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DEVELOPMENT OF METHODS FOR DETERMINATION SESQUITERPENIC ACIDS IN STRESSPROTECTIVE PHARMACOLOGICAL AGENT WITH EXTRACTS OF VALERIAN, LEMON BALM AND GLYCINE

Methods for identification and quantification of sesquiterpenic acids in stressprotective pharmacological agent with extracts of valerian, lemon balm and glycine have been developed. The basic verification characteristics have been studied. It is shown that the techniques are suitable for control the content of sesquiterpenic acids in stressprotective pharmacological agent with extracts of valerian, lemon balm and glycine.

Key words: tablets; dry extracts of valerian and lemon balm; glycine; sesquiterpenic acids; thin layer chromatography; high performance liquid chromatography

INTRODUCTION

Medicines from valerian are widely used in nervous excitement of various origins (emotional stress, intense mental stress, menopausal and other functional disorders of the central nervous system) disorders of sleep quality and sleep process, neurocirculatory dystonia and functional arrhythmia, spasm of neurotic gastrointestinal tract. Experimentally proved that they reduce the reflex excitability in the central nervous system and strengthen the processes of inhibition in the cerebral cortex. One way to improve the efficiency of valerian is the creation of combined preparations containing substances that can potentiate the sedative activity of valerian. Promising for this purpose is their combination with balm and glycine (inhibitory neurotransmitter type of action and control of metabolic processes in the central nervous system). The combination of extracts of valerian, lemon balm and glycine Ukraine protected by patent for utility model number 62461, Bulletin number 16 from 25.08.2011 year.

Medicinal herbs and preparations based valerian described in DF XI, European, British, American, Japanese, Indian, Ukrainian and Belarusian pharmacopoeias. In Ukraine, edition 1.2 SPU quality control of raw materials valerian carried out by DB XI and he called for microscopic analysis of raw valerian. At the same time European, British Pharmacopoeia and other leading world already identified valerenic acid and its derivatives, first identifying only the content of essential oils, and later quantitative value valerenic and acetoxyvalerenic acids, which are markers for substances valerian. For the first time in the European Pharmacopoeia 2005 edition introduced a quantitative determination of the amount

sesquiterpenic acids in the roots of valerian. According to European and British pharmacopoeias essential oil content in rhizomes with roots must be at least 5 ml/kg (0.5 %) for the entire stock or at least 3 ml/kg (0.3 %) – for raw materials, cut, and at least 0.17 % sesquiterpene acids (whole raw) or not less than 0.10 % (incised stock). To determine the amount sesquiterpenic acids recommended by high performance liquid chromatography using as a standard sample extract of valerian standardized dry. In 1.2 SPU quality raw valerian is controlled by the content of sesquiterpenic acids based on valerenic acid. For their quantitative determination using HPLC method using internal standard – dantron and used to identify the TLC method with the use of substances markers – fluorescein and sudan red G [4, 3, 9, 10].

The aim of our study was to develop techniques for the identification and quantification of sesquiterpenic acids in stressprotective pharmacological agent with extracts of valerian, lemon balm and glycine.

MATERIALS AND METHODS

Among BAR valerian, which should be stored in its extracts over time and their presence indicate suitability for extract expiration date are sesquiterpene acids, particularly valerene and acetoxyvalerene. To identify valerenic acid elected by TLC (Method A) in a solvent system described in 1.2 and SPU HPLC method (method B) in terms of quantitative determination. For quantification of sesquiterpene acids chosen by HPLC using chromatographic system described in the SPU.

Method A.

Test solution. 1.5 g of powder pounded tablets placed in a volumetric flask 25.0 ml, 6.0 ml shake with *methanol* R for 15 min and filtered. The flask and the filter

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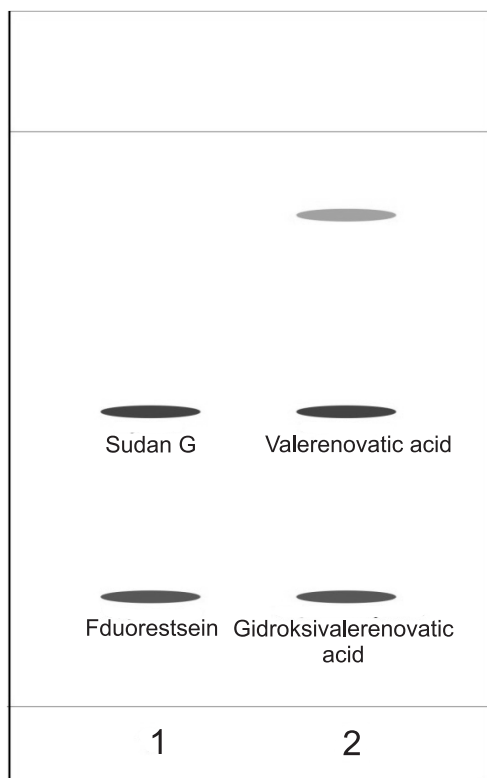


Fig. 1. Scheme of thin-layer chromatograms tablets based on extracts of valerian and lemon balm and glycine in terms of identification sesquiterpenic acids: 1 – reference solution, 2 – test solution.

washed with small portions of methanol to obtain 5 ml of the filtrate. The filtrate was evaporated to a volume of about 2 ml, add 3 ml of 100 g/l potassium hydroxide R and shake with two portions, 5 ml each, of methylene chloride R. After separation of the lower layer is drained. The aqueous layer was heated in a water bath at 40 °C for 10 min, cooled, diluted hydrochloric acid is added to acid reaction R and shake with two portions, 5 ml each of methylene chloride R. The combined lower layers filtered over anhydrous sodium sulfate R. The resulting filtrate was evaporated to dryness, the residue was dissolved in 1.0 ml of methylene chloride R.

Reference solution. 5 mg of fluorescein R and 5 mg of sudan red G R dissolved in 20.0 ml of methanol R.

At the starting line of the chromatographic plate with a layer of silica gel P micro syringeput 20 ml test solution, a 1 cm strip and parallel applied 20 ml solution. Plates coated samples are dried in air and immersed in saturated chromatographic chamber with a mixture of solvent glacial acetic acid R – acetate R – hexane R (0.5: 35: 65).

When the solvent front will be held 10 cm from the starting line, the plate is removed from the camera and viewed in daylight. In the chromatogram obtained with reference solution should occur: in the middle part – the red zone, corresponding sudan red G, at the bottom – a greenish-yellow, the corresponding fluorescein. Plate sprayed with a solution of aldehyde anise R, heated at a tempera-

ture of 100 to 105 °C for 5-10 min and browsing in daylight.

In the chromatogram obtained with the test solution are detected, violet-blue zone corresponding hydroxyvalerenic acid level zone corresponding fluorescein in the chromatogram obtained with reference solution, and violet zone corresponding valerenic acid at sudan red G in the chromatogram obtained with reference solution. In the chromatogram obtained with the test solution on top of the other are detected, less intense band of pink to purple.

The analysis results are valid only if the following requirements test "checks their chromatographic system".

The sequence of zones in the chromatogram of the test solution (2) and solution (1) in terms of TLC analysis are shown in Fig. 1.

Check the suitability of the chromatographic system. Chromatographic system is considered as eligible if:

- In the chromatogram obtained with reference solution in visible light clearly visible stain fluorescein and sudan red G;
- In the chromatogram obtained with reference solution Rf fluorescein stain and sudan red G are respectively – 0.18-0.22 and 0.76-0.80.

Method B.

The relative retention times and acetoxyvalerenic, valerenic acid (relative substance) in the chromatogram of the test solution obtained in terms of quantitative determination should be about 0.7 to acetoxyvalerenic acid and 1.2 for valerenic acid.

Scheme chromatograms of methanol extract tablets with extracts of valerian, lemon balm and glycine in terms of quantification of sesquiterpenic acids shown in Fig. 2-4.

Using chromatography conditions described in 1.2 SPU to quantify the sesquiterpene acids valerian roots, we studied the chromatographic behavior of valerian extract, lemon balm extract, glycine and tablets with extracts of valerian, lemon balm and glycine. Established that the selected conditions in the extract of valerian and tablets with extracts of valerian, lemon balm and glycine identified acetoxyvalerenic and valerenic acid relative retention times that meet the requirements of the monograph "Valerian root". The corresponding chromatograms are shown in Fig. 2-4.

At the same time found that in selected circumstances chromatography at a wavelength of 220 nm detection chromatograms of glycine and lemon balm extract at times hold valerenic and acetoxyvalerenic acids not found any substance – having a form of chromatogram zero line. This is natural, since glycine, the simplest amino acid as no chromophore groups that have absorption in the UV region of the spectrum. In lemon balm extract active ingredients are hydroxycinnamic acids that have intense absorption bands in the region 320-330 nm. Therefore, they are not detected at a wavelength of 220 nm, and thus do not interfere with the identification and

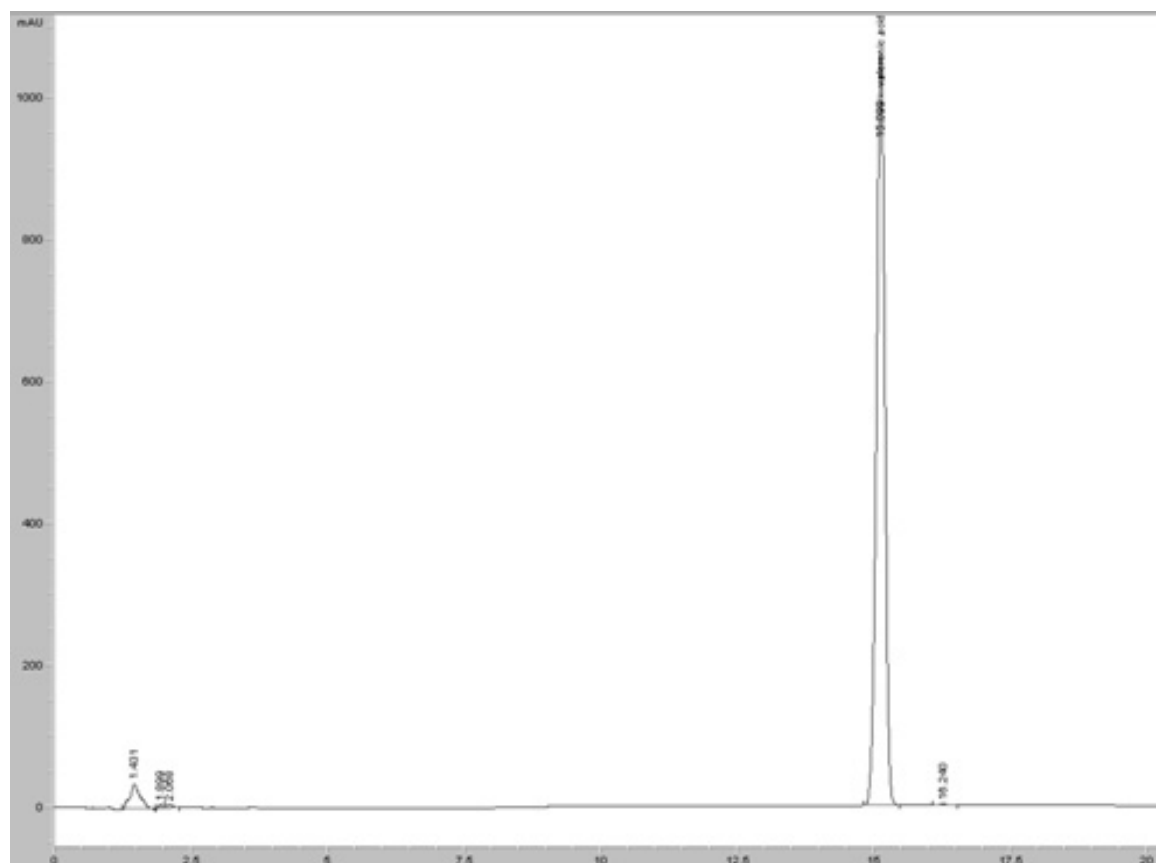


Fig. 2. Chromatogram methanol solution of acid valerenic.

quantitative determination valerenic and acetoxyvalerenic acids.

The next stage of the study was to develop a method of sample preparation tablets with extracts of valerian, lemon balm and glycine for the quantitative determination by HPLC. Established that removal of methanol by heating under reflux allows you to fully translate into solution determined by BAS valerian powder pounded tablets. Method of assay below.

Test solution. 0.5000 g of powder pounded tablets placed in a 100 ml flask with thin sections, add 20 ml of *anhydrous methanol R*, stirred, heated in a water bath under reflux for 30 minutes, cooled and filtered. The filter residue was washed with 20 ml of *anhydrous methanol R*. Combine the filtrates and dilute *methanol anhydrous R* to 50 ml.

Reference solution. The solution was prepared immediately before use, protect from glare. 30 mg sub-

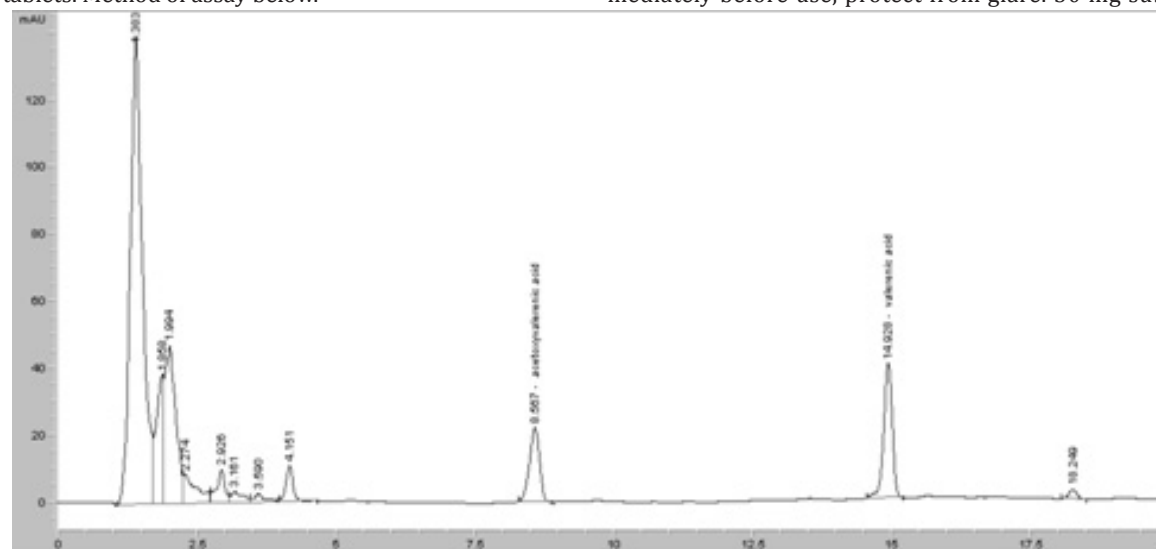


Fig. 3. Chromatogram methanol solution of valerian extract.

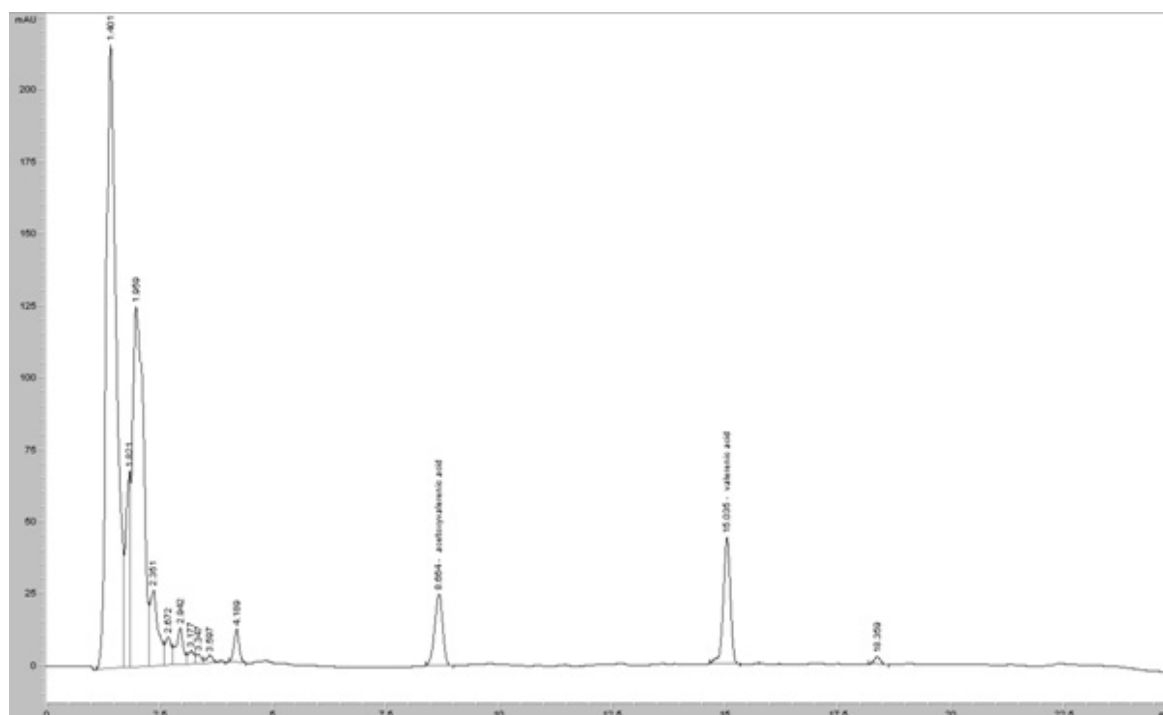


Fig. 4. Chromatogram obtained with the test solution of methanol extract tablets with extracts of valerian, lemon balm and glycine.

stance P dissolved in *anhydrous methanol R* and dilute with the same solvent to 100.0 ml. 5.0 ml of *anhydrous methanol R* prove to the volume of 50.0 ml.

Chromatography is performed on liquid chromatograph with UV detector under the following conditions:

- Column stainless steel measuring 0.25 m × 3 mm filled with octadecylsilyl silica gel for chromatography R with a particle size of 5 microns;
- Mobile phase A: acetonitrile R – solution of 5 g/L phosphoric acid P (20 : 80);
- Mobile phase B: acetonitrile R – a solution of 5 g/L phosphoric acid P (80 : 20);
- Temperature: 25 °C;
- The rate of mobile phase 1.5 ml/min in the following gradient elution mode: Time, min mobile phase A

Time, min	Mobile phase A (% V/v)	Mobile phase B (% V/v)
0-5	55	45
5-18	55→20	45→80
18-20	20	80
20-22	20→55	80→45

- Detection wavelength at 220 nm;
- The volume of sample injected 20 µl.

Chromatographed test solution and the solution turns comparison, getting five chromatogram for each solution.

Acetoxyvaleric acid content (X_1), in milligrams, measured by the average weight of the tablet, calculated using the formula:

$$X_1 = \frac{A_2 \cdot m_1 \cdot 115.1}{A_1 \cdot m_2} \cdot b.$$

Content valeric acid (X_2), in milligrams, measured by the average weight of the tablet, calculated using the formula:

$$X_2 = \frac{A_3 \cdot m_1 \cdot 80.9}{A_1 \cdot m_2} \cdot b.$$

Content sum sesquiterpenic acids (X_3), in milligrams, measured by the average weight of the tablet in terms of valeric acid, calculated using the formula:

$$X_3 = \left[\frac{A_2 \cdot 115.1}{A_1} + \frac{A_3 \cdot 80.9}{A_1} \right] \cdot \frac{m_1}{m_2} \cdot b,$$

where: A_1 – average size substance peak in the chromatogram obtained with reference solution;

A_2 – the average peak area in the chromatogram acetoxyvaleric acid test solution;

A_3 – the average peak area valeric acid in the chromatogram obtained with the test solution;

m_1 – mass substance sample taken for solution comparison, in grams;

m_2 – mass of sample powder pounded tablets, in grams;

b – the average weight of the tablet, in grams.

Since we used a technique chromatographic conditions which are described in SPU, then it was necessary to not validate but verify the methods that examine a limited number of validated characteristics, namely linearity, robustness and convergence.

We have researched the linear method. Methods must be linear in the range of minimum content to the maximum possible. To do this, we have made valerian extract

Table 1

RESULTS LINEARITY OF ASSAY SESQUITERPENIC ACIDS

C, mkg/ml	C _{norm} %	A _{measured}	A _{norm}	Requirements and eligibility criterion	
8.40	50	181.1	49.3	The equation of the line: $A_{\text{norm}} = 1.0057 \times S_{\text{norm}} - 0.5508$ Slope: $b = 1.0057$ The point of intersection with the ordinate axis: $a = -0.5508$ The correlation coefficient: $r = 0.9999$	
10.08	60	225.9	60.5		
11.76	70	260.6	69.8		
13.44	80	297.6	79.7		
16.80	100.0	373.4	100.0		
21.00	125	469.3	125.7		
25.20	150	557.2	149.2		
29.40	175	657.1	176.0		
33.6	200	748.9	200.6		
The correlation coefficient: $r = 0.9999$				$r > 0.9993$	Performed
The residual variance $S_0^2 = 0.3586$ The residual standard deviation $S_0 = 0.5988$				$S_0/b \leq 2.53$	Performed
The confidence interval is constant a : $D_a = 0.9240$ Freemember $a = -0.5508$				$> a \leq 3.072$	performed statistical criteria and practical insignificance

methanol solutions with different breeding – by 2 times less to 2.5 times the amount of concentration sesquiterpenic acids located in the test solution to be tested in the quantitative determination. The results linearity are shown in Table 1.

Table 2 shows the results of accuracy and convergence of methodology for determining the amount of sesquiterpenic acids by HPLC.

RESULTS AND DISCUSSION

As a result of studies found that the method is characterized by strict linearity (correlation coefficient greater than 0.99 and a small residual sum of squared deviations) over a wide range (from 50 to 250 % in normalized coordinates). A plot of the peak area of concentration is almost through the origin (the point of intersection with the ordinate axis -0.5508), indicating no effect of background or excipients in the area of the peaks of these substances in these conditions.

The results of the test are talking about convergence methods because none of the results largest error has not gone more than 4.8 % (the maximum permitted under the requirements SPU). Thus, the method of determining the content of sesquiterpenic acids given in HPLC for their determination in raw valerian has been adapted by us to analyze the tablets with extracts of valerian, lemon balm and glycine. A partial verification techniques, namely tested linearity and convergence, as performed by the eligibility criteria regarding these characteristics validated in accordance with the requirements of SPU.

CONCLUSIONS

1. Methods of identification sesquiterpenic acids in stress-protective pharmacological agent with extracts of valerian, lemon balm and glycine have been developed.
2. By HPLC method of assay content sesquiterpenic acids composed tablets with extracts of valerian,

RESULTS OF ACCURACY AND CONVERGENCE OF METHODS FOR DETERMINATION OF SESQUITERPENIC ACIDS BY HPLC

Nº s/n	C _{i input} %	C _{i output} %	$Z = C_{i \text{ output}} \cdot 100 / C_{i \text{ input}} \%$
1	50	49.3	98.6
2	60	60.5	100.8
3	70	69.8	99.7
4	80	79.7	99.6
5	100	100	100
6	125	125.7	100.6
7	150	149.2	99.5
8	175	176.0	100.6
9	200	200.6	100.3
Mean, \bar{Z}			99.97
The relative standard deviation, $S_z\%$			0.6982
The relative confidence interval, $\Delta_z\%$			1.2983
Critical values for convergence results $\Delta_z\% \leq 4.8$		≤ 4.8	performed
Criterion statistical insignificance bias $\delta\% = \bar{Z} - 100 \leq \Delta_z / \sqrt{n}$		≤ 0.43	performed
Criterion practical insignificance bias $\delta\% = \bar{Z} - 100 \leq 0.32 \times \Delta_{AS}$		≤ 1.536	performed
The overall conclusion of the method			correct

lemon balm and glycine. A verification of methodology for performance, linearity, convergence, precision has been made.

3. The results of these studies will be used to develop methods of standardization stressprotective pharmacological agent with extracts of valerian, lemon balm and glycine.

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УДК 615.243.3 – 615.451.1**Л. С. Логойда****РАЗРАБОТКА МЕТОДИК ОПРЕДЕЛЕНИЯ СЕСКВИТЕРПЕНОВЫХ КИСЛОТ В СТРЕСПРОТЕКТИВНОМ ФАРМАКОЛОГИЧЕСКОМ СРЕДСТВЕ С ЭКСТРАКТОМ ВАЛЕРИАНЫ, МЕЛИССЫ И ГЛИЦИНА**

Разработаны методики идентификации и количественного определения сесквитерпеновых кислот в стресспротективном фармакологическом средстве с экстрактами валерианы, мелиссы и глицина. Проведено верификацию методик. Показано, что методики пригодны для контроля содержания сесквитерпеновых кислот в стресспротективном фармакологическом средстве с экстрактами валерианы, мелиссы и глицина.

Ключевые слова: таблетки; сухие экстракты валерианы и мелиссы; глицин; сесквитерпеновые кислоты; тонкослойная хроматография; высокоэффективная жидкостная хроматография

УДК 615.243.3 – 615.451.1**Л. С. Логойда****РОЗРОБКА МЕТОДИК ВИЗНАЧЕННЯ СЕСКВІТЕРПЕНОВИХ КИСЛОТ У СТРЕСПРОТЕКТИВНОМУ ФАРМАКОЛОГІЧНОМУ ЗАСОБІ З ЕКСТРАКТАМИ ВАЛЕРІАНИ, МЕЛІСИ ТА ГЛІЦИНУ**

Розроблені методики ідентифікації та кількісного визначення сесквітерпенових кислот у стреспротективному фармакологічному засобі з екстрактами валеріани, меліси та гліцину. Проведено верифікацію методик. Показано, що методики придатні для контролю вмісту сесквітерпенових кислот у стреспротективному фармакологічному засобі з екстрактами валеріани, меліси та гліцину.

Ключові слова: таблетки; сухі екстракти валеріани і меліси; гліцин; сесквітерпенові кислоти; тонкошарова хроматографія; високоефективна рідинна хроматографія

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