UDC 615.457.074:54.062:543.544.5.068.7
DOI https://doi.org/10.32782/2226-2008-2024-4-14

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VALIDATION OF THE METHOD OF QUANTITATIVE DETERMINATION OF THE ACTIVE SUBSTANCE ((S)-2,6-DIAMINOHEXANOIC ACID 3-METHYL-1,2,4-TRIAZOLYL-5-THIOACETATE IN EYE DROPS

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The purpose of the present work is to validate the method of quantitative determination of the active substance ((S)-2,6-diaminohexanoic acid 3-methyl-1,2,4-triazolyl-5-thioacetate) in Angiolin 1% eye drops by the method of high-performance liquid chromatography.

Materials and methods. Certified substances were used: Angiolin, working standard sample, sodium chloride, purified water as auxiliary substances, liquid chromatograph with UV detector; column Hypersil ODS C-18250 X 4.6 with a particle size of 5 μm.

Results. During the determination of validation parameters, it is established that the method is characterized by sufficient correctness, since the criterion of insignificance of the systematic error of the method is fulfilled. The systematic error of the method satisfies the requirements of statistical and practical insignificance. The high value of the correlation coefficient r=0.9999 satisfies the requirements of the acceptance criterion (r=0.99810) and confirms the linearity of the dependence between the amount of Angiolin taken and found in the range from 80% to 120%, following its nominal content in the preparation. The requirements for parameters of linear dependence (a, SD0/b, r) of the Angiolin determination method are met in the entire concentration range from 80% to 120% of the nominal value.

Conclusions. The method of determining Angiolin by HPLC in the range of application of the method meets the acceptance criteria for validation characteristics: specificity, correctness, precision (convergence) and linearity. The total predicted uncertainty of the analyses results does not exceed the critical value regulated by the SPhU, and can be entered into the project of Quality Control Methods.

Key words: eye drops, analysis, quantitative determination, high-performance liquid chromatography, validation.

УДК 615.457.074:54.062:543.544.5.068.7

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ВАЛІДАЦІЯ МЕТОДИКИ КІЛЬКІСНОГО ВИЗНАЧЕННЯ ДІЮЧОЇ РЕЧОВИНИ ((S)-2,6-ДІАМІНОГЕКСАНОВОЇ КИСЛОТИ 3-МЕТИЛ-1,2,4-ТРИАЗОЛІЛ-5-ТІОАЦЕТАТУ В ОЧНИХ КРАПЛЯХ

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Процедура валідації передбачена для того, щоб аналітична методика посіла гідне місце у системі забезпечення якості, відповідала своєму призначенню та гарантувала достовірні результати аналізу.

У ході досліджень встановлено, що методика визначення Ангіоліну методом високоефективної рідинної хроматографії в діапазоні застосування методики відповідає критеріям прийнятності для валідаційних характеристик: специфічність, правильність, прецизійність та лінійність. Методика характеризується достатньою правильністю, оскільки виконується критерій незначущості систематичної похибки методики. Систематична похибка методики задовольняє вимоги статистичної та практичної незначущості. Повна прогнозована невизначеність результатів аналізів не перевищує критичне значення, регламентоване ДФУ.

Ключові слова: очні краплі, аналіз, кількісне визначення, високоефективна рідинна хроматографія, валідація.

Introduction. According to the Ministry of Health of Ukraine, eye diseases are now the sixth most common disease. Due to the severity of the consequences of eye diseases and the social costs of compensating blindness and poor vision, such pathologies require a lot of resources and continuous prevention and treatment. In today's context, given the military operations taking place in Ukraine, various injuries to the visual apparatus are one of the most complex clinical and social problems. Bruises and burns of the eye rank second only to penetrating injuries among injuries to the structures of the visual apparatus, accounting for 20%–42.2% of cases. One of the most urgent tasks of medicine and pharmacy is the development of new ophthalmic drugs, namely eye drops, which remain the most common and convenient form of medication due to their ease of use. A new compound derived from 1,2,4-triazole was synthesized at the Department of Pharmaceutical, Organic and Bioorganic Chemistry of Zaporizhzhia State Medical and Pharmaceutical University together with scientists of scientific and pedagogical institutions of higher education and under the leadership of Professor I.A. Mazur. It refers to ((S)-2,6-diaminohexanoic acid 3-methyl-1,2,4-triazolyl-5-thioacetate), with the conventional name "Angiolin", which exhibits anti-inflammatory, wound-healing and reparative effects [1].

Nowadays, high-performance liquid chromatography is widely used in pharmaceutical analysis for standardizing finished dosage forms. The advantage of HPLC over other methods is its versatility and accuracy. In previous studies, a technique for quantitative determination of Angiolin eye drops by HPLC was developed. According to the requirements of the SPhU, the next logical step was the validation of the developed methodology according to the following indicators: specificity, linearity, range of application, accuracy, correctness, and robustness [4–6].

The purpose of the present work is to validate the method of quantitative determination of the active substance ((S)-2,6-diaminohexanoic acid 3-methyl-1,2,4-triazolyl-5-thioacetate) in Angiolin 1% eye drops by the method of high-performance liquid chromatography.

Materials and methods. During studies at the Department of Pharmaceutical, Organic and Bioorganic Chemistry of ZSMPhU, six series of Angiolin eye drops were produced. Certified substances were used: Angiolin (manufacturer: State Enterprise "Factory of Chemical Reagents" of the Scientific and Technological Complex "Institute of Single Crystals" of the National Academy of Sciences of Ukraine, series 2451117) and a working standard sample (SE "Factory of Chemical Reagents" of the Scientific and Technological Complex "Institute of Single Crystals" of the National Academy of Sciences of Ukraine, Ukraine); methylcellulose (series 26101197551, manufacturer: Weissenborn, Germany) sodium chloride, purified water were used as auxiliary substances. The study was carried out under the following conditions: liquid chromatograph with UV detector; column Hypersil ODS C-18250 X 4.6 with a particle size of 5 μm. Limits: the content of ((S)-2,6-diaminohexanoic acid 3-methyl-1,2,4-triazolyl-5-thioacetate) in 1% eye drops of the drug should be from 0.98 g to 1.02 g per 100 ml. The determination of the active substance was carried out according to the methodology developed by us, which is given below:

Solutions are used freshly prepared.

Tested solution: 5.0 ml of the medicinal product (Angiolin eye drops) is brought to 50.0 ml with water and mixed. 10.0 ml of the resulting solution is brought up to 50.0 ml with the mobile phase and mixed.

Reference solution (a). Dissolve about 0.05 g (precise weight) of the working standard sample of Angiolin in water, adjust the volume of the solution to 50.0 ml with the same solvent and mix.

Reference solution (b). 10.0 ml of the reference solution (a) is brought up to 50.0 ml with the mobile phase and mixed.

Chromatography is carried out on a liquid chromatograph with a UV detector under the conditions described in the "Accompanied impurities" test:

– column Hypersil ODS C-18 250 X 4.6 with a particle size of 5 μm, or similar, for which the requirements of the test "Checking the suitability of the chromatographic system" are met;

– mobile phase: a mixture of acetonitrile – phosphate buffer solution pH 3.0 (5:95), degassed by any convenient method.

– velocity of the mobile phase: 1.0 ml/min;

– column temperature: 30ºC;

– detection: spectrophotometric at a wavelength of 246 nm;

– sample volume to be introduced: 20 μl;

Chromatograph the test solution and the reference solution (b), obtaining at least 3 chromatograms for each.

A chromatographic system is considered suitable if, for reference solution (b):

– efficiency of the chromatographic system: calculated according to the peak of Angiolin, there should be at least 3500 theoretical plates;

– peak symmetry factor calculated for the Angiolin peak should be from 0.8 to 1.5;

– relative standard deviation of the Angiolin peak areas from all chromatograms must meet the requirements of the SPhU.

The chromatogram of the studied solution and the standard sample are shown in Figure 1 and 2.

The content of Angiolin in 1% eye drops, in percent, is calculated by the formula:

$$X = \frac{S_1 \cdot m_0 \cdot 50 \cdot 10 \cdot 50 \cdot P \cdot 100}{S_0 \cdot 5 \cdot 50 \cdot 10 \cdot 50 \cdot 100} = \frac{S_1 \cdot m_0 \cdot P \cdot 100\%}{S_0 \cdot 500},$$

where

S_1 – average value of Angiolin peak areas, calculated from the chromatograms of the tested solution;

S_0 – average value of Angiolin peak areas, calculated from the chromatograms of the reference solution (b);

m_0 – weight of Angiolin standard sample, in milligrams;

P – content of Angiolin in the standard sample, which is specified in the certificate, in percent.

The suitability criteria of the validation characteristics of the method were calculated for a 5% tolerance of the content of active substances in the preparation. For this purpose, 9 solutions were prepared according to the following method: X ml of the medicinal product (Angiolin 1% eye drops) were diluted with water to 50.0 ml and mixed. 10.0 ml of the resulting solution is brought up to 50.0 ml with the mobile phase and mixed. The weight of

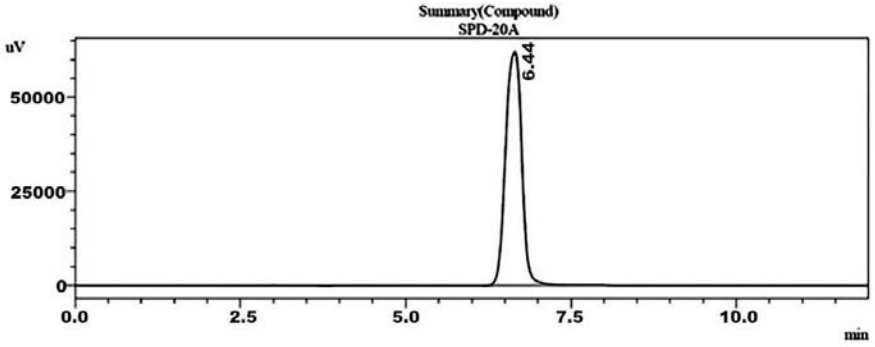

Fig. 1. Chromatogram of the tested solution

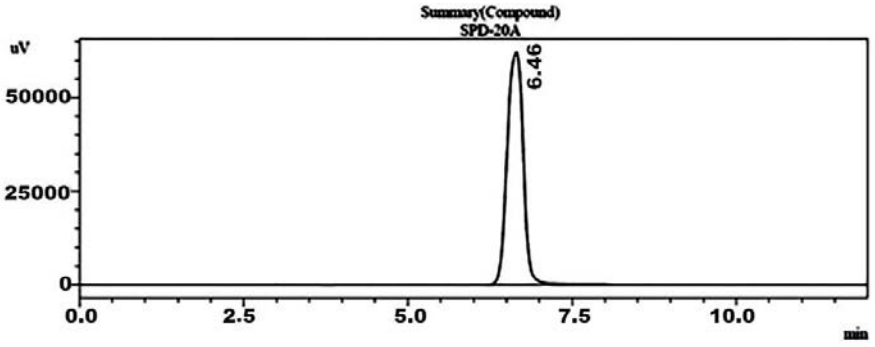

Fig. 2. Chromatogram of the standard sample

the sample, eye drops, in milliliters and the percentage content of the nominal value are given in table 1.

Table 1

Model solutions

Model solution №	Angiolin	
	Weight of the sample (ml)	Percentage content of the nominal value
1	4.00	80
2	4.25	85
3	4.50	90
4	4.75	95
5	5.00	100
6	5.25	105
7	5.50	110
8	5.75	115
9	6.00	120

Results. *Specificity.* After preparing the solutions according to the method presented above, we carry out their analysis using the method of high-performance liquid chromatography. Acceptability criteria are the following: 1) On the chromatograms of the "placebo" solution of the drug (sample 0), there should be no peaks with a retention time coinciding with the retention time of Angiolin on the chromatograms of the tested solution; 2) The retention time of the Angiolin peak on the chromatograms of the tested solution must coincide with the retention time of the Angiolin peak on the chromatograms of the reference eye drop solution. The specificity of the identification method and quantification of Angiolin in 1% eye drops is shown in Figure 3.

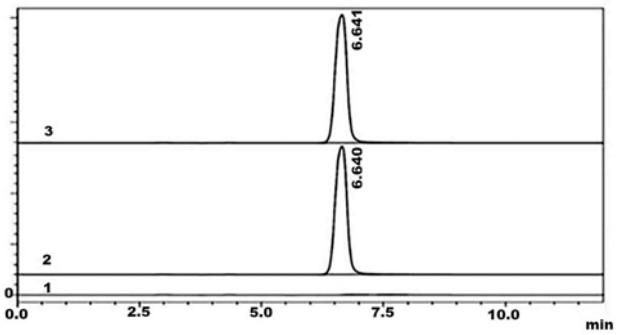

Fig. 3. Chromatograms of solutions: 1 – a "placebo" solution, 2 – a test solution of Angiolin in 1% eye drops

The specificity of the method of quantitative and qualitative determination of Angiolin in 1% eye drops is confirmed by the fact that:

– on the chromatogram of the "placebo" solution there are no peaks with the retention time of the Angiolin peak;

– on the chromatograms of the tested solution and the reference solution of Angiolin in 1% eye drops, the retention time of Angiolin is the same.

Based on all of the above, it can be stated that the identification method and quantitative determination of Angiolin in 1% eye drops by the method of high-performance liquid chromatography is specific [2; 3].

Correctness and precision. Correctness and precision characteristics were investigated on model solutions of the drug with Angiolin concentrations corresponding to 80%, 85%, 90%, 95%, 100%, 105%, 110%, 115% and 120% of nominal content (Figure 4 for Angiolin solution with a 100% concentration).

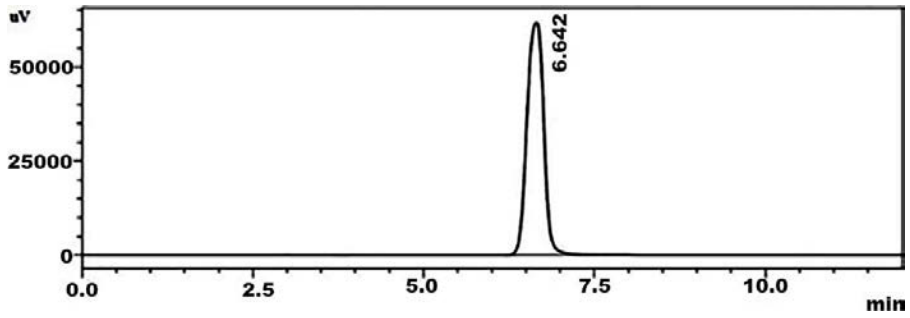

Fig. 4. Chromatogram of model solution for Angiolin solution with a 100% concentration of nominal content

The linearity characteristic was studied in the range of Angiolin concentrations from 80% to 120% in relation to the nominal value.

The graph of linear dependence is presented in Figure 5, and the results of calculations of linear dependence parameters in table 2.

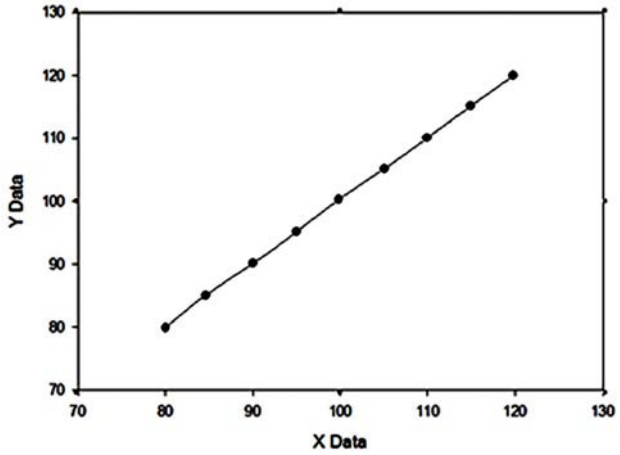

Fig. 5. Linear dependence of the detected concentration of Angiolin on its introduced concentration in normalized coordinates

Table 2

Metrological characteristics of the linear dependence of the detected concentration of Angiolin on its introduced concentration

Parameters	Value	Requirements 1	Requirements 2	Conclusion
B	1.0020			
S_b	0.0050			
A	-|0.0993|	≤ |0.25|	≤ |2.7|	Sustained by 1 criterion
S_a	0.50			
SD_0	0.1897			
SD_0/b	0.1893	≤ |0.85|		Fulfilled
R	0.9999	> |0.99810|		Fulfilled

As can be seen from the presented data, the requirements for linear dependence parameters are fulfilled, that is, the linearity of the method of quantitative determination of Angiolin by the method of high-performance liquid chromatography is confirmed in the concentration range from 80% to 120% of the nominal value for the content limits of ± 5%.

Correctness characterizes the degree of correspondence between the known content of the substance to be determined in the solution and its content in the solution, which is determined by this method.

Convergence characterizes the *precision* of the technique when it is carried out under the same conditions over a short period of time. At this stage, the convergence is investigated on 9 model mixtures, which cover the range of application of the technique.

The correctness and convergence of the methodology was checked by the "entered-found" method. The results of quantitative determination of Angiolin in model solutions in the area of analytical concentrations and the results of calculations of metrological characteristics are shown in table 3.

From the data presented in the table, it follows that the method of quantitative determination of Angiolin by the HPLC method is characterized by sufficient correctness and convergence (precision) over the entire range of concentrations (from 80% to 120%) and is correct.

As evidenced by the data given in table 4, in the range of Angiolin concentrations from 80% to 120% in relation to the nominal concentration, the method of its quantitative determination does not have a significant systematic error.

Evaluation of the methodology: the expected uncertainty of the sample preparation consisted of the uncertainty of the weight of the drug and the weight that was taken for the preparation of the reference solution, bringing the solutions to the mark and taking aliquots. Calculations and uncertainty values of the sample preparation procedure are given in table 4.

The obtained results (given in the table 4) showed that the uncertainty of sample preparation is significant.

To confirm the fulfilment of the requirements for maxRSD, the actual values of the relative standard deviation for the areas of the Angiolin peaks were calculated (table 5).

Discussion. After analyzing the obtained results, it can be said that the method is characterized by adequate convergence, since the values of the relative confidence intervals of the found endothelin values ΔZ exceed the critical value for the convergence of the results (1.6%). In addition, the method is characterized by adequate correctness, since the criterion of insignificant systematic errors of the method is met. The systematic errors of the method meet the requirements of statistical and practical insignificance. The high value of the correlation coefficient r=0.9999 meets the requirements of the acceptance criterion (r=0.99810) and confirms that the linear correlation between the extracted and found angiogenic

Table 3

Analysis results of model solutions containing from 80% to 120% of Angiolin in relation to the nominal concentration, and their statistical processing

Solution	Weight of the sample	Intro-duced in % of nominal concentration (Xi, fact., %)	Peak areas	Found in % of nominal concentration (Yi, %)	Found in % to entered Zi=100·(Yi/Xi)
1	0.0846	84.60	731114	84.96	100.42
2	0.0901	90.10	824993	90.11	100.01
3	0.0953	95.3	919219	95.36	100.06
4	0.1001	100.10	1016904	100.25	100.15
5	0.1049	104.9	1123583	104.94	100.04
6	0.1099	109.90	1230321	109.95	100.05
7	0.1152	115.2	1344939	115.32	100.10
8	0.1196	119.60	1460396	119.65	100.04
	Average, Zcp, % =				100.10
	Relative standard deviation, RSDz, % =				0.20
	Relative confidence interval Δz %=t (95%, 9 – 1) x RSDz=1.86 x 0.20 =				0.39
	Critical value for the convergence results ΔAs, % =				1.6
Systematic error d					0.10
Criterion of insignificance of systematic error: 1) statistical insignificance: δ<Δz : √9=0.39/3=0.13 % > 0.1 % If not fulfilled 1), then δ ≤ maxδ: 2) practical insignificance: δ% ≤ 0.32 × 1.6=0,51 % > 0,10 %					Fulfilled Fulfilled
General conclusion about the method					Correct

Table 4

Calculation of the uncertainty of the sample preparation for the method of quantitative determination of Angiolin

Sample preparation operation	Parameter for calculation formula	Uncertainty (Δ), %
Reference solution		
Taking the weight of Angiolin	m_o=100 mg	0.2 %
Bringing the volume of the solution in the volumetric flask to 100.0 ml to the mark	100	0.13 %
Taking the solution with a pipette 10 ml	10	0.26 %
Bringing to volume 50 ml	50	0.18 %
Tested solution		
Taking the weight	m=100 mg	0.2 %
Bringing the volume of the solution in the volumetric flask to 100.0 ml to the mark	100	0.12 %
Taking the solution with a pipette 10 ml	10	0.25 %
Bringing to volume 50 ml	50	0.17 %

amounts is in the range of 80% to 120%, depending on the nominal content in its preparation. The linear correlation parameters (a, SD0/b, r) of the angiotensin determination method require that the angiotensin concentrations are within the entire concentration range from 80% to 120% of the nominal value.

Conclusions. The method for the determination of Angiolin using HPLC met the acceptance criteria for the validated properties within the method's scope: specificity, accuracy, precision (convergence), and linearity. The overall prediction uncertainty of the analytical results did not exceed the critical values established by the SPhU and could be included in the project "Quality Control Methods" [6].

Table 5

Relative standard deviation for the areas of the Angiolin peaks (*S*)

	Angiolin	
	S_0^*	S_1^{**}
	1019623	1017975
	1016167	1015767
	1017912	1016342
***RSD*, %**	0.149	0.115
RSD_{max}, % (n_0=3, B=5 %)	0.63	

$^*S_0$ – the area of the Angiolin peak obtained from the chromatograms of the reference solution
$^*S_1$ – the area of the Angiolin peak obtained from the chromatograms of the tested solution

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Надійшла до редакції 29.03.2024 р.
Прийнята до друку 28.11.2024 р.
Електронна адреса для листування farm_chem@bigmir.net