MINISTRY OF HEALTH OF THE REPUBLIC OF MOLDOVA

NICOLAE TESTEMIȚANU STATE UNIVERSITY OF MEDICINE AND PHARMACY

Faculty of Pharmacy

Department of Pharmaceutical and Toxicological Chemistry

Livia UNCU, Elena DONICI

VALIDATION OF METHODS OF ANALYSIS

Methodical recommendation

Chisinau, 2023

MINISTRY OF HEALTH OF THE REPUBLIC OF MOLDOVA

***NICOLAE TESTEMITANU* STATE UNIVERSITY OF MEDICINE AND PHARMACY**

Faculty of Pharmacy

Department of Pharmaceutical and Toxicological Chemistry

Livia UNCU, Elena DONICI

VALIDATION OF METHODS OF ANALYSIS

Methodical recommendation

SRL "Foxtrot"
Chisinau, 2023

Approved by the Council of the Quality Management of
Nicolae Testemițanu SUMPh
(Minutes No. 7 of 30.06.2023)

Authors:
Livia Uncu, PhD, associate professor
Elena Donici, PhD, university assistant

Reviewers:
Valica Vladimir, dr. hab. pharm. sc., university professor, head of the Department of Pharmaceutical and Toxicological Chemistry, *Nicolae Testemițanu* SUMPh;
Treapițîna Tatiana, PhD, associate professor, Department of Pharmaceutical and Toxicological Chemistry, *Nicolae Testemițanu* SUMPh.

Under the editorship of the author: Livia Uncu

Tipărit la: SRL "Foxtrot"

DESCRIEREA CIP A CAMEREI NAȚIONALE A CĂRȚII DIN REPUBLICA MOLDOVA

Uncu, Livia.
Validation of methods of analysis: Methodical recommendation / Livia Uncu, Elena Donici; under the editorship: Livia Uncu; Ministry of Health of the Republic of Moldova, Nicolae Testemitanu State University of Medicine and Pharmacy, Faculty of Pharmacy, Department of Pharmaceutical and Toxicological Chemistry. – Chişinău: [S. n.], 2023 (Foxtrot). – 48 p.: tab.
Bibliogr.: p. 40-44 (30 tit.). – [100] ex.
ISBN 978-9975-89-281-0.
615.3:543(076)
U 46

CONTENT

PREFACE 4

ABBREVIATIONS 5
INTRODUCTION 6

SUBJECTS FOR THE INDIVIDUAL PREPARATION OF THE STUDENT BASED ON THE THEORETICAL MATERIAL 8
INFORMATIONAL MATERIAL 9
The process of validation of an analytical method. General aspects 9

Validation parameters of a method of analysis 10

Methodological peculiarities of validation parameters 17

Interpretation of the statistical results of the validation. Calculation methods in the validation process 22
Peculiarities of validation of some common methods applied in drug analysis 25
The practical approach of a general protocol for validation of an analytical method 30
TASKS FOR INDIVIDUAL WORK 37

BIBLIOGRAPHY 40

Annex 1. Work sheet. Validation of an assay method. Results of the "Linearity" parameter study 45
Annex 2. Work sheet. Validation of an assay method. Results of the "Accuracy" parameter study 47
Annex 3. Work sheet. Validation of an assay method. Results of the "Precision" parameter study 48
Annex 4. Work sheet. Validation of a dosing method. Results of the study of the parameter "Robustness" 48

PREFACE

The development of analytical methods with applicability to medicines involves instantly verifying and confirming the fact that the method (or analytical procedure) corresponds exactly to the purpose for which it was designed/developed. In other words, an analytical method must be validated. The concept of validation can also be interpreted as a methodology of scientific accreditation of an analysis method. The main purpose of the validation is to select the best and most accessible methods of detection and dosing of a drug substance/pharmaceutical impurity/degradation products. The validation process is based on objective data and performance, all determinations being made in strict accordance with the regulations in the field of drug analysis and control.

The methodological recommendation Validation of analysis methods is designed as teaching material for students of the study program 0916. Pharmacy, residents of pharmaceutical specializations, master's students, doctoral students, pharmacists, but it can also be a support for specialists in related fields or anyone interested in the field of validation of analysis methods.

Authors.

ABBREVIATIONS

ed.	– edition
etc.	– etcetera
exp.	– experimental
FDA	– Food and Drug Administration
GLP	– Good Laboratory Practice
HPLC	–High Performance Liquid Chromatography
ICH	–International Harmonization Conference
ISO	– International Organization for Standardization
min.	– minute(s)
UV-Vis	– ultraviolet-visibile

INTRODUCTION

The importance of validating an analytical procedure is defined by the assurance of obtaining reliable and repeatable results for routine and stability analysis.

From an ethical point of view, the validation of a method is important because patients trust the results obtained in the laboratory by analyzing a drug that they are not in the power to do themselves. Analytical pharmacists, on the other hand, in the process of validating a method, will apply all aspects of science to obtain reliable results.

Likewise, from a commercial point of view, it is very important to be sure that a method will give accurate and precise results before it is carried out. However, there is also a shortcoming of the validation of an analysis method in this regard: errors may be detected after the measurements are made and the experiments will need to be repeated. In the commercial environment, where the manufacturer has the duty to ensure the quality of the drug before it is released to the consumer, validation takes on part of this responsibility.

In some areas, validation of methods according to GLP is a regulatory requirement and is mandatory for certain types of research. Thus, it is mandatory to validate accredited methods according to the ISO standard. Evaluating the performance parameters of a method during the validation process provides data that shows which parts of the method are stable as well as where the weak points are. Validation helps design and implement appropriate quality control procedures. The data obtained from the validation of a method provide

information that allows the comparability of the results of the samples analyzed in different laboratories.

Purpose: to be able to define the principles of the validation of an analysis method and acquaint the general working methodologies.

Recommended minimum duration

To study the topic, 2 laboratory works (6 hours) are given.

Stages of studying the theme:

1. Theoretical preparation for the fulfillment of the determined goals.
2. Carrying out practical assignments.
3. Current knowledge assessment.

Objectives of the theme:

1. On the basis of consulting the course support and specialized literature to acquire the general notions regarding the validation of an analysis method;
2. Knowledge of the general aspects of the validation process of an analytical method;
3. Knowledge and appreciation of the validation parameters of an analysis method;
4. Preparation of a validation report.

SUBJECTS FOR THE INDIVIDUAL PREPARATION OF THE STUDENT BASED ON THE THEORETICAL MATERIAL

1. Definition of validation of an analytical method. Specify the validation parameters.
2. The role of validation in pharmaceutical analysis.
3. Specificity – validation parameter. Definition, characterization and application.
4. Linearity – validation parameter. Definition, characterization and application.
5. Accuracy – validation parameter. Definition, characterization and application.
6. Accuracy – validation parameter. Definition, characterization and application.
7. Detection limit – validation parameter. Definition, characterization and application.
8. Lower limit of quantification – validation parameter. Definition, characterization and application.
9. Robustness – validation parameter. Definition, characterization and application.
10. Preparation of a validation report.

INFORMATIONAL MATERIAL

The process of validation of an analytical method. General aspects

Validation of an analytical method, according to the United States Pharmacopoeia 31, is "the process of determining through laboratory studies whether that method meets the conditions for the analytical applications for which it was developed."

Thus, the validation process has the role of verifying and establishing which are the limits of variability between which the method will present specific, accurate and precise results.

In the field of pharmaceutical analysis, the process of validating a method is regulated in validation guidelines. For the first time, in 1987 the FDA issued practical guidelines on the main principles of validation and on the presentation of samples and data analyzes related to the validation of methods. In 1993, within the ICH guide, the first generalized recommendations regarding the validation of analysis methods appeared, these documents being published in 1994 and treated in more detail in 1995.

Currently, the process of validating an analysis method is regulated in the following normative acts:

- ICH Q2R1 guide: for analytical procedures and validation; The ICH Q2R2 guideline is under approval, available for discussion;
- European Pharmacopoeia, ed. the eleventh;
- Pharmacopoeia of the United States of America: 1225 Validation of compendial methods;
- Food and Drug Administration of the United States of America: Guide "Validation of methods of analysis for drugs and biological

substances";

- Center for the Evaluation and Research of Medicines: Guide "Validation of a Bioanalytical Method".

The ICH quality guidelines (The International Conference of Harmonization of Technical Requirements) reflect a harmonized approach to ensuring the quality control of medicines. They complement and explain the requirements of the Pharmacopoeia monographs. Guidelines Q2(R1) and Q2(R2) Validation of Analytical Procedures/Validation of Analytical Procedures) identify the validation parameters required for a variety of analytical methods. It also describes the characteristics that must be taken into account when validating analytical procedures.

Validation parameters of a method of analysis

Any newly developed analysis method must be validated, but also those that have already been developed and included in pharmacopoeias or other recognized analytical documents, but have undergone small changes in the working technique, also need to be revalidated. Thus, it must be checked in real conditions if an analysis method will give precise and accurate results using well-documented normative acts. When carrying out the process of validation or revalidation of a method, the parameters included in the ICH guidelines, which address the validation of analysis methods, will be taken into account first of all (figure 1).

The choice of validation parameters to be tested is influenced by:

- the purpose of analytical measurements (dosage of a medicinal substance, impurity determinations, stability studies, determinations of biological environments, etc.);
- analytical procedure;
- the nature and concentration of the analyte;
- the nature of the matrix;
- regulations in the field.

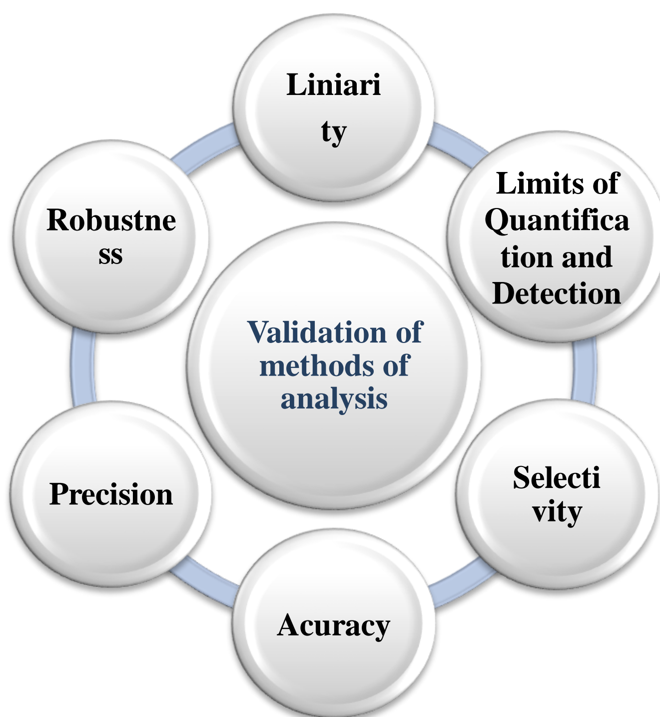

Figure 1. Validation parameters of an analytical method

Table 1 lists the most important parameters for the validation of different types of analytical procedures according to ICH Guideline Q2(R1). The same parameters will be used in the case of revalidation, which may be required in the following circumstances:

- changes in the synthesis of the medicinal substance;
- changes in the composition of the medicine;
- changes in the analytical method.

Table 1. Validation parameters for different types of analysis methods

Analysis methods/ Validation parameters	Identification method	Impurity determination method		Assay method
		Identification of impurities	Quantitative determination of impurities	
Specificity	+	+	+	+
Accuracy			+	+
Precision: Repeatability Intermediate precision			+ + (1)	+ + (1)
Limit of Detection	+	+	(2)	+ (3)
Limit of Quantification			+	+ (3)
Linearity			+	
Robustness	+	+	+	+

"+" Mandatory
(1) In cases where the reproducibility parameter will be achieved, the intermediate precision it's not necessary;
(2) The detection limit in some situations may be necessary;
(3) If the method is developed at low concentrations, towards the detection limit of the method, it is mandatory to determine the detection limit and the lower limit of quantification. If methods are applied at high concentrations, then these two parameters do not have to be determined.

Specificity. In the ICH Q2(R1) Guide, specificity is defined as follows: "the ability of the method to evaluate the substance to be analyzed in the presence of other compounds that may be present, such as: impurities, excipients, etc." This parameter is used both in the methods intended for the identification and for the determination of the purity and quantitative content of a medicinal substance:

- in the case of an identification method, specificity aims to demonstrate that the method is capable of detecting the medicinal substance with certainty, even in the presence of specific and very similar impurities according to the chemical structure;
- in the case of a method used for the purpose of determining purity, the specificity comes to ensure the purity of an active substance, by detecting and measuring the exact amount of chemically related impurities, solvents, inorganic impurities, etc., if they are present in the medicinal substance;
- for the quantitative determination of a medicinal substance, specificity is a parameter that contributes to demonstrating that the method will be able to detect with certainty and quantify with accuracy and precision the medicinal substance in the presence of other substances such as: excipients, solvents, possible impurities, within accessible limits, etc.

The linearity of an analysis method is a parameter that indicates the possibility of the method to obtain results directly proportional to the concentration of the medicinal substance. Linearity is important for the quantitative determination of an active substance or an

impurity, as well as in the case of the dissolution test within a certain well-defined range, since for each batch of medicine the content of the active substance and potential impurities may vary slightly, which is why concentrations above and below that expected value must be determined correctly.

Linearity should be evaluated as follows:

• at least 5 analyte concentrations are used, each concentration being carried out and analyzed at least 3 times, and the results obtained must be analyzed statistically, most often by performing regression analysis using the least squares method;

• **r** (correlation coefficient) to be between 0.99 and 1.00 (preferably higher than 0.999, but this criterion is not sufficient);

• the linear regression graph (calibration or calibration right) being an average, then the coefficient of variation % of the points compared to the regression line must be lower than a limit value imposed by the regulations in the field; for example in pharmaceutical analysis it is 2.0% at the lower limit of quantification;

• the residuals (the difference between the concentration calculated from the regression line and the theoretical concentration, %) must not have a tendency to increase or decrease with increasing concentration and be randomly distributed in a certain range from 0; also, their values must fall within the limits imposed by the regulations in the field.

In a linear regression line $y = mx + c$, the regression coefficient is the constant "m" which represents the rate of change of one variable "y" as a function of the change in the other variable "x" (hence the

slope), while "c" is the Y-intercept. Thus, m is the slope and c is the intercept of the ordinates.

Precision (fidelity) is a validation parameter that represents the degree of agreement between test results when the method is repeated several times. Precision is usually expressed as the standard deviation or relative standard deviation (coefficient of variation) of a series of measurements and should be no more than 2.0%.

The precision of a method is of three types, and for each type, the standard deviation, relative standard deviation, and confidence interval are determined:

- *repeatability,* which is performed by repeating the method in a laboratory over a short period of time, using the same analyst with the same equipment. Repeatability should be assessed using at least nine determinations covering the range of the method. Thus, as a rule, it is done on three concentrations and three replicates of each concentration or using at least six determinations at 100% of the sample concentration;
- *intermediate precision,* which is the result of within-laboratory variations due to random events such as different days, different analysts, different equipment, etc.
- *reproducibility,* which expresses the precision between laboratories, being demonstrated through an interlaboratory study.

The accuracy of an analytical method is the closeness of the results obtained by that method to the actual or true value. It is recommended that accuracy be determined using a minimum of 9

determinations at at least 3 concentration levels covering the specified range: 3 concentrations of 3 replicates each.

The detection limit is a validation parameter that is determined by analyzing samples of known analyte concentration and establishing that minimum level at which the analyte can be detected, but not necessarily quantified as a precise value, under the previous experimental conditions. Usually, the limit of detection is expressed in the unit of measurement of the concentration of the medicinal substance in the sample.

Depending on the device used for analysis, the nature of the medicinal substance and the nature of the method, there are a number of possibilities for determining the detection limit:

- visual observation;
- signal-noise ratio;
- the standard deviation of the answer;
- the standard deviation of the slope of the linearity graph.

To determine the detection limit by using the value of the standard deviation of the slope of the calibration line, which was built to the linearity parameter, the formula for calculating the ratio of the standard deviation of the intercepts of the calibration curves to the slope of the calibration line is applied.

The lower limit of quantification is a characteristic of the validation process of an analytical method, which signifies the lowest drug concentration in a sample with which the corresponding precision and accuracy are estimated under the stated experimental conditions.

Similar to the limit of detection, the following methods are recommended for estimating the limit of quantification:

- visual assessment;
- signal-noise ratio;
- the standard deviation of the answer;
- the standard deviation of the slope of the linearity graph.

To determine the quantification limit by using the value of the standard deviation of the slope of the calibration line, which was built to the linearity parameter, the formula for calculating the ratio of the standard deviation of the response to the mean of the slopes of the calibration curves is applied.

Robustness is a parameter that measures the ability of an analysis method to remain unchanged to small changes in method parameters. The variable parameters of the method are different, depending on the type of method that needs to be validated, and for their selection the work technique will be taken into account.

Methodological peculiarities of validation parameters

Specificity. Although this parameter was initially intended for studies evaluating the purity and determining the quality of drugs or active substances, it is now also mandatory for quantitative research. To check specificity the placebo solution is measured in the same way as the standard solvent solutions. The placebo solution contains the auxiliary substances in the pharmaceutical form, but without the active substance(s). Solvent standard solutions are the standard solutions obtained with the reference substance in the appropriate solvent.

Linearity represents the most important stage of the validation of a dosing method and expresses the proportionality of the instrumental signal with the amount existing in the sample, over a range of concentrations where accuracy, precision and linearity are acceptable. The range of concentrations in which the linearity is checked is usually the range of concentrations in which the samples to be analyzed are expected to fall. Work in parallel with solvent standard solutions (standard solutions obtained with the reference substance in the appropriate solvent) and labeled placebo solutions (standard solutions obtained from placebo samples labeled with the reference substance) obtained on different days or by different analysts. Work at a minimum of 5 concentration levels (k=5 groups), each concentration being performed and analyzed 3 times (n=3) by different analysts or on 3 different days for the same concentration. Thus, the total number of realizations for k=5 and n=3 is equal to 15 for the standard solvent solutions as well as for the labeled placebo solutions, totaling 30 results.

Accuracy expresses the closeness of the result obtained with that method to the true (theoretical) value. It can be determined by serially marking standard samples of concentrations in the range considered for linearity testing with known amounts of analyte of a placebo (an artificially created matrix that reproduces the analyte matrix in the samples to be analyzed). Work is done at 5 concentration levels around the value of interest, each concentration level being performed at least 3 times. It is expressed as the recovery of the analyte from the

reconstituted pharmaceutical form, using as a reference system the calibration curve obtained with standard solutions.

The precision is verified starting from determinations performed both on labeled placebo solutions (standard solutions obtained from placebo samples labeled with reference substance) and on standard solutions in solvent (standard solutions obtained with the reference substance in the appropriate solvent). We work at a concentration level that would result for the solution obtained by processing the drug sample (100% concentration level – corresponding 100% to the concentration level of the sample, also known as the concentration level expected in the sample to be analyzed).

To check the repeatability, n=6 solutions are prepared and analyzed by k=3 different analysts or on k=3 different days, both as standard solutions in solvent and as labeled placebo solutions.

Limit of Detection is possible to be determined by several methods, depending on the type of method:

a. *Visual assessment* can be used for both non-instrumental and instrumental methods by analyzing samples with known analyte concentrations to establish the minimum level at which the analyte can be reliably detected.
b. *Signal-to-noise ratio.* This method can only be applied to analytical procedures that exhibit baseline noise. Determination of the signal-to-noise ratio is performed by comparing the measured signals from samples with known low concentrations of analyte to those of blank samples and establishing the minimum concentration at which the analyte

can be reliably detected. A signal-to-noise ratio between 3 or 2:1 is generally considered acceptable for estimating the detection limit.

c. *The standard deviation of the response and the standard deviation of the slope of the linearity plot.* The detection limit can be calculated by the calculation formula (1):

$$LOD = \frac{3{,}3 \cdot SD}{a}, \quad \text{in which:} \tag{1}$$

LOD – Limit of Detection;

SD – the Standard Deviation of the intercept;

a – the slope of the calibration line.

The quantitation limit can be determined by the same methods as the detection limit:

a. *Visual assessment* can be used for non-instrumental methods, but also for instrumental ones. The lower limit of quantification is generally determined by analyzing samples with known analyte concentrations and establishing the minimum level at which the analyte can be quantified with acceptable accuracy and precision.

b. *Signal-to-noise ratio.* As with the determination of the Limit of Detection, this method can only be applied to analytical procedures that exhibit baseline noise. The determination of the signal-to-noise ratio is performed by comparing the signals measured from samples with known low concentrations of analyte to those of blank samples and establishing the minimum concentration at which the analyte

can be reliably quantified. A typical signal-to-noise ratio is 10:1.

c. *The standard deviation of the response and the standard deviation of the slope of the linearity plot.*

The quantitation limit was determined using the calculation formula (2):

$$QL = \frac{10 \cdot SD}{a},$$ in which: (2)

QL – quantitation limit;

SD – the standard deviation of the intercept;

a – the slope of the calibration line.

Robustness. A consequence of the robustness assessment should be the establishment of a series of system suitability parameters (eg resolution test) to ensure that the validity of the analytical method is maintained whenever it is to be used.

Examples of typical variations are:

- stability of analytical solutions;
- extraction time.

Some examples of typical variations for liquid chromatography can be:

- the influence of pH variations in a mobile phase;
- the influence of variations in the composition of the mobile phase;
- different columns (different lots and/or suppliers);

- temperature;
- the debit.

Some examples of typical variations for gas chromatography can be:

- different columns (different batches and/or suppliers);
- temperature;
- the debit.

Interpretation of the statistical results of the validation. Calculation methods in the validation process

Specificity. The method is considered specific if the placebo solution does not show a measurable signal, for example, in the UV-Vis spectrophotometric method, there is no absorbance at the maximum absorption wavelength of the analyte, and in chromatographic methods, there is no interference at the retention time of the substance to be analyzed.

Linearity. The characteristics of the calibration lines are determined for:

- right obtained with labeled placebo solutions (standard solutions obtained from placebo samples labeled with reference substance);
- right obtained with standard solutions.

Next step, the following is determined:

1. The Pearson correlation coefficient, which must be greater than 0.99, demonstrating that the line approximates a linear variation of the analytical signal with concentration.

2. Application of test **C** to verify the homogeneity of the dispersions between the 5 concentration levels for each calibration line. If $C_{calculated} < C_{theroretical}$, then the variances of the groups of determinations are homogeneous, with a risk of 5%.
3. The application of the **F** test for:
   a) checking the validity of the regression line (compare the adjustment errors with the experimental ones). If Fcalculated < Ftheoretical, then Fcalculated is not significant and the adjustments are valid at the 5% risk level, the right being valid;
   b) testing the existence of a significant slope (compares the variations due to the regression with the fitting and experimental errors). The slopes of the 2 lines must be significant (Y must increase significantly with the increase of X, the slope must not tend to 0). If $F_{calculated} > F_{theoretical}$, then $F_{calculated}$ is significant. The slope will characterize a linear dependence with a risk of 5%.
4. The application of the **t** test for:
   a) comparing the slopes of the 2 straight lines. If $t_{calculated} < t_{theoretical}$, then the 2 slopes do not differ significantly, at a 5% risk level;
   b) comparing the ordinates at the origin of the 2 lines. If tcalculated < ttheoretical, then the 2 ordinates at the origin do not differ significantly, at a 5% risk level.

Accuracy is checked by:

1. test **C**, with the help of which the homogeneity of the finding in the 3 groups of determinations obtained on the samples with marked

placebo is tested (standard solutions obtained from placebo samples marked with reference substance). The variances of the different groups are homogeneous at a significance threshold of 5%, if $C_{experimental} < C_{critical}$;

2. the **F** test, with the help of which the retrieval average is validated. If $F_{experimental} < F_{critical}$, then the mean accuracy (retrieval) of the method is valid at a significance limit of 5%;
3. the calculation of the average relative recovery by which the percentage accuracy is expressed, which must be between the limits of 98% and 102%.

Precision is checked by:

1. test **C**, with the help of the development, the homogeneity of the finding is tested in the 3 groups of determinations obtained on the samples with marked placebo (standard solutions obtained from placebo samples marked with reference substance) and standard solutions in solvent. The variances of the different groups are homogeneous at a significance threshold of 5%, if Cexp < Ccritic;
2. calculation of the coefficient of variation of repeatability and intermediate precision according to the calculation formulas (3, 4):

$$CVr\% = \frac{S_r}{\bar{X}} \cdot 100\% , \qquad \text{in which:} \qquad (3)$$

CVr – coefficient of variation of repeatability, %;

S_r^2 – repeatability variance (within a group of determinations);

$\bar{\bar{X}}$ – average of all determinations.

$$CVR\% = \sqrt{\frac{S_r^2 + S_g^2}{\overline{\overline{X}}}} \cdot 100\% , \qquad \text{in which:} \qquad (4)$$

CVR – the coefficient of variation of the intermediate precision, %;

S_r^2 – repeatability variance (within a group of determinations);

S_g^2 – the variance between groups of determinations;

$\overline{\overline{X}}$ – average of all determinations.

The coefficient of variation of repeatability and intermediate precision must not exceed the limit value of 2%.

Robustness is a parameter that measures the ability of an analysis method to remain unchanged to small changes in method parameters. The variable parameters of the method are different, depending on the type of method that needs to be validated, and for their selection the work technique will be taken into account.

Peculiarities of validation of some common methods applied in drug analysis

UV-Vis spectrophotometry

Validation of the UV-Vis spectrophotometric method is essential to ensure the accuracy and reliability of the results obtained with this analytical technique. Here are some of the validation features of the UV-Vis spectrophotometric method:

- *Linearity*. It is verified that the method has a linear relationship between the concentration of the substance of interest and the

measured absorbance or transmittance. This is done by constructing a calibration curve using reference solutions with known analyte concentrations.

- *Reproducibility and precision*. Evaluation of the ability of the method to provide consistent and accurate results under repetitive conditions. This involves repeatedly measuring the same samples and calculating precision errors.
- *Limit of detection and limit of quantification*. It is determined to establish the lowest analyte concentrations that can be accurately detected and quantified using the UV-Vis method.
- *Specificity*. It is determined whether the method can distinguish the analyte from other substances that may be present in the sample. This may require testing for potential interference.
- *Accuracy*. It is determined how close the measured values are to the actual concentration values. This is usually done by reference sample analysis or by using certified samples.
- *Stability of the solution*. It is checked how stable the analytical solution is over time, especially if measurements are made over several hours.
- *Robustness*. The ability of the method to provide valid results in the face of minor variations in experimental conditions, such as changes in temperature or pH, is verified.
- *Response to spectral lines*. It is determined that the detector and light source used in the UV-Vis instrument are properly

calibrated and cover the range of wavelengths relevant to the analysis.

- *Adequate documentation.* Detailed recording and documentation of all validation steps, as well as standard operating procedures, is essential to ensure reproducibility and verifiability of results.
- *Uncertainty assessment.* Determine the uncertainty associated with measurements made using the UV-Vis method to assess the accuracy of the results.

HPLC method

The HPLC method is widely applied in drug analysis. For its validation, basic parameters are recommended, such as:

- *Specificity.* One of the most important features of HPLC validation is ensuring the specificity of the method. This implies the ability of the method to separate and identify compounds of interest from a complex mixture. It is verified that the method can accurately distinguish the analyte from other co-eluted substances.
- *Linearity.* It is verified that the response of the HPLC detector is proportional to the concentration of the analyte. This is done by measuring the response of the detector to known analyte concentrations and creating a linear calibration curve.
- *Precision.* It refers to the reproducibility of the results. This can be assessed by measuring the standard deviation of multiple replicates of the same sample or by determining the intermediate precision and repeatability precision.

- *Limit of detection and limit of quantification*. These values represent the lowest analyte concentrations that can be reliably detected and quantified by the HPLC method. Determining these limits is essential to assess the sensitivity of the method.
- *Accuracy*. It is checked how close the measured values are to the actual value of the analyte concentration. This can be done by analyzing reference samples or by comparing with other methods of analysis.
- *Repeatability and reproducibility*. Repeatability refers to precision within the same laboratory, using the same method and equipment, while reproducibility refers to precision between different laboratories.
- *Stability of solutions and equipment*. The stability of the test solutions and equipment over time is checked to ensure that there are no significant changes that may affect the results.
- *Robustness*. The ability of the method to produce consistent results despite small variations in experimental conditions, such as variations in temperature or solvent flow, is verified.
- *Interference*. Any possible interferences from other substances or impurities that may affect the results of the analysis are identified.
- *Adequate documentation*. Detailed records of all validation procedures, data obtained and analysis protocol are made to demonstrate method compliance with quality requirements and applicable regulations.

Validation of a titrimetric method of assay

Here are some key features and aspects of validating a titrimetric assay method:

- *Specificity*. The titrimetric method must be specific for the analyte or chemical compound of interest. This means that the method must only react with the target analyte and not be influenced by other substances present in the analysis matrix.
- *Linearity*. Validation should include evaluation of method linearity. This involves testing the method over a range of known analyte concentrations to ensure that the instrumental response or required volume of titrant varies linearly with concentration.
- *Precision.* Repeatability (reproducibility within the same laboratory) and reproducibility (reproducibility between different laboratories) tests can be performed to assess the precision of the method.
- *Accuracy*. Reference sources or alternative methods may be used to verify the accuracy of the method.
- *Sensitivity*. It is determined whether the method can detect small variations in concentration. The more sensitive a method is, the lower concentrations of the analyte it can detect.
- *Limit of detection and limit of quantification* are determined.
- *Robustness*. The influence of small variations in experimental conditions such as temperature, pH and titrant concentration is tested.

- *Checking the titration curve*. Validation should include verification of the titration curve and determination of critical parameters such as the equivalence point or end point of the titration.

The practical approach of a general protocol for validation of an analytical method

The validation procedure involves a very rigorous description of all the stages of sample preparation, preparation of the equipment and apparatus used, the way of performing the experiments. For familiarization with the basic stages of the validation of an instrumental analysis method, a brief description of a validation study is proposed.

Validation study of an HPLC method for the determination of isohydrafural and methyluracil in combined ointment

The aim of the study is to develop and validate an HPLC method for the quantitative determination of isohydrafural (figure 2) and methyluracil (figure 3) in the combined ointment.

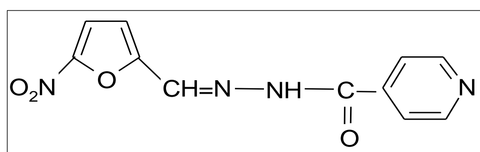

Figure 2. Chemical structure of isohydrafural

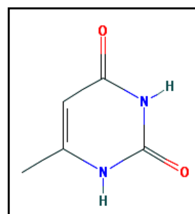

Figure 3. Chemical structure of methyluracil

Materials and methods

The researched drug: The combined ointment containing isohydrafural and methyluracil; 100 g of ointment contains: active

substances: isohydrafural 0.1 g, methyluracil 4.0 g, excipients: polyethylene glycol 400, polyethylene glycol 1500.

Reference substances: isohydrafural (synthesized in the laboratory, batch 00.01.112.10), pharmacopoeia standard methyluracil.

Equipment:

- Liquid Chromatograph Agilent 1220 Infinity LC Technologies,
- Nucleosil 100 C18 chromatographic column, with dimensions 4x150 mm, particle size 10 μm;
- mobile phase: the mixture of methanol and purified water in the ratio 25:75;
- UV-Vis detection at a wavelength of 260 nm;
- mobile phase flow rate: 1.0 ml/minute;
- temperature of the chromatographic column: $+30^0$C.

The working methodology

The HPLC method is most often used for combined pharmaceutical forms. Based on the mechanism of separating components from a mixture, it allows the identification and dosing of active principles at the same time. Impurities can also be accurately detected.

Working techniques for obtaining the solutions necessary for method validation:

Preparation of isohydrafural stock standard solution: 0.01 g (exact mass) of isohydrafural working standard was transferred to a 100 ml volumetric flask, it was added about 30 mL of mobile phase

and was stired until dissolved. Then it was brought up to the mobile phase level.

Preparation of methyluracil stock standard solution: 0.4 g (exact mass) of methyluracil standard was transferred to a 100 ml volumetric flask, it was added about 30 mL of mobile phase and was stired until dissolved. Then it wass brought up to the mobile phase level.

Preparation of the placebo sample. about 1.0 g (exact mass) of placebo is transferred to a porcelain cup, to which 10 ml of mobile phase is added and heated at 30°C until melting. The obtained sample is cooled, filtered through a membrane filter (0.45 μm) and transferred to a 50 ml volumetric flask. The extraction is repeated twice with 15 ml each of mobile phase. The samples obtained are combined with the first extraction and brought to the mobile phase quota. 5 ml of the obtained solution are placed in a graduated flask with a capacity of 10 ml and filled up to the mark with mobile phase.

Obtaining of calibration standard solutions for linearity determination of D_1 curve of isohydrafural: it was prepared 3 different series of solutions, each series with 5 concentration levels of standard solutions with concentrations of 8, 9, 10, 11 and 12 μg/ml in diluted placebo .

Obtaining of calibration standard solutions for linearity determination of D_2 curve of isohydrafural: it was prepared 3 different series of solutions, each series with 5 concentration levels of standard solutions with concentrations of 8, 9, 10, 11 and 12 μg/ml mobile phase (the solvent of the isohydrafural stock standard solution).

Obtaining of calibration standard solutions for linearity determination of D_1 curve of methyluracil: it was prepared 3 different series of solutions, each series with 5 concentration levels of standard solutions with concentrations of 320, 360, 400, 440 and 480 µg/ml in diluted placebo .

Obtaining of calibration standard solutions for linearity determination of D_2 curve of methyluracil: it was prepared 3 different series of solutions, each series with 5 concentration levels of standard solutions with concentrations of 320, 360, 400, 440 and 480 µg/ml mobile phase (the solvent of the isohydrafural stock standard solution).

Preparation of the sample solution: about 1.0 g (exact mass) of the ointment was transferred to a porcelain cup, to which 10 ml of mobile phase was added and heated to 30°C until the ointment was melted. The sample was cooled to room temperature and filtered through a membrane filter (0.45 µm). The filtrate was transfered to a 50 ml volumetric flask. The extraction was repeated twice with 15 ml each of mobile phase. The obtained samples were added to the first extraction and made up to the mark with mobile phase.

Preparation of sample of isohydrafural 80% for accuracy determination. 2.5 ml of sample solution and 0.3 ml of isohydrafural stock standard solution were placed in a 10 ml volumetric flask. It was mixed well and brought up to volume with mobile phase.

Preparation of sample of isohydrafural 100% for accuracy determination. 2.5 ml of sample solution and 0.5 ml of isohydrafural

stock standard solution were placed in a 10 ml volumetric flask. It was mixed well and bring to volume with mobile phase.

Preparation of sample of isohydrafural 120% for accuracy determination. 2.5 ml of sample solution and 0.7 ml of isohydrafural stock standard solution were placed in a 10 ml volumetric flask. It was mixed well and bring to volume with mobile phase.

Preparation of sample of methyluracil 80% for accuracy determination. 2.5 ml sample solution and 3.0 ml methyluracil stock standard solution were placed in a 10 ml volumetric flask. It was mixed well and bring to volume with mobile phase.

Preparation of sample of methyluracil 100% for accuracy determination. 2.5 ml sample solution and 5.0 ml methyluracil stock standard solution were placed in a 10 ml volumetric flask. It was mixed well and bring to volume with mobile phase.

Preparation of sample of methyluracil 120% for accuracy determination. 2.5 ml of sample solution and 7.0 ml of methyluracil stock standard solution were placed in a 10 ml volumetric flask. It was mixed well and bring to volume with mobile phase.

Preparation of the sample for determination of precision: 5 ml of the sample solution was transferred into a 10 ml volumetric flask and was brought to the level with the mobile phase.

HPLC method validation and results processing

Specificity. The placebo sample and the standard solutions of the active substances are injected into the HPLC system. The retention time for each drug substance is determined. It will be investigated whether a chromatographic peak at the same retention time as that of

isohydrafural or methyluracil is observed in the chromatogram of the placebo solution. If an additional peak is seen that distorts or disrupts the isohydrafural or methyluracil peak then the method is not specific.

Linearity. The series of solutions for lines D_1 and lines D_2 of isohydrafural and methyluracil are injected. Record each chromatographic peak area as a function of series, concentration level, and replicate level of each concentration level. Plot the calibration lines (chromatographic peak area versus concentration) for each of the replicates of a concentration level and for each of the 3 different data series, respectively. Linearity is statistically evaluated for each of the 2 lines of isohydrafural and methyluracil by applying the **C** test (homogeneity of variances of some groups of determinations), the **F** test (testing the validity of the line and the existence of a significant slope), the **t** test (comparing the slopes and, respectively, interceptions, it is also checked if the validated lines pass through the origin).

Detection limit. It is determined by repeatedly injecting solutions of isohydrafural and methyluracil (in mobile phase) until the ratio of the analytical signal given by the chromatographic peak to the background noise is 3. The limit of detection can also be calculated by formula (1).

The quantitation limit. It is determined by repeated injection of isohydrafural and methyluracil solutions (in placebo and mobile phase) until the ratio of the analytical signal given by the chromatographic peak to the background noise is 10. The limit of quantitation can also be calculated by formula (2).

Accuracy. Chromatographic peak areas are measured for the 3 levels and replicates corresponding to the accuracy check for isohydrafural and methyluracil solutions prepared in diluted placebo. The recovered concentrations are determined using the calibration curve equation D_2. The validation of the accuracy verification data is done by applying the **C** (homogeneity of the variances of some groups of determinations) and **F** (comparison of intra-group and intergroup variances) tests. The mean recovery is calculated which must be between the limits of 98 and 102%.

Precision. Measure the chromatographic peak areas for the 3 series of solutions of isohydrafural and methyluracil obtained in diluted placebo and calculate the corresponding concentrations by reference to the right of D_1. The same is done for the other 3 series of isohydrafural and methyluracil obtained in the mobile phase, using the right D_2 as a reference. The validation of the accuracy verification data is done by applying the **C** test. The coefficient of variation corresponding to the *CVr* repeatability and that of the intermediate precision *CVR* is calculated, which must be below 2% in both situations.

Robustness is determined by varying the chromatographic conditions: the flow rate of the mobile phase by ±1 ml/min, the amount of methanol in the mobile phase by ±2% and the column temperature by ±50C. The coefficient of variation is calculated which must be less 2%.

TASKS FOR INDIVIDUAL WORK

Based on the experimental validation data, the calculations will be performed and the worksheets in *Annexes 1, 2, 3* and *4* will be completed.

PROBLEM 1. Investigate the linearity validation parameter for a UV-Vis spectrophotometric method developed for dosing a drug substance at a 5% significance level:

1.1. Linear regression line obtained with the reconstituted pharmaceutical form (D_1)

Level of C	X, µg/ml	Y, Absorbance	$X - X_{med}$	$(X - X_{med})^2$	$Y - Y_{med}$	$(Y - Y_{med})^2$	$(X-X_{med})*$ $*(Y-Y_{med})$
60%							
80%							
100%							
120%							
140%							
X_{mean}=		**Y_{mean} =**		**Sum =**		**Sum =**	**Sum =**

1.2. Linear regression line obtained with the reference substance (D_2)

Level of C	X, µg/ml	Y, Absorbance	$X - X_{med}$	$(X - X_{med})^2$	$Y - Y_{med}$	$(Y - Y_{med})^2$	$(X-X_{med})*$ $*(Y-Y_{med})$
60%							
80%							
100%							
120%							
140%							
X_{mean}=		**Y_{mean} =**		**Sum =**		**Sum =**	**Sum =**

PROBLEM 2. Investigate the accuracy validation parameter for a UV-Vis spectrophotometric method developed for dosing a drug substance at a significance level of 5%, as determined at 3 concentration levels: 80%, 100%, and 120% by the standard addition method , and the theoretical concentration of the active substance will be calculated using the linear regression equation established at the linearity parameter in Problem 1:

Concentration levels	**A**	**C_p**	**C_a**	**C_t**	**Recovery, %** **R = [(Ct – Cp)/Ca]*100%**
80%					
100%					
120%					
Mean, %					

PROBLEM 3. Investigate the repeatability validation parameter for a UV-Vis spectrophotometric method developed for dosing a drug substance at a significance level of 5%, and the theoretical concentration of the active substance will be calculated using the linear regression equation established at the linearity parameter in Problem 1:

Nr.	Absorbance, Y	Concentration, X	$X - X_{mean}$	$(X - X_{mean})^2$
1.				
2.				
3.				
4.				
5.				
6.				
7.				
		X_{mean} =		Sum =

PROBLEM 4. Investigate the robustness validation parameter for a UV-Vis spectrophotometric method developed for the dosage of a drug substance, if the investigations were performed by ±1 nm variation in the maximum wavelength of light absorbed by the sample (373 nm) at a 5% significance level:

The wavelength	Absorbance, Y_{ij}	$Y_{ij} - Y_{mean}$	$(Y - Y_{mean})^2$
	Y_{mean} =		Sum =

BIBLIOGRAPHY

1. Achimaş C. A. Metodologia cercetării ştiinţifice medicale, Editura Medicală Universitară „Iuliu Haţieganu” Cluj-Napoca, 1999.
2. Bibire, N., Vieriu, M., Panainte, A., Agoraei, L., Uncu, L. et al. A new High Performance Liquid Chromatographic Analysis Method for Ciprofloxacin. In: Revista de chimie. 2015, nr. 9(66), pp. 1463-1466. ISSN 0034-7752. (IF: 0,956).
3. Bojiţă M., Roman L., Săndulescu R., Oprean R. Analiza şi Controlul medicamentelor, vol. I. - Cluj-Napoca: Editura Intelcredo, 2003.
4. Bojiţă M., Roman L., Săndulescu R., Oprean R. Analiza şi Controlul medicamentelor, vol. II. - Cluj-Napoca: Editura Intelcredo, 2003.
5. British Pharmacopoeia. – London, 2009.
6. Chan C.C., Lam H., Lee Y.C., Zhang X. Analytical method validation and instrument performance verification, Wiley Interscience, 2004.
7. Colectiv de autori: Guide de validation analitique. Rapport d’une commission SFSTP. STP Pharma Pratiques, 1992, 2(4), p. 205-239.
8. Dehelean C. A., Danciu C., Simu G. M., Șoica C. M. Elemente de metodologia cercetării științifice, Ed. Hippocrate, Timișoara, 2013.
9. Donici E. Elaborarea și validarea metodei spectrofotometrice în ultraviolet şi vizibil de dozare a fluocinolonului acetonid dintr-un

unguent combinat: studiu experimental. În: *Moldovan Journal of Health Sciences. Revista de Ştiinţe ale Sănătăţii din Moldova*. 2017, vol. 13, nr.3, pp. 53-58. ISSN 2345-146.

10. DONICI, E. Elaborarea metodei spectrofotometrice UV-VIS de determinare cantitativă a izohidrafuralului, metiluracilului și benzocainei din unguentul combinat. În: *Buletinul Academiei de Științe a Moldovei. Științe Medicale*. Chișinău, 2016, 1(50), pp. 245-248. ISSN 1857-0011.
11. Ermer J., Miller J. H. McB. Method Validation in Pharmaceutical Analysis, Wiley-VCH, 2005.
12. European Pharmacopoeia - 8th edition. Council of Europe, 67075 Strasbourg Cedex, France – 2013.
13. Farmacopea Română. Ediţia X-a –Bucureşti: Editura medicală, 1993.-1315 p.
14. Imre S., Ion V., Muntean D.-L. Ghid practic de metodologia cercetării în științele farmaceutice și conexe, Târgu Mureș: University Press, 2019.
15. Kumar R. Research Methodology. A Step-by-Step Guide for Beginners, Sage Publications, 2005.
16. Mullins E. Statistics for the Quality Control Chemistry Laboratory, The Royal Society for Chemistry, 2003.
17. Oprean R., Rozet E., Dewe E., Boulanger B., Hubert Ph. Ghid de validare a procedurilor analitice cantitative, Editura Medicală Universitară Iuliu Hațieganu, Cluj-Napoca, 2007.

18. Popa L. Elemente de metodologia cercetării ştiinţifice în domeniul farmaceutic, Ediţia a II-a revizuită şi adăugită, Editura Printech Bucureşti, 2005.
19. Roman L., Bojiţă M., Săndulescu R., Muntean D.L. Validarea metodelor analitice, Editura Medicală, 2007.
20. Rus, L.M., Donici, E., Valica, V., Prisacari, V., Tomuță, I., Șepeli, D., Hegheș S.C., Iuga, A.C., Uncu, L. Development, physical-chemical characterization and *in vitro* antibacterial activity evaluation of a fixed-dose combination isohydrafural-methyluracil hydrophilic ointment. *Farmacia*. 2019, 67(5), 857-865. DOI:10.31925/farmacia.2019.5.15. ISSN (p) 0014-8237, ISSN (e) 2065-0019.
21. Themistocles P. H., Christian G. D., Koupparis M. A., Macheras P. E. Quantitative Calculations in Pharmaceutical Practice and Research, VCH Publishers, New York, 1993.
22. Uncu, L, Donici, E., Valica, V., Vîslouh, O., Gonciar, V., Parii, S. Development and validation of an assay method for ciprofloxacin hydrochloride determination in combination ear drops. *Chemistry Journal of Moldova*. 2019, DOI: http://dx.doi.org/10.19261/cjm.2019.607. ISSN (p) 1857-1727, ISSN (e) 2345-1688.
23. Uncu, L., Evtodienco, V., Mazur, E., Donici, E., Valica, V. Validation of the spectrophotometric method for the dosing of some combined capsules. *Moldovan Medical Journal.* October, 2021, 64(4), 2021. pp. 10-16. ISSN 2537-6373 (Print). ISSN

2537-6381 (Online) Disponibil: DOI: https://doi.org/10.52418/moldovan-med-j.64-4.21.02 .

24. Uncu, A. Development and validation of the high-pressure liquid chromatographic method for the quantitative determination of propylthiohinothiadiazole. *Moldovan Medical Journal.* September 2020;63(3):3, 32-37. DOI: https://10.5281/zenodo.3958549
25. Uncu, L., Vîrlan, V., Mazur, E., Donici, E., Valica, V. Validarea metodei spectrofotometrice de dozare a unor picături auriculare combinate. In: Materialele Congresului Național de Farmacie, Ed. XVIII-a, 15-17 septembrie, 2021, Oradea, România. Oradea: Editura Universității din Oradea, 2021, p. 28. I-SBN 978-606-10-2144-4. Disponibil: https://cnfronline.ro/images/Brosura_Congres_CNFR_2021_ISBN.pdf
26. Valica V., Uncu L., Donici E., Treapițîna T., Mazur E., Ștefaneț T. Chimie farmaceutică; sub redacția: Vladimir Valica. Volumul I., Chișinău: Garomont-Studio, 2022 -330 p. –ISBN 978-9975-162-37-1
27. Vieriu, M., Tantaru, G., Apostu, M., Panainte, A.D., Agoroaei, L., Uncu, L. et al. A New Spectrometric Method for Quantitative Determination through Molecular Absorption of Lisinopril. In: Revista de chimie. 2015, nr. 10(66), pp.1563-1566. ISSN 0034-7752. (IF 0,956).
28. European Medicines Agency ICH: Q2 (R1) Validation of analytical procedures: Text and methodology step, 1995. URL:

https://database.ich.org/sites/default/files/Q2%28R1%29%20Guideline.pdf (accesat la 15.10.2022).

29. European Medicines Agency: VICH GL1 Validation of analytical procedures: definition and terminology, 1998. URL: https://database.ich.org/sites/default/files/Q2%28R1%29%20Guideline.pdf (accesat la 15.10.2022).
30. Introduction to method validation. Disponibil la: https://www.lgcgroup.com/media/1509/introduction-to-method-validation.pdf [accesat la 12.11.2022].

Annex 1. Work sheet

Validation of an assay method. Results of the "Linearity" parameter study

D_1 – linear regression line obtained with labeled placebo solutions
D_2 – linear regression line obtained with standard solutions in solvent

Nr.	Statistical Parameter/Test	Line D_1	Line D_2	The critical value of the statistical parameter (5% risk)	Conclusions
A.	**Parameters of the calibration line equation:**				The two lines describe/do not describe a linear dependence
1.	The field of concentrations, mg/ml				
2.	Slope	$a_1 =$	$a_2 =$		
3.	Interception	$b_1 =$	$b_2 =$		
4.	The correlation coefficient	$r_1 =$	$r_2 =$	0,99 – 1,00	
B.	**Homogeneity of variants test (C – Cochran test)**	$C_{exp} =$	$C_{exp} =$	C(0,05;5;2) = 0,8412	The variants of the determination groups are/are not homogeneous
C.	**Test for existence of a significant slope (Fisher F test)**	$F_{exp} =$	$F_{exp} =$	F(0,05;1;13) = 4,67	The slope characterizes/does not characterize a linear dependence
D.	**Validity test of the linear regression line (compares fitting errors with experimental ones: Fisher F test)**	$F_{exp} =$	$F_{exp} =$	F(0,05;3;10) = 3,71	Adjustments are/are not valid; the line is/is not valid
E.	**Comparison test of the slopes of the lines (t-test)**	$t_{exp} =$		t(0,05;26) = 2,056	The 2 slopes do not differ/differ significantly

<table>
<tr><td rowspan="2">F.</td><td>Test for comparing the ordinates at the origin with 0 (t-test)</td><td>$t_{exp} =$</td><td>$t_{exp} =$</td><td>t(0,05;13) = 2,16</td><td>The ordinate at the origin differs/does not differ significantly from 0</td></tr>
<tr><td>Ordinate comparison test at the origin (t-test)</td><td colspan="2">$t_{exp} =$</td><td>t(0,05;26) = 2,056</td><td>The 2 ordinates at the origin differ/do not differ significantly</td></tr>
</table>

Annex 2. Work sheet

Validation of an assay method. Results of the "Accuracy" parameter study

Statistical Parameter/Test	**The obtained results**	**The critical value of the statistical parameter (5% risk)**	**Conclusions**
The field of concentrations, mg/ml			
Test for homogeneity of within-group variances (C – Cochran's test)	C_{exp} =	C(0,05;3;2) = 0,9669	Intra-group variations are/are not homogeneous
Mean validity test (Fisher F test)	F_{exp} =	F(0,05;2;6) = 5,14	The average accuracy (recovery) of the method is/is not valid
Average recovery, %	R =	[98 ÷ 102]	Mean accuracy (retrieval) of the method falls/does not fall within the acceptance range

Annex 3. Work sheet

Validation of an assay method. Results of the "Precision" parameter study

Statistical Parameter/Test	**The obtained results**	**The critical value of the statistical parameter (5% risk)**	**Conclusions**
The field of concentrations, mg/ml			
Homogeneity of variance test (C – Cochran test)	C_{exp} =	C(0,05;3;5) = 0,7457	Variations are/are not homogeneous
Coefficient of variation of repeatability, %	CVr =	[-2 ÷ 2]	The method is/is not precise
Coefficient of variation of intermediate precision, %	CVR =	[-2 ÷ 2]	

Annex 4. Work sheet

Validation of a dosing method. Results of the study of the parameter "Robustness"

The wavelength	**Absorbance, Y_{ij}**	**Standard deviation**	**Coefficient of variation, %**
		$s = \sqrt{\frac{\sum_{i=1}^{N}(Y - Ymean)^2}{N-1}}$	$CV\% = \frac{s}{Y_{mean}} \cdot 100\%$ [-2 ÷ 2]
The method is/is not robust			