not achieved
Phosphate buffer pH 6.8				
80	37.0	51.0	Not achieved	Not achieved
70	25.6	30.6	53.1	Not achieved
60	18.8	21.5	29.5	Not achieved
50	13.9	15.5	19.8	32.8

The Table 2.6 shows how technologically important is standardization of particle size. Thus, 70% of dissolution degree for average particle size of 0.075 mm is achieved in 0.1 M hydrochloric acid in 31.1 minutes, for 0.13 mm - in 38.5 minutes, for 0.238 mm - in 107.5 minutes and for 0.408 mm is not achieved at all at the studied dissolution time range (up to 3 hours).

2.3.6. Study of actual distribution of industrial fensuccinal substance batches on particle size

The Figure 2.3 shows that all three studied industrial fensuccinal batches are a mixture of the two fractions with the modes of distribution (particle size) about 35 and 75 μm (0.035 and 0.075 mm). Given that the proportions of these fractions found are approximately equally in the different batches, it indicates the two-stage crystallization of substances from the mother liquor. This leads to heterogeneity of the substance on particle size, which can cause problems with tableting, as well as degrade the standardization of tablets by the "Dissolution" test.

Table 2.7

Fractional composition of the industrial batches of fensuccinal substanes

	Batch 010504	Batch 020504	Batch 030504

Measurements	1	2	3	1	2	3	1	2	3
Average particle size, μm:									
by particle number	51.8	52.0	51.9	52.0	52.0	52.0	52.0	51.9	52.1
by particle weight	185.1	155.2	154.5	151.6	162.4	153.0	155.0	162.7	135.4
Proportion of fraction in % > 100 μm:									
by particle number	20.9	11.5	12.1	11.4	14.5	11.8	11.7	14.1	7.0
by particle weight	86.7	73.6	73.5	71.9	76.9	78.0	73.2	76.9	60.1
Proportion of fraction in % > 160 μm:									
by particle number	7.2	2.6	2.8	2.5	3.7	3.9	2.6	3.6	1.2
by particle weight	61.3	41.5	40.8	39.0	46.0	58.8	40.9	45.9	29.0
Maxima by particle number, *μm:*									
The first	34.8	32.8	37	32.8	36.7	36.7	32.8	36.7	32.8
The second	79.7	71.4	71.4	71.4	71.4	71.4	71.4	71.4	71.4
Maxima by particle weight, *μm*	-	138.8	138.8	138.8	155.1	138.8	138.8	155.1	111.2

The Table 2.7 shows that the average particle size is 52 μm (by particle number), that would seem a good indicator (as it is found above, the critical value of the average particle size is L_{crit} = 65 μm). However, the average particle size by mass (it is important for technology) varies from 135 to 185 microns, i.e. well above the critical value of L_{crit} = 65 μm.

The proportion of more than 100 μm fraction of particles does not exceed 20%, which is a good indicator. At the same time, the mass proportion of a fraction more than 100 microns in this case typically exceeds 70%. Even the fraction with the particle size above 0.16 mm has generally a proportion above 40%. This can lead to irreproducible results of "Dissolution" test for tablets, made of this substance.

2.4. Conclusions

1. The particle size of the poorly soluble substances can significantly affect the dissolution rate and, therefore, is in need of standardization. In particular, it is important when comparing the dissolution profiles of solid dosage forms in *in vitro* bioequivalent studies.
2. The dependence of the solubility of the fensuccinal substance on the dissolution time and particle size is similar to the dependence of the dissolution degree on time and the layer thickness for ointments and suppositories.
3. The dependence of solubility of the substance on dissolution time is well described with one-compartment exponential model. For practical purposes, it can be used the simpler Langmuir model, which can be transformed to the linear form.
4. The solubility of the fensuccinal substance exponentially decreases with increasing in particle size.
5. The proposed general equation of the dependence of solubility of fensuccinal substance on dissolution time and particle size well describes the experiment and allows to determine the maximum (equilibrium) value of solubility and other technological characteristics of solubility.
6. It is developed the expression for the critical average particle size, below which there is no significant increase in solubility through technologically acceptable dissolution time.
7. There are calculated the critical average particle sizes for the studied dissolution media and the average value for all media (65 μm), which corresponds to the studied fraction of 50-100 microns. This confirms the conclusion, obtained previously for suspension suppositories, that reducing the size of particles below 100 μm does not lead to the further growth of the dissolution.
8. When determining the fractional composition of substances, it is shown the expediency of recalculation of results to a weight proportion of the individual fractions. It is proposed to control the weight proportion of fractions of active pharmaceutical ingredients with a particle size of more than 100 μm.

3. DESCRIPTION OF DISSOLUTION PROFILES IN AN *IN VITRO* BIO-EQUIVALENCE STUDY [68]

Investigation of dissolution profiles of target components is an important part of bioequivalence studies *in vitro* of solid dosage generic drugs [63]. These studies are similar to the "Dissolution" test [33] and are particularly critical at the stage of development of manufacturing technique of these generic drugs.

Hence the mathematical description of the dissolution (release) profiles (curves) is of interest. The equations obtained for a developed generic drug can be used to compare with the original drug one and assessing the maximum dissolution degree (G_o) for the technological composition in this dissolution medium.

This chapter considers the mathematical description of the dissolution profiles (curves) in bioequivalence studies *in vitro* under development of generic drugs.

3.1. Theory

As illustrated in Chapter 1 of Addendum 2, dissolution (release) profiles of active substances from ointments and suppositories are well described by the two-parametric exponential model:

$$G(t) = G_o \cdot \left(1 - e^{-k \cdot t}\right). \tag{3.1}$$

Here *G(t)* is a dissolution degree (usually as per cent of nominal content in the drug product) for the dissolution time *t*. The parameter G_o has a sense of a maximum dissolution (release) degree for the infinitive dissolution time, *k* is a factor of a dissolution speed.

The exponential model (3.1) may not be brought to the linear form and to find its parameters we want to use a nonlinear least squares method (see Chapter 1 of Addendum 2). In practice, therefore, more convenient is the two-parametric Langmuir model:

$$G(t) = \frac{G_o \cdot k \cdot t}{1 + k \cdot t}. \tag{3.2}$$

It can be considered as an expansion of the exponential model (3.1) into Maclaurin series (see Chapter 1 of Addendum 2). Unlike the exponential model (3.1), the Langmuir model (3.2) can be easy transformed to the linear form (see Chapter 1 of Addendum 2):

$$Y = \frac{1}{G(t)} = \left(\frac{1}{G_o}\right) + \left(\frac{1}{G_o \cdot k}\right) \cdot \frac{1}{t} = a + b \cdot X. \quad X = \frac{1}{t}. \tag{3.3}$$

G_o and k values can be easy calculated from the parameters of the equation (3.3) after obtaining them by the least squares method [26]:

$$G_o = \frac{1}{a}, \quad k = \frac{a}{b}. \tag{3.4}$$

Equations of (3.1-3.4) are well established for a description of an active substance release from ointments and suppositories (see Chapter 1 of Addendum 2) as well as describing the dependence of solubility of substances on their particle size (see Chapter 2 Addendum 2). So it's only natural to assume that they are correct to describe the dissolution profiles of tablets within the bioequivalence studies *in vitro*.

It should be noted that the processing of experimental dependencies *G(t)* of dissolution degree on time by the exponential model (3.1) with the nonlinear least squares method (NLLSM) gives us immediately G_o and k values, their standard deviations $SD(G_o)$ and $SD(k)$, the residual standard deviation $SD_{rest}(G)$ of the experimental values $G_{exp}(t)$ around the calculated line $G_{calc}(t)$ and the correlation coefficient $R(G)$ in the terms of G. Note that just these values are of interest for the practical description of the experimental data.

At the same time, processing of experimental dependencies *G(t)* with the linearized Langmuir model (3.3) gives us coefficients a and b of the linear relationship (3.3), their standard deviations SD_a and SD_b, the residual standard deviation $SD_{rest}(1/G)$ of the experimental points (*1/G*) around the line (3.3) and the correlation coefficient $R(1/G)$ in terms of *1/G*. As can be seen, the parameters of the Langmuir model (3.3) cannot be compared with the appropriate parameters of the exponential model (3.1) that does not allow comparing the effectiveness of the experimental data descriptions by these various models.

To transform the Langmuir model (3.3) parameters to the same form as for the exponential model (3.1), get the equation (3.4) parameters G_o and k. Differentiate (as a function of several random variables) the ratios of (3.4), we can obtain the expression of the connection between $SD(G_o)$, $SD(k)$ and SD_a, SD_b (see Chapter 2 of Add. 2):

Langmuir model:

$$SD(G_o) = \frac{SD_a}{a^2}. \tag{3.5}$$

Langmuir model:

$$SD(k) = k \cdot \sqrt{\frac{SD_a^2}{a^2} + \frac{SD_b^2}{b^2}}. \tag{3.6}$$

Getting with the equations of (3.2, 3.4) the calculated G_{calc} values for all n time points and subtracting from them the experimental G_{exp} values, find the residual standard deviation of $SD_{rest}(G)$ in terms of G:

Langmuir model:

$$SD_{rest}(G) = \sqrt{\frac{(G_{calc} - G_{\exp})^2}{n-2}}. \quad (3.7)$$

Here 2 is a number of parameters of the line (3.3).

Find the correlation coefficient $R(G)$ of the line (3.3) in terms of G from the next equation [26]:

Langmuir model:

$$R(G) = \sqrt{1 - \frac{SD_G^2}{SD_{rest}(G)}}. \quad (3.8)$$

Here SD_G is a standard deviation.

The equations of (3.4-3.8) allow comparing the effectiveness of descriptions of experimental dissolution data with the exponential and Langmuir models.

3.2. Experiment

Choice of objects to study. It is reasonable to check applicability of ratios (3.1-3.4) for different ranges of dissolution time (t), dissolution degrees (G) and dissolution points (n), as well as for different formulations and doses of drug products and dissolution media. Suitable objects for this are the different technological compositions of levothyroxine sodium tablets, obtained during optimization of the drug product formulation to be bioequivalent to the original preparation (see Table 3.1). As can be seen from Table 3.1, the compositions meet all of these requirements.

Table 3.1

Objects of study

Composition number	Levothyroxine sodium dose, μ/tablet	pH of the dissolution medium	Range of dissolution time, t minute	Range of dissolution degree, G%	Number of points of dissolution curve, n
1	25	1.2	15-60	57.7 - 86.8	4
2	25	0.01 M HCl	15-60	99.6 - 104.8	4
3	25	4.5	300-480	55.2 - 67.6	4
4	25	1.2	15-120	50.6 - 83.8	5
5	25	6.8	15-120	42.9 - 70.9	5

6	150	6.8	15-120	45.9 - 80.5	5
7	25	6.8	15-90	41.2 - 67.5	6
8	150	1.2	15-90	49.8 - 84.2	6
9	25	4.5	15-120	31.3 - 56.9	7
10	150	4.5	15-480	27.6 - 72.9	11

Analytical procedure. As an analytical procedure in studying the dissolution profiles, we used the USP HPLC procedure for sodium levothyroxine tablets (test 3) [68]. Dissolution media (Table 3.1) and the number of test tablets (12) of each sample were selected in accordance with the SPU requirements [63, section 6.2.3].

Results. Dissolution profiles (curves) *G(t)* of dependence of the dissolution degree *G%* on time *t* (min) are presented in Table 3.2. This table shows also the absolute standard deviations SD_i (in absolute percentage), dissolution degrees *G* for each point (of 12 tablets), that allows to evaluate the statistical correctness of the obtained experimental *G* values. In addition, on the base of SD_i values, for each curve we also calculated the pooled standard deviation SD_{pool} with the ratio [26]:

$$SD_{pool} = \sqrt{\frac{\sum_{i=1}^{g} \nu_i \cdot SD_i^2}{\sum_{i=1}^{g} \nu_i}}. \qquad (3.9)$$

Here: ν_i is a number of freedom degrees, which in the case of SD_i values is equal to $\nu = g - 1 = 12 - 1 = 11$ for all compositions. In the case of pooling of the residual standard deviations SD_{rest} of different compositions, the number of degrees of freedom can be different for different compositions and is equal to $\nu_i = n_i - 2$, where *n* is a number of points of a dissolution curve.

The SD_{pool} values allow us to evaluate the statistical correctness of the used dissolution models (Langmuir and exponential), so they are also represented in the Table 3.3-3.4. The pooled mean values of all compositions, calculated by the ratio (3.9), are presented in the Tables 3.3-3.4 for SD_{pool} and SD_{rest} as well. In addition, the mean values of correlation coefficients *R(G)* for all compositions are given as well.

The results of experimental data processing for linearized Langmuir model (3.3) are presented in the Table 3.3. A typical line (3.3) is presented in the Figure 3.1.

Table 3.2

Experimental dissolution profiles for different compositions of levothyroxine tablets (*SD* – standard deviations of 12 tablets)

Compo-sition N	Dissolution degree *G* % for time *t* (minute)													SD_{pool}
t →	15	30	45	60	75	90	120	180	240	300	360	420	480	
1	51.7	69.9	82.1	86.8										
SD	5.9	5.0	4.0	4.2										9.6
2	99.6	100.9	104.0	104.8										
SD	3.0	1.6	1.5	0.7										2.7
3										55.2	62.5	65.2	67.6	
SD										3.5	2.2	1.4	1.2	2.6
4	50.6	71.6		80.6		81.7	83.8							
SD	1.9	1.8		3.5		2.0	3.1							2.8
5	42.9	58.5		66.9		70.1	70.9							
SD	3.0	2.5		2.4		1.9	1.9							2.3
6	45.9	61.5		72.3		75.2	80.5							
SD	3.4	1.9		1.3		1.8	1.6							1.9
7	41.2	52.3	58.3	64.2	66.4	67.5								
SD	3.4	2.2	2.7	2.4	3.9	3.5								2.8
8	49.8	65.6	73.6	77.9	82.7	84.2								
SD	5.6	4.0	2.2	1.9	3.2	2.0								3.0
9	31.3	41.7	47.7	50.6	51.9	53.9	56.9							
SD	4.1	3.1	2.3	1.9	1.4	1.5	1.1							2.1
10	27.6	43.7		55.9		62.3	64.9	69.5	69.7	70.2	71.6	72.2	73.0	
SD	2.1	1.9		0.6		2.5	1.7	1.2	1.9	1.1	1.7	1.7	1.8	1.8

Table 3.3

Results of experimental data processing on the Langmuir model the with least squares method

Compo-sition number	Parameters of the line $1/G = A + B\cdot(1/t)$						Calculated parameters of the relationship $G = G_o\cdot k\cdot t/(1+k\cdot t)$						
	A	SD_A	B	SD_b	SD_{rest}	R	$G_o = 1/A$	$SD(G_o)$	$k=A/B$	$SD(k)$	$SD_{rest}(G)$	$R(G)$	SD_{pool}
1	0.0089	0.00020	0.158	0.0050	0.0002	0.999	113.0	0.056	0.056	0.0022	1.1	0.997	4.8
2	0.0094	0.00012	0.0096	0.0039	0.0001	0.911	105.9	0.980	0.980	0.40	1.2	0.863	1.9
3	0.0091	0.00106	2.64	0.39	0.0004	0.978	110.0	0.003	0.003	0.00065	1.4	0.965	2.3
4	0.0104	0.00048	0.134	0.014	0.0024	0.984	96.1	0.077	0.077	0.0088	3.5	0.967	2.5
5	0.0124	0.00033	0.159	0.0094	0.0005	0.995	80.6	0.078	0.078	0.0051	1.7	0.989	2.3
6	0.0113	0.00017	0.156	0.0048	0.0002	0.999	88.8	0.072	0.072	0.0025	1.2	0.996	2.1
7	0.0130	0.00024	0.172	0.0072	0.0003	0.996	77.0	0.075	0.075	0.0035	1.1	0.994	3.1
8	0.0103	0.00007	0.0148	0.0022	0.0001	1.000	97.4	0.695	0.695	0.10	0.64	0.999	3.4
9	0.0158	0.00011	0.243	0.0037	0.0002	0.999	63.5	0.065	0.065	0.0011	0.50	0.998	2.4
10	0.0127	0.00022	0.341	0.0093	0.0006	0.997	78.8	0.037	0.037	0.0012	1.7	0.993	1.7
Pooled and mean values:											**1.6**	**0,976**	**2.8**

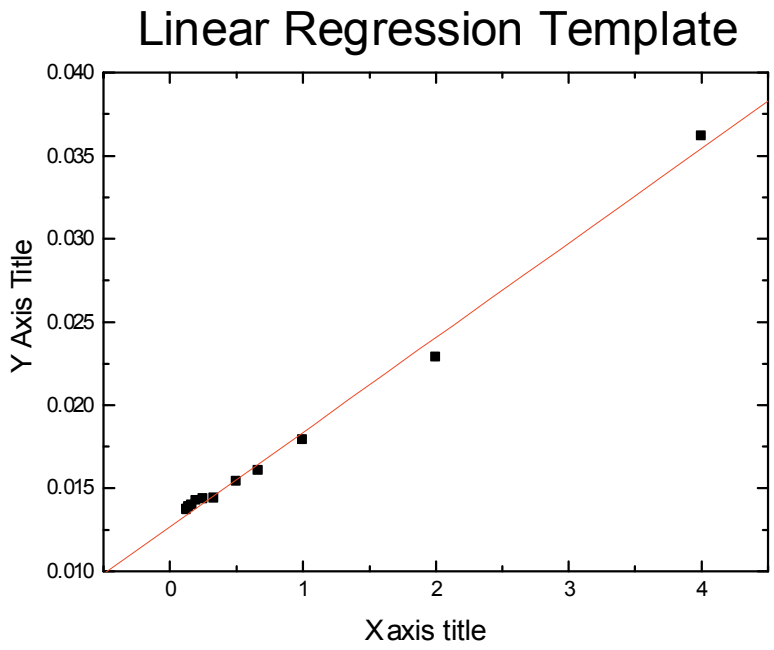


Figure 3.1. The typical linear regression for the linearized Langmuir model (3.3) for tablet composition N 10.

The parameters of the equation (3.3) have no interest per se. So we calculated values of G_o , k, $SD(G_o)$, $SD(k)$, $SD_{rest}(G)$ and $R(G)$ with the ratios (3.4-3.8), which are also presented in the Table 3.3. This allows us to evaluate the statistical correctness of the Langmuir model (3.2-3.3) and compare it with the exponential model (3.1).

The Table 3.4 illustrates the processing results for the exponential model (3.1) using the nonlinear least squares method (NLSM). It should be noted that the use of NLSM for the exponential model with small degrees of freedom (for 4 points the number of degrees of freedom is equal to 4-2 = 2) has certain difficulties with the volatility of calculations.

Table 3.4

The results of experimental data processing for the exponential model using a nonlinear least squares method

Object number	G_o	SD_{Go}	k	SD_k	SD_{rest}	R	SD_{pool}
1	89.4	2.4	0.055	0.005	2.0	0.992	4.8
2	103.4	1.2	0.22	0.04	2.0	0.601	1.9
3	80.0	4.5	0.0041	0.0006	1.2	0.974	2.3
4	82.9	0.6	0.064	0.002	1.0	0.997	2.5
5	70.0	0.7	0.062	0.003	1.1	0.996	2.3
6	77.1	1.9	0.056	0.005	2.9	0.978	2.1

7	66.3	1.7	0.057	0.006	2.5	0.968	3.1
8	82.5	1.7	0.056	0.005	2.6	0.980	3.4
9	54.3	1.1	0.051	0.004	1.9	0.977	2.4
10	70.2	0.9	1.77	0.12	2.4	0.986	1.7
Pooled and mean values:					**2.2**	**0.945**	**2.8**

3.3. Results and discussion

According to the SPU requirements [63], the standard deviations from the mean, as a percentage of the nominal content of the analyte in the dosage unit, should be not more than 20% for the first point in the dissolution kinetic curve and not more than 10% for all other control points. As can be seen from the Table 3.2, these requirement swith a large reserve is performed on all points and all studied compositions, indicating compliance of the received kinetic curves with the SPU requirements.

The Table 3.3 shows, that the residual standard deviations SD_{rest} of the Langmuir model for almost all compositions are significantly less SD_{pool}. This is especially evident on the mean values: SD_{rest} = 1.6% is 1.8 times lower than SD_{pool} = 2.8%. That illustrates the statistical accuracy of the Langmuir model, as the scatter of points around the curve does not exceed the statistical uncertainty of individual values (G). In all cases (different compositions, dissolution media, time ranges and degrees of dilution) we have high correlation coefficients $R(G)$ (above 0.965) and low residual standard deviations $SD_{rest}(G)$ (usually below 1.7%). It confirms the adequacy of the description of the kinetic dissolution curves with the Langmuir model. Some worse correlation coefficient value (0.863) is observed for the formulation 2. This is due to the small number of freedom degrees (4-2 = 2) and to the very narrow interval of G value variation - from 99.6 to 104.8% (see the Table 3.1). As it follows from the expression of (3.8) for the correlation coefficient $R(G)$, it leads to a reduction of $R(G)$.

The same can be said about the exponential model (see the Table 3.4). The residual standard deviations SD_{rest} are usually significantly lower than SD_{pool} and SD_{rest}; a combined value for SD_{rest} (2.2%) is 1.3 times less than combined value for SD_{pool} (2.8%).

It is of interest a comparison of the Langmuir and exponential models. For this purpose it is convenient to use the pooled values on all compositions. As can be seen from the comparison of the Table 3.3-3.4, the residual standard deviation for the Langmuir model (1.6%) is 1.3 times less than for the exponential model (2.2%) and the mean correlation coefficient (0.976) is significantly higher than for the exponential model (0.945). Thus, it can be said that to describe the dissolution profiles in the *in vitro* bioequivalence study is preferable to use a simple Langmuir model compared to the exponential model.

Conclusions

The dissolution profiles in the *in vitro* bioequivalence study are well described with the two-parametric exponential and Langmuir models. It is shown the adequacy of the description with them of the experimental dissolution profiles for different compositions of levothyroxine tablets, dissolution media, time ranges and dissolution degrees. It is shown that the simple Langmuir model gives better results than the exponential one.

Table 3.2

Experimental dissolution profiles for different compositions of levothyroxine tablets (*SD* – standard deviations of 12 tablets)

Composition N	Dissolution degree *G* % for time *t* (minute)													SD_{pool}
t →	15	30	45	60	75	90	120	180	240	300	360	420	480	
1	51.7	69.9	82.1	86.8										
SD	5.9	5.0	4.0	4.2										9.6
2	99.6	100.9	104.0	104.8										
SD	3.0	1.6	1.5	0.7										2.7
3										55.2	62.5	65.2	67.6	
SD										3.5	2.2	1.4	1.2	2.6
4	50.6	71.6		80.6		81.7	83.8							
SD	1.9	1.8		3.5		2.0	3.1							2.8
5	42.9	58.5		66.9		70.1	70.9							
SD	3.0	2.5		2.4		1.9	1.9							2.3
6	45.9	61.5		72.3		75.2	80.5							
SD	3.4	1.9		1.3		1.8	1.6							1.9
7	41.2	52.3	58.3	64.2	66.4	67.5								
SD	3.4	2.2	2.7	2.4	3.9	3.5								2.8
8	49.8	65.6	73.6	77.9	82.7	84.2								
SD	5.6	4.0	2.2	1.9	3.2	2.0								3.0
9	31.3	41.7	47.7	50.6	51.9	53.9	56.9							
SD	4.1	3.1	2.3	1.9	1.4	1.5	1.1							2.1
10	27.6	43.7		55.9		62.3	64.9	69.5	69.7	70.2	71.6	72.2	73.0	
SD	2.1	1.9		0.6		2.5	1.7	1.2	1.9	1.1	1.7	1.7	1.8	1.8

Table 3.3

Results of experimental data processing on the Langmuir model the with least squares method

Compo-sition number	Parameters of the line $1/G = A + B\cdot(1/t)$						Calculated parameters of the relationship $G = G_o\cdot k\cdot t/(1+k\cdot t)$						
	A	SD_A	B	SD_b	SD_{rest}	R	$G_o = 1/A$	$SD(G_o)$	$k=A/B$	$SD(k)$	$SD_{rest}(G)$	$R(G)$	SD_{pool}
1	0.0089	0.00020	0.158	0.0050	0.0002	0.999	113.0	0.056	0.056	0.0022	1.1	0.997	4.8
2	0.0094	0.00012	0.0096	0.0039	0.0001	0.911	105.9	0.980	0.980	0.40	1.2	0.863	1.9
3	0.0091	0.00106	2.64	0.39	0.0004	0.978	110.0	0.003	0.003	0.00065	1.4	0.965	2.3
4	0.0104	0.00048	0.134	0.014	0.0024	0.984	96.1	0.077	0.077	0.0088	3.5	0.967	2.5
5	0.0124	0.00033	0.159	0.0094	0.0005	0.995	80.6	0.078	0.078	0.0051	1.7	0.989	2.3
6	0.0113	0.00017	0.156	0.0048	0.0002	0.999	88.8	0.072	0.072	0.0025	1.2	0.996	2.1
7	0.0130	0.00024	0.172	0.0072	0.0003	0.996	77.0	0.075	0.075	0.0035	1.1	0.994	3.1
8	0.0103	0.00007	0.0148	0.0022	0.0001	1.000	97.4	0.695	0.695	0.10	0.64	0.999	3.4
9	0.0158	0.00011	0.243	0.0037	0.0002	0.999	63.5	0.065	0.065	0.0011	0.50	0.998	2.4
10	0.0127	0.00022	0.341	0.0093	0.0006	0.997	78.8	0.037	0.037	0.0012	1.7	0.993	1.7
										Pooled and mean values:	**1.6**	**0,976**	**2.8**

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STANDARDIZED VALIDATION SCHEMES FOR DRUG QUALITY CONTROL PROCEDURES

2nd Edition

Translated from Russian

Gryzodub Oleksandr Ivanovich (27.12.1948). Doctor of Chemical Sciences (1990), professor (1996), director of the Pharmacopoeial Centre (Ukraine). Author over 350 scientific publications. Main scientific directions: standardization, analysis and quality co0ntrol of medicines. One of the principal ideologists of the National system of medicines standardization in Ukraine. Scientific supervisor of the Ukrainian State Pharmacopoeia development.

This monograph presents a systematic consideration of the theoretical basis of the standardized schemes for drug quality control procedures, as well as the specific features of their application to all basic quantitative pharmacopoeial tests: assay, related substances control, residual solvents control, “Dissolution”, “Content uniformity”, *in vitro* bioequivalence study. There are used the main pharmacopoeial analytical methods: UV-VIS spectrophotometry, liquid and gas chromatography, atomic absorption spectrophotometry, titration.

The standardized schemes are developed for all basic options of standardization: reference standard method, calibration graph method, standard addition method, specific absorbance method.

The specificity of validation of quality control procedures of summarized drugs are discussed as well.

All developed standardized procedures are illustrated by examples of their application to validation of quality control procedures of real pharmaceutical objects.

СТАНДАРТИЗОВАННЫЕ ПРОЦЕДУРЫ ВАЛИДАЦИИ МЕТОДИК КОНТРОЛЯ КАЧЕСТВА ЛЕКАРСТВЕННЫХ СРЕДСТВ

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В данной монографии представлено систематическое изложение теоретической базы стандартизованных процедуры валидации методик контроля качества ЛС, а также специфика их применения для всех основных количественных фармакопейных испытаний: количественное определение, контроль сопутствующих примесей, контроль остаточных растворителей, тест «Растворение», «Однородность содержания», исследование биоэквивалентности “in vitro”. При этом используются основные фармакопейные методы анализа: спектрофотометрия в ультрафиолетовой и видимой областях, жидкостная и газовая хроматографии, атомно-абсорбционная спектрофотометрия, титрование. Все разработанные стандартизованные процедуры иллюстрируются примерами их применения к валидации методик контроля качества реальных фармацевтических объектов.