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DEVELOPMENT AND VALIDATION OF THE HPLC-PROCEDURES OF PHENYTOIN DETERMINATION IN BLOOD IN THE VARIANT OF THE METHOD OF STANDARD

The set of procedures of phenytoin quantitative determination in blood by the method of high-performance liquid chromatography using amphiphylic solvents (acetone and acetonitrile) under the conditions of aqueous phase saturation by ammonium sulphate has been developed; acetonitrile application in the acid medium (pH = 2) 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 and toxicological analysis.

Key words: validation, bioanalytical methods, high-performance liquid chromatography, phenytoin, method of standard.

THE PROBLEM STATEMENT

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 [7, 8, 10, 11].

ANALYSIS OF THE LAST RESEARCHES AND PUBLICATIONS

The available international guidances on carrying out validation of bioanalytical methods [8, 10] 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 and toxicological analysis 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 [3], 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 and toxicological analysis in the variant of the method of standard [4, 5, 12].

ALLOCATION OF THE UNSETTLED BEFORE PARTS OF THE COMMON PROBLEM

The developed approaches were successfully applied to procedures using optical methods of analysis [9], and it is interesting to approve these validation procedures on chromatographic methods of analysis.

THE ARTICLE PURPOSE STATEMENT

The purpose of the paper is developing the set of procedures of phenytoin quantitative determination in blood using different procedures of sample preparation based on HPLC-method offered before [1]; carrying out validation of the offered methods for choosing the optimal procedure of sample preparation provided effective phenytoin 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 phenytoin HPLC-determination in blood.
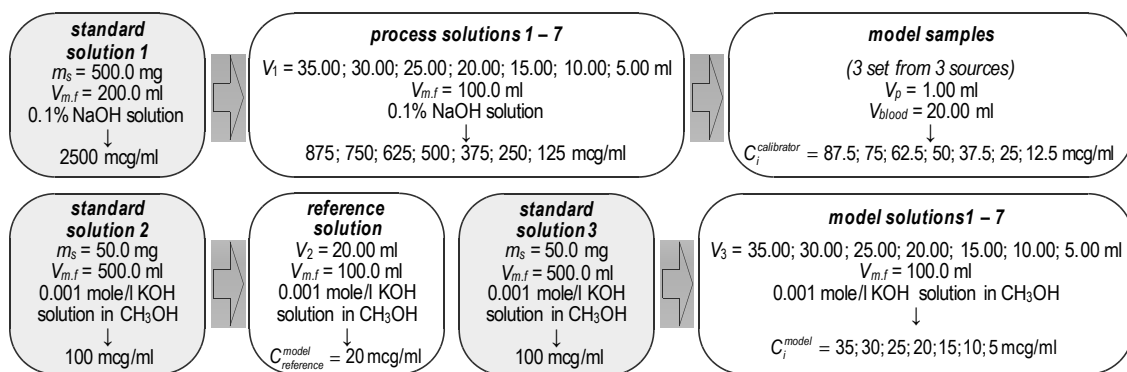
THE MAIN INFORMATION STATEMENT

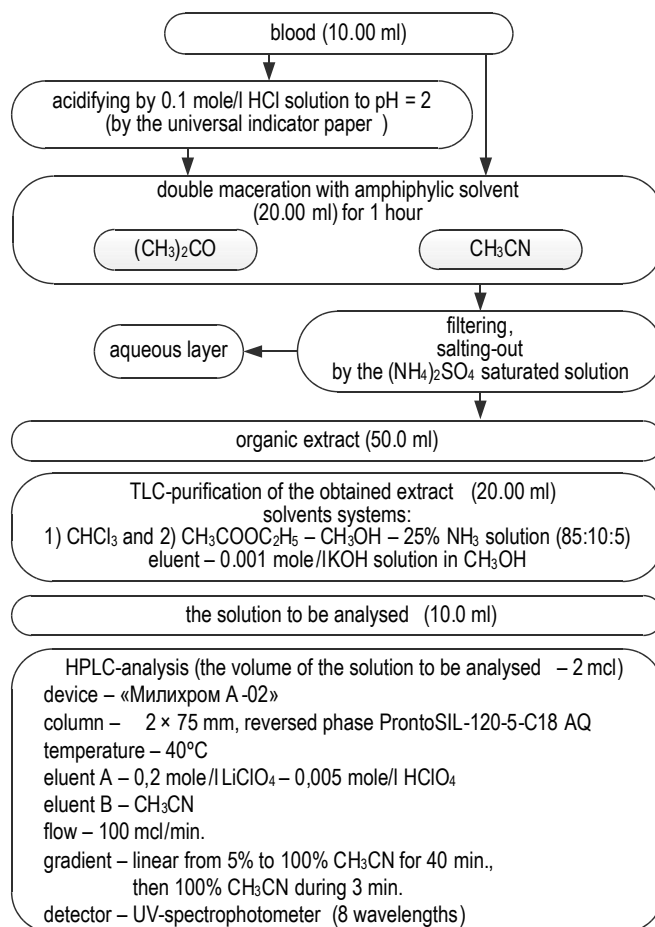Phenytoin of pharmacopoeial purity was used in the experiment. The procedure of preparation

of standard, process and model solutions, and also model samples is presented on *Scheme 1*.

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

The model (see *Scheme 1*) and also blank-samples were analysed for each developed procedure; the blank-samples were prepared in the following way: 5 samples (10.00 ml) of the blood obtained

standard solution 1
m_s = 500.0 mg
$V_{m.f}$ = 200.0 ml
0.1% NaOH solution
↓
2500 mcg/ml

process solutions 1 – 7
V_1 = 35.00; 30.00; 25.00; 20.00; 15.00; 10.00; 5.00 ml
$V_{m.f}$ = 100.0 ml
0.1% NaOH solution
↓
875; 750; 625; 500; 375; 250; 125 mcg/ml

model samples
(3 set from 3 sources)
V_p = 1.00 ml
V_{blood} = 20.00 ml
↓
$C_i^{calibrator}$ = 87.5; 75; 62.5; 50; 37.5; 25; 12.5 mcg/ml

standard solution 2
m_s = 50.0 mg
$V_{m.f}$ = 500.0 ml
0.001 mole/l KOH solution in CH_3OH
↓
100 mcg/ml

reference solution
V_2 = 20.00 ml
$V_{m.f}$ = 100.0 ml
0.001 mole/l KOH solution in CH_3OH
↓
$C_{reference}^{model}$ = 20 mcg/ml

standard solution 3
m_s = 50.0 mg
$V_{m.f}$ = 500.0 ml
0.001 mole/l KOH solution in CH_3OH
↓
100 mcg/ml

model solutions 1 – 7
V_3 = 35.00; 30.00; 25.00; 20.00; 15.00; 10.00; 5.00 ml
$V_{m.f}$ = 100.0 ml
0.001 mole/l KOH solution in CH_3OH
↓
C_i^{model} = 35; 30; 25; 20; 15; 10; 5 mcg/ml

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

blood (10.00 ml)
↓
acidifying by 0.1 mole/l HCl solution to pH = 2
(by the universal indicator paper)
↓
double maceration with amphiphylic solvent
(20.00 ml) for 1 hour
$(CH_3)_2CO$ CH_3CN
↓
filtering,
salting-out
by the $(NH_4)_2SO_4$ saturated solution → aqueous layer
↓
organic extract (50.0 ml)
↓
TLC-purification of the obtained extract   (20.00 ml)
solvents systems:
1) $CHCl_3$ and 2) $CH_3COOC_2H_5$ – CH_3OH – 25% NH_3 solution (85:10:5)
eluent – 0.001 mole/l KOH solution in CH_3OH
↓
the solution to be analysed (10.0 ml)
↓
HPLC-analysis (the volume of the solution to be analysed – 2 mcl)
device – «Милихром А-02»
column –   2 × 75 mm, reversed phase ProntoSIL-120-5-C18 AQ
temperature – 40ºC
eluent A – 0,2 mole/l $LiClO_4$ – 0,005 mole/l $HClO_4$
eluent B – CH_3CN
flow – 100 mcl/min.
gradient – linear from 5% to 100% CH_3CN for 40 min.,
            then 100% CH_3CN during 3 min.
detector – UV-spectrophotometer (8 wavelengths)

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

from the different sources, 1.00 ml of 0.1% sodium hydroxide solution 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 repeated injections − the relative standard deviation of the mean **RSD$_{nom}$** calculated towards the nominal value of peak area **S$_{nom}$** should not exceed:

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

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

where $\max\Delta_{As}$ − is the extreme relative uncertainty of the procedure of analysis, $\max\Delta_{As} = 20\%$ [4, 8]; $\overline{S}_{25\%}$ − 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).

RESULTS AND THEIR DISCUSSION

The HPLC-method for phenytoin determination was developed by authors before [1] and its specificity in relation to other medicines, which were pharmacological analogues of phenytoin, was shown. This method was applied for estimation of efficiency of phenytoin isolation from blood by maceration with 0.1 mole/l hydrochloric acid solution and subsequent extraction with chloroform in the acid medium (pH = 2) − the recovery was equal to ~60% [1].

In the present paper it has been suggested to carry out phenytoin 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, 6, 7]. Such amphiphylic solvents as acetone and acetonitrile 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 acid medium (pH = 2) − as in the method offered before [1] − and without previous blood processing.

Thus, the development of the set of HPLC-methods of phenytoin 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 phenytoin determination in blood we have carried out validation of all developed procedure 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 [4, 5, 12].

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 phenytoin concentration in blood [7] − 50 mcg/ml − is accepted.

The methods validation has been carried out at the first stage using model solutions (*Scheme 3*) and proceeding from two approaches [4]:

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 results Δ_{As}.

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 phenytoin quantitative 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 phenytoin quantification.

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*.
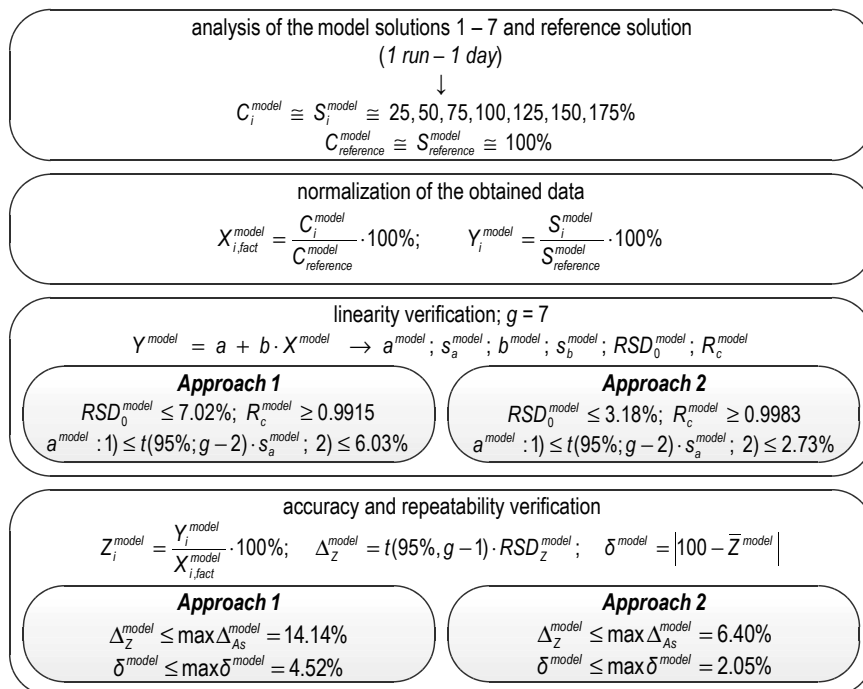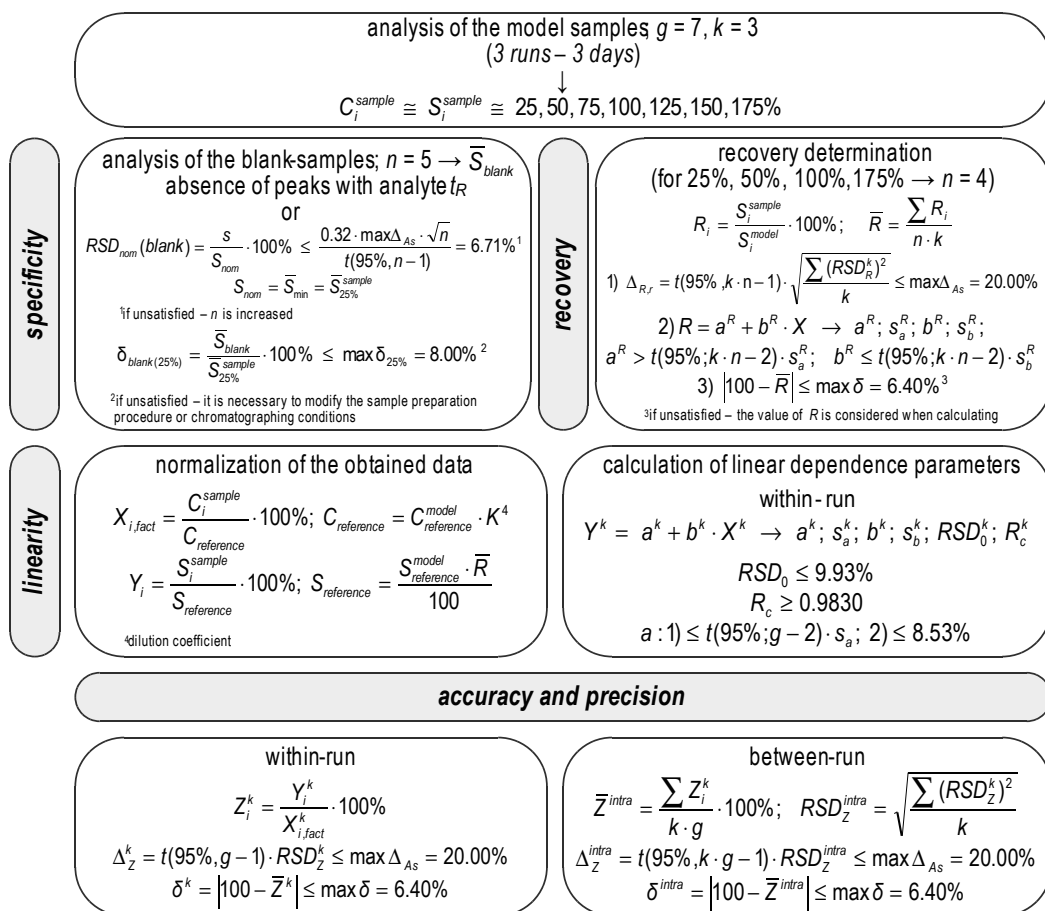
analysis of the model solutions 1 – 7 and reference solution
(*1 run – 1 day*)
↓
$$C_i^{model} \cong S_i^{model} \cong 25, 50, 75, 100, 125, 150, 175\%$$
$$C_{reference}^{model} \cong S_{reference}^{model} \cong 100\%$$

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
$$RSD_0^{model} \leq 7.02\%; \; R_c^{model} \geq 0.9915$$
$$a^{model} : 1) \leq t(95\%; g-2) \cdot s_a^{model}; \; 2) \leq 6.03\%$$

Approach 2
$$RSD_0^{model} \leq 3.18\%; \; R_c^{model} \geq 0.9983$$
$$a^{model} : 1) \leq t(95\%; g-2) \cdot s_a^{model}; \; 2) \leq 2.73\%$$

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

Approach 1
$$\Delta_Z^{model} \leq \max \Delta_{As}^{model} = 14.14\%$$
$$\delta^{model} \leq \max \delta^{model} = 4.52\%$$

Approach 2
$$\Delta_Z^{model} \leq \max \Delta_{As}^{model} = 6.40\%$$
$$\delta^{model} \leq \max \delta^{model} = 2.05\%$$

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

analysis of the model samples, $g = 7$, $k = 3$
(*3 runs – 3 days*)
↓
$$C_i^{sample} \cong S_i^{sample} \cong 25, 50, 75, 100, 125, 150, 175\%$$

specificity

analysis of the blank-samples; $n = 5 \rightarrow \overline{S}_{blank}$
absence of peaks with analyte t_R
or
$$RSD_{nom}(blank) = \frac{s}{S_{nom}} \cdot 100\% \leq \frac{0.32 \cdot \max \Delta_{As} \cdot \sqrt{n}}{t(95\%, n-1)} = 6.71\%^{[1]}$$
$$S_{nom} = \overline{S}_{min} = \overline{S}_{25\%}^{sample}$$
[1]if unsatisfied – n is increased
$$\delta_{blank(25\%)} = \frac{\overline{S}_{blank}}{\overline{S}_{25\%}^{sample}} \cdot 100\% \leq \max \delta_{25\%} = 8.00\%^{[2]}$$
[2]if unsatisfied – it is necessary to modify the sample preparation procedure or chromatographing conditions

recovery

recovery determination
(for 25%, 50%, 100%, 175% → $n = 4$)
$$R_i = \frac{S_i^{sample}}{S_i^{model}} \cdot 100\%; \quad \overline{R} = \frac{\sum R_i}{n \cdot k}$$
$$1) \; \Delta_{R,r} = t(95\%, k \cdot n - 1) \cdot \sqrt{\frac{\sum (RSD_R^k)^2}{k}} \leq \max \Delta_{As} = 20.00\%$$
$$2) \; R = a^R + b^R \cdot X \rightarrow a^R; s_a^R; b^R; s_b^R;$$
$$a^R > t(95\%; k \cdot n - 2) \cdot s_a^R; \quad b^R \leq t(95\%; k \cdot n - 2) \cdot s_b^R$$
$$3) \; \left| 100 - \overline{R} \right| \leq \max \delta = 6.40\%^{[3]}$$
[3]if unsatisfied – the value of R is considered when calculating

linearity

normalization of the obtained data
$$X_{i,fact} = \frac{C_i^{sample}}{C_{reference}} \cdot 100\%; \; C_{reference} = C_{reference}^{model} \cdot K^{[4]}$$
$$Y_i = \frac{S_i^{sample}}{S_{reference}} \cdot 100\%; \; S_{reference} = \frac{S_{reference}^{model} \cdot \overline{R}}{100}$$
[4]dilution coefficient

calculation of linear dependence parameters
within - run
$$Y^k = a^k + b^k \cdot X^k \rightarrow a^k; s_a^k; b^k; s_b^k; RSD_0^k; R_c^k$$
$$RSD_0 \leq 9.93\%$$
$$R_c \geq 0.9830$$
$$a : 1) \leq t(95\%; g-2) \cdot s_a; \; 2) \leq 8.53\%$$

accuracy and precision

within-run
$$Z_i^k = \frac{Y_i^k}{X_{i,fact}^k} \cdot 100\%$$
$$\Delta_Z^k = t(95\%, g-1) \cdot RSD_Z^k \leq \max \Delta_{As} = 20.00\%$$
$$\delta^k = \left| 100 - \overline{Z}^k \right| \leq \max \delta = 6.40\%$$

between-run
$$\overline{Z}^{intra} = \frac{\sum Z_i^k}{k \cdot g} \cdot 100\%; \quad RSD_Z^{intra} = \sqrt{\frac{\sum (RSD_Z^k)^2}{k}}$$
$$\Delta_Z^{intra} = t(95\%, k \cdot g - 1) \cdot RSD_Z^{intra} \leq \max \Delta_{As} = 20.00\%$$
$$\delta^{intra} = \left| 100 - \overline{Z}^{intra} \right| \leq \max \delta = 6.40\%$$

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

Table 1

THE TOTAL RESULTS OF VALIDATION OF PHENYTOIN 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}
		1.012	0.013	−0.139	1.280	1.375	0.9997
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}
		101.44		2.02		1.44	4.07
acceptability criterion	Approach 1	–		–		$\le 4.52\%$	$\le 14.14\%$
						satisfied	satisfied
	Approach 2	–		–		$\le 2.05\%$	$\le 6.40\%$
						satisfied	satisfied

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

The results of analysis show the absence of peaks with the retention time, which is coincident with (or near to) the phenytoin 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.

By results the recovery study the best efficiency of phenytoin isolation from blood is noted in the case of the experiment carrying out at pH = 2 and using acetonitrile. The reproducibility of recovery values satisfies the acceptability criteria for all variants of methods.

For the method with acetonitrile application at pH = 2 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 phenytoin extraction from blood and low value of the method uncertainty allow to consider the method with acetonitrile application in the acid medium as optimal for sample preparation of blood to further HPLC-determination of phenytoin.

CONCLUSIONS AND FURTHER RESEARCHES OUTLOOK

1. The set of HPLC-procedures of phenytoin quantitative determination in blood using amphiphylic solvents (acetone and acetonitrile) for analyte isolation from matrix without acidifying and at pH = 2 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 acid medium (pH = 2) is optimal for phenytoin 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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Table 2

THE TOTAL RESULTS OF VALIDATION
OF HPLC-METHODS OF PHENYTOIN DETERMINATION IN BLOOD

Parameter	Solvent — $(CH_3)_2CO$ pH = 2	$(CH_3)_2CO$ pH ≈ 6		CH_3CN pH = 2	CH_3CN pH ≈ 6		acceptability criterion
recovery							
$\bar{R}$	83.79	78.01		94.96	85.64		–
$\Delta_{R,r}$	8.75	8.78		7.32	7.00		≤ 20.00%
b^R / s_b^R	0.030 / 0.018	0.029 / 0.017		0.028 / 0.018	0.024 / 0.016		$b^R \leq 1.812 \cdot s_b^R$
a^R / s_b^R	81.17 / 1.93	75.46 / 1.77		92.48 / 1.92	83.58 / 1.66		$a^R \leq 1.812 \cdot s_b^R$
$\lvert 100 - \bar{R} \rvert$	16.21	21.99		5.04	14.36		≤ 6.40%
linearity							
pH = 2 · a^k	−2.389	−5.170	−3.436	−1.776	−1.334	−4.404	$a \leq 2.015 \cdot s_a$; $a \leq 8.53\%$
pH = 2 · s_a^k	3.354	2.206	3.553	1.999	2.536	1.004	
pH = 2 · b^k	1.053	1.089	1.075	1.039	1.039	1.068	
pH = 2 · s_b^k	0.030	0.020	0.032	0.018	0.023	0.009	
pH = 2 · RSD_0^k	3.969	2.611	4.204	2.366	3.000	1.188	≤ 9.93%
pH = 2 · R_c^k	0.9980	0.9992	0.9978	0.9993	0.9988	0.9998	≥ 0.9830
pH ≈ 6 · a^k	−2.290	−2.854	−5.474	−1.031	−3.871	−2.057	$a \leq 2.015 \cdot s_a$; $a \leq 8.53\%$
pH ≈ 6 · s_a^k	3.135	3.244	2.286	1.500	1.034	2.627	
pH ≈ 6 · b^k	1.052	1.071	1.092	1.026	1.062	1.048	
pH ≈ 6 · s_b^k	0.028	0.029	0.020	0.013	0.009	0.023	
pH ≈ 6 · RSD_0^k	3.709	3.838	2.705	1.775	1.224	3.109	≤ 9.93%
pH ≈ 6 · R_c^k	0.9982	0.9982	0.9991	0.9996	0.9998	0.9987	≥ 0.9830
precision and accuracy (within-run)							
pH = 2 · $\bar{Z}^k$	102.79	101.79	103.60	102.22	102.60	100.57	–
pH = 2 · δ^k	2.79	1.79	3.60	2.22	2.60	0.57	≤ 6.40%
pH = 2 · RSD_z^k	3.63	4.89	4.47	2.69	3.67	4.16	–
pH = 2 · Δ_z^k	7.05	9.5	8.69	5.23	7.13	8.08	≤ 20.00%
pH ≈ 6 · $\bar{Z}^k$	102.77	103.87	101.77	101.81	100.72	102.61	–
pH ≈ 6 · δ^k	2.77	3.87	1.77	1.81	0.72	2.61	≤ 6.40%
pH ≈ 6 · RSD_z^k	3.40	3.89	4.98	2.51	3.95	3.73	–
pH ≈ 6 · Δ_z^k	6.61	7.56	9.68	4.88	7.68	7.25	≤ 20.00%
precision and accuracy (between-run)							
$\bar{Z}^{intra}$	102.73	102.80		101.80	101.71		–
δ^{intra}	2.73	2.80		1.80	1.71		≤ 6.40%
RSD_z^{intra}	4.37	4.15		3.56	3.46		–
Δ_z^{intra}	7.54	7.16		6.14	5.97		≤ 20.00%

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РОЗРОБКА ТА ВАЛІДАЦІЯ ВЕРХ-МЕТОДИК ВИЗНАЧЕННЯ ФЕНІТОЇНУ В КРОВІ У ВАРІАНТІ МЕТОДУ СТАНДАРТУ

Розроблено серію методик кількісного визначення фенітоїну в крові методом високоефективної рідинної хроматографії з використанням амфіфільних розчинників (ацетону та ацетонітрилу) в умовах насичення водної фази амонію сульфатом – оптимальним є використання ацетонітрилу в кислому середовищі (pH = 2). Проведено валідацію розроблених методик та показано можливість використання для визначення методу стандарту з метою раціоналізації проведення кількісних визначень в судово-токсикологічному аналізі.

Ключові слова: валідація, біоаналітичні методики, високоефективна рідинна хроматографія, фенітоїн, метод стандарту.

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РАЗРАБОТКА И ВАЛИДАЦИЯ ВЭЖХ-МЕТОДИК ОПРЕДЕЛЕНИЯ ФЕНИТОИНА В КРОВИ В ВАРИАНТЕ МЕТОДА СТАНДАРТА

Разработана серия методик количественного определения фенитоина в крови методом высокоэффективной жидкостной хроматографии с использованием амфифильных растворителей (ацетона и ацетонитрила) в условиях насыщения водной фазы аммония сульфатом — оптимальным является использование ацетонитрила в кислой среде (pH = 2). Проведена валидация разработанных методик и показана возможность использования для определения метода стандарта с целью рационализации проведения количественных определений в судебно-токсикологическом анализе.

Ключевые слова: валидация, биоаналитические методики, высокоэффективная жидкостная хроматография, фенитоин, метод стандарта

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Якість, стандартизація і сертифікація в фармації