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DETERMINING ACCURACY IN VALIDATION OF UV-SPECTROPHOTOMETRIC METHODS OF QUANTITATIVE MEASUREMENT IN FORENSIC TOXICOLOGICAL ANALYSIS

Criteria and procedures for evaluating acceptability of accuracy offered by UV-spectrophotometric methods of quantitative measurement of analytes in biological fluids used in forensic toxicological analysis have been determined. The accuracy evaluation is suggested for carrying out in two stages – on test solutions (no matrix) and on a matrix sample, whereas also in terms of two levels, i.e. within-run and between-run. The suggested approaches have been tested with respect to UV-spectrophotometric methods of quantitative measurement of doxylamine in blood.

Key words: validation; accuracy; acceptability criteria; UV-spectrophotometry; doxylamine; bioanalytical methods

INTRODUCTION

This article is the continuation of authors' research [3-5, 12, 13] 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 "accuracy".

The purpose of this paper is to analyse the present approaches to determination and acceptability estimation of validation parameter "accuracy" according to the requirements of the international guidances [7-11] and, respectively, to form the determination procedure and criteria for acceptability estimation of accuracy 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, and also to test the offered approaches by the example of UV-spectrophotometric method of doxylamine quantitative determination in blood.

MATERIALS AND METHODS

The process solutions: 1000.0 mg of doxylamine succinate were placed in the measuring flask with the capacity of 250.0 ml, dissolved in distilled water and the solution was diluted to the volume with the same solvent (the standard solution 1, the concentration was 4000 mcg/ml). 32.50; 30.00; 25.00; 20.00; 15.00; 10.00 and 5.00 ml respectively of the doxylamine succinate standard solution 1 were placed using burette in seven measuring flasks with the capacity of 100.0 ml and the solutions were diluted to the volume with distilled water (the process solutions 1, 2, 3, 4, 5, 6 and 7 respectively, the concentrations were 1300, 1200, 1000, 800, 600, 400 and 200 mcg/ml respectively).

400.0 mg of doxylamine succinate were placed in the measuring flask with the capacity of 100.0 ml, dissolved in distilled water and the solution was diluted to the volume with the same solvent (the standard solution 2, the concentration was 4000 mcg/ml). 32.50; 30.00; 20.00; 10.00 and 5.00 ml respectively of the doxylamine succinate standard solution 2 were placed using burette in five measuring flasks with the capacity of 100.0 ml and the solutions were diluted to the volume with distilled water (the process solutions 8, 9, 10, 11 and 12 respectively, the concentrations were 1300, 1200, 800, 400 and 200 mcg/ml respectively).

The model solutions: 100.0 mg of doxylamine succinate were placed in the measuring flask with the capacity of 500.0 ml, dissolved in the 0.1 mole/l hydrochloric acid solution and the solution was diluted to the volume with the same solvent (the standard solution 3, the concentration was 200 mcg/ml). 26.00; 24.00; 20.00; 16.00; 12.00; 8.00 and 4.00 ml respectively of the doxylamine succinate standard solution 3 were placed using burette in seven measuring flasks with the capacity of 100.0 ml and the solutions were diluted to the volume with the 0.1 mole/l hydrochloric acid solution (the model solutions 1, 2, 3, 4, 5, 6 and 7 respectively, the concentrations were 1300, 1200, 1000, 800, 600, 400 and 200 mcg/ml respectively).

The reference solution: 400.0 mg of doxylamine succinate were placed in the measuring flask with the capacity

Table 1

REQUIREMENTS TO THE ACCURACY 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

of 100.0 ml, dissolved in the 0.1 mole/l hydrochloric acid solution and the solution was diluted to the volume with the same solvent (the standard solution 4, the concentration was 4000 mcg/ml). 18.00 ml of the doxylamine succinate standard solution 4 were placed using burette in measuring flask with the capacity of 100.0 ml and the solution was diluted to the volume with the 0.1 mole/l hydrochloric acid solution (the standard solution 5, the concentration was 720 mcg/ml). 2.00 ml of the doxylamine succinate standard solution 5 were placed in measuring flask with the capacity of 50.0 ml and the solution was diluted to the volume with the 0.1 mole/l hydrochloric acid solution (the reference solution, the concentration was 28.8 mcg/ml).

The calibration samples (calibrators): 3 lines in 7 samples (20.00 ml) of model blood (matrix) obtained from three different sources, which were spiked with 1.00 ml of the process solutions 1-7 respectively.

The model samples: 3 lines in 5 samples (20.00 ml) of model blood obtained from three different sources, which were spiked with 1.00 ml of the process solutions 8-12 respectively.

The solutions to be analysed: the solutions obtained by the method to be validated [5] for the calibration and model samples.

The absorbance of the solutions to be analysed, model solutions and reference solution was measured 3 times with taking out the cell at the wavelength of 262 nm by the spectrophotometer СФ-46 in the cell with the layer thickness of 10 mm. The 0.1 mole/l hydrochloric acid solution was used as the compensation solution.

RESULTS AND DISCUSSION

The ***accuracy*** of an analytical procedure expresses the closeness of agreement between the value, which is accepted either as a conventional true value or an accepted reference value, and the value found (ICH) [10].

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], "Standard Practices for Method Validation in Forensic Toxicology" (SWGTOX, 2012) [11], "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) [8] and "Guideline on validation of bioanalytical methods" (EMA, 2011) [9], and in all guidances the formulation of ICH [10] with more precise definitions is in the basis of its definition. Thus, in the UNODC guidance [8], in the first place, the accuracy is called "ability of the procedure to get the true result" and "measure of systematic error of the procedure".

As regards the procedure of accuracy 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. The information about number of concentration levels used for verification of accuracy and number of replicates for each concentration level has been resulted in Tab. 1.

The requirements to the number of concentration levels *g* used for accuracy 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 (Tab. 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 [9] and SWGTOX [11] 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 % [9] or 80 % [11] of the upper point of calibration curve (high sample); the medium sample is chosen as a middle of the method application range [9] or as a middle point between low and high samples [11].

As well as when linearity determination the requirements to the number of replicates for each concentration level (Tab. 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 [9] insists on replicate "analysis of samples", in the UNODC guidance [8] it is a question of "replicates", and in the SWGTOX paper [11] 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 [9], UNODC [8] and SWGTOX [11] papers even differ in the number of such runs (see Tab. 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 [9] suggests to carry out five replicates for each concentration level only within the first run (verification of within-run accuracy), for other runs one sample for each concentration level is analysed (verification of between-run accuracy), but then the question arises: how should the values of responses be averaged for verification of between-run accuracy? 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.

The UNODC [8] and SWGTOX [11] guidances do not consider determination of accuracy at the within-run and between-run levels – in all cases it is suggested to process the mean results calculated taking into account all obtained values for each concentration level.

As already discussed before [13] there are not clarity and unity in the texts of the considered papers [7-9, 11] 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 accuracy – using the mean values of responses or each obtained, using combined curve or within each run?

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

All considered papers [7-9, 11] suggest to determine accuracy 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 [7-9, 11] it is recommended to present the accuracy in percent – differences concern only the value expressed in percent – "found/spiked" or "(found – spiked)/spiked", but in all cases the difference of "found" from "spiked" should be within 15 % of the true value for all concentration levels, except the LLOQ, for which such difference should be within 20 % of the true value.

For accuracy estimation the FDA guidance [7] also suggests to calculate so-called "back-calculated" concentrations of the samples used for plotting the calibration model and advances the following requirements to them:

- the deviation of the calculated concentration from nominal for the standard sample corresponding to the lower limit of quantification (LLOQ) should not exceed 20 %;
- the deviations for standard samples, which are differ from LLOQ, should not exceed 15 %.

Thus a minimum of 4 from 6 standard samples should satisfy the given criterion, including LLOQ and standard sample of the highest concentration.

The EMA guidance [9] suggests to use the same approach with the same requirements to deviations of the calculated concentrations of calibration samples from their nominal concentration, but at least 75 % of standard samples (but not less than six concentration levels) should satisfy this criterion. In the case the replicates are used at least 50 % of calibration samples should satisfy this criterion.

Thus, the stated approaches to carrying out the experiment on accuracy 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 "accuracy" for the methods used in forensic and toxicological analysis, particularly, for UV-spectrophotometric methods of analytes quantitative determination in biological liquids.

The domestic developments [1, 2] 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 "accuracy" 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 [1] for forming the procedure determination and acceptability criteria for accuracy 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 [7-9, 11] on the work exactly by this method.

As it has been stated before [4, 5, 12, 13], 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 [1, 2] – 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 analyte ($C_{reference}$) 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 [12] 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 [5]. As 100 % in the normalized coordinates it has been suggested earlier [13] 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 [13] to understand the complete carrying out the replicate experiment under the concept "replicate".

We suggest to carry out accuracy confirmation of the method at two levels – within-run and between-run. According to the recommendations given in [2] 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 accuracy with carrying out the experiment on determination of specificity, recovery and linearity.

Determination of within-run accuracy. We recommend to confirm within-run accuracy simultaneously with determination of within-run linearity 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.

Determination of between-run accuracy. We recommend to confirm between-run accuracy in two stages:

- 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;
- 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 [12], 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 accuracy 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 accuracy, and also on its specificity and recovery.

The calculated values of concentrations of calibration and model samples X_{calc}, % are used for calculation of the parameter "found/spiked" *RR*, %:

$$RR,\% = \frac{X_{i,calc}}{X_{i,fact}} \cdot 100. \qquad (1)$$

The mean value of this parameter $\overline{RR}$, % for each group of measurements is used for calculation of systematic error δ, which should be insignificant against extreme uncertainty of analysis Δ_{As}, % [2], i. e. should not exceed extreme systematic error maxδ. Thus, in accordance with [2] it is possible to write down the following ratio:

$$\delta,\% = |100 - \overline{RR}| \le \max\delta = 0.32 \cdot \max\Delta_{As} = \\ = 0.32 \cdot 20\ \%\ [8] = 6.40\ \%, \qquad (2)$$

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

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 accuracy of the method using model solutions.

To verify the method accuracy by model solutions we offer to calculate the concentrations of these model solutions X_{calc}^{model}, % using the respective linear dependence. The obtained values of X_{calc}^{model}, % are used for calculation of systematic error δ^{model} in accordance with formulae (1) and (2).

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 $\Delta_{sample\ preparation}$,

therefore the total uncertainty of the method can be written down in following way [1, 2]:

$$\Delta_{As} = \sqrt{(\Delta_{As}^{model})^2 + \Delta_{sample\ \ preparation}^2} \le \\ \le \max \Delta_{As} = 20\ \%\ [8]. \qquad (3)$$

Table 2

RESULTS OF ACCURACY DETERMINATION FOR UV-SPECTROPHOTOMETRIC METHOD OF DOXYLAMINE SUCCINATE QUANTITATIVE DETERMINATION BY MODEL SOLUTIONS

Factual concentration of doxylamine succinate in model solution (C_{st} = 28.8 mcg/ml)		Absorbance (A_{st} = 0.801)	Found in % to standard absorbance Y_i^{model}, %	Calculated concentration of doxylamine succinate in model solution $X_{i,calc}^{model}$, %	$RR,\% = \frac{X_{i,calc}^{model}}{X_{i,fact}^{model}} \cdot 100$
$C_{i,fact}^{model}$, mcg/ml	$X_{i,fact}^{model}$, %				
D = 25-175 % (g = 7)					
8.00	27.78	0.226	28.21	28.35	102.05
16.00	55.56	0.444	55.43	55.70	100.26
24.00	83.33	0.657	82.02	82.42	98.91
32.00	111.11	0.890	111.11	111.66	100.49
40.00	138.89	1.121	139.95	140.64	101.26
48.00	166.67	1.348	168.29	169.12	101.47
52.00	180.56	1.421	177.40	178.27	98.73
				$\overline{RR}^{model}$, %	100.45
		$\delta^{model} = \lvert 100 - \overline{RR}^{model} \rvert$	approach 1	≤4.52 %	0.45
			approach 2	≤2.05 %	
D = 25-150 % (g = 6)					
8.00	27.78	0.226	28.21	27.90	100.43
16.00	55.56	0.444	55.43	54.82	98.67
24.00	83.33	0.657	82.02	81.12	97.34
32.00	111.11	0.890	111.11	109.89	98.90
40.00	138.89	1.121	139.95	138.41	99.65
48.00	166.67	1.348	168.29	166.44	99.86
			$\overline{RR}^{model}$, %		99.14
		$\delta^{model} = \lvert 100 - \overline{RR}^{model} \rvert$	approach 1	≤4.52 %	0.86
			approach 2	≤2.05 %	
D = 25-125 % (g = 5)					
8.00	27.78	0.226	28.21	28.07	101.04
16.00	55.56	0.444	55.43	55.15	99.27
24.00	83.33	0.657	82.02	81.61	97.94
32.00	111.11	0.890	111.11	110.56	99.50
40.00	138.89	1.121	139.95	139.25	100.26
				$\overline{RR}^{model}$, %	99.60
		$\delta^{model} = \lvert 100 - \overline{RR}^{model} \rvert$	approach 1	≤4.52 %	0.40
			approach 2	≤2.05 %	

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

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

$$\max \Delta_{As}^{model} = \max \Delta_{sample\ \ preparation}. \quad (4)$$

Then:

$$\max \Delta_{As}^{model} = \max \Delta_{sample\ \ preparation} \leq \leq \max \Delta_{As} / \sqrt{2} = 0.707 \cdot \max \Delta_{As}, \quad (5)$$

$$\delta^{model}, \% = \lvert 100 - \overline{RR}^{model} \rvert \leq \max \delta^{model} = 0.32 \cdot \max \Delta_{As}^{model} = 0.32 \cdot 0.707 \cdot \max \Delta_{As} = 0.32 \cdot 0.707 \cdot 20\ \%\ [8] = 4.52\ \%. \quad (6)$$

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

$$\Delta_{As}^{model} \leq \max\ \Delta_{As}^{model} = 0.32 \cdot \max \Delta_{As}, \quad (7)$$

$$\delta^{model}, \% = \lvert 100 - \overline{RR}^{model} \rvert \leq \max \delta^{model} = = 0.32 \cdot \max \Delta_{As}^{model} = 0.32 \cdot 0.32 \cdot \max \Delta_{As} = 0.32 \cdot 0.32 \cdot 20\ \%\ [8] = 2.05\ \%. \quad (8)$$

For illustration of the offered approaches to accuracy determination and estimation UV-spectrophotometric method of doxylamine quantitative determination in blood [5] was used; the lethal doxylamine concentration in blood [6] – 25 mg/l (that corresponds to 36 mg/l of doxylamine succinate) has been accepted as 100 %.

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Table 3

RESULTS OF WITHIN-RUN ACCURACY DETERMINATION FOR UV-SPECTROPHOTOMETRIC METHOD OF DOXYLAMINE QUANTITATIVE DETERMINATION IN BLOOD WITHOUT PRELIMINARY TLC-PURIFICATION

Factual concentration of doxylamine succinate in blood (C_{st} = 36 mcg/ml)		Absorbance ($A_{st} = \frac{A_{reference} \cdot R}{100} = 0.532$)			Found in % to standard absorbance Y_i, %			Calculated concentration of doxylamine succinate in blood $X_{i,calc}$, %			$RR,\% = \frac{X_{i,calc}}{X_{i,fact}} \cdot 100$		
$C_{i,fact}$, mcg/ml	$X_{i,fact}$, %	1st day	2nd day	3d day	1st day	2nd day	3d day	1st day	2nd day	3d day	1st day	2nd day	3d day
D = 25-175 % (g = 7)													
10.00	27.78	0.222	0.195	0.205	41.73	36.65	38.53	26.82	26.38	26.59	96.54	94.96	95.72
20.00	55.56	0.368	0.347	0.352	69.17	65.23	66.17	54.45	54.76	54.09	98.00	98.56	97.35
30.00	83.33	0.545	0.526	0.537	102.44	98.87	100.94	87.95	88.17	88.69	105.54	105.81	106.43
40.00	111.11	0.662	0.644	0.654	124.44	121.05	122.93	110.11	110.19	110.57	99.10	99.17	99.51
50.00	138.89	0.795	0.779	0.783	149.44	146.43	147.18	135.28	135.40	134.70	97.40	97.49	96.98
60.00	166.67	0.986	0.970	0.979	185.34	182.33	184.02	171.44	171.05	171.36	102.86	102.63	102.81
65.00	180.56	1.021	1.008	1.015	191.92	189.47	190.79	178.06	178.14	178.09	98.62	98.66	98.63
										$\overline{RR}$, %	99.72	99.61	99.63
										δ = |100 – $\overline{RR}$|≤6.40 %	0.28	0.39	0.37
D = 25-150 % (g = 6)													
10.00	27.78	0.222	0.195	0.205	41.73	36.65	38.53	27.48	27.02	27.24	98.92	97.26	98.06
20.00	55.56	0.368	0.347	0.352	69.17	65.23	66.17	54.67	54.96	54.32	98.40	98.92	97.77
30.00	83.33	0.545	0.526	0.537	102.44	98.87	100.94	87.65	87.84	88.37	105.18	105.41	106.05
40.00	111.11	0.662	0.644	0.654	124.44	121.05	122.93	109.45	109.52	109.91	98.51	98.57	98.92
50.00	138.89	0.795	0.779	0.783	149.44	146.43	147.18	134.23	134.33	133.66	96.64	96.72	96.23
60.00	166.67	0.986	0.970	0.979	185.34	182.33	184.02	169.81	169.43	169.74	101.88	101.66	101.84
										$\overline{RR}$, %	99.92	99.76	99.81
										δ = |100 – $\overline{RR}$|≤6.40 %	0.08	0.24	0.19
D = 25 – 125 % (g = 5)													
10.00	27.78	0.222	0.195	0.205	41.73	36.65	38.53	26.15	25.85	25.94	94.13	93.05	93.38
20.00	55.56	0.368	0.347	0.352	69.17	65.23	66.17	54.29	54.69	53.94	97.71	98.43	97.08
30.00	83.33	0.545	0.526	0.537	102.44	98.87	100.94	88.42	88.64	89.17	106.11	106.37	107.01
40.00	111.11	0.662	0.644	0.654	124.44	121.05	122.93	110.98	111.02	111.45	99.88	99.92	100.31
50.00	138.89	0.795	0.779	0.783	149.44	146.43	147.18	136.62	136.63	136.02	98.37	98.37	97.93
										$\overline{RR}$, %	99.24	99.23	99.14
										δ = |100 – $\overline{RR}$|≤6.40 %	0.76	0.77	0.86

Table 4

RESULTS OF BETWEEN-RUN ACCURACY DETERMINATION FOR UV-SPECTROPHOTOMETRIC METHOD OF DOXYLAMINE QUANTITATIVE DETERMINATION IN BLOOD WITHOUT PRELIMINARY TLC-PURIFICATION

Calculation by calibration samples						Calculation by model samples					
Factual concentration of doxylamine succinate in blood (C_{st} = 36 mcg/ml)		Absorbance ($A_{st} = \frac{A_{reference} \cdot R}{100} = 0.532$)	Found in % to standard absorbance Y_i, %	Calculated concentration of doxylamine succinate in blood $X_{i,calc}$, %	*RR*, %	Factual concentration of doxylamine succinate in blood (C_{st} = 36 mcg/ml)		Absorbance ($A_{st} = \frac{A_{reference} \cdot R}{100} = 0.532$)	Found in % to standard absorbance Y_i, %	Calculated concentration of doxylamine succinate in blood $X_{i,calc}$, %	*RR*, %
$C_{i,fact}$, mcg/ml	$X_{i,fact}$, %					$C_{i,fact}$, mcg/ml	$X_{i,fact}$, %				
1	2	3	4	5	6	7	8	9	10	11	12
D = 25-175 % (g = 7)											
10.00	27.78	0.207	38.91	26.55	95.57	10.00	27.78	0.188	35.34	22.99	82.76
20.00	55.56	0.356	66.92	54.50	98.09	10.00	27.78	0.231	43.42	31.05	111.77
30.00	83.33	0.536	100.75	88.26	105.92	10.00	27.78	0.235	44.17	31.80	114.47
40.00	111.11	0.653	122.74	110.21	99.19	20.00	55.56	0.336	63.16	50.75	91.34
50.00	138.89	0.786	147.74	135.16	97.31	20.00	55.56	0.377	70.86	58.43	105.17
60.00	166.67	0.978	183.83	171.18	102.71	20.00	55.56	0.332	62.41	50.00	89.99
65.00	180.56	1.015	190.79	178.12	98.65	40.00	111.11	0.633	118.98	106.46	95.81
						40.00	111.11	0.675	126.88	114.34	102.91
						40.00	111.11	0.671	126.13	113.59	102.23
						65.00	180.56	1.034	194.36	181.69	100.63
						65.00	180.56	0.998	187.59	174.93	96.88
						65.00	180.56	1.040	195.49	182.82	101.25
				$\overline{RR}$, %	99.63					$\overline{RR}$, %	99.60
				$\delta = \lvert 100 - \overline{RR} \rvert \leq 6.40$ %	0.37					$\delta = \lvert 100 - \overline{RR} \rvert \leq 6.40$ %	0.40
D = 25 – 150 % (g = 6)											
10.00	27.78	0.207	38.91	27.21	97.95	10.00	27.78	0.188	35.34	23.70	85.31
20.00	55.56	0.356	66.92	54.75	98.54	10.00	27.78	0.231	43.42	31.64	113.89
30.00	83.33	0.536	100.75	88.01	105.62	10.00	27.78	0.235	44.17	32.38	116.56
40.00	111.11	0.653	122.74	109.63	98.67	20.00	55.56	0.336	63.16	51.05	91.88
50.00	138.89	0.786	147.74	134.22	96.64	20.00	55.56	0.377	70.86	58.62	105.51
60.00	166.67	0.978	183.83	169.70	101.82	20.00	55.56	0.332	62.41	50.31	90.55
						40.00	111.11	0.633	118.98	105.94	95.35
						40.00	111.11	0.675	126.88	113.71	102.34
						40.00	111.11	0.671	126.13	112.97	101.67

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Table 4 continued

1	2	3	4	5	6	7	8	9	12	11	12
						60.00	166.67	0.995	187.03	172.85	103.71
						60.00	166.67	0.955	179.51	165.46	99.27
						60.00	166.67	0.965	181.39	167.30	100.38
					$\overline{RR}$, %	99.87				$\overline{RR}$, %	100.54
					δ = |100 – $\overline{RR}$|≤6.40 %	0.13				δ = |100 – $\overline{RR}$|≤6.40 %	0.54
					D = 25-125 % (g = 5)						
10.00	27.78	0.207	38.91	25.93	93.34	10.00	27.78	0.188	35.34	22.31	80.31
20.00	55.56	0.356	66.92	54.37	97.86	10.00	27.78	0.231	43.42	30.51	109.83
30.00	83.33	0.536	100.75	88.72	106.47	10.00	27.78	0.235	44.17	31.27	112.56
40.00	111.11	0.653	122.74	111.04	99.94	20.00	55.56	0.336	63.16	50.55	90.98
50.00	138.89	0.786	147.74	136.42	98.22	20.00	55.56	0.377	70.86	58.37	105.06
						20.00	55.56	0.332	62.41	49.79	89.61
						40.00	111.11	0.633	118.98	107.22	96.50
						40.00	111.11	0.675	126.88	115.24	103.72
						40.00	111.11	0.671	126.13	114.48	103.03
					$\overline{RR}$, %	99.17				$\overline{RR}$, %	99.07
					δ = |100 – $\overline{RR}$|≤6.40 %	0.83				δ = |100 – $\overline{RR}$|≤6.40 %	0.93

Table 5

RESULTS OF WITHIN-RUN ACCURACY DETERMINATION FOR UV-SPECTROPHOTOMETRIC METHOD OF DOXYLAMINE QUANTITATIVE DETERMINATION IN BLOOD WITH PRELIMINARY TLC-PURIFICATION

Factual concentration of doxylamine succinate in blood (C_{st} = 36 mcg/ml)		Absorbance ($A_{st} = \frac{A_{reference} \cdot R}{100} = 0.510$)			Found in % to standard absorbance Y_i, %			Calculated concentration of doxylamine succinate in blood $X_{i,calc}$, %			$RR,\% = \frac{X_{i,calc}}{X_{i,fact}} \cdot 100$		
$C_{i,fact}$, mcg/ml	$X_{i,fact}$, %	1st day	2nd day	3d day	1st day	2nd day	3d day	1st day	2nd day	3d day	1st day	2nd day	3d day
1	2	3	4	5	6	7	8	9	10	11	12	13	14
D = 25-175 % (g = 7)													
10.00	27.78	0.159	0.147	0.151	31.18	28.82	29.61	30.87	28.56	29.26	111.12	102.81	105.33
20.00	55.56	0.297	0.295	0.287	58.24	57.84	56.27	57.66	57.32	55.60	103.78	103.17	100.07
30.00	83.33	0.415	0.420	0.410	81.37	82.35	80.39	80.56	81.62	79.44	96.68	97.95	95.33
40.00	111.11	0.583	0.579	0.573	114.31	113.53	112.35	113.18	112.52	111.02	101.86	101.27	99.92

Table 5 continued

1	2	3	4	5	6	7	8	9	10	11	12	13	14
50.00	138.89	0.704	0.698	0.695	138.04	136.86	136.27	136.67	135.64	134.65	98.40	97.66	96.95
60.00	166.67	0.882	0.874	0.877	172.94	171.37	171.96	171.23	169.84	169.92	102.74	101.90	101.95
65.00	180.56	0.937	0.931	0.929	183.73	182.55	182.16	181.91	180.92	180.00	100.75	100.20	99.69
										$\overline{RR}$, %	102.19	100.71	99.89
										$\delta = \lvert 100 - \overline{RR} \rvert \leq 6.40$ %	2.19	0.71	0.11
D = 25-150 % (g = 6)													
10.00	27.78	0.159	0.147	0.151	31.18	28.82	29.61	30.90	28.56	29.26	111.23	102.81	105.33
20.00	55.56	0.297	0.295	0.287	58.24	57.84	56.27	57.72	57.32	55.60	103.89	103.17	100.07
30.00	83.33	0.415	0.420	0.410	81.37	82.35	80.39	80.64	81.62	79.44	96.77	97.95	95.33
40.00	111.11	0.583	0.579	0.573	114.31	113.53	112.35	113.29	112.52	111.02	101.96	101.27	99.92
50.00	138.89	0.704	0.698	0.695	138.04	136.86	136.27	136.81	135.64	134.65	98.50	97.66	96.95
60.00	166.67	0.882	0.874	0.877	172.94	171.37	171.96	171.40	169.84	169.92	102.84	101.90	101.95
										$\overline{RR}$, %	102.53	100.79	99.93
$\delta = \lvert 100 - \overline{RR} \rvert \leq 6.40$ %											2.53	0.79	0.07
D = 25 – 125 % (g = 5)													
10.00	27.78	0.159	0.147	0.151	31.18	28.82	29.61	32.08	29.47	30.53	115.48	106.08	109.90
20.00	55.56	0.297	0.295	0.287	58.24	57.84	56.27	59.92	59.14	58.01	107.85	106.44	104.41
30.00	83.33	0.415	0.420	0.410	81.37	82.35	80.39	83.71	84.20	82.88	100.46	101.04	99.46
40.00	111.11	0.583	0.579	0.573	114.31	113.53	112.35	117.60	116.08	115.82	105.84	104.47	104.24
50.00	138.89	0.704	0.698	0.695	138.04	136.86	136.27	142.02	139.94	140.48	102.25	100.76	101.14
										$\overline{RR}$, %	106.38	103.76	103.83
										$\delta = \lvert 100 - \overline{RR} \rvert \leq 6.40$ %	6.38	3.76	3.83

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Table 6

RESULTS OF BETWEEN-RUN ACCURACY DETERMINATION FOR UV-SPECTROPHOTOMETRIC METHOD OF DOXYLAMINE QUANTITATIVE DETERMINATION IN BLOOD WITH PRELIMINARY TLC-PURIFICATION

Calculation by calibration samples						Calculation by model samples					
Factual concentration of doxylamine succinate in blood (C_{st} = 36 mcg/ml)		Absorbance ($A_{st} = \frac{A_{reference} \cdot R}{100} = 0.510$)	Found in % to standard absorbance Y_i, %	Calculated concentration of doxylamine succinate in blood $X_{i,calc}$, %	RR, %	Factual concentration of doxylamine succinate in blood (C_{st} = 36 mcg/ml)		Absorbance ($A_{st} = \frac{A_{reference} \cdot R}{100} = 0.510$)	Found in % to standard absorbance Y_i, %	Calculated concentration of doxylamine succinate in blood $X_{i,calc}$, %	RR, %
$C_{i,fact}$, mcg/ml	$X_{i,fact}$, %					$C_{i,fact}$, mcg/ml	$X_{i,fact}$, %				
1	2	3	4	5	6	7	8	9	10	11	12
D = 25-175 % (g = 7)											
10.00	27.78	0.152	29.80	29.48	106.12	10.00	27.78	0.138	27.06	26.77	96.36
20.00	55.56	0.293	57.45	56.82	102.27	10.00	27.78	0.154	30.20	29.87	107.52
30.00	83.33	0.415	81.37	80.48	96.58	10.00	27.78	0.158	30.98	30.64	110.30
40.00	111.11	0.578	113.33	112.10	100.89	20.00	55.56	0.279	54.71	54.11	97.39
50.00	138.89	0.699	137.06	135.57	97.61	20.00	55.56	0.290	56.86	56.24	101.22
60.00	166.67	0.878	172.16	170.29	102.17	20.00	55.56	0.297	58.24	57.61	103.69
65.00	180.56	0.933	182.94	180.95	100.22	40.00	111.11	0.592	116.08	114.82	103.34
						40.00	111.11	0.566	110.98	109.77	98.79
						40.00	111.11	0.582	114.12	112.88	101.59
						65.00	180.56	0.944	185.10	183.09	101.40
						65.00	180.56	0.923	180.98	179.01	99.14
						65.00	180.56	0.917	179.80	177.84	98.49
				$\overline{RR}$, %	100.84					$\overline{RR}$, %	101.60
				$\delta = \lvert 100 - \overline{RR} \rvert \leq 6.40$ %	0.84					$\delta = \lvert 100 - \overline{RR} \rvert \leq 6.40$ %	1.60
D = 25-150 % (g = 6)											
10.00	27.78	0.152	29.80	29.48	106.12	10.00	27.78	0.138	27.06	26.77	96.36
20.00	55.56	0.293	57.45	56.82	102.27	10.00	27.78	0.154	30.20	29.87	107.52
30.00	83.33	0.415	81.37	80.48	96.58	10.00	27.78	0.158	30.98	30.64	110.30
40.00	111.11	0.578	113.33	112.10	100.89	20.00	55.56	0.279	54.71	54.11	97.39
50.00	138.89	0.699	137.06	135.57	97.61	20.00	55.56	0.290	56.86	56.24	101.22
60.00	166.67	0.878	172.16	170.29	102.17	20.00	55.56	0.297	58.24	57.61	103.69
						40.00	111.11	0.592	116.08	114.82	103.34
						40.00	111.11	0.566	110.98	109.77	98.79
						40.00	111.11	0.582	114.12	112.88	101.59

Table 6 continued

1	2	3	4	5	6	7	8	9	10	11	12
						60.00	166.67	0.865	169.61	167.76	100.65
						60.00	166.67	0.887	173.92	172.03	103.22
						60.00	166.67	0.893	175.10	173.19	103.91
				$\overline{RR}$, %	100.94					$\overline{RR}$, %	102.33
				$\delta = \lvert 100 - \overline{RR} \rvert \leq 6.40$ %	0.94					$\delta = \lvert 100 - \overline{RR} \rvert \leq 6.40$ %	2.33
D = 25-125 % (g = 5)											
10.00	27.78	0.152	29.80	30.63	110.26	10.00	27.78	0.138	27.06	27.81	100.11
20.00	55.56	0.293	57.45	59.04	106.26	10.00	27.78	0.154	30.20	31.04	111.74
30.00	83.33	0.415	81.37	83.63	100.36	10.00	27.78	0.158	30.98	31.84	114.61
40.00	111.11	0.578	113.33	116.47	104.82	20.00	55.56	0.279	54.71	56.23	101.21
50.00	138.89	0.699	137.06	140.86	101.42	20.00	55.56	0.290	56.86	58.44	105.18
						20.00	55.56	0.297	58.24	59.86	107.74
						40.00	111.11	0.592	116.08	119.30	107.37
						40.00	111.11	0.566	110.98	114.06	102.66
						40.00	111.11	0.582	114.12	117.29	105.56
				$\overline{RR}$, %	104.62					$\overline{RR}$, %	106.24
				$\delta = \lvert 100 - \overline{RR} \rvert \leq 6.40$ %	4.62					$\delta = \lvert 100 - \overline{RR} \rvert \leq 6.40$ %	6.24

The results of measuring the absorbance values of model solutions, calculated values of concentrations of model solutions and values *RR,* % for different ranges of method application are given in Tab. 2. The data of Table 2 about the value δ^{model} are the evidence that the requirements to systematic error are satisfied both for *Approach 1* and *Approach 2*.

The results of measuring the absorbance values for calibration and model samples, respective values X_{calc}, % and values *RR,* % for different ranges of method application are given in Tab. 3-6. It is obviously from the data given in Tab. 3-6 that the requirements to systematic error δ are satisfied for all offered variants of ranges of method application and for both variants of the method – with TLC-purification and without it. For the application range of 25-125 % in the case of carrying out the analysis with preliminary TLC-purification the value of systematic error reaches critical number – 6.38 % and 6.24 %, therefore it is better to use more wide range of application for this variant of the method.

CONCLUSIONS

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

- application of the normalized coordinates;
- accuracy confirmation of the method is carried out in two directions – by model solutions (without matrix) and by matrix samples;
- verification of the method accuracy by model solutions is carried out by calculation of their concentrations using the respective linear dependence;
- estimation of the method accuracy by matrix samples is carried out at two levels – within-run and between-run – using calibration and model samples;
- determination of within-run accuracy is carried out in the way of calculating the concentrations of calibration samples for each run by individual values of absorbance using the linear dependence obtained for this run;
- determination of between-run accuracy is carried out in two stages – by calculation of the concentrations of model samples and mean concentrations of calibration samples using the linear dependence obtained by the mean values of parallel runs;
- investigations of model samples are carried out for three replicate runs; the samples of biological matrix are obtained from the different source; for D = 25-125 % each run consists of 3 model samples (concentrations correspond to the points of 25 %, 50 % and 100 % in the normalized coordinates), for D = = 25-150 % and 25-175 % – of 4 samples (concentrations correspond to the points of 25 %, 50 % and 150 % or 175 % in the normalized coordinates);
- the calculated values X_{calc}, % and X_{calc}^{model}, % are used for calculation of δ and δ^{model} respectively;
- the acceptability criteria have been offered for estimation of value δ^{model} within two approaches based on: 1) assumption of equality of the uncertainty of sample preparation procedure and the uncertainty of analyte quantitative determination in model solutions ($\delta^{model} \le 4.52$ %); 2) assumption of insignificance of the uncertainty of analyte quantitative determination in model solutions ($\delta^{model} \le 2{,}05$ %);
- it is proceeded from insignificance of systematic error for estimation of value δ (δ ≤ 6.40 %).

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ВИЗНАЧЕННЯ ПРАВИЛЬНОСТІ В ХОДІ ВАЛІДАЦІЇ УФ-СПЕКТРОФОТОМЕТРИЧНИХ МЕТОДИК КІЛЬКІСНОГО ВИЗНАЧЕННЯ В СУДОВО-ТОКСИКОЛОГІЧНОМУ АНАЛІЗІ

Сформовані критерії та процедура оцінки прийнятності правильності УФ-спектрофотометричних методик кількісного визначення аналітів у біологічних рідинах, що застосовуються в судово-токсикологічному аналізі. Оцінку правильності запропоновано проводити в два етапи – на модельних розчинах (без матриці) і на зразках матриці та на двох рівнях – within-run і between-run. Запропоновані підходи апробовані на УФ-спектрофотометричній методиці кількісного визначення доксиламіну в крові.

Ключові слова: валідація; правильність; критерії прийнятності; УФ-спектрофотометрія; доксиламін; біоаналітичні методики

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ОПРЕДЕЛЕНИЕ ПРАВИЛЬНОСТИ В ХОДЕ ВАЛИДАЦИИ УФ-СПЕКТРОФОТОМЕТРИЧЕСКИХ МЕТОДИК КОЛИЧЕСТВЕННОГО ОПРЕДЕЛЕНИЯ В СУДЕБНО-ТОКСИКОЛОГИЧЕСКОМ АНАЛИЗЕ

Сформированы критерии и процедура оценки приемлемости правильности УФ-спектрофотометрических методик количественного определения аналитов в биологических жидкостях, применяемых в судебно-токсикологическом анализе. Оценку правильности предложено проводить в два этапа – на модельных растворах (без матрицы) и на образцах матрицы и на двух уровнях – within-run и between-run. Предложенные подходы апробированы на УФ-спектрофотометрической методике количественного определения доксиламина в крови.

Ключевые слова: валидация; правильность; критерии приемлемости; УФ-спектрофотометрия; доксиламин; биоаналитические методики

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