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APPROACHES TO DETERMINATION OF PRECISION FOR UV-SPECTROPHOTOMETRIC METHODS

OF QUANTITATIVE DETERMINATION IN FORENSIC AND TOXICOLOGICAL ANALYSIS

This article is the continuation of authors' research [1-5] in the field of development of the approaches to validation of quantitative determination methods for purposes of forensic and toxicological analysis and devoted to the questions of the determination procedure development and formation of the acceptability criteria for validation parameter «precision».

ANNOTATION

The criteria and procedure of acceptability estimation of precision for UV-spectrophotometric methods of analytes quantitative determination in biological fluids used in forensic and toxicological analysis have been formed. It has been suggested to estimate the precision in two stages – by model solutions (without matrix) and by matrix samples – and at two levels – within-run and between-run.

Keywords: precision, UV-spectrophotometric methods, method of calibration curve, of forensic toxicological analysis.

INTRODUCTION

The purpose of this paper is to analyse the present approaches to determination and acceptability estimation of validation parameter «precision» according to the requirements of the international guidances [6-10] and to form the determination procedure and criteria for acceptability estimation of precision when carrying out the validation of UV-spectrophotometric methods of quantitative determination for forensic and toxicological analysis in the variant of the method of calibration curve.

INVESTIGATION METHODS

Such methods of scientific research as analysis, synthesis, systematic analysis, mathematical statistics, comparison and summarising were used.

RESULTS AND DISCUSSION

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 (ICH) [6]. The precision of an analytical procedure is usually expressed as the variance, standard deviation or relative standard deviation of a series of measurements [6].

Precision may be considered at three levels: repeatability, intermediate precision and reproducibility. Repeatability describes the precision under the same operating conditions over a short interval of time. Intermediate precision expresses within-laboratories variations: different days, different analysts, different equipment, etc. Reproducibility describes the precision between laboratories (collaborative studies, usually applied to standardization of methodology) [6].

This parameter is present in all guidances, which give the directed recommendations in regard to validation of bioanalytical methods, – «Guidance for Industry: Bioanalytical method validation» (U.S. FDA, 2001) [7], «Guideline on validation of bioanalytical methods» (EMA, 2011) [8], «Guidance for the Validation of Analytical Methodology and Calibration of Equipment used for Testing of illicit Drugs in Seized Materials and Biological Specimens» (UNODC, 2009) [9] and «Standard Practices for Method Validation in Forensic Toxicology» (SWGTOX, 2012) [10], and in all guidances the formulation of ICH [6] with more precise definitions is in the basis of its definition –

in the UNODC guidance [9], for example, the precision is called «reflection of random error of the procedure».

It is necessary also to note that only this document considers precision at the levels of repeatability and reproducibility entering these terms in the text of guidance, other papers talk about within-run and between-run precision – for one and several analytical run respectively.

As regards the procedure of precision determination for bioanalytical methods, all guidances recommend with this purpose to carry out the analysis of the special (not calibration) samples containing the known amounts of analyte, and it is accentuated that it is possible to use the same samples as for precision verification with this purpose. The information about number of concentration levels used for verification of precision and number of replicates for each concentration level has been resulted in Table 1.

Table 1 – Requirements to the precision determination according to the FDA, EMA, UNODC and SWGTOX papers

Paper	The number of runs (days)	The number of concentration levels	The number of replicates
FDA	–	not less 3	not less 5
EMA	not less 3 (not less 2)	not less 4	not less 5
UNODC	not less 3	not less 3	not less 3
SWGTOX	not less 5	not less 3	not less 3

The requirements to the number of concentration levels g used for precision verification are similar on the whole – not less than three, and only the EMA guidance [9] says about a minimum of four values of concentration (see Table 1); as regards the position of these concentration levels within the range of method application, in all papers it is a question of «low, medium and high» concentrations. The EMA [8] and SWGTOX [10] guidances concretize these recommendations – it is a question of the lower limit of quantification (LLOQ), concentrations within three times the LLOQ (low sample) and at least 75% [8] or 80% [10] of the upper point of calibration curve (high sample); the medium sample is chosen as a middle of the method application range [8] or as a middle point between low and high samples [10].

As well as when linearity determination the requirements to the number of replicates for each concentration level (Table 1) are also considerably differed, and it is also not clear, what is meant under the term «replicate» – replicate experiment or replicate measurement? The FDA guidance [7] says about replicate «determinations», EMA [8] insists on replicate «analysis of samples», in the UNODC guidance [9] it is a question of «replicates», and in the SWGTOX paper [10] it is recommended to carry out «triplicate measurements».

As for the number of runs/days – the FDA guidance [7] does not discuss this question generally, the EMA [8], UNODC [9] and SWGTOX [10] papers even differ in the number of such runs (see Table 1), but are a unit that «replicates» are carried out within one run and the mean values obtained for each concentration level are used in calculations.

The EMA guidance [8] suggests to carry out five replicates for each concentration level only within the first run (verification of within-run precision), for other runs one sample for each concentration level is analysed (verification of between-run precision), but then the question arises: how should the data be processed for verification of between-run precision? In our opinion, it is incorrect to use 5 values from the first run and only in one for two another – the numbers of samples to be analysed within each run should be the same.

As already discussed before [5] there are not clarity and unity in the texts of the considered papers [7-10] in regard to the data that should be used for determination of calibration model – it is offered to plot either combined calibration curve using the mean values of responses for each concentration level, or combined calibration curve using all values of responses for each concentration level, and also individual calibration curve for each analytical run. Thereby the next question appears: how should the concentrations of model samples be calculated when verifying precision – using combined curve or within each run?

Separately in the EMA guidance [8] it is accentuated that the samples used for verification of precision should be spiked by analyte independently from the calibration samples using separately prepared solutions, and the UNODC gui-dance [9], in addition, insists that the concentrations of these samples should differ from the samples used for plotting the calibration curve.

All considered papers [7-10] suggest to determine precision using such biological matrix, for which the method is developed, but do not specify, from which sources the matrix is taken – from one or from different.

In all guidances [7-10] it is recommended to present the results of precision determination in the form of relative standard deviation in percent – in all cases its value should be within 15% for all concentration levels, except the LLOQ, for which the extreme value of 20% is set.

Thus, the stated approaches to carrying out the experiment on precision confirmation when validating bioanalytical methods have ample quantity of the differences and contradictions and, in addition, are too bulky, especially taking into account the procedures of sample preparation used in domestic forensic and toxicological analysis. That leads to the necessity of elaboration of the uniform approaches to the determination procedure and acceptability estimation of the validation parameter «precision» for the methods used in forensic and toxicological analysis, particularly, for UV-spectrophotometric methods of analytes quantitative determination in biological liquids.

The domestic developments [11,12] in the field of validation of analysis methods for medicines foresee the very clear and definite order of determination and acceptability criteria for the parameter «precision» within the developed validation standardized procedures. Therefore it has been suggested to be guided by the mentioned domestic developments and, particularly, by the approaches to methods

validation in the variant of the method of calibration curve given in [12] for forming the procedure determination and acceptability criteria for precision when carrying out the validation of UV-spectrophotometric methods of quantitative determination for forensic and toxicological analysis. The choice of the method of calibration curve is dictated by the primary orientation of all studied international guidances [7-10] on the work exactly by this method.

As it has been stated before [2-5], for validation of UV-spectrophotometric methods of quantitative determination for forensic and toxicological analysis we use the normalized coordinates (i. e. transition from the equation of $A_i = b_1 \cdot C_i + a_1$ type to the equation of $Y_i = b_2 \cdot X_i + a_2$ type), which advantages of application are widely reported [11,12] – the validation characteristics obtained in the normalized coordinates do not depend on the specificity of concrete analyte and can be regulated easier. In our case the expressions for the normalized coordinates have such appearance:

$$X_i = \frac{C_i}{C_{st}} \cdot 100\%, \quad C_{st} = C_{reference};$$

$$Y_i = \frac{A_i}{A_{st}} \cdot 100\%, \quad A_{st} = \frac{A_{reference} \cdot R}{100}.$$

I. e. for normalization of the obtained experimental data the reference solution with the concentration of ana-lyte ($C_{reference}$) corresponded to its concentration in the end solution to be spectrophotometric measured under the condition of zero losses for the point of 100% in the normalized coordinates is used; the absorbance of such reference solution ($A_{reference}$) is corrected by the value of recovery R obtained at the preliminary stage of validation [4] and is used for normalization of absorbance values. Such approach is needed for decline of influence of the systematic error introduced by the components of blank sample, which significance has been shown at the preliminary stage of validation [3]. As 100% in the normalized coordinates it has been suggested earlier [5] to accept the mean toxic or lethal analyte concentration in biological liquid – depending on the purposes and tasks, for which the developed methods is intended.

It has been suggested earlier [5] to understand the complete carrying out the replicate experiment under the concept «replicate».

We suggest to carry out precision confirmation of the method at two levels – within-run (repeatability) and between-run (intermediate precision), and, in our mind, it is necessary to specify both terms for the purpose of ensuring the relative unity in the questions of terminology. According to the recommendations given in [11] the validation experiment should be as much as rational, and, as a result, its volume should be minimized, therefore we recommend to combine carrying out the experiment on verification of precision with carrying out the experiment on determination of specificity, recovery, linearity and accuracy.

For forming the acceptability criteria for repeatability the approach offered in [12] has been used – the total uncertainty of analysis results Δ_{As} for the method of calibration curve is determined by several factors, among them the main ones are:

- the uncertainty associated with calibration curve, Δ_{cal};
- the uncertainty directly associated with the sample to be analyse, Δ_{sample}; it is caused by the uncertainty of measuring its absorbance and sample preparation.

Therefore the total uncertainty of the method can be written down in following way [11,12]:

$$\Delta_{As} = \sqrt{\Delta^2_{cal} + \Delta^2_{sample}} \le max\ \Delta_{As} = 20\%\ [9]. \quad (1)$$

The approach based on assumption of their equality has been offered in paper [12] for regulation of values Δ_{cal} and Δ_{sample}, i. e.:

$$\max \Delta_{cal} = \max \Delta_{sample}. \quad (2)$$

Then:

$$\max \Delta_{cal} = \max \Delta_{sample} \le max\ \Delta_{sample} = \sqrt{2} = 0.707 \cdot max\ \Delta F_{As}. \quad (3)$$

Determination of within-run precision (repeatability). We recommend to confirm within-run precision simultaneously with determination of within-run linearity and within-run accuracy in the way of calculating the concentrations of calibration samples X_{calc}, % for each run by individual values of absorbance using the linear dependence obtained for this run.

The calculated values of concentrations of calibration samples X_{calc}, % are used for calculation of the para-meter «found/spiked» RR, %:

$$RR, \% = \frac{X_{i,\ cals}}{X_{i,\ fact}} \cdot 100. \quad (4)$$

The values RR, %, for each run of measurements are used for calculation of relative standard deviation of the value RR – RSD_{RR} and, respectively, the uncertainty of repeatability of single result of value RR $\Delta_{RR} = \Delta_{sample}$, which should not exceed extreme uncertainty of analysis of the sample to be analyse max Δ_{sample}. Thus, in accordance with [12] it is possible to write down the following ratio:

$$\Delta_{RR} = \Delta_{sample} = t(95\%, n - 1) \cdot RSD_{RR} \le max\ \Delta_{sample} = 0.707 \cdot max\ \Delta_{As}. \quad (5)$$

that is the criterion of acceptability for verification of the method precision.

Determination of between-run (intermediate) precision. We recommend to confirm between-run precision in three stages:

- in accordance with recommendations [12] to calculate the difference between the mean values in different days, which should be insignificant against extreme uncertainty of analysis of the sample to be analyse max Δ_{sample}:

$$|\overline{RR}_1 - \overline{RR}_2|, |\overline{RR}_1 - \overline{RR}_3|, |\overline{RR}_3 - \overline{RR}_2| \leq 0.32 \cdot \Delta_{sample} = 0.32 \cdot 0.707 \cdot 20\% \text{ [9]} = 4.52\%; \quad (6)$$

• to calculate the mean concentrations of calibration samples X_{calc}, % by the mean values of absorbance using the linear dependence obtained by the mean values of parallel runs;

it is also necessary to calculate RR, %, and Δ_{RR} for this stage, and then to check the criterion (5) fulfilment;

• to calculate X_{calc}, %, for model samples (concentrations correspond to the points of 25%, 50%, 100%, 150% and 175% in the normalized coordinates), which are used for determination of specificity and recovery at the preliminary stage of validation, by means of the linear dependence obtained by the mean values of parallel runs;

At this stage the necessity of tight regulation of the origin of matrix used for preparation of model samples appears. As it has been already discussed before [4], the different degree of analyte extraction from the matrix, which, in turn, mainly depends on two reasons – the analyte amount in the matrix and the state of matrix – is the critical factor for making the decision about suitability or unsuitability of the method for further application.

In order to estimate the influence of these two factors on precision of the method to be validated we suggest to carry out the investigations for three replicate runs, each one consists of 3 (for D = 25-125%) or 4 (for D = 25-150% and 25-175%) samples of biological matrix obtained from the same source, i. e. for analysis of each run the individual source of biological matrix is used. We recommend to carry out the analysis of runs in different days (one day is one run) – such approach allows to avoid the necessity to store the samples of biological matrix and give the possibility to estimate the influence of analyte amount and changing the matrix on the method precision, and also on its specificity and recovery.

It is also necessary to calculate RR, %, and Δ_{RR}^{intra} for this stage, and then to check the following criterion fulfilment:

$$\Delta_{RR}^{intra} = \Delta_{sample}^{intra} = t(95\%, n - 1) \cdot RSD_{RR}^{intra} \leq max\ \Delta_{As}. \quad (7)$$

The relative mildness of the criterion (7) as compared with the criterion (5) is dictated by that Δ_{RR}^{intra} at the third stage reflects the total error – and due to changing the matrix, and due to the different amount of analyte in the sample, and the error of determination of the linear dependence parameters – unlike Δ_{sample} at the second stage, where the influence of matrix changing is not taken into account.

It is necessary separately to discuss the following question: development of methods of analytes quantitative determination in biological liquids is carried out at the first stage using model solutions (without matrix) – linear dependence is plotted, linearity parameters are calculated, presence and significance of systematic error are verified, etc. This process also should be regulated somewise and the verification procedure and acceptability criteria should be elaborated for precision of the method using model solutions.

To verify the method precision by model solutions we offer to calculate the concentrations of these model solutions X_{cals}^{model}, %, using the respective linear dependence. The obtained values of X_{calc}^{model}, % are used for calculation of RR^{model}, Δ_{sample}^{model} % and value ; it is possible to offer the acceptability criterion for Δ_{sample}^{model} proceeding from the following reasoning.

It is possible to present the total uncertainty of analysis results Δ_{As} for methods of analyte quantitative determination in biological liquids by way of two components:

• the uncertainty of analyte quantitative determination in model solutions Δ_{As}^{model};

• the uncertainty of sample preparation procedure $\Delta_{\text{sample preparation}}$;

therefore the total uncertainty of the method can be written down in following way [11,12]:

$$\Delta_{As} = \sqrt{(\Delta_{AS}^{msdel})^2 + \Delta^2_{sample\ preperation}} \leq max\ \Delta_{As} = 20\% \text{ [9]}. \quad (8)$$

In turn the uncertainty of analyte quantitative determination Δ_{As}^{model} in model solutions is determined by:

• the uncertainty associated with deviations from linearity of calibration curve plotted by model solutions, Δ_{cal}^{model} ;

• the uncertainty of analysis of a single model solution, Δ_{sample}^{model} ;

therefore the uncertainty of analyte quantitative determination in model solutions can be written down in following way [11,12]:

$$\Delta_{As}^{model} = \sqrt{(\Delta_{cal}^{model})^2 + (\Delta_{sample}^{model})^2} \leq max\ \Delta_{As}^{model}. \quad (9)$$

It is possible to offer 2 approaches for regulation of the value and, respectively, Δ_{sample}^{model} .

Approach 1: the uncertainty of sample preparation procedure is equal to the uncertainty of analyte quantitative determination in model solutions, i. e.:

$$max\ \Delta_{As}^{model} = max\ \Delta_{sample\ preperetion}. \quad (10)$$

Then:

$$max\ \Delta_{As}^{model} = max\ \Delta_{sample\ preperetion} \leq max\ \Delta_{As} \sqrt{2} = 0.707 \cdot max\ \Delta_{As}. \quad (11)$$

and taking into account (2):

$$max\ \Delta_{sample}^{model} = max\ \Delta_{As}^{model} / \sqrt{2} = 0.707 \cdot max\ \Delta_{As}^{model} = 0.707 \cdot max\ \Delta_{As} = 0.707 \cdot 0.707 \cdot 20\% \text{ [9]} = 10\%. \quad (12)$$

Approach 2: the uncertainty of analyte quantitative determination in model solutions is insignificant against the total uncertainty of analysis results Δ_{As}, i. e.:

$$\Delta_{As}^{model} \leq max\ \Delta_{As}^{model} = 0.32 \cdot max\ \Delta_{As}, \quad (13)$$

$$max\ \Delta_{sample}^{model} = max\ \Delta_{As}^{model} \sqrt{2} = 0.707 \cdot max\ \Delta_{As}^{model} = 0.707 \cdot 0.32 \cdot max\ \Delta_{As} = 0.707 \cdot 0.32 \cdot 20\%\ [9] = 4.52\%. \quad (14)$$

CONCLUSIONS

Thus, the criteria and procedure of acceptability estimation of precision for UV-spectrophotometric methods of analytes quantitative determination in biological fluids used in forensic and toxicological analysis have been offered.

ТҮЙІН

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СОТТЫҚ-ТОКСИКОЛОГИЯЛЫҚ ТАЛДАУ КЕЗІНДЕ УФ-СПЕКТРОФОТОМЕТРИЯЛЫҚ ӘДІСТЕРДІ САНДЫҚ АНЫҚТАУДЫҢ ПРЕЦИЗИОНДЫЛЫҒЫН АНЫҚТАУ ТӘСІЛДЕРІ

Соттық-токсикологиялық талдау кезінде пайдаланылатын биологиялық сұйықтықтағы аналиттерді сандық анықтау әдістері прецизиондылығының тиімділігін бағалау өлшемдері мен шаралары қалыптастырылды. Прецизиондылықты бағалауды екі кезеңде: модельді ерітінділерде (матрицасыз) және матрица үлгілерінде, сонымен қатар екі – within-run менbetween-run дәрежеделерінде өткізу ұсынылды.

Түйін сөздер: прецизиондылық, УФ-спектрофотометриялықәдістер, калибрлік қисық әдіс, соттық-токсикологиялық талдау.

РЕЗЮМЕ

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ПОДХОДЫ К ОПРЕДЕЛЕНИЮ ПРЕЦИЗИОННОСТИ ДЛЯ УФ-СПЕКТРОФОТОМЕТРИЧЕСКИХ МЕТОДИК КОЛИЧЕСТВЕННОГО ОПРЕДЕЛЕНИЯ В СУДЕБНО-ТОКСИКОЛОГИЧЕСКОМ АНАЛИЗЕ

Сформированы критерии и процедура оценки приемлемости прецизионности УФ-спектрофотометрических методик количественного определения аналитов в биологических жидкостях, применяемых в судебно-токсикологическом анализе. Оценку прецизионности предложено проводить в два этапа: на модельных растворах (без матрицы) и образцах матрицы, а также на двух уровнях – within-run и between-run.

Ключевые слова: прецизионность, УФ-спектрофотометрические методики, метод калибровочной кривой, судебно-токсикологический анализ. ■

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