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Development and validation of the methods of captopril spectrophotometric determination in blood by the reaction with the Ellman reagent

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Key words: *Validation Studies, Analitical Chemistry Techniques, Captopril, Ellman's reagent, Spectrophotometry.*

Aim. To rationalize quantitative determinations in forensic and toxicological analysis, the possibility to use the method of standard for analytes quantitative determination in biological fluids has been studied.

Methods and results. The series of spectrophotometric methods of captopril quantitative determination in blood based on reaction with the Ellman reagent has been developed using amphiphylic solvents (isopropanol, acetonitrile, methanol) in condition of aqueous phase saturation by ammonium sulphate. Validation of the developed methods in the variant of the method of standard has been carried out, conformity of the obtained values of validation parameters to the acceptability criteria has been shown.

Conclusion. It has been set that acetonitrile application in the acid medium (pH=2) is optimal.

Розробка та валідація методик спектрофотометричного визначення каптоприлу у крові за реакцією з реактивом Еллмана

Л. Ю. Клименко, З. В. Шовкова, О. Є. Микитенко, А. Г. Каплаушенко

З метою раціоналізації здійснення кількісних визначень у судово-токсикологічному аналізі вивчили можливість використання методу стандарту для кількісного визначення аналітів у біологічних рідинах. Розробили серію спектрофотометричних методик кількісного визначення каптоприлу у крові, що ґрунтуються на реакції з реактивом Еллмана, з використанням амфіфільних розчинників (ізопропанолу, ацетонітрилу, метанолу) в умовах насичення водної фази амонію сульфатом. Виконали валідацію методик у варіанті методу стандарту, показали відповідність значень валідаційних параметрів, які отримали, до критеріїв прийнятності. Встановили, що оптимальним є використання ацетонітрилу в кислому середовищі (pH = 2).

Ключові слова: *валідація, біоаналітичні методики, каптоприл, реактив Еллмана, спектрофотометрія.*

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Разработка и валидация методик спектрофотометрического определения каптоприла в крови по реакции с реактивом Эллмана

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С целью рационализации проведения количественных определений в судебно-токсикологическом анализе изучена возможность использования метода стандарта в ходе количественного определения аналитов в биологических жидкостях. Разработана серия спектрофотометрических методик количественного определения каптоприла в крови, основанных на реакции с реактивом Эллмана, с использованием амфифильных растворителей (изопропанола, ацетонитрила, метанола) в условиях насыщения водной фазы аммония сульфатом. Проведена валидация разработанных методик в варианте метода стандарта, показано соответствие полученных значений валидационных параметров критериям приемлемости. Установлено, что оптимально использование ацетонитрила в кислой среде (pH=2).

Ключевые слова: *валидация, биоаналитические методики, каптоприл, реактив Эллмана, спектрофотометрия.*

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The necessity of analytical methods' validation currently becomes the vital and widely discussed problem in analytical toxicology [1,2].

The available international guidance on carrying out validation of bioanalytical methods [3,4] are reckoned, firstly, on using exclusively chromatographic methods of analysis, and secondly, on application of the method of calibration curve that implies carrying out a lot of routine analyses in practical work. Single examinations are more widespread in practice of forensic and toxicological analysis, and application of the method of standard is more justified in this situation.

Purpose of the work:

- developing the series of methods of captopril quantitative determination in blood using different procedures of sample preparation based on spectrophotometric methods offered before [5] by reaction with the Ellman reagent;
- carrying out validation of the offered methods and estimating the possibility of the method of standard application for captopril spectrophotometric determination in blood;
- choosing the optimal procedure of sample preparation which provides effective captopril isolation from blood and low content of co-extracted substances in the obtained extracts at the minimum value of the method.

Materials and methods

Captopril of pharmacopoeia purity was used in the experiment. The order of preparation of standard, process and model solutions, and also model samples are presented on *fig. 1*.

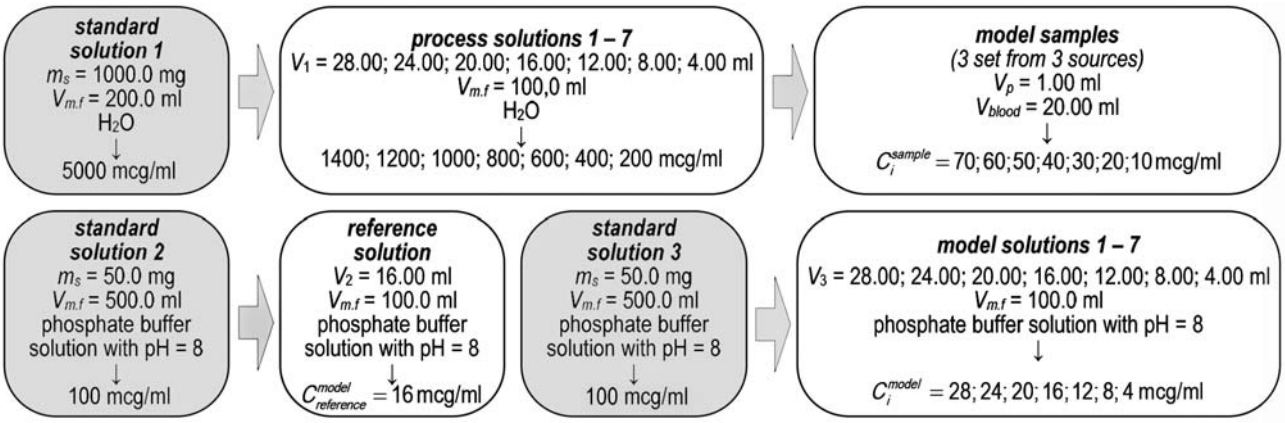

Fig. 1. The procedure of solutions and samples preparation for validation of spectrophotometric methods of captopril determination in blood.

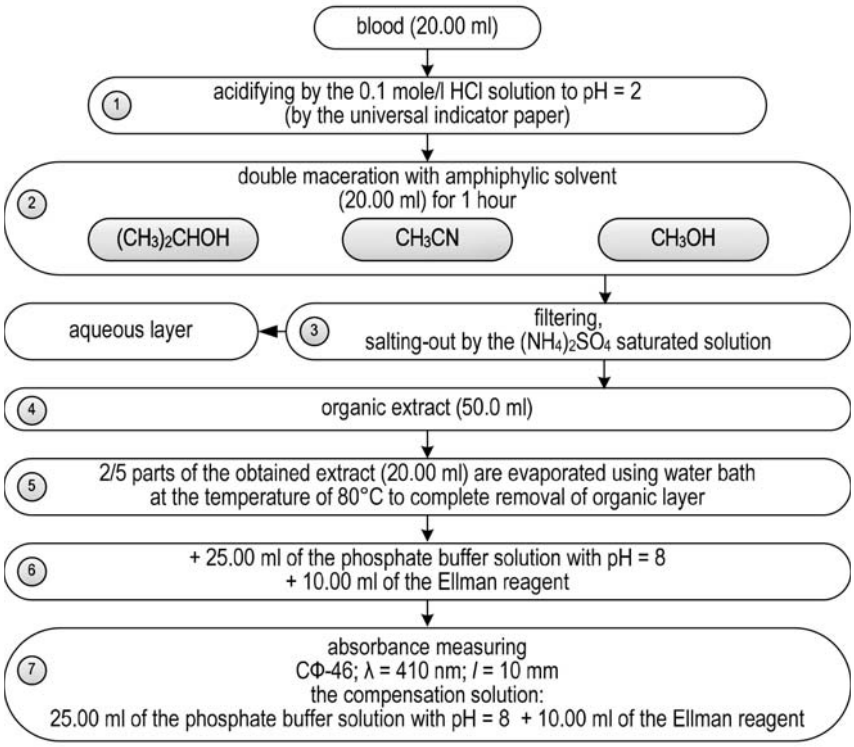

Fig. 2. The main stages of the methods of captopril spectrophotometric determination in blood.

The design of experiment on development of methods of captopril spectrophotometric determination in blood by reaction with the Ellman reagent is presented on *fig. 2*.

The procedure of the Ellman reagent preparation: 50.0 mg of 5,5'-dithio-bis(2-nitrobenzoic acid) were placed in the measuring flask with the capacity of 100.0 ml, a little amount of the phosphate buffer solution with pH = 6.8 and 2.5 ml of the 0,1 mole/l sodium hydroxide solution were added to complete dissolution of 5,5'-dithio-bis(2-nitrobenzoic acid), the solution was diluted to the volume with the phosphate buffer solution with pH = 6.8. The Ellman reagent pH was controlled with the help of ionometer pH-150 (the pH value of the solution should not exceed 7.0).

The model (see *fig 1*) and blank-samples were analysed for each developed method; the blank-samples were prepared in the following way: 1) 5 samples (20.00 ml) of the blood obtained from the different sources, 1.00 ml of distilled water were added into them; 2) 3 samples (20.00 ml) of distilled water.

The absorbance of the solutions was measured 3 times with randomization of cell position.

Results and discussion

The approaches to validation of quantitative determination methods for forensic and toxicological analysis as to the instrument of optimal method development within the assigned purpose have been offered by us before [6 – 8] – the procedures of experiment carrying out properly and acceptability criteria of the obtained results have been developed in the variant of the method of standard.

For captopril determination the spectrophotometric method based on the photometric reaction with the Ellman reagent at pH = 8 [5] has been developed by us before and the procedure

of mentioned analyte isolation from blood by maceration with the 1 mole/l hydrochloric acid solution and subsequent extraction with the mixture of chloroform and isopropanol (8:2) in the acid medium (pH = 2) [9] – the efficiency of captopril isolation from blood using this procedure has been evaluated by means of the spectrophotometric method mentioned above and is equal to ~75%.

In the present work it has been suggested to carry out captopril isolation from blood by its maceration with amphiphylic solvents and subsequent separation of organic layer under the conditions of aqueous phase saturation by electrolyte for increasing the efficiency; this approach enjoys wide popularity in modern forensic and toxicological analysis [2, 10]. Such amphiphylic solvents as methanol, isopropanol and acetonitrile were used in the experiment; ammonium sulphate was applied as electrolyte for saturation of aqueous phase. Isolation was carried out in the acid medium (pH = 2) – as in the method offered before [9].

Thus, the development of the series of methods of captopril determination in blood using the method of spectrophotometry by reaction with the Ellman reagent has been become the result of this stage of investigations; the methods differ by the procedures of sample preparation (*fig. 2*).

For choosing the optimal methods of captopril determination in blood we carried out their validation 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 [6–8].

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 absorbance of 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 absorbance values of 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 captopril concentration in blood [2] – 40 mcg/ml – is accepted.

The methods validation was carried out at the first stage using model solutions (*fig. 3*) and proceeding from two approaches [6]:

Approach 1: the uncertainty of analyte quantitative determination in model solutions Δ_{As}^{model} is insignificant against the total uncertainty of analysis results.

Approach 2: the uncertainty of sample preparation procedure is equal to the uncertainty of analyte quantitative determination in model solutions Δ_{As}^{model}.

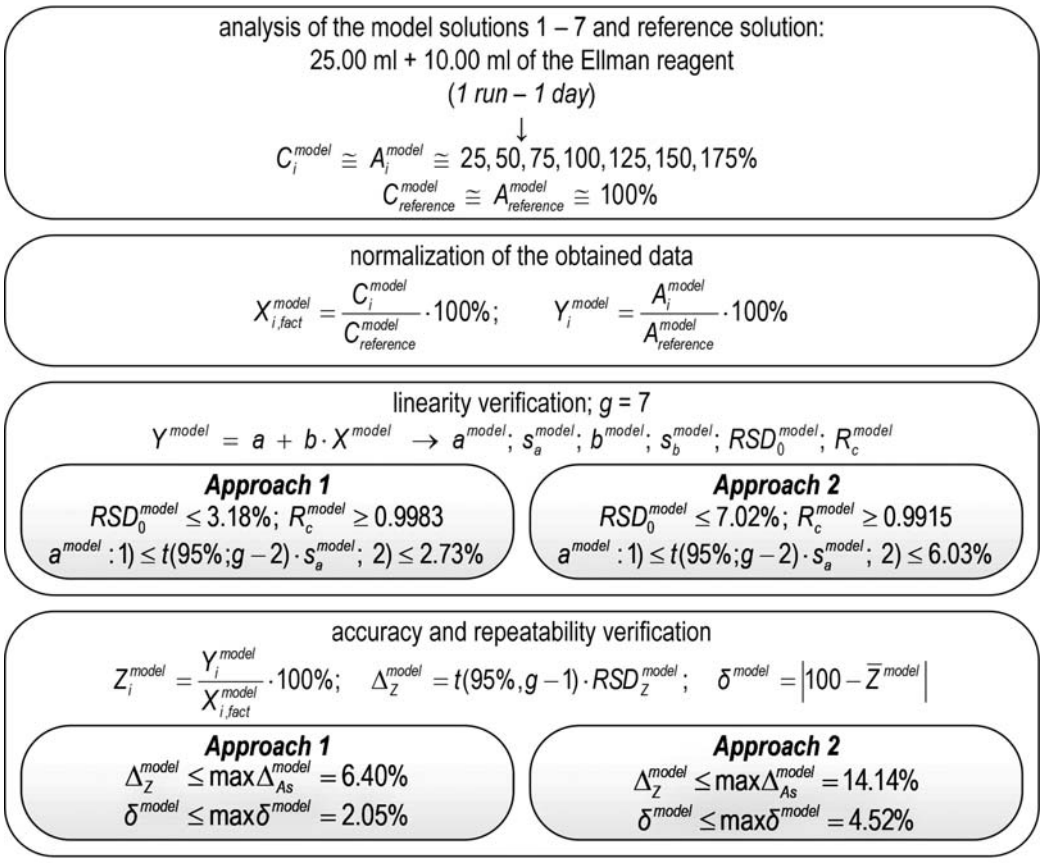

Fig. 3. The stages of validation of spectrophotometric method of captopril determination using model solutions.

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 method of captopril quantitative determination by the method of spectrophotometry by reaction with the Ellman reagent in the variant of the method of standard; at the same time it is necessary to note that the requirements to accuracy are fulfilled only within *Approach 2*.

Thus, it is possible to recommend the method of captopril quantitative determination by the method of spectrophotometry by reaction with the Ellman reagent to further application in forensic toxicology with the purpose of development of the methods of biological objects analysis for captopril quantification.

Table 1

The total results of validation of spectrophotometric method of captopril determination by reaction with the Ellman reagent, which were obtained using model solutions

linearity		Parameter					
		b^{model}	s_b^{model}	a^{model}	s_a^{model}	RSD_0^{model}	RSD_c^{model}
		1.019	0.011	0.130	1.257	1.487	0,9997
acceptability criterion	Approach 1	–	–	$a^{model} \le 2.73\%$	$a^{model} \le 2.015 \cdot s_a^{model}$	≤ 3,18%	≥ 0,9983
				satisfied	satisfied	satisfied	satisfied
	Approach 2	–	–	$a^{model} \le 6.03\%$	$a^{model} \le 2.015 \cdot s_a^{model}$	≤ 7,02%	≥ 0,9915
				satisfied	satisfied	satisfied	satisfied
accuracy and repeatability		Parameter					
		$\bar{Z}^{model}$		RSD_z^{model}	δ^{model}	Δ_z^{model}	
		102.54		2.24	2.54	4.35	
acceptability criterion	Approach 1	–		–	≤ 2.05%	≤ 6.40%	
					unsatisfied	satisfied	
	Approach 2	–		–	≤ 4.52%	≤ 14.14%	
					satisfied	satisfied	

Table 2

The total results of validation of spectrophotometric methods of captopril determination in blood by reaction with the Ellman reagent

Parameter	Solvent									acceptability criterion
	$(CH_3)_2CHOH$			CH_3CN			CH_3OH			
specificity										
$\bar{A}_{blank}$	0.019			0.020			0.024			–
RSD_{nom}(blank)	5.41			4.87			5.73			≤ 6.71%
δ_{blank}(25%/50%)	9.80 / 5.23			9.64 / 5.12			12.33 / 6.73			≤ 8.00% / 6.40%
$\bar{A}_{procedure}$	0.005			0.005			0.006			–
$\delta_{procedure(25\%)}$	2.70			2.39			3.14			$\le 0.32 \cdot \delta_{blank}$
recovery										
$\bar{R}$	90.40			96.80			86.80			–
$\Delta_{R,r}$	8.72			4.38			9.96			≤ 20.00%
b^R / s_b^R	–0.002 / 0.021			0.010 / 0.012			0.031 / 0.021			$b^R \le 1{,}812 \cdot s_b^R$
a^R / s_a^R	90.57 / 2.20			95.97 / 1.21			84.06 / 2.19			$a^R \le 1{,}812 \cdot s_a^R$
I100–$\bar{R}$I	9.60			3.20			13.20			≤ 6.40%
linearity										
a^k	–0.023	–1.193	1.721	–1.114	–2,050	–0,914	–3,321	–0,951	–0,021	$a \le 2{,}015 \cdot s_a$
s^k_a	1.476	2.558	2.448	2.195	3,626	2,032	2,454	2,637	2,712	a≤ 8,53%
b^k	1.027	1.032	0.990	1.037	1,045	1,026	1,063	1,041	1,019	
s^k_b	0.013	0.023	0.022	0.020	0,032	0,018	0,022	0,024	0,024	
RSD^k_0	1.747	3.026	2.897	2.598	4,290	2,404	2,903	3,121	3,209	≤ 9,93%
R^k	0.9996	0.9988	0.9988	0.9991	0,9976	0,9992	0,9989	0,9987	0,9986	≥ 0,9830
accuracy										
$\bar{Z}^k$	102.51	102.34	102.36	102.78	102,61	101,80	101,68	103,12	102,75	–
δ^k	2.51	2.34	2.36	2.78	2,61	1,80	1,68	3,12	2,75	≤ 6,40%
$\bar{Z}^{intra}$	102.40			102.40			102.52			–
δ^{intra}	2.40			2.40			2.52			≤ 6.40%
precision										
RSD^k_z	2.95	3.44	6.44	2.93	3,72	2,75	4,68	3,37	4,41	–
Δ_z	5.73	6.68	12.51	5.69	7,23	5,34	9,09	6,55	8,57	≤ 20,00%
RSD_z^{intra}	4.55			3.16			4.19			–
Δ_z^{intra}	7.85			5.45			7.23			≤ 20.00%

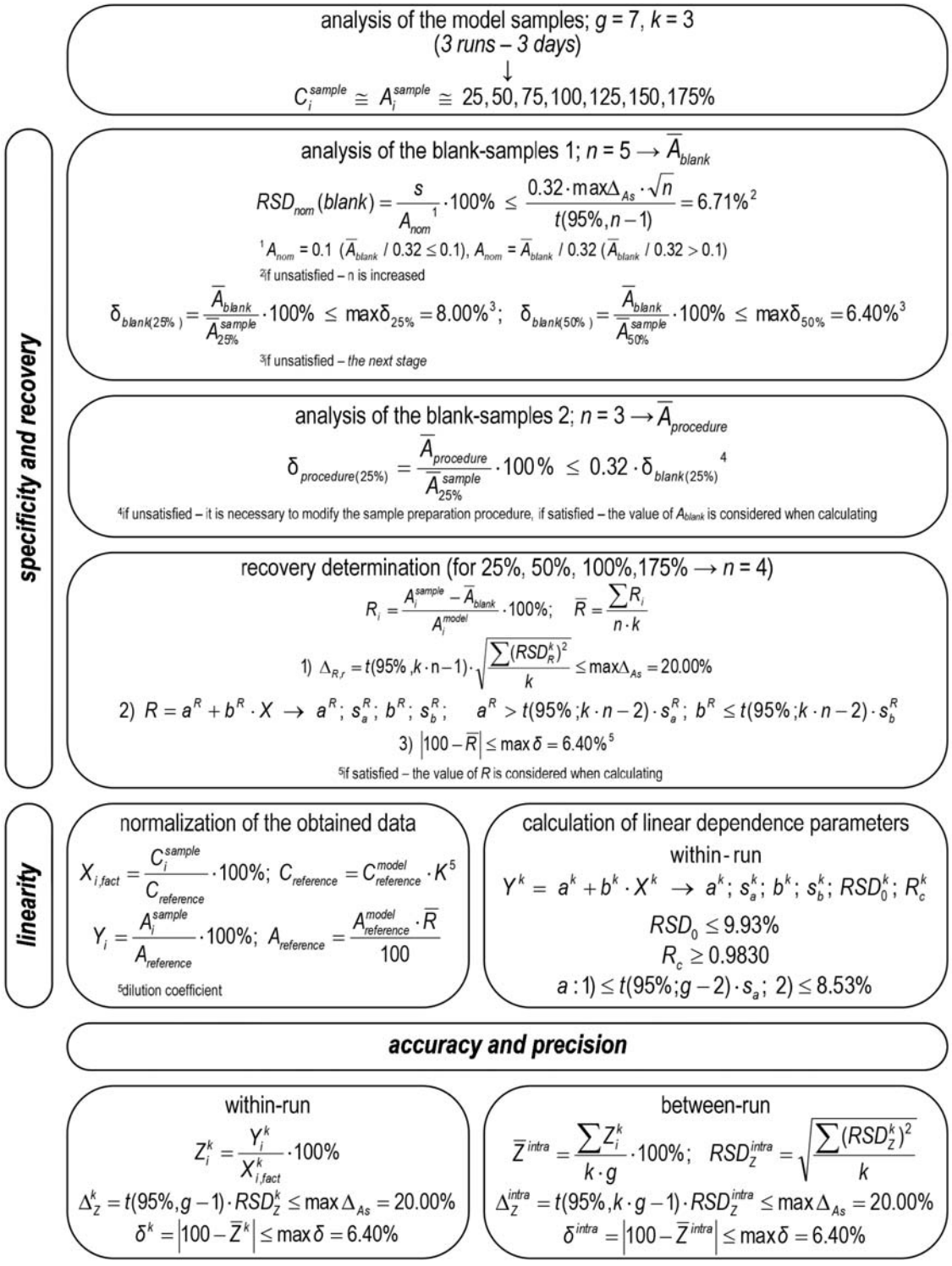

Fig. 4. The stages of validation of spectrophotometric methods of captopril determination in blood using model samples.

At the second stage the methods validation was carried out using model samples (*fig. 4*).

The total results of validation are presented in *table 2*.

The results of specificity study show that carrying out captopril isolation from blood using amphiphylic solvents provides low contribution of biological matrix components into the absorbance of the sample to be analysed. It is possible to point to the conclusion about high efficiency of captopril isolation from blood – not less than 90% – by the results of recovery study. The method with acetonitrile application is characterized by the best extraction efficiency.

The values reproducibility for recovery and blank-samples absorbance satisfy the acceptability criteria for all variants of the methods. The absorbance values obtained for the blank-samples 2 are the evidence of the correct choice of sample preparation procedure for all considered cases.

On the whole, all examined methods are characterized by the acceptable parameters of linearity, accuracy and precision, and the obtained data are the evidence of application possibility of the method of standard for captopril spectrophotometric determination in blood by reaction with the Ellman reagent.

For the method with acetonitrile application processing the results of experiment was carried out both with correction by the R value and without it. It was necessary to note that absence of such correction did not lead to worsening of the method validation parameters, but the method systematic error changed its sign (Z^{intel}= 99.09%).

Conclusions

Thus, we have developed the series of spectrophotometric methods of captopril quantitative determination in blood by reaction with the Ellman reagent using amphiphylic solvents (isopropanol, acetonitrile, methanol) for analyte isolation from matrix under the conditions of aqueous phase saturation by ammonium sulphate. Acetonitrile application in the acid medium (pH = 2) is optimal – contribution of matrix components into the absorbance of the sample to be analysed does not exceed 10%, extraction efficiency is ~97%.

Validation of the developed methods has been carried out using the approaches offered before by us and the possibility of the method of standard application for determination has been shown.

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