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VALIDATION OF UV-SPECTROPHOTOMETRIC METHOD OF DOXYLAMINE QUANTITATIVE DETERMINATION IN BLOOD: LINEARITY

©L. Yu. Klymenko, S. M. Trut, E. Yu. Akhmedov

National University of Pharmacy, Kharkiv

Summary: the approaches to choice of the method application range, to the quantity of concentration levels in the range and parallel experiments for each level, and also to the procedure of carrying out the experiment on linearity determination, which were offered in our previous paper, have been tested by the example of UV-spectrophotometric method of doxylamine quantitative determination in blood.

Key words: validation, linearity, range, UV-spectrophotometry, doxylamine, bioanalytical methods.

Introduction. This article is the continuation of authors' research [1 – 5] in the field of development of the approaches to validation of methods of quantitative determination for purposes of forensic and toxicological analysis and devoted to the problem of range choosing and validation parameter «linearity» determination.

The purpose of this paper is to test the approaches to choosing the method application range and to the procedure of linearity determination, which have been offered in our previous paper [5], by the example of UV-spectrophotometric method of doxylamine quantitative determination in blood.

Investigation methods. *The method to be validated*: 20.00 ml of blood are coated with 10.00 ml of the 10% trichloroacetic acid aqueous solution, mixed and left for 1 hour when constant shaking. The mixture is centrifuged (during 5 minutes at 5000 rpm), the supernatant liquid is poured off and diluted to the volume of 30 ml with distilled water, its pH is checked (should be equal to 2) and the mixture is extracted with chloroform three times by portions of 10.00 ml. The obtained chloroform extracts are separated and are not researched in the sequel. The aqueous layer is alkalified by the 50% sodium hydroxide solution to pH = 11 and extracted with chloroform three times by portions of 10.00 ml (if stable emulsions are formed centrifugation is applied (during 5 minutes at 5000 rpm)). «Alkaline» chloroform extracts are combined and filtered through the paper filter («red label») with 1 g of sodium sulphate anhydrous in the measuring flask with the capacity of 50.0 ml, and diluted to the volume with chloroform. Then the investigation is carried out in two ways:

1) 2/5 of the obtained chloroform extract (20.00 ml) are evaporated using water-bath at the temperature of 80°C to complete removal of organic layer. The dry residue is dissolved in 10.00 ml (r0.5 to the volume of blood taken for analysis) of the 0.1 mole/l hydrochloric acid solution.

2) 2/5 of the obtained chloroform extract (20.00 ml) are evaporated using water-bath at the temperature of 80°C to complete removal of organic layer; the dry residue is dissolved in ~0.5 ml of chloroform and applied quantitatively on the start line of the «Sorbfil» PTLC-IIB chromatographic plate (the plates have been processed preliminary with the 0.1 mole/l potassium hydroxide solution in methanol and then dried out at 110°C for 30 minutes) in the form of stripe 2 cm wide. Near 10 mcl of the doxylamine succinate standard chloroform solution (concentration is 1 mg/ml) are applied in point («testifier»). The plate is eluted in chloroform twice. After drying the plate is eluted using the mixture of chloroform and methanol (90:10) as a mobile phase, dried out, and the «testifier» stripe is developed with the Dragendorff reagent and the spot of brown colour in the area of R_f = 0.5 – 0.7 is observed. The sorbent is carefully removed from the plate part with area of 3 cm × 1 cm opposite the spot of «testifier» by scalpel in the glass bottle. 10.00 ml of the 0.1 mole/l hydrochloric acid solution are added into the bottle and the bottle content is shaken during 5 minutes, then filtered in the measuring flask with the capacity of 10.0 ml (r0.5 to the volume of blood taken for analysis) and diluted to the volume through the filter («red label») with the same solvent.

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).

The reference solution: 400.0 mg of doxylamine succinate were placed in the measuring flask with the capacity 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 2, the concentration was 4000 mcg/ml). 18.00 ml of the doxylamine succinate standard solution 2 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 3, the concentration was 720 mcg/ml). 2.00 ml of the doxylamine succinate standard solution 3 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): 6 lines in 7 samples (20.00 ml) of model blood (matrix) obtained from the different sources, which were spiked with 1.00 ml of the process solutions 1 – 7 respectively.

The solutions to be analysed: the solutions obtained by the method to be validated for the calibration samples.

The absorbance of the solutions to be analysed and the 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. Earlier [5] the following procedure of linearity confirmation for UV-spectrophotometric methods of analytes quantitative determination in biological fluids used in forensic and toxicological analysis has been offered:

- application of the normalized coordinates (normalization by the reference solution, which absorbance is corrected by the value of recovery);
- the application ranges are 25 – 125%, 25 – 150%, 25 – 175%; as 100% the mean toxic or lethal analyte concentration in biological liquid is accepted;
- the number of concentration levels is g = 5, 6 or 7 (depending on the chosen application range) in constant increments of 25%;
- the number of «replicates» – replicate experiments – for each concentration level is determined by the results of calculation of $s_{nom,r}$ value, which acceptability estimation is carried out according to the following criterion:

$$s_{nom,r}(sample) \le \max s_{nom,r} = 0.707 \cdot \max\Delta_{As} \cdot \sqrt{n} / t(95\%, n-1).$$

- each replicate experiment is carried out within individual run/day using the matrix samples obtained from the same source;
- calculation of the parameters of linear dependence is carried out for each run (within-run (within-day) linearity) and by the mean values of replicate experiments (between-run (between-day) linearity).

For illustration of the offered approaches to linearity determination UV-spectrophotometric method of doxylamine quantitative determination in blood 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%.

Results of determination of the mean values of absorbance for calibration samples calculated in the way of successive averaging the values obtained in 6 replicate runs are given in Tables 1 and 2. The values of $s_{nom,r}$ are specified for all values; it is shown that they do not exceed the calculated values of $\max s_{nom,r}$.

The factual concentrations of doxylamine succinate in blood and the mean values of absorbance, which are respective to them, are normalized in the way offered above. The obtained values of X_i, % and Y_i, % are used for linearity determination; the calculated parameters of linear dependences of $Y = b \cdot X + a$ type are resulted in Tables 3 and 4.

The data resulted in Tables 3 and 4 show obviously that international guidances [7 – 10] suggest excessively strict requirements to the number of concentration levels and replicates for each level – there are not substantial changes in values of metrological performance for linear dependences when some their decreasing, and the time costs are reduced twice at least.

Conclusions. The approaches to choosing the range and to the procedure of carrying out the experiment on linearity determination, which were offered in our previous paper, have been tested by the example of UV-spectrophotometric method of doxylamine quantitative determination in blood. The results have shown the adequacy of formed approaches.

Table 1. The mean values of absorbance of calibration samples for UV-spectrophotometric method of doxylamine quantitative determination in blood without preliminary TLC-purification

№ blood sample	Theoretical concentration of doxylamine in blood		Theoretical concentration of doxylamine succinate in blood $C_{i,theor}$, mcg/ml	Factual concentration of doxylamine succinate in blood $C_{i,fact}$, mcg/ml (C_{st} = 36 mcg/ml)	Factual concentration of doxylamine succinate in blood $X_{i,fact}$, %	Mean absorbance ($s_{nom,r}$)				Found in % to standard absorbance Y_i, % $(A_{st} = \frac{A_{reference} \cdot R}{100} = 0.532)$			
	$X_{i,theor}$, %	$C_{i,theor}$, mcg/ml				$n = 3$	$n = 4$	$n = 5$	$n = 6$	$n = 3$	$n = 4$	$n = 5$	$n = 6$
1	25	6.25	9.00	10.00	27.78	0.207 (7.28%)	0.202 (8.49%)	0.208 (9.63%)	0.213 (10.80%)	38.91	37.97	39.10	40.04
2	50	12.50	18.00	20.00	55.56	0.356 (5.85%)	0.351 (7.71%)	0.356 (8.83%)	0.352 (9.76%)	66.92	65.98	66.92	66.17
3	75	18.75	27.00	30.00	83.33	0.536 (5.18%)	0.541 (7.58%)	0.536 (8.73%)	0.539 (9.12%)	100.75	101.69	100.75	101.32
4	100	25.00	36.00	40.00	111.11	0.653 (4.72%)	0.648 (7.33%)	0.654 (8.66%)	0.657 (8.87%)	122.74	121.80	122.93	123.50
5	125	31.25	45.00	50.00	138.89	0.786 (4.61%)	0.791 (7.39%)	0.786 (8.00%)	0.790 (9.22%)	147.74	148.68	147.74	148.50
6	150	37.50	54.00	60.00	166.67	0.978 (4.09%)	0.982 (6.07%)	0.977 (8.05%)	0.973 (9.09%)	183.83	184.59	183.65	182.89
7	175	43.75	63.00	65.00	180.56	1.015 (3.65%)	1.020 (6.63%)	1.015 (7.33%)	1.019 (8.85%)	190.79	191.73	190.79	191.54
					$\max s_{nom,r}$	8.39%	12.02%	14.83%	17.19%				

Table 2. The mean values of absorbance of calibration samples for UV-spectrophotometric method of doxylamine quantitative determination in blood with preliminary TLC-purification

№ blood sample	Theoretical concentration of doxylamine in blood		Theoretical concentration of doxylamine succinate in blood $C_{i,theor}$, mcg/ml	Factual concentration of doxylamine succinate in blood $C_{i,fact}$, mcg/ml (C_{st} = 36 mcg/ml)	Factual concentration of doxylamine succinate in blood $X_{i,fact}$, %	Mean absorbance ($s_{nom,r}$)				Found in % to standard absorbance Y_i, % $(A_{st} = \frac{A_{reference} \cdot R}{100} = 0.510)$			
	$X_{i,theor}$, %	$C_{i,theor}$, mcg/ml				$n = 3$	$n = 4$	$n = 5$	$n = 6$	$n = 3$	$n = 4$	$n = 5$	$n = 6$
1	25	6.25	9.00	10.00	27.78	0.152 (6.08%)	0.149 (9.54%)	0.152 (10.28%)	0.154 (11.53%)	29.80	29.22	29.80	30.20
2	50	12.50	18.00	20.00	55.56	0.293 (5.18%)	0.290 (9.18%)	0.293 (10.03%)	0.295 (10.99%)	57.45	56.86	57.45	57.84
3	75	18.75	27.00	30.00	83.33	0.415 (5.01%)	0.411 (9.00%)	0.415 (9.98%)	0.412 (10.79%)	81.37	80.59	81.37	80.78
4	100	25.00	36.00	40.00	111.11	0.578 (5.03%)	0.582 (8.90%)	0.579 (9.87%)	0.581 (10.65%)	113.33	114.12	113.53	113.92
5	125	31.25	45.00	50.00	138.89	0.699 (4.95%)	0.696 (8.47%)	0.698 (8.91%)	0.701 (10.45%)	137.06	136.47	136.86	137.45
6	150	37.50	54.00	60.00	166.67	0.878 (4.37%)	0.875 (8.14%)	0.877 (8.55%)	0.880 (10.37%)	172.16	171.57	171.96	172.55
7	175	43.75	63.00	65.00	180.56	0.933 (3.98%)	0.936 (7.53%)	0.933 (8.19%)	0.930 (10.20%)	182.94	183.53	182.94	182.35
					$\max s_{nom,r}$	8.39%	12.02%	14.83%	17.19%				

Table 3. Metrological performance of calibration straight line $Y = b \cdot X + a$ for UV-spectrophotometric method of doxylamine quantitative determination in blood without preliminary TLC-purification

Characteristic	Analytical range of the method application											
	D = 25 – 125% (g = 5)				D = 25 – 150% (g = 6)				D = 25 – 175% (g = 7)			
	n = 3	n = 4	n = 5	n = 6	n = 3	n = 4	n = 5	n = 6	n = 3	n = 4	n = 5	n = 6
b	0.985	0.998	0.984	0.987	1.017	1.030	1.016	1.012	1.002	1.014	1.001	1.000
s_b	0.041	0.048	0.041	0.042	0.033	0.036	0.032	0.031	0.026	0.029	0.026	0.024
a	13.365	12.049	13.498	13.628	11.242	9.986	11.441	12.060	12.309	11.074	12.480	12.875
s_a	3.769	4.397	3.737	3.871	3.546	3.922	3.490	3.337	3.210	3.509	3.153	2.943
s_0	3.593	4.193	3.563	3.691	3.809	4.212	3.749	3.585	3.694	4.039	3.629	3.387
R_c	0.9974	0.9966	0.9975	0.9973	0.9979	0.9975	0.9980	0.9981	0.9983	0.9980	0.9983	0.9985

Table 4. Metrological performance of calibration straight line $Y = b \cdot X + a$ for UV-spectrophotometric method of doxylamine quantitative determination in blood with preliminary TLC-purification

Characteristic	Analytical range of the method application											
	D = 25 – 125% (g = 5)				D = 25 – 150% (g = 6)				D = 25 – 175% (g = 7)			
	n = 3	n = 4	n = 5	n = 6	n = 3	n = 4	n = 5	n = 6	n = 3	n = 4	n = 5	n = 6
b	0.973	0.978	0.973	0.974	1.011	1.012	1.009	1.012	1.011	1.016	1.010	1.009
s_b	0.024	0.031	0.025	0.029	0.027	0.028	0.027	0.029	0.020	0.021	0.020	0.022
a	2.679	1.921	2.739	2.861	0.268	–0.274	0.374	0.416	0.220	–0.541	0.287	0.613
s_a	2.204	2.873	2.317	2.700	2.875	3.058	2.892	3.139	2.401	2.571	2.416	2.631
s_0	2.102	2.739	2.210	2.574	3.089	3.285	3.107	3.372	2.763	2.959	2.781	3.028
R_c	0.9991	0.9985	0.9990	0.9986	0.9986	0.9984	0.9986	0.9984	0.9990	0.9989	0.9990	0.9988

Literature

1. Клименко Л. Ю. Анализ подходов к определению специфичности / селективности при проведении валидации аналитических методик в судебно-токсикологическом анализе / Л. Ю. Клименко, Г. П. Петюнин // Укр. мед. альм. – 2013. – Т. 16, №1. – С. 47 – 49.
2. Клименко Л. Ю. Подходы к определению специфичности/селективности при валидации УФ-спектрофотометрических методик количественного определения в судебно-токсикологическом анализе / Л. Ю. Клименко, Г. П. Петюнин, Т. А. Костина // Фармация Казахстана. – 2013. – №8. – С. 53 – 56.
3. Модификация и валидация УФ-спектрофотометрической методики количественного определения доксиламина в крови: специфичность /селективность / Л. Ю. Клименко, С. Н. Трут, Г. П. Петюнин, И. М. Иванчук // Укр. журн. клін. та лаборатор. медицини. – 2013. – Т. 8, №4. – С. 191 – 199.
4. Validation of UV-spectrophotometric methods of quantitative determination in forensic and toxicological analysis: recovery / L. Yu. Klimenko, S. M. Trut, G. P. Petyunin, I. M. Ivanchuk // Фармация Казахстана. – 2013. – №12. – С. 42 – 48.
5. Development of approaches to validation of UV-spectrophotometric methods of quantitative determination in forensic and toxicological analysis: linearity and range / L. Yu. Klimenko, G. P. Petyunin // Фармацевтичний часопис. – 2014. – №1 (30). – С. 41 – 52.
6. Clarke's analysis of drugs and poisons in pharmaceuticals, body fluids and postmortem material: 4th ed. / edited by A. C. Moffat, M. D. Osselton, B. Widdop. – London: Pharmaceutical Press, 2011. – 2609 p.
7. Guidance for Industry: Bioanalytical Method Validation / U.S. Department of Health and Human Services, Food and Drug Administration (FDA), Center for Drug Evolution and Research (CDER), Center for Veterinary Medicine (CVM). – Washington, DC: U.S. Government Printing Office, 2001. – 22 p.
8. Standard Practices for Method Validation in Forensic Toxicology (draft) / Scientific Working Group for Forensic Toxicology (SWGTOX). – 2012. – 52 p.
9. Guidance for the Validation of Analytical Methodology and Calibration of Equipment used for Testing of Illicit Drugs in Seized Materials and Biological Specimens / United Nations Office on Drugs and Crime, Laboratory and Scientific Section. – New York: United Nations, 2009. – 70 p.
10. Guideline on bioanalytical method validation / European Medicines Agency. Committee for Medicinal Products for Human Use (CHMP). – London, 2009. – 22 p.

ВАЛІДАЦІЯ УФ-СПЕКТРОФОТОМЕТРИЧНОЇ МЕТОДИКИ КІЛЬКІСНОГО ВИЗНАЧЕННЯ ДОКСИЛАМІНУ В КРОВІ: ЛІНІЙНІСТЬ

Л. Ю. Клименко, С. М. Трут, Е. Ю. Ахмедов

Національний фармацевтичний університет, Харків

Резюме: підходи до вибору діапазону застосування методики, кількості концентраційних рівнів всередині діапазону та паралельних дослідів для кожного такого рівня, а також до процедури проведення експерименту з визначення лінійності, запропоновані в нашій попередній роботі, апробовано на прикладі УФ-спектрофотометричної методики кількісного визначення доксиламіну в крові.

Ключові слова: валідація, лінійність, діапазон застосування, УФ-спектрофотометрія, доксиламін, біоаналітичні методики.

ВАЛИДАЦИЯ УФ-СПЕКТРОФОТОМЕТРИЧЕСКОЙ МЕТОДИКИ КОЛИЧЕСТВЕННОГО ОПРЕДЕЛЕНИЯ ДОКСИЛАМИНА В КРОВИ: ЛИНЕЙНОСТЬ

Л. Ю. Клименко, С. Н. Трут, Э. Ю. Ахмедов

Национальный фармацевтический университет, Харьков

Резюме: подходы к выбору диапазона применения методики, количества концентрационных уровней внутри диапазона и параллельных опытов для каждого такого уровня, а также к процедуре проведения эксперимента по определению линейности, предложенные в нашей предыдущей работе, апробированы на примере УФ-спектрофотометрической методики количественного определения доксиламина в крови.

Ключевые слова: валидация, линейность, диапазон применения, УФ-спектрофотометрия, доксиламин, биоаналитические методики.

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