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DEVELOPMENT OF HPLC METHOD FOR QUANTITATIVE DETERMINATION OF EPIMIDIN - NEW PERSPECTIVE API WITH ANTICONVULSIVE ACTIVITY

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Мета. Розробка оптимальної, високоточної та відтворюваної методики кількісного визначення основної речовини в субстанції Епімідину – 1-(4-methoxyphenyl)-5-[2-[4-(4-methoxyphenyl)piperazin-1-yl]-2-oxo-ethyl]pyrazolo[3,4-d]pyrimidin-4-one методом високоефективної рідинної хроматографії.

Матеріали і методи. Високоефективну рідинну хроматографію (ВЕРХ) проведено використовуючи систему Shimadzu Nexera X2 LC-30AD (Shimadzu, Японія), оснащений діодним матричним детектором SPD-M20A (DAD). Колонка ACE C18, розміром 250×4,6 мм, фірми YMC з передколонукою, з розміром часток 5 мкм, заповнену силікагелем октилсилільним для хроматографії Р. В роботі використовувалися ацетонітрил та трифтороцтову кислоту класу HPLC (Sigma-Aldrich GmbH, Швейцарія), інші хімічні речовини та розчинники були аналітичного сорту. У дослідженні використовували аналітичний посуд класу А, що відповідає вимогам ДФУ.

Результати. Встановлено наступні оптимальні умови хроматографічного розподілу: колонка C18 (250*4,6 мм); швидкість рухомої фази 1 мл/хв; температура термостату колонки 35 °С; об'єм інжекції 10 мкл; рухома фаза А – 0,1 % трифтороцтова кислота; рухома фаза В - ацетонітрил Р; довжина хвилі детектування 270 нм, час утримання досліджуваної сполуки становить 7.22 хв. Продуктивність колонки була визначена для її основних показників, таких як кількість теоретичних тарілок (більше 25410) і коефіцієнт симетрії (близько 1,00). Методику було апробовано на вплив різних факторів, таких як швидкість потоку, склад рухомої фази та температура термостату колонки. Встановлено, що вплив цих факторів є незначущим та не впливає на результати, отримані за цією методикою. Методика була валідована згідно з рекомендаціями ДФУ за параметрами специфічності, лінійності, правильності, прецизійності, робастності (стабільність).

Висновки. Вперше розроблена високоточна та відтворювана методика кількісного визначення основної речовини у субстанції Епімідину з протисудомною активністю методом високоефективної рідинної хроматографії. Стандартизовано умови проведення хроматографічного аналізу (ВЕРХ). Встановлено вимоги до тесту «Перевірка придатності хроматографічної системи». Статистична обробка результатів експерименту свідчить, що відносна невизначеність середнього результату знаходиться у допустимих межах. Коректність методики підтверджено валідаційними дослідженнями. Розроблена методика буде використана для фармацевтичної розробки та стандартизації лікарської форми

Ключові слова: Епімідин, піразолопіримідин, антиконвульсант, кількісне визначення, ВЕРХ

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1. Introduction

In the treatment of epilepsy and other pathological conditions accompanied by convulsions, the use of drugs remains the leading method of treatment. Despite the wide choice of antiepileptic drugs (AEDs) with different mechanisms of action, including prolonged, satisfactory control of seizures is achieved in only 65–70 % of patients [1]. It is the development of refractory forms of epilepsy [2], the need for simultaneous administration of several AEDs, the lack of drugs that would meet the criteria of high efficacy with a favourable safety profile, prompts the search for anticonvulsants among new classes of compounds. In the context of the above problem, a promising API with anticonvulsant activity was synthesized – (4-methoxyphenyl)-5-[2-[4-(4-methoxyphenyl)piperazin-1-yl]-2-oxo-ethyl]pyrazolo[3,4-d]pyrimidin-4-one, which gave the code name "Epimidin" (Fig. 1) [3].

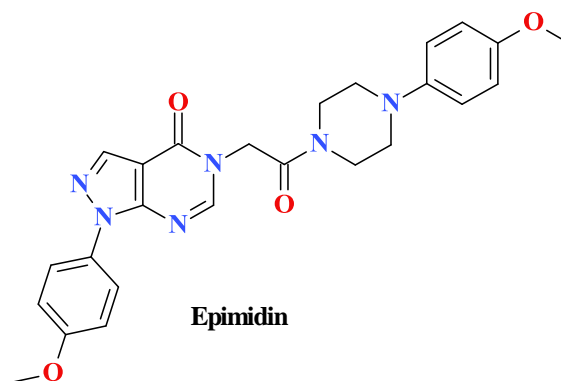


Fig. 1. The promising anticonvulsant – (4-methoxyphenyl)-5-[2-[4-(4-methoxyphenyl)piperazin-1-yl]-2-oxo-ethyl]pyrazolo[3,4-d]pyrimidin-4-one (Epimidin)

The strong anticonvulsant activity of Epimidin has been proven in various models of seizures: on the pentylenetetrazole (PTZ) model at a dose of 200 mg / kg exceeds the effect of the comparison drug sodium valproate at a dose of 300 mg / kg [3, 4], a pronounced effect on seizures caused by caffeine and picrotoxin, moderate - on strychnine convulsions [5]; high efficiency under conditions of experimental chronic epileptogenesis – model of PTZ-kindling; on the model of the maximum electroshock shows moderate activity. Wide range of effective anticonvulsant doses, favourable concomitant pharmacological profile: weak sedative and anxiolytic effect without muscle relaxant properties, positive effect on cognitive functions, no effect on depressive behaviour, no antihypoxic properties [5] and belonging to the V class of toxicity justifies the need for further study of Epimidin as a potential API.

The introduction of a new API or drug in medical practice is impossible without the development of methods for its analysis [6, 7]. A necessary and important stage of implementation is the development of an effective, unified, optimal, high-precision, reproducible and rapid method of quantitative determination of the active substance in the substance. To quantify pyrimidine and pyrazolopyrimidine derivatives, scientists use a variety of physicochemical methods, including ion chromatography [8], thin-layer chromatography and HPLC [9, 10], gas chromatography [11], polarography [12], spectrofluorimetry [13], etc. The subjects of the study were substances, various dosage forms and body fluids [14, 15]. High-performance liquid chromatography remains a priority for all subjects. The use of HPLC for the identification, separation and quantification of the hypnotic drug zaleplon – 3-(3-cyanopyrazolo[1,5-a]pyrimidin-7-yl)-N-ethylacetanilide has been widely demonstrated. Use of different mobile phases, such as methanol – water [13], acetonitrile – deionized water [9], ammonium formate buffer – acetonitrile [14], 5 % acetonitrile, 95 % formic acid 0.1 % to a ratio of 80–20 % [15], methanol – ammonium acetate buffer [16], the use of UV and MS detection allowed to determine zaleplon in different dosage forms and in blood plasma. It is due to the wide possibilities of HPLC method that it was chosen to quantify Epimidin.

The aim of the research. Development of a method for the quantitative determination of Epimidin in a substance using the method of high performance liquid chromatography.

2. Planning (methodology) of research

The State Pharmacopoeia of Ukraine recommends the use of direct objective methods for the quantification of APIs, such as, for example, titrimetric methods. Previously, a method for the quantitative determination of Epimidin by the method of nitrogen determination in organic compounds after mineralization with sulfuric acid was developed, which is described in the general monograph of SPhU 2.5.9 [17]. But the developed method is unacceptable for dosage forms, as excipients will interfere with the definition. Therefore, for subsequent pharmaceutical development in the dosage form, it was proposed to determine Epimidin using the method of high performance liquid chromatography. The presented

study included the following stages: selection of optimal chromatography conditions and development of a technique based on high performance liquid chromatography, conducting an experiment to assess the suitability of the chromatographic system and predict the uncertainty of the analysis results, and validation according to SPhU requirements.

3. Materials and methods

Liquid chromatography separation was performed using a Shimadzu Nexera X2 LC-30AD HPLC system (Shimadzu, Japan) composed of a quaternary pump, an on-line degasser, a column temperature controller, the SIL-30AC autosampler (Shimadzu, Japan); the CTO20AC thermostat (Shimadzu, Japan) as well as the SPDM20A diode array detector (DAD). Other instruments such as Ultrasonic Cleaner Set for ultra-sonication using (Wise Clean WUC-A06H, Witeg Labortechnik GmbH, Germany), Libra UniBloc AUW120D (Shimadzu Analytical Scale, Japan); class A analytical vassals that meets requirements of the SPhU (SPhU, 2015) were used in the investigation. HPLC grade acetonitrile (Sigma-Aldrich GmbH, Switzerland) were used in the analysis work. HPLC grade water was obtained from a water purifying system (Millipore, Bedford, MA, USA). Other chemicals and solvents were of analytical grade.

Quantification of Epimidin by HPLC was performed on an ACE C18 column (250x4.6 mm, particle size 5 µm) with a pre-column filled with octylsilyl silica gel for chromatography P, for which the conditions of suitability of the chromatographic system are met. A binary system of mobile phase solvents was used: solvent A (0.1 % trifluoroacetic acid) and solvent B (acetonitrile P). The following profile of the linear gradient elution was used: 0–9 min 65→50 % A, 35→50 % B; 9–11 min 50 % A, 50 % B; 11–12 min 50→65 % A, 50→35 % B; 12–15 min 65 % A, 35 % B. The flow rate was maintained at 1ml/min with UV detection at 270 nm. The sample injection volume was 10 µl and the column temperature was maintained at 35 °C.

Test solution. 60 mg (precisely weighed) of the substance KS 78553 is placed in a volumetric flask with a capacity of 250.0 ml, add 30.0 ml of DMSO, treated in an ultrasonic bath for 15 minutes before dissolution, bring to the mark with methanol P. Take 20.0 ml of the resulting solution and make up to volume 50.0 ml with methanol P. Filter through a membrane filter with a pore diameter of not more than 0.45 µm. The freshly prepared solution is used.

Comparison solution. 60 mg (precisely weighed) of the standard substance KS 78553 is placed in a volumetric flask with a capacity of 250.0 ml, add 30.0 ml of DMSO, treated in an ultrasonic bath for 15 minutes before dissolution, bring to the mark methanol P. Take 20.0 ml of the resulting solution and make up to volume. 50.0 ml of methanol P. Filter through a membrane filter with a pore diameter of not more than 0.45 µm. The solution is used freshly prepared.

4. Result

Under these conditions, the release time of the main peak of Epimidin was 7.22 minutes. Fig. 2 shows the chromatogram of the test solution.

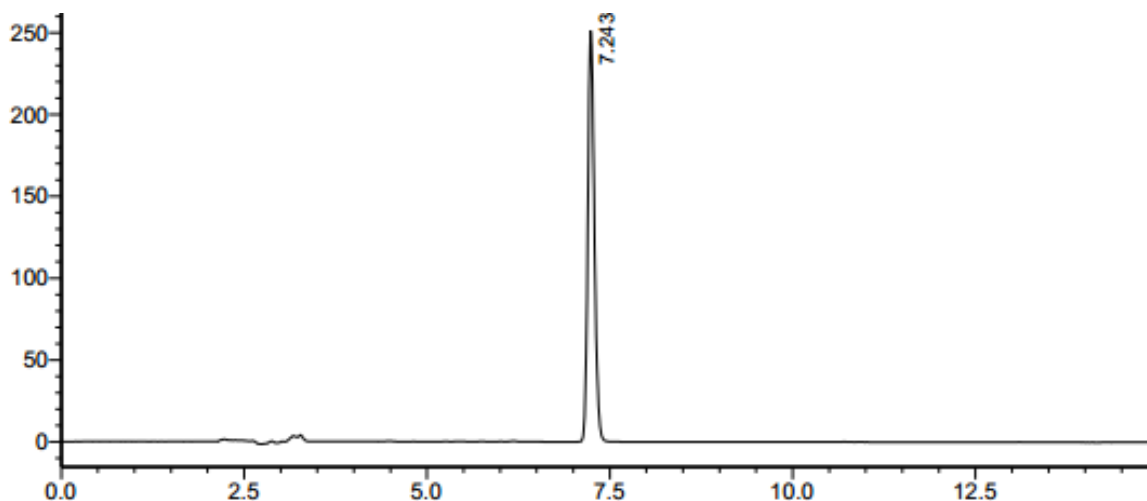


Fig. 2. Chromatogram of the tested solution of Epimidin

The chromatographic system is considered suitable if the following conditions are met for the reference solution:

- the efficiency of the chromatographic column, calculated at the peak of the basic substance, must be at least 3000 theoretical plates;

- the peak symmetry coefficient should be in the range from 0.8 to 1.5;

- the relative standard deviation for peak areas should not exceed 1.0 %, calculated from 5 injections. Quantitative characteristics of the suitability of the chromatographic system of the Epimidin comparison solution are given in Table 1.

Table 1

Characteristics of the chromatographic system for the comparison solution

Substance	Efficiency (number of theoretical plates)	Symmetry factor	Relative standard deviation, %
Epimidin	25410	1.18	0.06

Prognosis of analysis results uncertainty

The maximum allowable total relative uncertainty of the substance analysis method ΔAS % is related to the symmetrical tolerances of the content of the analyte according to the specification (B). That is:

$$\Delta AS \leq \frac{B_H - B_L}{2} \times 0.32.$$

The quantitative content of its components in the substance of Epimidin should be within ± 5 %, therefore:

$$\Delta AS \leq \frac{95 - 105}{2} \times 0.32 = 1.6 \%$$

Calculation of uncertainty of sample preparation is given in Table 2.

Table 2

Calculation of sample preparation uncertainty (Δsp)

Operation of sample	Value	Uncertainty (Δ), %
<i>Test solution</i>		
Weigh (m)	60 mg	0.33
Uncertainty of weighing	0.2 mg	
Bringing to volume	250 ml	0.12
Aliquot	20 ml	0.18
Bringing to volume	50 ml	0.17
<i>Comparison solution</i>		
Weigh (m) (m)	40 mg	0.25
Uncertainty of weighing	0.2 mg	
Bringing to volume	250 ml	0.12
Aliquot	20 ml	0.18
Bringing to volume	50 ml	0.17
<i>Complete uncertainty of sample preparation Δsp %</i>		0.60
<i>Uncertainty of the final analytical operation ΔFAO (liquid chromatography)*</i>		1.35
<i>Complete uncertainty of the method of analysis ΔAS %</i> $\Delta AS \% = \sqrt{(\Delta sp \%)^2 + (\Delta FAO \%)^2}$		1.48

Determination of the uncertainty of the final analytical operation Δ_{FAO} is performed for the test solution and the comparison solution. When calculating confidence intervals use a one-sided Student's ratio for a probability of 95 % and the corresponding number of degrees of freedom. Confidence intervals for the comparison solution and the test solution are calculated for the average of 5 results (maximum number of measurements according to the MQC of the drug).

$$\Delta_{FAO}^{cm} = \frac{1}{\sqrt{5}} \times t(95\%, n-1) \times RSD,$$

$$\Delta_{FAO}^{cm} = \frac{1}{\sqrt{5}} \times t(95\%, n-1) \times RSD.$$

According to the requirements of the suitability of the chromatographic system of the determination method, the relative standard deviation of five parallel determinations should not exceed 1.0 %. At $n = 5$, $t(95\%, n-1) = 2.1318$:

$$\Delta_{FAO}^{cm} = \frac{1}{\sqrt{5}} \times 2.1318 \times 1.0\% = 0.9534,$$

$$\Delta_{FAO}^{smp} = \frac{1}{\sqrt{5}} \times 2.1318 \times 1.0\% = 0.9534.$$

Total uncertainty of the final analytical operation:

$$\Delta_{FAO} = \sqrt{(\Delta_{FAO}^{smp})^2 + (\Delta_{FAO}^{cm})^2} = 1.35.$$

Complete uncertainty of the analysis method $\Delta_{AS} \%$:

$$\Delta_{AS} = \sqrt{(\Delta_{sp})^2 + (\Delta_{sp})^2} = 1.50\%,$$

$$\Delta_{AS} = \sqrt{(\Delta_{sp})^2 + (\Delta_{sp})^2} = 1.49\%.$$

Validation study

To confirm the correctness of the proposed method, validation studies were performed in accordance with the requirements of SPhU [17]. The following validation parameters were calculated: specificity, linearity, correctness, precision, robustness (stability).

Specificity. The retention times of the Epimidin peak on the chromatogram of the test solution correspond to the retention time of the Epimidin peak on the chromatogram of the reference solution – approximately 7.22 min (Fig. 2–3).

Linearity. To confirm the linearity of the method, 9 model solutions were prepared, the concentration of which varies evenly within the range of application (step – 5 %).

$X_i = C_i / C_{st} \cdot 100$ and $Y_i = S_i / S_{st} \cdot 100$ (Tables 2, 3).

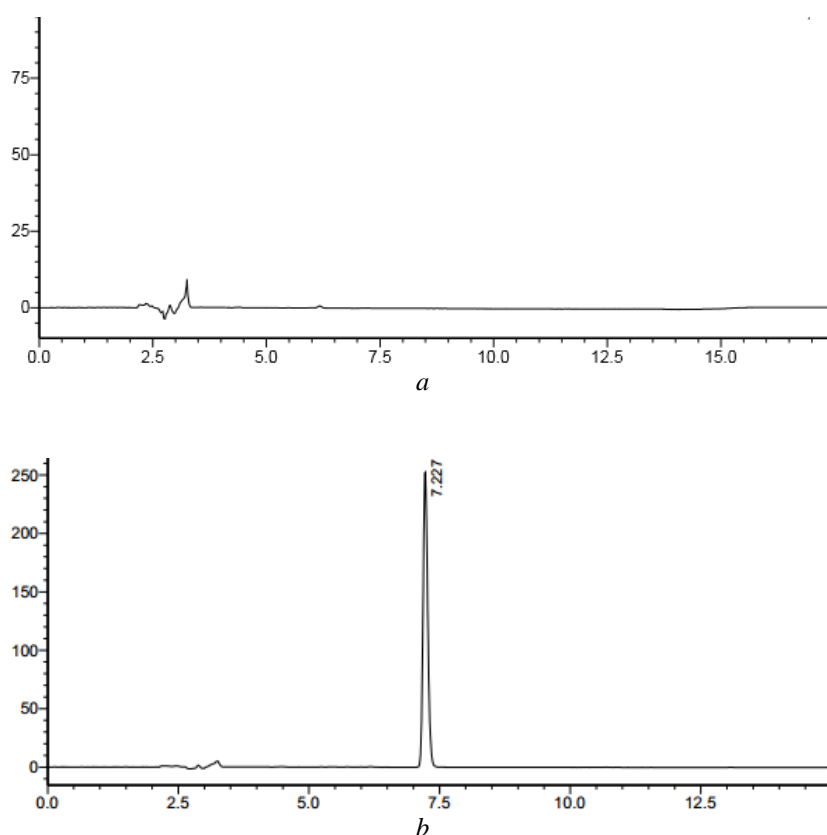


Fig. 3. The chromatograms of solutions: *a* – solvent (blank-chromatogram); *b* – comparison solution

Calculation of linearity parameters of the method of quantitative determination of Epimedin

No.	C, %	C (mg/ml)	C _{i/st}	The average value of the peak area	S _{i/st}
1	80	0.0768	80.0	1269455	80.20
2	85	0.0816	85.0	1348517	85.20
3	90	0.0864	90.0	1425586	90.07
4	95	0.0912	95.0	1508682	95.32
5	100	0.096	100.0	1577694	99.68
6	105	0.1008	105.0	1657269	104.70
7	110	0.1056	110.0	1734989	109.61
8	115	0.1104	115.0	1819394	114.95
9	120	0.1152	120.0	1895002	119.72
Standard	100	0.096	100.0	1582834	100,00

In Fig. 4 presented a graph of the linear dependence of the analytical signal on the actual concentration of the Epimedin solution, constructed in normalized coordinates, based on the data in the Table 4.

Data of linearity verification of the method of quantitative determination of Epimedin are presented in Table 4.

Correctness. To determine the correctness within the range of use of the analytical method, 9 test solutions were prepared in compliance with all stages of the analytical method. The calculation of the parameters of correctness and the conclusion about the correctness of the method is given in Table 5.

The correctness of the method was performed according to two criteria – practical and statistical insignificance, which were determined during experimental studies (Table 6).

Intra-laboratory precision. To determine, the results of a study of 6 tests of one sample performed by two analysts on different days during one working week using different measuring vessels were used. Determination of the parameters of intralaboratory precision are given in Table 7, and the results of the precision assessment in Table 8.

Robustness (stability). The study of the stability of the test solution was performed after 24 hours, and the results are shown in Table 9.

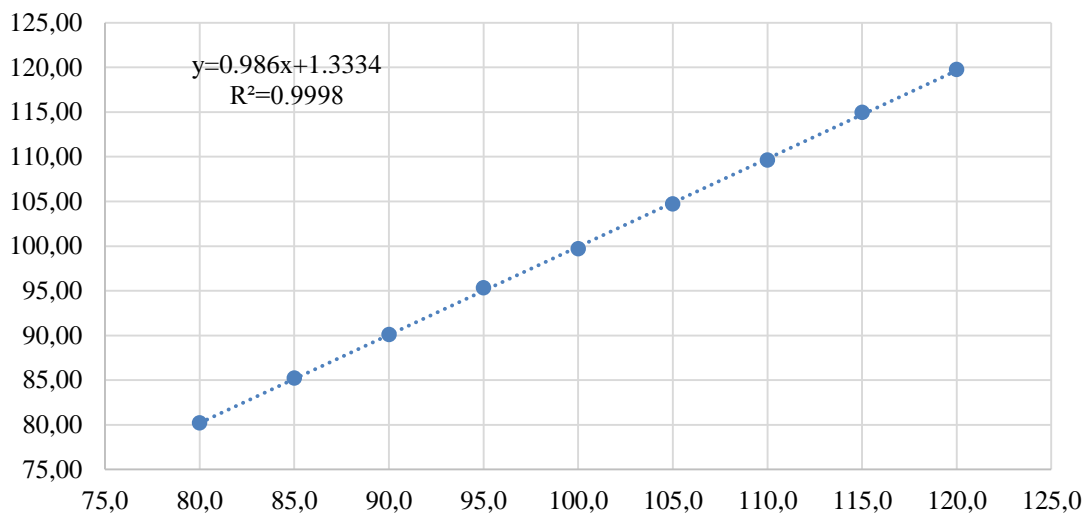


Fig. 4. Graph of the linear dependence of the analytical signal on the actual concentration of the Epimedin solution

Table 4

Data of linearity verification of the method of quantitative determination of Epimedin

№	Parameter	Requirements	The value obtained	Fulfillment of the criterion
1	a	≤4.8	0.986	Executed
2	S ₀	≤1.58	0.24	Executed
3	r	>0.9933	0.9998	Executed

Table 5

Calculation of parameters of method correctness

Number of solutions	Concentrations of components		
	Introduced in % to the concentration of the comparison solution (C_i/C_{st})*100 %	Found in % to the concentration of the comparison solution (S_i/S_{st})*100 %	Found in % to introduced $Z_i=(S_i/S_{st})*100/(C_i/C_{st})$
Solution 1	80	80.20	100.25
Solution 2	85	85.20	100.23
Solution 3	90	90.07	100.07
Solution 4	95	95.32	100.33
Solution 5	100	99.68	99.68
Solution 6	105	104.70	99.72
Solution 7	110	109.61	99.65
Solution 8	115	114.95	99.95
Solution 9	120	119.72	99.77
average Z, %			99.96
relative standard deviation, Sz %			0.27
relative confidence interval $\Delta Z=t(95\%,8)*s_z=1.8595*s_z$			0.50
critical for convergence of results $\Delta\% \leq$			1.6
systematic error $\delta\% = Z_{av}-100 $			0.04
Criterion of statistical insignificance $\delta\% \leq \Delta Z/3 = 0.50/3 = 0.16$ (0,07 ≤ 0,16); If not met, the criterion of practical insignificance, $\delta\% \leq 0.512$ (0.04 ≤ 0.512)			Executed
General conclusion about the method			Correct

Table 6

The results of the assessment of correctness by two criteria

Parameter	Value	Requirements for statistical insignificance	Requirements for practical insignificance	Fulfillment of the criterion
$ \bar{Z} - 100 $	0.04	≤ 0.16	≤ 0.512	Executed according to two criteria

Table 7

Determination of parameters of intralaboratory precision

No.	Analyst No. 1	Analyst No. 2
1	99.87	99.72
2	99.44	99.11
3	99.34	99.50
4	99.37	99.48
5	99.38	99.59
6	99.36	99.13
Mean	99.46	99.42
Dispersion, s^2	0.068	0.105
General average		99.45
Relative standard deviation, RSD %		0.21
Confidence interval, ($\Delta_{intra}=t(95\%, m*n-1)*RSD, \%=1,7956 *RSD, \%$)		0.42

Table 8

The results of the assessment of intra-laboratory precision

Parameter	Criteria requirements	The obtained value	Fulfillment of the criterion
Δ_{intra}	≤ 1.6	0.42	Executed

Table 9

Determination of the stability of Epimidin solution

Solution	The average S value of the peak of the freshly prepared solution	The average value of the S peak of the solution after 24 hours	Parameter changes, as a percentage after 24 hours
Standard	1564919	1558997	0.38
Test	1513057	1508408	0.31

5. Discussion

The proposed method allows you to reliably and confidently identify and quantify Epimidin. The complete uncertainty of the analysis method ΔAS % less than max ΔAS (1.48 % < max ΔAS = 1.6 %;) is calculated, which meets the requirements for this parameter [17]. Therefore, the uncertainty of sample preparation and analysis in general should provide sufficient measurement accuracy. The developed method of quantitative determination of Epimidin meets the requirements of “System suitability test criteria for chromatographic methods” in terms of parameters: the efficiency of the chromatographic column, the coefficient of separation of peaks on the chromatogram, the rate of peak asymmetry. It is established that all validation parameters meet the necessary eligibility criteria [17]. The specificity of the technique was confirmed by comparing the chromatograms of the comparison solution, the test solution and the chromatogram of the blank solution (solvent). No peaks were found on the blank chromatogram, the retention time of which would coincide with the retention time of the peak of the test compound. Comparison of chromatograms shows that in the conditions of the method, the determination is not hindered by either the solvent or the mobile phase, which indicates the specificity of the method. The method of quantification should be linear within the range of application, which should overlap the possible values of the concentrations of the active substance. SPhU sets the range of application of methods for quantification of 80–120 %. Requirements for the linearity of the method were performed over the entire concentration range for Epimidin, and the correlation coefficient was 0.9998. The correctness of the method was performed according to practical and statistical insignificance. The results of the assessment of intra-laboratory

precision showed the compliance of the obtained values of the confidence interval of the average result of the acceptability criterion ($\Delta \% = 0,42 \leq 1.60$). The differences between the obtained values of the peak areas should not exceed the criterion of insignificance compared to the maximum allowable uncertainty of the analysis results (ΔAS , insig), i.e. 0.512 %. According to the results of the determination, it is established that for optimal chromatography conditions it is necessary to use a freshly prepared comparison solution within 24 hours.

Study limitations. In the course of our study, there were difficulties in selecting the optimal chromatography conditions due to the poor solubility of the test substance in most organic solvents and insolubility in water.

Prospects for further research. The proposed technique can be used in the process of pharmaceutical development and standardization of the dosage form.

5. Conclusions

An analytical method for the quantitative determination of Epimidin - API with pronounced anticonvulsant activity has been developed. Chromatographic analysis (HPLC) conditions are standardized according to the requirements of SPhU. The requirements for the test “System suitability test criteria for chromatographic methods” are set. Statistical processing of the experimental results shows that the complete uncertainty of the average result is within acceptable limits. For the method of quantitative determination of Epimidin, such validation parameters as specificity, linearity, accuracy, precision and robustness were studied.

Conflict of interests

There are no conflicts of interest regarding this study.

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