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Development and validation of HPLC-procedures of doxylamine determination in blood in the variant of the method of standard

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ABSTRACT

The set of procedures of doxylamine quantitative determination in blood by the method of high-performance liquid chromatography using amphiphylic solvents (isopropanol, acetonitrile, methanol) under the conditions of aqueous phase saturation by ammonium sulphate has been developed; acetonitrile application in the weak-acid medium (pH = 5) is optimal. Validation of the developed procedures has been carried out in the variant of the method of standard and the possibility of application of the method of standard for determination has been shown with the purpose of rationalization of quantitative determinations carrying out in forensic toxicology.

Keywords: validation, bioanalytical methods, high-performance liquid chromatography, doxylamine, method of standard

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INTRODUCTION

Development of strong medicines determination procedures in human biological fluids for application in forensic and clinical toxicology is one of the actual problems of pharmaceutical science, but validation of such analytical procedures becomes much more vital and widely discussed problem of analytical toxicology in the past decade [1 – 7].

The available international guidance's on carrying out validation of bioanalytical methods [6, 7] are reckoned on the experiment performance in the variant of the method of calibration curve that implies carrying out a lot of routine analyses in practical work. In forensic toxicology we often meet with single examinations, and various biological fluids, organs and tissues are sent for the examinations, i.e. it is necessary to determine analyte quantitatively in some various biological objects, and the necessity of carrying out such determination can arise rarely enough. In such situation plotting the calibration curve for each matrix demands quite nonrational investment of time, and to the moment of obtaining the results of analysis they can become irrelevant.

Taking into account the experience of standardized validation procedures development in Ukraine [8], we offered the approaches to determination and estimation of such main validation parameters as specificity, recovery, linearity, precision and accuracy for procedures of analyte quantitative determination in biological fluids applied in forensic toxicology in the variant of the method of standard [9 – 11].

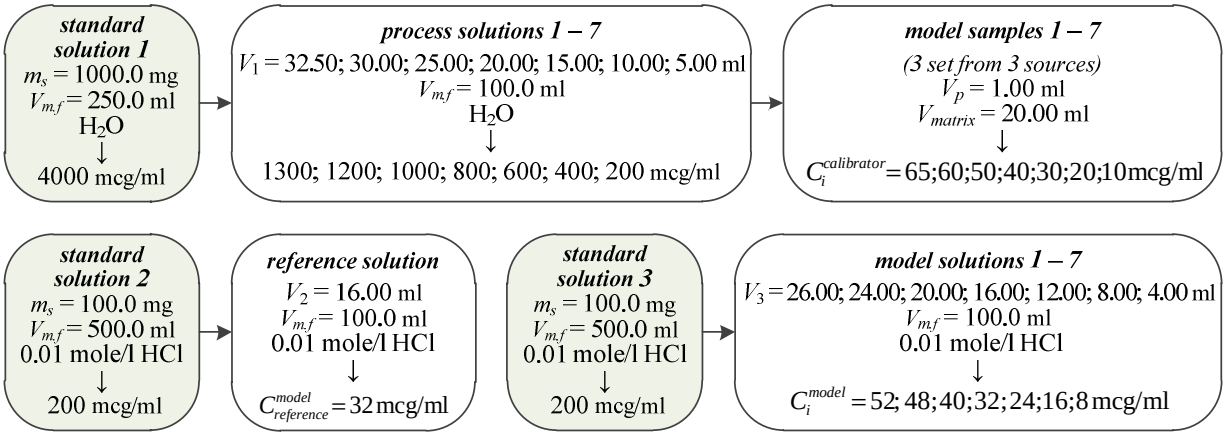
The developed approaches were successfully applied to procedures using optical methods of analysis [11], and it is interesting to approve these validation procedures on chromatographic methods of analysis.

The purpose of the paper is developing the set of procedures of doxylamine quantitative determination in blood using different procedures of sample preparation based on HPLC-method offered before [12], carrying out validation of the offered methods for choosing the optimal procedure of sample preparation provided effective doxylamine isolation from blood and low content of co-extracted substances in the obtained extracts at the minimum value of the

method uncertainty, and also estimating the possibility of the method of standard application for doxylamine HPLC-determination in blood.

EXPERIMENTAL SECTION

Doxylamine of pharmacopoeial purity was used in the experiment. The procedure of preparation of standard, process and model solutions, and also model samples is presented on *Scheme 1*.



Scheme 1. **The order of solutions and samples preparation for validation of doxylamine determination procedures in blood by the method of HPLC**

The design of experiment on development of procedures of doxylamine determination in blood by the method of HPLC is presented on *Scheme 2*.

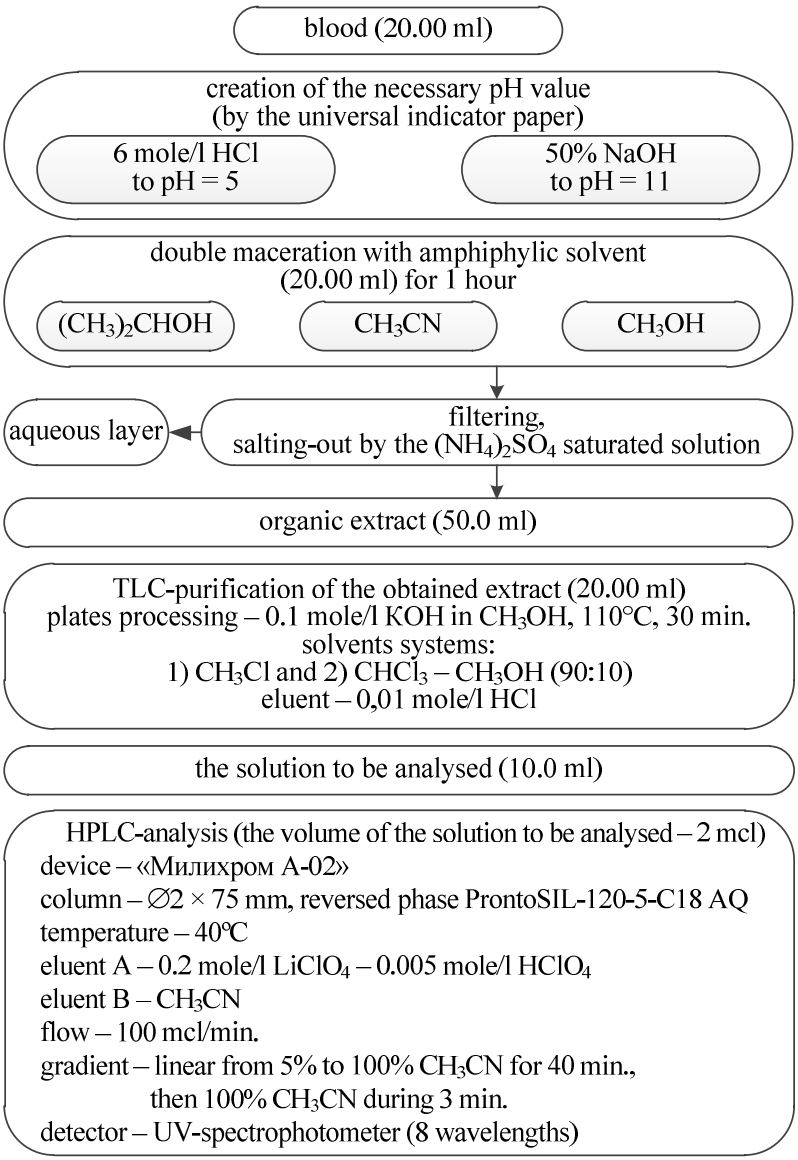
The model (see *Scheme 1*) and also blank-samples and blank-solutions were analysed for each developed procedure; the blank-samples were prepared in the following way: 5 samples (20.00 ml) of the blood obtained from the different sources, 1.00 ml of distilled water were added into them.

Each solution to be analysed was chromatographed 3 times or, as required, more following the our offered requirements to repeatability of peaks areas *S*for replicateinjections – the relative standard deviation of the mean RSD_{nom} calculated towards the nominal value of peak area S_{nom}should not exceed:

$$RSD_{nom} = \frac{s}{S_{nom}} \cdot 100\% \le \frac{0.1 \cdot \max\Delta_{As} \cdot \sqrt{n}}{t(95\%, n-1)} = \begin{Bmatrix} 1.47\%;\ n=3 \\ 1.88\%;\ n=4 \\ 2.22\%;\ n=5 \\ 2.52\%;\ n=6 \end{Bmatrix};$$

$$S_{nom} = S_{\min} = \overline{S}_{25\%},$$

where $\max\Delta_{As}$ – is the extreme relative uncertainty of the procedure of analysis, $\max\Delta_{As}$ = 20% [6]; $\overline{S}_{25\%}$ – the mean peak area obtained when analysing the respective solutions with the analyte concentration corresponded to the point of 25% in the normalized coordinates (see explanations in the text).



***Scheme* 2. The main stages of the procedures of doxylamine determination in blood by the method of HPLC**

RESULTS AND DISCUSSION

The HPLC-method for doxylamine determination using the system of HPLC-analyzer«МилихромА-02» was developed by authors before [12]; the retention time for doxylamine was 11.85 min. This method was applied for estimation of efficiency of doxylamine isolation from blood by maceration with 10% trichloroacetic acid solution and subsequent extraction with chloroform in the alkaline medium (pH = 11) – the recovery was equal to ~75% [13].

In this paper it has been suggested to carry out doxylamine isolation from using amphiphylic solvents with subsequent separation of organic layer under the conditions of aqueous phase saturation by electrolyte; this approach enjoys wide popularity in modern forensic and toxicological analysis [5, 14]. Such amphiphylic solvents as isopropanol, acetonitrile and methanol have been used in the experiment; ammonium sulphate has been applied as electrolyte for saturation of aqueous phase.

Isolation has been carried out in the alkaline (pH = 11) and weak-acid medium (pH = 5); carrying out isolation of analytes from biological objects in the weak-acid medium results in decreasing of co-extraction processes of biological matrix components in a number of cases [5, 14]. It is necessary to note that application of amphiphylicsolvents and saturated solution of ammonium sulfate allows to maintain the isolation efficiency of substances of base character in the weak-acid medium at the same level as in the alkaline medium – it is conditioned by shift of pH real value in alkaline side for mixtures of electrolytes saturated solutions with amphiphylicsolvents [15].

Thus, the development of the set of HPLC-methods of doxylamine determination in blood has become the result of this stage of investigations; the methods differ by the procedures of sample preparation (see *Scheme* 2).

For choosing the optimal method of doxylamine determination in blood we have carried out validation of all developed procedures by such parameters as specificity, recovery, linearity, accuracy, repeatability and intermediate precision according to the approaches offered by us in the variant of the method of standard [9 – 11].

The validation procedure foresees application of the normalized coordinates. For normalization of the obtained experimental data the reference solution with the concentration of analyte corresponded to its concentration in the end solution to be analysed under the condition of zero losses for the point of 100% in the normalized coordinates is used. The peak area for reference solution is corrected taking into account the value of recovery *R*, which significance and value has been showed at the preliminary stage of validation, and is used for normalization of peak areas for the model samples.

The range of the methods application is D = 25 – 175%; the number of concentration levels is g = 7 in constant increments of 25%; as 100% the mean toxic doxylamine concentration in blood [5] is accepted.

The methods validation has been carried out at the first stage using model solutions (*Scheme 3*) and proceeding from two approaches [9]:

*Approach 1:*the uncertainty of sample preparation procedure is equal to the uncertainty of analyte quantitative determination in model solutions Δ_{As}^{model}.

*Approach 2:*the uncertainty of analyte quantitative determination in model solutions Δ_{As}^{model} is insignificant against the total uncertainty of analysis resultsΔ_{As}.

analysis of the model solutions 1 – 7 and reference solution

(*1 run – 1 day*)

$$C_i^{model} \cong S_i^{model} \cong 25,50,75,100,125,150,175\%$$

$$C_{reference}^{model} \cong S_{reference}^{model} \cong 100\%$$

normalization of the obtained data

$$X_{i,fact}^{model} = \frac{C_i^{model}}{C_{reference}^{model}} \cdot 100\%; \qquad Y_i^{model} = \frac{S_i^{model}}{S_{reference}^{model}} \cdot 100\%$$

linearity verification; $g = 7$

$$Y^{model} = a + b \cdot X^{model} \rightarrow a^{model};\ s_a^{model};\ b^{model};\ s_b^{model};\ RSD_0^{model};\ R_c^{model}$$

Approach 1

$$RSD_0^{model} \le 7.02\%;\ R_c^{model} \ge 0.9915$$

$$a^{model}: 1) \le t(95\%; g-2) \cdot s_a^{model};\ 2) \le 6.03\%$$

Approach 2

$$RSD_0^{model} \le 3.18\%;\ R_c^{model} \ge 0.9983$$

$$a^{model}: 1) \le t(95\%; g-2) \cdot s_a^{model};\ 2) \le 2.73\%$$

accuracy and repeatability verification

$$Z_i^{model} = \frac{Y_i^{model}}{X_{i,fact}^{model}} \cdot 100\%; \quad \Delta_Z^{model} = t(95\%, g-1) \cdot RSD_Z^{model}; \quad \delta^{model} = \left|100 - \bar{Z}^{model}\right|$$

Approach 1

$$\Delta_Z^{model} \le \max\Delta_{As}^{model} = 14.14\%$$

$$\delta^{model} \le \max\delta^{model} = 4.52\%$$

Approach 2

$$\Delta_Z^{model} \le \max\Delta_{As}^{model} = 6.40\%$$

$$\delta^{model} \le \max\delta^{model} = 2.05\%$$

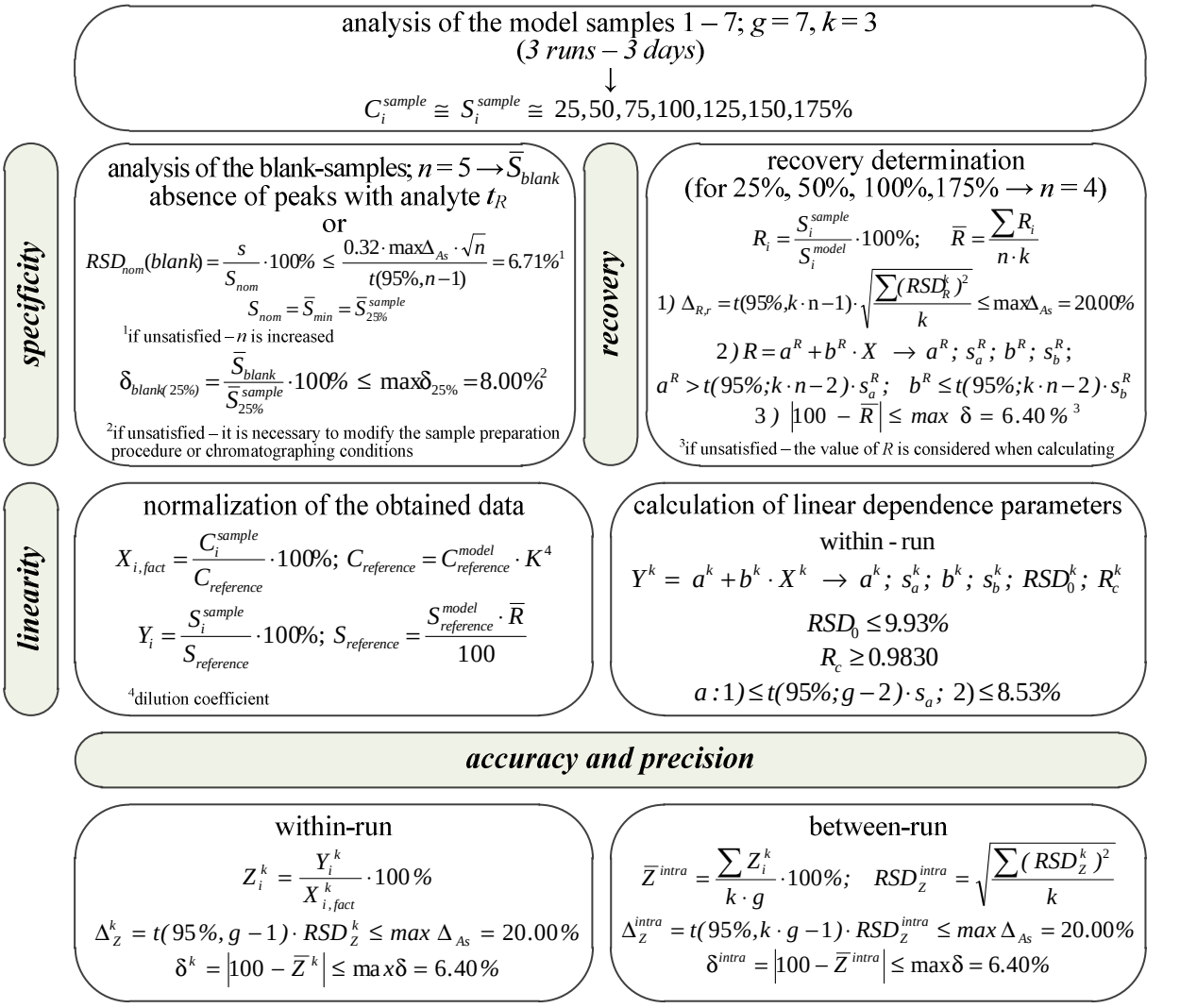
***Scheme* 3. The stages of validation of HPLC-method of doxylamine determination using model solutions**

The total results of validation are presented in *Table 1* and allow to point to the conclusion about acceptable linearity, accuracy and repeatability of the HPLC-procedure of doxylaminequantitative determination in the variant of the method of standard both for *Approach 1* and *Approach 2* that gives the possibility to recommend it to further application in forensic toxicology with the purpose of development of the methods of biological objects analysis for doxylaminequantification.

Table 1 **The total results of validation of doxylamine determination procedure by the method of HPLC, which were obtained using model solutions**

linearity		Parameter					
		b^{model}	s_b^{model}	a^{model}	s_a^{model}	RSD_0^{model}	R_c^{model}
		1.000	0.010	0.726	1.357	1.561	0.9998
acceptability criterion	*Approach 1*	–	–	$a^{model} \le 6.03\%$	$a^{model} \le 2.015 \cdot s_a^{model}$	$\le 7.02\%$	≥ 0.9915
				satisfied	satisfied	satisfied	satisfied
	Approach 2	–	–	$a^{model} \le 2.73\%$	$a^{model} \le 2.015 \cdot s_a^{model}$	$\le 3.18\%$	≥ 0.9983
				satisfied	satisfied	satisfied	satisfied

accuracy and repeatability		Parameter			
		$\overline{Z}^{model}$	RSD_Z^{model}	δ^{model}	Δ_Z^{model}
		100.91	1.44	0.91	2.80
acceptability criterion	*Approach 1*	–	–	$\le 4.52\%$	$\le 14.14\%$
				satisfied	satisfied
	Approach 2	–	–	$\le 2.05\%$	$\le 6.40\%$
				satisfied	satisfied



***Scheme* 4. The stages of validation of HPLC-methods of doxylaminedetermination in blood using model samples**

At the second stage the methods validation has been carried out using model samples – the determination procedure and acceptability criteria are presented at *Scheme 4*.

For specificity investigation for the developed procedures as for the components of biological matrix we have determined the sum of peaks areas on the chromatograms of blank-samples within 11thand 12th minutes – $\overline{S}_{blank}$.

The maximum peak area for doxylamineis observed in the case of detection at the wavelength of 210 nm, but at the same wavelength the sum of peaks areas is maximum on the chromatograms of blank-samples. At the same time the less intensive doxylamine peak at λ = 260 nm (this wavelength is the nearest to characteristic doxylamine in UV-range of spectrum) is accompanied by the absence of peaks with the retention time, which is coincident with (or near to) the doxylamine retention time, on the chromatograms of blank-samples for all variants of procedures of analyte isolation from blood that points to the conclusion about acceptable specificity of the developed methods as for the components of biological matrix when using 260 nm as a working wavelength.

The absence of peaks with the retention time, which is coincident with (or near to) the doxylamine retention time, on the chromatograms of blank-solutions for all wave lengths used for detection in the described HPLC-system is the evidence of the correct choice of sample preparation procedure for all considered cases, i. e. the sample preparation procedure does not influence on the results of analysis.

It is necessary to note that in all cases carrying out doxylamine isolation from blood at pH = 5 provides lower sum of peaks areas on the chromatograms of blank-samples than in the case of alkaline pH using; at the same time by the results of recovery study small decreasing of doxylamine isolation efficiency from blood – within 3 – 5% – is noted under these conditions. The methods with acetonitrile application are characterized by the best extraction efficiency.

The reproducibility of recovery values (*Table 2*) satisfies the acceptability criteria for all variants of methods.

Table 2 The results of recovery determination for HPLC-methods of doxylamine quantification in blood

Parameter	Solvent						Acceptability criterion
	$(CH_3)_2CHOH$		CH_3CN		CH_3OH		
	pH = 5	pH = 11	pH = 5	pH = 11	pH = 5	pH = 11	
$\overline{R}$	78.14	83.18	92.45	94.10	86.85	87.52	–
$\Delta_{R,r}$	10.12	9.56	7.56	8.54	9.45	8.65	≤ 20.00%
b^R	0.03	0.02	0.03	0.03	–0.01	0.00	$b^R \le 1.812 \cdot s_b^R$
s_b^R	0.02	0.02	0.02	0.02	0.02	0.02	
a^R	75.30	81.63	90.39	90.99	87.09	84.77	$a^R > 1.812 \cdot s_a^R$
s_a^R	1.75	2.31	1.54	1.85	2.21	2.01	
$\lvert 100 - \overline{R} \rvert$	21.86	16.82	7.55	5.90	13.15	12.48	≤ 6.40%

Taking into account the data about specificity of the developed procedures the investigations of linearity, accuracy and precision have been carried out only for the set of procedures with application of the weak-acid medium for doxylamine isolation from blood – the total results are presented in *Table 3*.

For the method with acetonitrile application at pH = 5 calculation of linearity, accuracy and precision parameters has been carried out both with correction by the *R* value and without it – absence of such correction does not lead to significant worsening of the method validation parameters.

On the whole, all examined methods are characterized by the acceptable parameters of linearity, accuracy and precision, but high efficiency of doxylamine extraction from blood and low value of the method uncertainty allow to consider the method with acetonitrile application in the weak-acid medium as optimal for sample preparation of blood to further HPLC-determination of doxylamine.

Table3 The results of linearity, accuracy and precision determination for HPLC-methods of doxylamine quantification in blood (pH = 5)

Parameter	$(CH_3)_2CHOH$			CH_3CN			CH_3OH			Acceptability criterion
linearity										
a^k	–3.739	–2.422	–2.507	–2.639	–0.810	–1.497	–2.131	–1.502	0.457	$a \le 2.015 \cdot s_a$
s_a^k	1.388	2.039	2.720	3.049	3.029	2.696	2.795	4.129	2.840	$a \le 8.53\%$
b^k	1.067	1.049	1.047	1.036	1.023	1.023	1.007	0.997	0.988	
s_b^k	0.013	0.019	0.025	0.028	0.028	0.025	0.026	0.038	0.026	–
RSD_0^k	1.598	2.346	3.130	3.509	3.486	3.103	3.216	4.752	3.269	≤ 9.93%
R_c^k	0.9996	0.9992	0.9986	0.9982	0.9982	0.9985	0.9984	0.9964	0.9983	≥ 0.9830
accuracy										
$\bar{Z}^k$	101.38	101.58	99.98	99.93	101.33	101.05	96.82	97.26	99.11	–
δ^k	1.38	1.58	0.02	0.07	1.33	1.05	3.18	2.74	0.89	≤ 6.40%
$\bar{Z}^{intra}$	100.98			100.77			97.73			–
δ^{intra}	0.98			0.77			2.27			≤ 6.40%
precision										
RSD_Z^k	4.07	4.12	7.36	3.73	3.32	5.97	6.45	4.61	3.03	–
Δ_Z^k	7.91	8.01	14.30	7.25	6.45	11.60	12.53	8.96	5.89	≤ 20.00%
RSD_Z^{intra}	5.41			4.50			4.90			–
Δ_Z^{intra}	9.33			7.76			8.45			≤ 20.00%

CONCLUSION

1. The set of HPLC-procedures of doxylamine quantitative determination in blood using amphiphylic solvents (isopropanol, acetonitrile, methanol) for analyte isolation from matrix in the weak-acid and alkaline medium with further separation of organic layer under the conditions of aqueous phase saturation by ammonium sulphate has been developed.
2. Validation of the developed procedures has been carried out and it has been set that acetonitrile application in the weak-acid medium (pH = 5) is optimal for doxylamine determination in blood – the extraction efficiency is maximal and equal to ~97%, and parameters of linearity, accuracy and precision are optimal.
3. The possibility of application of the offered approaches to validation of quantitative determination procedures for forensic and toxicological analysis in the variant of the method of standard has been shown for validation of procedures using the method of high-performance liquid chromatography.

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