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FACULTY OF PHARMACY
DEPARTMENT OF ANALYTICAL, PHYSICAL AND COLLOID CHEMISTRY
Speciality - 226 "Pharmacy, industrial pharmacy"

GRADUATION QUALIFICATION WORK

Topic "**OPTIMIZATION OF THIOTRIAZALIN QUANTITATIVE DETERMINATION BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY**»

Performed by: 5th year higher education student, group 9601fa
Branch of knowledge 22 "Health care"
Educational program "Pharmacy"
Mehrdad Jafarifard

Supervisor: Head of the Department of Analytical, Physical and Colloid Chemistry, Candidate of Chemical Sciences, Zaitseva Galyna Mikolaivna

Reviewer: Head of the Department of Medicinal Chemistry and Toxicology, Doctor of Medicine, Professor Nizhenkovska Iryna Volodymyrivna

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CONTENT

LIST OF TERMS, SYMBOLS AND ABBREVIATIONS

HPLC is high performance liquid chromatography

DFU - state pharmacopoeia of Ukraine

NMU - Bogomolets National Medical University

GLP – good laboratory practice

GMP - good manufacturing practice

ISO is an international organization for standardization

TLF is a solid dosage form

LZ is a medicine

g - gram

IR spectrum - infrared spectrum

μl - microliter

ml - milliliter

nm is a nanometer

T. b. - boiling point

T. m. - melting point

UV spectrum – ultraviolet absorption spectrum

C^0 - degrees Celsius

INTRODUCTION

The thiotriazoline compound is a synthetic drug that was synthesized in Ukraine on the basis of the Zaporizhzhya Medical Institute in the 80s of the last century by a group of scientists led by Professor I.A. Mazur. In medicine and pharmacy, medicinal products primarily have cardio- and hepatoprotector against antioxidant, membrane-stabilizing, anti-seminal, antiviral and regenerative effects [1].

Thiotriazoline is on the list of vitally necessary medicines, beginning to occupy a worthy place among domestic pharmacological preparations, and every year this medicine is used more and more. Forms of use of this medicinal disease. Today, hospitals prescribe the drug to patients in the form of tablets, injections, eye drops, vaginal suppositories and ointments, therefore, in connection with the spread of the scope of thiotriazoline, one of the modern analytical tasks is the control of the amount of the active substance thiotriazoline in the drug [2-9].

Actuality : Search for new methods and optimization of known methods for multi-assay determination of thiotriazoline in various dosage forms.

Goal: to optimize the method of quantitative determination of thiotriazoline in solid and liquid dosage forms by high performance liquid chromatography (HPLC).

Task:

1. To analyze the sources of scientific information regarding the application, physico-chemical and pharmacological properties, mechanism of action and metabolism of thiotriazoline, methods of identification and quantitative determination of thiotriazoline.

2. Choose the method of quantitative determination of thiotriazoline in dosage forms based on biblio-semantic research and optimize it to the conditions of conducting the experiment.

3. To carry out a partial validation of the optimized method for the quantitative determination of thiotriazoline.

Research methods: Bibliosemantic, HPLC (high performance liquid chromatography) process.

The novelty and significance of the obtained results: The optimal conditions for the quantitative determination of thiotriazoline by the method of high-performance chromatography using a Shimadzu liquid chromatograph with UV detector and a chromatographic steel column (250x4.6) mm, filled with Nucleosil C18 (100-5) were determined.

The method of chromatographic determination of thiotriazoline in liquid and solid dosage forms was optimized, tested and validated in terms of specificity, linearity, precision, and correctness.

Approbation of research results:

Zaitseva G., Reva T., Jafarifard M. OPTIMIZATION OF THE METHOD OF THIOTRIAZOLINE QUANTITATIVE DETERMINATION BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY. / The 13th International scientific and practical conference “Information and innovative technologies in the development of society” (April 02 – 05, 2024) Athens, Greece. International Science Group. 2024. p.180-181.

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Structure of work: The work is presented at 43 pages, figures – 4, tables – 5, appendices – 3.

Chapter 1. Thiotriazoline, methods of determination

1.1 Application of thiotriazoline

The thiotriazoline is considered as original cardioprotector, which is prescribed to patients for the treatment of coronary heart disease, chronic hepatitis, cirrhosis, and pancreatitis.

Thiotriazoline is used for the manufacture of such dosage forms as tablets, injection solutions, eye drops, ointments, suppositories and for combined medicinal products. In the form of suppositories, they are used rectally, in the form of ointments - on the skin, in ophthalmology - in the form of eye drops [10].

The widespread use of triazolin in the creation of medicines is due to the fact that thiotriazolin has an antioxidant effect and inhibits oxidative stress and, therefore, restores the sensitivity of neuron receptors and improves the energy supply of the brain [10].

1.2. Physical-chemical properties of thiotriazoline.

The chemical name of thiotriazoline is morpholinium-3-methyl-1,2,4-triazoline-5-thioacetate.

Name according to IUPAC is Mopholine -5 -methyl -1,2,4- triazoline-5-thioacetate.

Molecular formula: $C_9H_{16}N_4O_3S$, molecular weight 253,252 g/mol (Fig. 1).

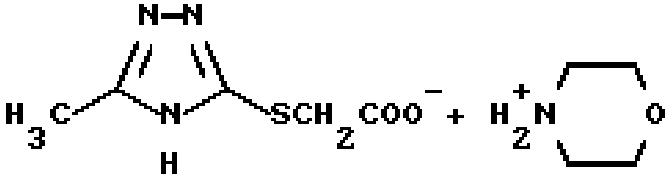
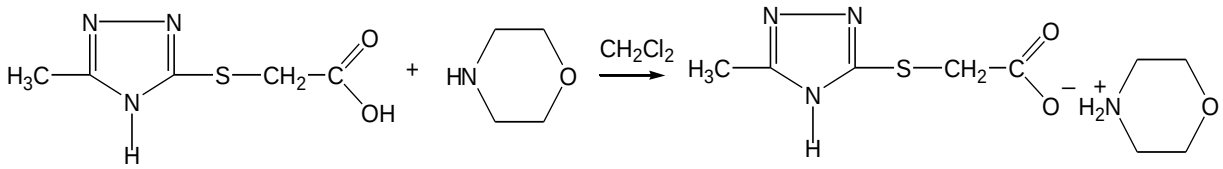


Fig. 1 Structural formula of Thiotriazoline

The thiotriazoline is crystalline compound of white color, sometimes with a grayish or pale yellow tint. It has a specific smell. Thiotriazoline is soluble in water, moderately soluble in alcohol, practically insoluble in acetone, hexane and chloroform [11].

It is recommended to store thiotriazoline without air access in a tightly closed chemical container.

Thiotriazoline is obtained according to the following scheme [11]:



To do this, methylene chloride CH_2Cl_2 is added to Morpholine, then 3-methyl-1,2,4-triazolyl-5-thioacetic acid (this compound is insoluble in CH_2Cl_2) and the mixture is stirred. As a result of the chemical reaction, a compound insoluble in CH_2Cl_2 (thiotriazoline) is formed. The solid phase is separated by filtration and washed on the filter with methylene chloride in order to remove possible impurities of morpholine.

1.3. Mechanism of action and metabolism of thiotriazoline

Thiotriazoline is absorbed at a sufficiently high rate after oral and parenteral administration, and the maximum concentration in the body is reached after 1 hour.

Thiotriazoline binds poorly to blood proteins, the highest concentrations are determined in the kidneys, myocardium, spleen, and liver. Bioavailability of the drug after oral administration is 53%, after rectal administration - 60%, after parenteral administration - 100%.

There are no data on metabolism. Thiotriazoline is excreted in the urine, the elimination half-life is 8 hours [12-18].

1.4. Pharmacological effects, side effects and overdose

It is known from scientific investigations and clinical studies that thiotriazoline inhibits damage to hepatocytes, regenerates the activity of hepatocytes, is able to normalize certain metabolic processes in the body (protein, lipid, pigment, etc.). Accelerates the secretion of bile and activates the antioxidant system in the body, due to the reaction of complex formation, oxygen free radicals are converted into a bound state, which, accordingly, preserves reserves of non-enzymatic antioxidant (tocopherol).

Thiotriazoline helps to increase the compensatory activity of anaerobic colisis and reduces inhibition of oxidation processes in the Krebs cycle. The membrane-stabilizing property of thiotriazoline manifests itself in the fact that during the spread of inflammatory reactions in patients, the release of biogenic amines into the blood is limited, and the manifestation of cytolytic syndrome in diseases of the disease is reduced.

Thiotriazoline is able to show antisemitic properties, therefore it activates the antioxidant system and reduces the process of lipid oxidation in certain areas of the myocardium, reduces areas of necrosis, inhibits the activity of the myocardium and reduces the sensitivity of heart muscles to catecholamines. of the above, thiotriazoline exhibits antiarrhythmic properties in both adults and children. When conducting clinical studies of the drug, it was found that this manifested substance can have an antiviral property in relation to hepatitis (B and C), but this property is still poorly studied, and is mainly explained by immunomodulatory properties. It is shown that thiotriazoline prevents the development of cerebral vasodilatation, which prevents the increase of local and total cerebral blood circulation. In addition, thiotriazoline stimulates reparative processes in diseases of mucous membranes and eyes [12-18].

The drug is capable of showing a side effect, and, first of all, it is:

- Various reactions (itching of the skin, anaphylactic shock, fever, inflammation);

- Nausea, vomiting;
- General weakness, dizziness;
- arterial hypertension and tachycardia;
- Dyspnea;
- Increased concentrations of potassium and sodium cations in urine.

In case of renal insufficiency, deficiencies and breastfeeding, taking thiotriazoline is not required [12-18].

1.5. Methods of identification and quantitative determination of thiotriazoline

Identification of thiotriazoline by chemical methods [11]:

Reaction to morpholine.

The mixture is mixed with concentrated lye, heated to half an hour. At the same time, litmus paper raised to the opening of the test tube turns blue.

Water pyrolysis.

When boiling substances with concentrated nitric acid, the group is revealed SO_4^{2-} by adding a barium salt solution. Turbidity is formed or a white precipitate falls out:

$$Ba^{2+} + SO_4^{2-} = BaSO_4.$$

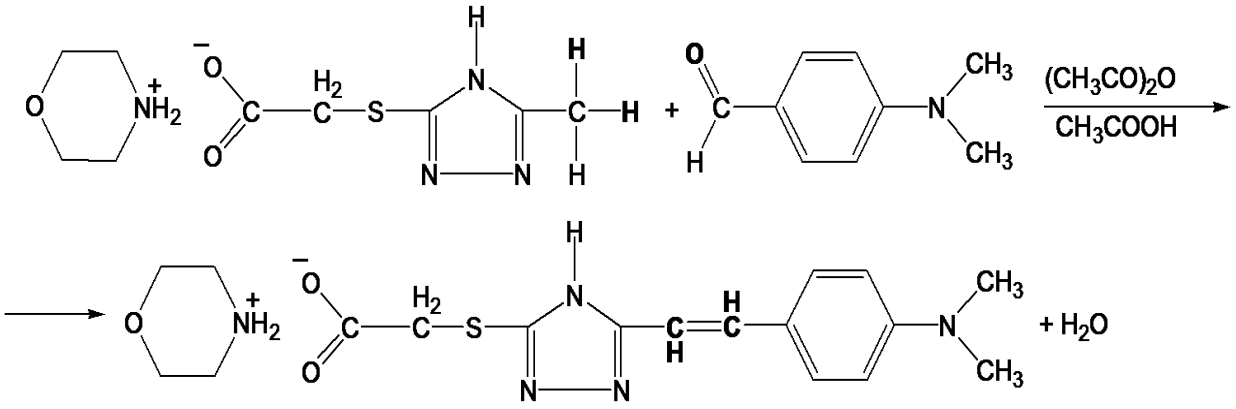
Dry pyrolysis.

The presence of sulfur is detected using a piece of paper impregnated with $Pb(CH_3COO)_2$ salt. Black PbS is formed:

$$Pb^{2+} + S^{2-} = PbS.$$

Reaction with p-dimethylaminobenzaldehyde.

An orange color is observed when p-dimethylaminobenzaldehyde is added to the preparation in an acetone-hydride medium and heated::



Quantitative thiotriazoline start :

By the method of acid-base titration with the preliminary release of Nitrogen according to the Kjeldahl method.

The method is based on the general determination of Nitrogen by mineralization of the substance. Potassium sulfate, copper sulfate and concentrated sulfuric acid are added to the solution and heated to boiling. As a result of the reaction, nitrogen transforms into ammonium hydrosulfate. After interaction with sodium hydroxide, free ammonia is formed. Ammonia gas is directed into a vessel with orthoboric acid. The following chemical reactions occur:

$NH_4HSO_4 + NaOH = NH_3 + Na_2SO_4 + 2\ H_2O$

$NH_3 + H_3BO_3 = NH_4BO_2 + H_2O$

$2NH_3 + 4\ H_3BO_3 = (NH_4)_2B_4O_7 + 5\ H_2O$

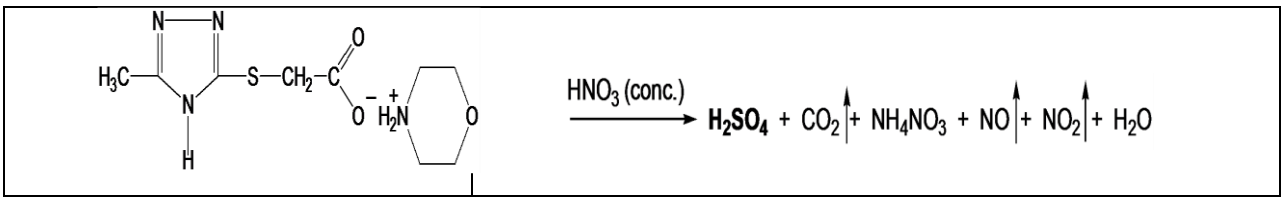
Salts are titrated with a solution of hydrochloric acid in the presence of a mixture of methyl red and methyl amber indicators:

$NH_4BO_2 + HCl + H_2O = NH_4Cl + H_3BO_3$

$(NH_4)_2B_4O_7 + 5\ H_2O + 2\ HCl = 2NH_4Cl + 4H_3BO_3$

Gravimetry

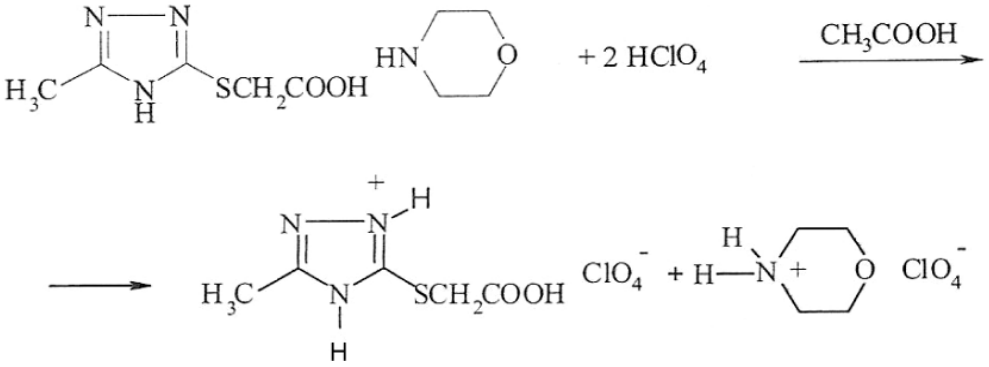
Gravimetric determination of thiotriazoline is carried out after preliminary treatment of the compound with concentrated nitric acid:



As a result, sulfuric acid is formed in an equivalent amount. Then sulfate anions are precipitated with barium salts, the resulting precipitated form is filtered, dried, calcined and weighed. Calculations are carried out using the gravimetric factor [11].

Non-aqueous titration method

According to DFU [19], the quantitative determination of thiotriazoline is carried out by the method of direct non-aqueous titration:



Thiotriazoline is also determined by amperometric titration using 12-molybdophosphate heteropolyacid as a titrant [19]. The method is sensitive and rapid.

Identification of thiotriazoline is carried out by physicochemical methods (by melting point, IR spectroscopy) [11].

Triazoline in various dosage forms is quantitatively determined spectrophotometrically in the UV region at a wavelength of 210-230 nm [20].

Several methods have been proposed for the quantitative determination of thiotriazoline in a mixture by the HPLC method [21-25].

1.6. The method of high performance liquid chromatography (HPLC)

High-performance liquid chromatography (HPLC) is a modern instrumental method of identification and separation of substances based on the theory of sorption. In the HPLC mobile and stationary phases are separated. The mobile phase is a liquid that is passed under high pressure through a chromatographic column filled with a stationary phase [26,27]. Different materials are offered as the stationary phase, which allows for variable studies. The most common stationary phase is silica gel, including chemically modified ones.

Determination of the content of the analyzed substance is carried out using the methods of absolute calibration or the method of the internal standard [26,27]. The absolute calibration method is based on determining the dependence of the area under the peak in the chromatogram of the analyzed substance on the concentration of the analyte. In other words, a series of standard solutions (solutions with a known concentration) are prepared to construct a gradation (calibration) graph. Each standard solution is chromatographed (carry out a series of determinations), determine the average value of the peak area for each concentration, and plot the graphical dependence of the area on the concentration. Such a graduation graph allows you to determine the content of the analyte in the samples.

In chemical, medical, and pharmaceutical practice, HPLC makes it possible to conduct research at the level of international GLP, GMP, and ISO standards [26,27].

In the process of conducting HPLC analysis, the researcher is faced with the question of choosing a mobile phase, since the mobile phase significantly affects the selectivity of the separation, the efficiency of the column, etc. Solvents are subject to certain requirements, namely: substances that act as solvents must dissolve well all the components of the mixture, must not chemically react with the components of the mixture and the solid phase (sorbent), enable the detector to recognize the sample. For example, the eluent should not absorb at the same wavelength as the analyte during UV detection of the sample. In addition, solvents

must be chemically inert, chemically pure. When separating multicomponent samples by the HPLC method, it is advisable to use mixtures of solvents as the mobile phase. When preparing and approving the HPLC technique, the researcher, as a rule, evaluates the viscosity of the solvent mixture, miscibility, reactivity, explosiveness, etc. Typically, the mobile phase is supplied under pressure and the most important aspect of HPLC is the high resolution that allows the analysis of batches of a mixture and if the sample consists of a mixture, high performance liquid chromatography allows the separation, identification and quantification of the components of the mixture. When appropriate conditions are created, the level of reproducibility reaches almost 100%.

Chapter 2. Experimental part.

The experimental part of the work was performed in the liquid chromatography laboratory of the Institute of Hygiene and Ecology of the Bogomolets NMU.

2.1. Materials and methods

2.1.1. The goal of the study

The goal of the study was to optimize the method of quantitative determination of thiotriazoline in solid and liquid dosage forms by the high-performance liquid chromatography (HPLC) and to test it.

2.1.2. Research objects

Solid and liquid dosage forms (tablets and solutions for injections), which include thiotriazoline, were chosen as the object of the study:

Sample 1, tablets.

Active substance - thiotriazoline, 1 tablet contains morpholinium salt of thiazotic acid in 100% substance - 200 mg, which is equivalent to 133 mg of thiazotic acid;

Excipients:

potato starch;

povidone;

powdered sugar;

microcrystalline cellulose;

calcium stearate.

Sample 2, solutions for injections.

Thiotriazoline solution for injections, 2.5%, 2 ml in an ampoule

Main physicochemical properties: transparent colorless or slightly yellowish liquid.

Active substance: 1 ml of solution contains 25 mg of morpholinium salt of thiazotic acid (thiotriazoline), calculated at 100%, which is equivalent to 16.6 mg of thiazotic acid

Excipients:

Water for injections.

Sample 2, solutions for injections

2.1.3. Chemical dishes and equipments

Chemical measuring vessels of accuracy class A.

Rotary evaporator for extraction.

Water bath.

Shimadzu liquid chromatograph with UV detector, factory number C20964330924CC, inventory number 010466981, calibration certificate number 3991 dated June 7, 2019, Appendix 1.

Chromatographic steel column (250×4.6) mm, filled with Nucleosil C18 (100-5) and a chromatographic steel pre-column (4×3) mm filled with Nucleosil C18 (100-5).

Microsyringe for HPLC with a capacity of 20 μl, produced by Hamilton.

Analytical laboratory scales with a measurement error of 0.0002 g Radwag® AS220.R2, factory No. 502964, inventory No. 104769236, calibration certificate No. 3816 dated May 30, 2019.

Paper filters de-ashed "red tape", diameter 150 mm.

2.1.4. Reagents

1. Pharmacopoeia standard sample of DFU Thiotriazolin Morpholinium tizotate Morpholine salt thiazotic acid (Tiotriazolin), catalog number T0406, registration number 357172-63-5.

2. Methanol, pure, for liquid chromatography.

3. 0.05 mol/l potassium dihydrogen phosphate solution.

4. Distilled water.

2.1.5. Techniques and conditions of chromatography

When developing and approving the methodology, we relied on the results of research works [21-25].

Preparation of mobile phase. Transfer 1.7 g of potassium dihydrogen phosphate into a 250 ml volumetric flask, add 100-150 ml of purified water, stir until the salt dissolves, add to the mark water and mix thoroughly.The solution is used immediately after preparation.

The rate of the mobile phase is 1 ml/min.

The detection wavelength is 220 nm.

Injection - 20 µl.

The temperature of the column is 30^0C.

2.1.6. Preparation of standard solution

A standard solution is prepared by dissolving the exact mass of 200 mg of a standard pharmacopoeial sample of thiotriazoline in 40-50 ml of water in a 100 ml volumetric flask and bringing it up to the mark with water.

The prepared solution is diluted 10 times. To do this, take 5 ml of standard solution by pipette, transfer it to a 50 ml volumetric flask, add purified water up to the mark and mix thoroughly. The solution is used immediately after preparation.

Preparation of solutions for the calibration graph .

A series of standard solutions containing of 80, 100, 120 μg/ml of thiotriazoline according to standard method of diluting of the solutions [26] was prepared. Using a pipette, the corresponding volume of the standard solution was transferred to a 100 volumetric flask, solution of 0.05 mol/l potassium dihydrogen phosphate was added. After mixing, the solution was chromatographed (solution volume 20 μl), and detected at wavelength of 220 nm.

2.1.7. Preparation of the solution of the studied sample**s**

Sample 1, solid dosage form.

To prepare the solution of sample 1 for chromatography, the sample preparation procedure was carried out as described in the section 2.2.

0.1 g of the prepared sample () was weighed on analytical balances, transferred to a 100 ml volumetric flask, dissolved in 0.05 mol/l potassium dihydrogen phosphate solution and brought up to the mark with this solution.

Sample 2, solution for injection

The contents of the 2 ml ampoule are quantitatively transferred to a 100 ml flask and added 20-30 ml of 0.05 mol/l potassium dihydrogen phosphate solution, stir the mixture and brought up to the mark with this solution.

2.1.8. Calculation of thiotrialoline content based on chromatographic analysis data

The content of thiotriazoline in the samples in mg according to the experimental data was calculated according to the standard formula [26]:

$$m, mg = R_2 \times m_1 \times A / R_1 \times m_2$$

where R_1 = the average value of the peak area of the standard solution;

R_2 = average value of the peak area of the sample;

m_1 = mass of the standard substance;

m_2 = sample mass;

A = purity of the standard, confirmed 1.

2.2. Sample preparation

Sample preparation of tableted forms (Sample 1) was carried out as standard, namely:

The tablet of sample 1 in water in a 0.5 liter volumetric flask was dissolved constantly shaken. The suspension after dissolution in a separatory funnel with a capacity of 1 l was placed. Extraction was carried out with ethyl acetate (three portions of 50 ml each). The organic fractions were combined and transferred to a 250 ml flask, dried with anhydrous sodium sulfate (the weight of the desiccant is approximately 20 g) for 24 hours. After drying, the extract in a distillation flask was placed and a standard concentration procedure to 0.1-0.3 ml was carried out. Dry sample residues by final evaporation in air were obtained.

2.3. Regeneration of the chromatographic column

In the course of a series of chromatographic determinations, the question of column regeneration sometimes arises. Therefore, the following simple technique was used to restore the chromatographic column:

1. Attach the chromatographic column in the reverse direction;

2. Turn on the reverse current of water and pass 25 ml of purified water at a rate of 0.5 ml/min;

3. Then the column is washed with organic solvents, for example, isopropanol and methylene chloride at a rate of 0.5 ml/min;

4. After that, the column is washed with 25 ml of hexane at a rate of 0.5 ml/min and again with isopropanol and methylene chloride;

5. After washing, the column is connected in the usual way and brought to equilibrium with the mobile phase.

2.4. Chromatographic column protection

To increase the life of any chromatographic column, it is necessary to periodically get rid of impurities that can accumulate on the solid phase and impair the resolution and distort the shape of the peaks. Technically, the best protection is to install a pre-column in line between the injector and the main column. As a rule, the pre-column is filled with the same solid phase that is used in the work.

In our study, a chromatographic steel (4x3) mm pre-column filled with Nucleosil C18(100-5)was used.

2.5. Statistical processing of chemical analysis results

The following metrological values are calculated:

The average value of the measurement $\bar{x}$ is calculated as the arithmetic mean of the experimental data:

$$\bar{x} = \frac{x_1 + x_2 + \cdots + x_n}{n},$$

where n is the number of parallel dimensions.

The value of measurement deviations x_i from the average value $\bar{x}$ - di is calculated according to the equation:

$$d_i = |x_i - \bar{x}|,$$

and the number of degrees of freedom (the number of independent variants) υ,-

$$\upsilon = n - 1,$$

where is the number of degrees of freedom.

Dispersion s^2 (measure of reproducibility (convergence) of results) and standard deviation s (measure of random error) were calculated according to equations respectively.

$$s^2 = \frac{\sum_{i=1}^{n}(x_i - \bar{x})^2}{\upsilon} = \frac{\sum_{i=1}^{n} d_i^2}{\upsilon},$$

$$s = \sqrt{s^2}$$

The results for which $|d_i| > 3s$, are gross errors with a given confidence probability. They are rejected.

The standard deviation of the average result is calculated according to the formula:

$$s_{\bar{x}} = \frac{s}{\sqrt{n}}.$$

Such metrological parameters as: relative dispersion, relative standard deviation and relative standard deviation of the average result are calculated according to the formulas:

$$s_r^2 = \frac{s^2}{\bar{x}^2}, \qquad s_r = \frac{s}{\bar{x}}, \qquad s_{\bar{x},r} = \frac{s_r}{\sqrt{n}}.$$

or as a percentage. In this case, they are denoted as RSD and:

$$RSD = s_r \cdot 100\%,$$

$$RSD_{\bar{x}} = s_{\bar{x},r} \cdot 100\%.$$

Dispersion, standard deviation and relative standard deviation characterize the reproducibility of the applied method of analysis.

If RSD 1−5% - the reproducibility of the measurement results is considered good, RSD 5−10% - satisfactory, RSD 10−15% - poor for the titrimetric method of analysis.

The confidence interval is the interval of values in which the true value of the analytical signal is located $\bar{x} - \Delta_{\bar{x}} \leq \mu \leq \bar{x} + \Delta_{\bar{x}}$.

with a certain value of statistical reliability (usually 95%) μ is calculated by the formula:

$$\bar{x} \pm t_{P,\upsilon} \frac{s}{\sqrt{n}} = \bar{x} \pm \Delta_{\bar{x}},$$

where $-\bar{x}$ average value, s – standard deviation, n – number of experimental data, $t_{P,\upsilon}$ – Student's coefficient or t-test, $\Delta_{\bar{x}}$ – half width of confidence interval.

The correctness of the obtained results is evaluated using the Student's test (t-test) by calculating the value of the Student's test using the formula:

$$t_{\text{експ.}} = \frac{|\bar{x} - \mu|}{s} \cdot \sqrt{n}$$

where – $\bar{x}$ average value, μ – true value of the sought value, s – standard deviation, n – number of experimental data.

If $t_{\text{експ.}} > t_{\text{табл.}}$,., then there is a systematic error.

If $t_{\text{експ.}} < t_{\text{табл.}}$., then the results do not contain a systematic error.

The value of the relative systematic error can be estimated by the formula:

$$.\delta = \frac{|\bar{x} - \mu|}{\mu} \cdot 100\%.$$

Chapter 3. Results and discussions

3.1. Selection of the optimal temperature regime of the chromatographic column and eluent

The choice of the temperature chromatographic regime when working out the methodology for the quantitative determination of thiotriazoline by the HPLC method is a primary task. To solve this problem, the dependence of the pressure change in the column on the temperature was investigated. The research was carried out on a standard solution with a concentration of 100 μg/ml. The results are shown in Fig. 2.

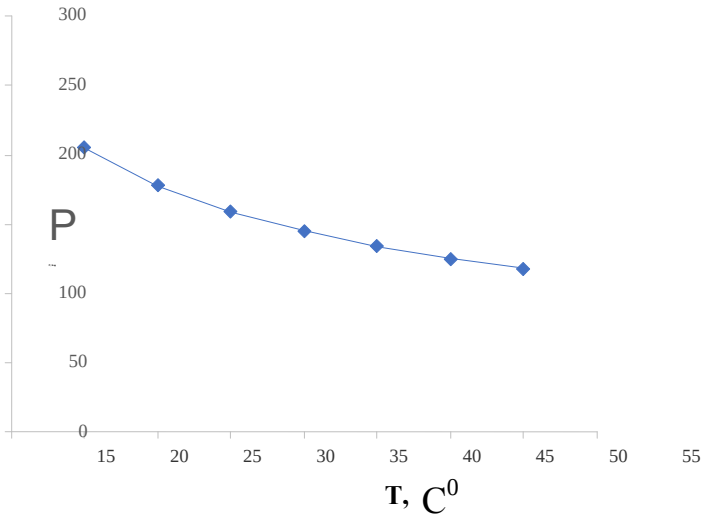


Fig. 2. Change in pressure in a chromatographic column Nucleosil C18 (100-5), (250×4.6) mm as a function of temperature. C (thiotriazoline)= 100 μg/ml.

As we can see from Fig. 2, as the temperature increases, the pressure in the column decreases. Therefore, 30℃ was chosen for further quantitative determination of thiotriazoline in dosage forms, since at this temperature the pressure decreases to acceptable values, at which there are no risks of destruction of the solid phase in the column as a result of hydrolysis.

Since thiotriazoline and excipients Sample 1 (tablet form) are well soluble in water, and Sample 2 (liquid dosage form) is a solution of thiotriazoline in water, it

is advisable to use a mobile phase - 0.05 M solution of potassium dihydrogen phosphate, proposed by the authors [25].

3.2. Selection of wavelength for detection

Based on analysis of scientific sources of information the selection of wavelength for the detection of thiotriazoline was carried out. It is known that thiotriazoline absorbs in the ultraviolet region of the spectrum when exposed to light. The range of the maximum absorption of thiotriazoline is achieved at 210-230 nm, depending on the chromatography technique [21-25]. Since the method proposed by the authors [25] was chosen as the basis, the wavelength for the detection of thiotriazoline was chosen to be 220 nm, as recommended in [21].

3.3. Graduation graph and statistical evaluation of linearity parameters

For the quantitative determination of the active substance thiotriazoline in solid and liquid dosage forms, it was necessary to construct a graduation graph. For this purpose, the dependence of the peak area on the concentration of standard solutions of standard thiotriazoline solution was determined (section 2.1.6). The results of the experiment are presented in Table 1.

Chromatograms of samples of standard solutions for clarity are presented in Appendix 2.

Metrological characteristics of the technique are established by statistical processing of experimental data.

The main steps of statistical processing of chemical analysis results:

1) checking the results for gross errors (misses);

2) finding the average value from reliable data of parallel determinations;

3) finding the standard deviation;

4) finding the tabular value of the Student's coefficient with a given confidence probability and a known number of degrees of freedom;

5) finding the half-width of the confidence interval for the average value and, accordingly, the interval values for it;

6) estimation of systematic error.

Table 1. Dependence of the peak area on the concentration of standard thiotriazoline solution.

Trial	Thiotriazoline's content, ml/ml	Peak area	Average peak area
1	80	5746263	5812807
2		5825358	
3		5845324	
4		5852287	
5		5855321	
6		5752288	
1	100	7329812	7395821
2		7426788	
3		7389654	
4		7377769	
5		7376634	
6		7474267	
1	120	8605456	8698954
2		8703871	
3		8889075	
4		8569698	
5		8745698	
6		8679925	

The obtained experimental data were evaluated for the presence of gross errors - deviation of the measurement results from the true value of the measured value.

1. The homogeneity of the experimental data (n=6) was checked using the Q-criterion:

a) for a concentration of 80 μg/ml

$$Q1 = \frac{5752288 - 5746263}{5855321 - 5746263} = 0.055$$

$$Q2 = = 0.028 \frac{5855321 - 5852287}{5855321 - 5746263}$$

Tabular value Q(0.95;6) = 0.56

Because Q1<0.56 and Q2< 0.56, we can conclude that there are no gross errors and the experimental data is homogeneous.

b) for a concentration of 100 μg/ml

$$Q1 = \frac{7376634 - 7329812}{7474267 - 7329812} = 0.32$$

$$Q2 = \frac{7474267 - 7426788}{7474267 - 7329812} = 0.33$$

Tabular value Q(0.95;6) = 0.56

Because Q1<0.56 and Q2< 0.56, we can conclude that there are no gross errors and the experimental data is homogeneous.

c) for a concentration of 120 μg/ml

$$Q1 = \frac{8605456 - 8569698}{8889075 - 8745698} = 0.11$$

$$Q2 = \frac{8889075 - 8745698}{8889075 - 8745698} = 0.45$$

Because Q1<0.56 and Q2< 0.56, we can conclude that there are no gross errors and the experimental data is homogeneous.

Therefore, we can use all experimental data to calculate the average value and build a calibration graph.

a) for a concentration of 80 μg/ml - $\bar{y}$=5812807

b) for a concentration of 100 μg/ml - $\bar{y}$=7395821

c) for a concentration of 120 μg/ml - $\bar{y}$=8698954

Where $\bar{y}$ - is average peak area.

The dependence of the peak area on the concentration of standard solutions is presented graphically in Fig. 3.

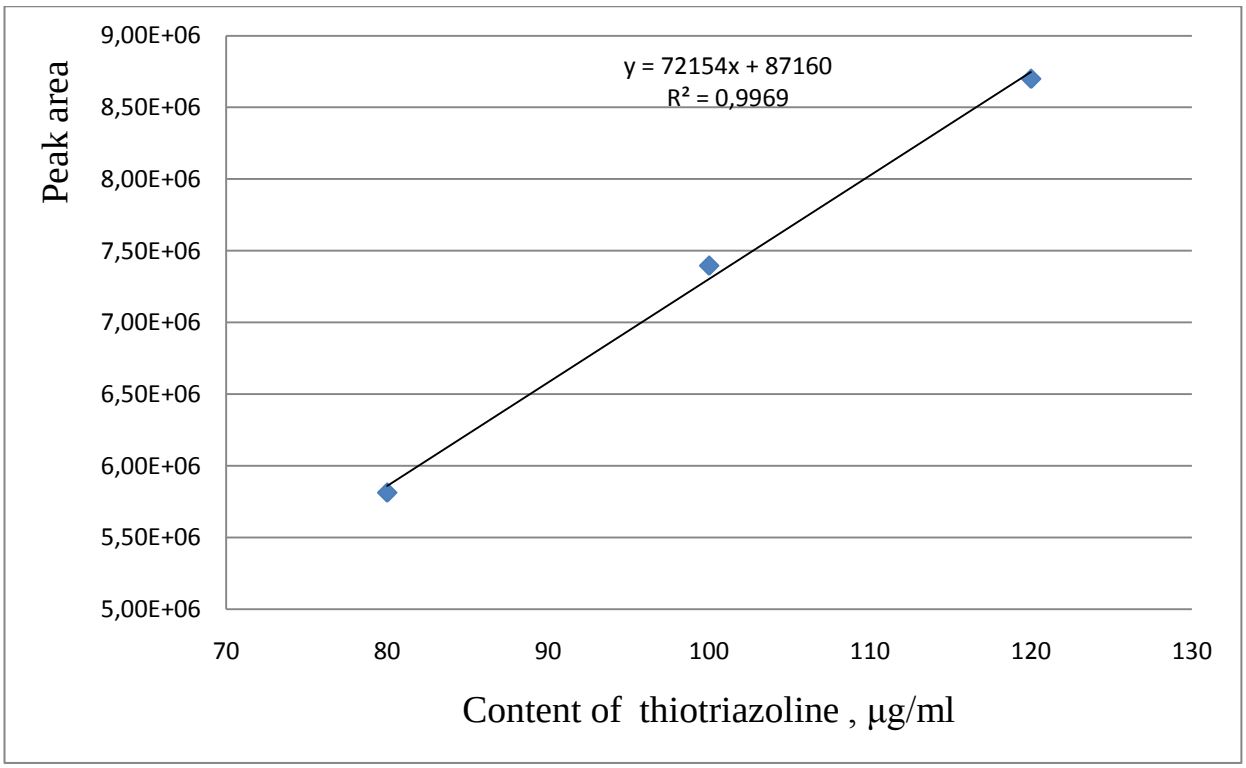


Fig. 3. Dependence of the area of the chromatographic peak of thiotriazoline on the concentration of thiotriazoline.

3.4. Determination of thiotriazoline's content in the samples

Determination of the content of the active substance thiotriazoline in the studied samples (medicine forms) was carried out as indicated in section 2.1.5. The solution of the studied Sample 1 was prepared after the sample preparation procedure, the solution of Sample 2 by direct dissolution in the eluent (section 2.1.7). Reagent solutions for analysis were prepared on the day of determination.

The quantitative content of thiotriazoline in solid and liquid dosage forms was determined according to the graduation schedule taking into account the dilution factor. The results of quantitative determination of thiotriazoline content in Samples 1 and 2 are given in Table 2.

Table 2 . Quantitative determination of thiotriazoline in samples 1 and 2.

Sample/ regulated content of thiotriazoline	Trial	Analysis results	
		Peak area	Content of thiotriazoline, mg
1, 200 mg/tablet	1	6703277	200.3
	2	6614801	201.5
	3	6724786	197.9
2, 25mg/ml	1	6388796	25.2
	2	6362801	25.8
	3	6298561	23.3

Chromatograms of sample solutions are shown in Fig.4.

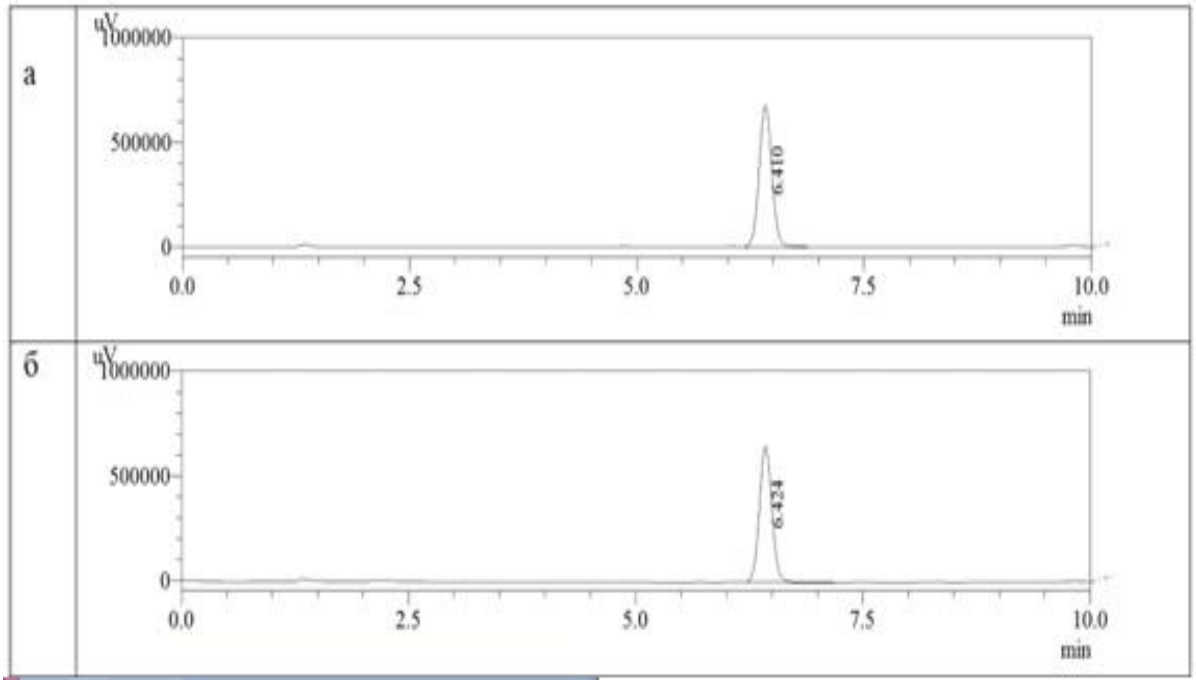


Fig. 4. Chromatograms of the solutions: 1 - Sample 1 , 2 - Sample 2,

3.5. Partial validation of the optimized method

Validation is the process of expert evaluation of methods, equipment, and products in accordance with the principles of good manufacturing practice [23-24]. It is a process that provides confirmation that the analytical technique used for a specific determination is appropriate for the intended use.

3.5.1. Verification of the specificity of the technique

Analytical methods should be specific [23-24], that is, be able to determine analytes (analyzed substances) in the presence of others.

We developed an alternative method for the quantitative determination of thiotriazoline in liquid and solid medicinal forms by the method of high-performance liquid chromatography directly on medicinal products sold by pharmaceutical institutions. Therefore, the results we obtained were compared with the content of thiotriazoline specified in the instructions for medical use.The results are presented in Table 2.

As you can see, the data in Table 2 correlate with those regulated according to the instructions. Thus, the proposed method can be considered specific.

3.5.2. The linearity of the technique

The linearity of the method in the range of concentrations from 80 to 120 μg/ml was studied. Measurements of the analytical signal of standard solutions prepared were performed with the number of injections n = 6.

The results of metrological evaluation of linear regression are as follows:

1. The linear function is described by the equation $y = 72154x + 87160$

2. Correlation coefficient $R^2 = 0.9969$,

which meets the requirements of the State Federal Statistical Office [17].

Thus, the proposed method satisfies all the calculated criteria [28, 29]. The method is linear in the investigated concentration range of 80-120 μg/ml.

3.5.3. Intra-laboratory precision

Intra-laboratory precision characterizes the influence on the results of the experiment of intra-laboratory variations: different days, different analysts, different equipment, etc. Reproducibility characterizes precision in an interlaboratory experiment. It is usually used to standardize the methodology [28, 29].

The intra-laboratory precision was evaluated based on the results of the studies conducted on different days.

Convergence assessment based on [28, 29] was carried out:

$|x_1 - x_n| < L\,(P, n) \cdot s,$

Where:

x_1 – the smallest value among the experimental data,

x_n – the largest value among the experimental data,

s – the standard deviation,

L (P, n) - the factor calculated by Pearson at the confidence probability P = 0.95 and the corresponding amount of experimental data n

The values of the L factor (95%, n) fare given below.

p	2	3	4
$L(P,n)$	2.77	3.31	3.65

The results of metrological evaluation of the convergence of experimental data are presented in Table 3 and Table 4.

Table 3. Results of the intra-laboratory accuracy assessment of the optimized method for determining the thiotriazoline content in Sample 1.

	Day 1	Day 2	Difference in results, %
	Active substance content, μg		
1	200.61	197.86	Not more than 2.0
2	198.76	198.12	Not more than 2.0
3	199.23	200.52	Not more than 2.0
Average value	199.53	198.83	
Results of intra-laboratory precision testing			
Pooled average	199.18		
Relative standard deviation (%)	0.59		
Student's coefficient (95%, 5)	2.5706		
Relative confidence interval (%)	1.51		
Critical value for convergence of results	1.51% < 2.00%		

Table 4. Results of the intra-laboratory accuracy assessment of the optimized method for determining the thiotriazoline content in Sample 2.

	Day 1	Day 2	Difference in results, %
	Active substance content, μg		
1	25,34	25.05	Not more than 2.0
2	25,14	25,16	Not more than 2.0
3	25,11	24.85	Not more than 2.0
Average value	25,20	25.02	
Results of intra-laboratory precision testing			
Pooled average	25,11		
Relative standard deviation (%)	0.64		
Student's coefficient (95%, 5)	2.5706		
Relative confidence interval (%)	1.63		
Critical value for convergence of results	1.63% < 2.00%		

The results of the intra-laboratory accuracy checking, presented in Table 3 and Table 4, allow to consider the results of determining the content of thiotriazoline in the samples by the proposed method as convergent.

3.5.4. The correctness of the technique

A conclusion on the correctness of the method can be formulated after establishing conclusions about the specificity, linearity and intra-laboratory precision of the proposed method [28,29].

Taking into account the above results, the optimized methodology for quantitative determination of thiotriazoline in samples 1 and 2 by HPLC can be considered correct.

3.6. Comparison of thiotriazoline's quantitative determination methods

According to previous scientific investigations, thiotriazoline in the substance and various mixtures (medicines) is quantitatively determined by non-aqueous titration and gravimetry (chapter 1.5.). From our point of view, the gravimetry is a time-consuming and energy-consuming method. Non-aqueous acid-base titration has a number of disadvantages, namely:

- High value of titration error (from 5 to 10 percent in determination);
- Standardization of titrant, titration in the presence of an indicator;
- Non-ecological.
- In some cases, violation of the stoichiometry of the reaction.

The HPLC method is modern, ecological, its highly authentic method and the advantage can be appreciated of the full automation of the process, ease of operation and processing of results, auto-validation system in accordance with the international requirements of GLP/GMP or ISO.

Table 5 presents the parameters of known methods for determining thiotriazoline in various medicinal products by HPLC and the optimized method proposed by us. As you can see, the methods differ in the type of column, the mobile phase, the temperature of chromatography, which is due to the nature of the components of the investigated objects.

Table 5. Comparison of methods of chromatographic determination of thiotriazoline

Parameters	Known methods					Optimized technique
	[21]	[22]	[23]	[24]	[25]	
Object of study	thiotriazoline + L-tryptophan	thiotriazoline +carbamazepine	thiotriazoline + L-arginine	thiotriazoline + L-arginine	thiotriazoline + piracetam	thiotriazoline
mobile phase velocity	1 ml / min	1 ml / min	1 ml / min	1 ml / min	1 ml / min	1 ml / min
analytical wavelength of the detector	220 nm	230 nm	220 nm	220 nm	210 nm	220 nm
eluent	20% methanol 80% phosphate buffer solution with pH = 3.0.	1.50% methanol, 50% 0.01M TBAHS 2.40% methanol, 60% 0.01M TBAHS 3.50% methanol 50% phosphate buffer solution with pH = 3.0.	aqueous solution of 3.4 g / l Bu_4NHSO_4 and 0.05% trifluoroacetic acid	an aqueous solution of 3.4 g/l Bu_4NHSO_4 and 0.05% trifluoroacetic acid.	0.05 M solution of potassium dihydrogen phosphate, degassed	0.05 M solution of potassium dihydrogen phosphate, degassed
Sample volume	10 µl	20 µl	20 µl	20 µl	20 µl	20 µl
Temperature	25^0C	25^0C	Not specified	Not specified	30^0C	30^0C
Column	Prontosil Eurobond C18, 250×4,6 мм particle diameter 5 µm	1.Prontosil Eurobond C18, 250×4,6 мм 2.Hypersil ODS C18, 250×4,6 мм particle diameter 5 µm	Hypersil ODS-C18-5u, 4.6 x 250 mm, particle diameter 5 µm	Hypersil ODS-C18-5u, 4.6 x 250 mm, particle diameter 5 µm	Waters, silica gel grafted 3(chlorodimethylallyl)propyl-N dodecylcarbamate, 3.9×150 mm,particle diameter 5 µm	Nucleosil C_{18} 100-5, 250×4,6 мм, particle diameter 5 µm Nucleosil C_{18} 100-5, 4×3 мм.

CONCLUSIONS

1 The sources of scientific information were analyzed and the method of high-performance liquid chromatography was chosen for the determination of thiotriazoline in medicines based on the bibliosemantic analysis.

2 The optimal conditions for the determination of thiotriazoline in solutions of drug samples were determined. using a Shimadzu liquid chromatograph with UV detector and a chromatographic column (250x4.6) mm, filled with Nucleosil C18 (100-5). The optimized method of quantitative determination of thiotriazoline in liquid and solid dosage forms was tested .

3 Validation of the developed method in terms of specificity, linearity, correctness and precision is in accordance with the acceptance criteria of the State Pharmacopoeia of Ukraine

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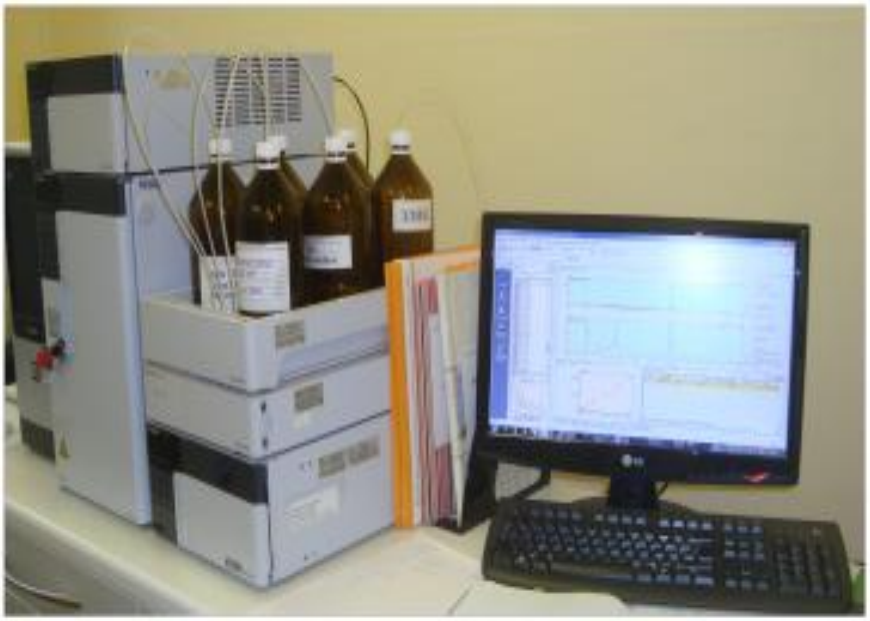
Appendices

Appendix 1

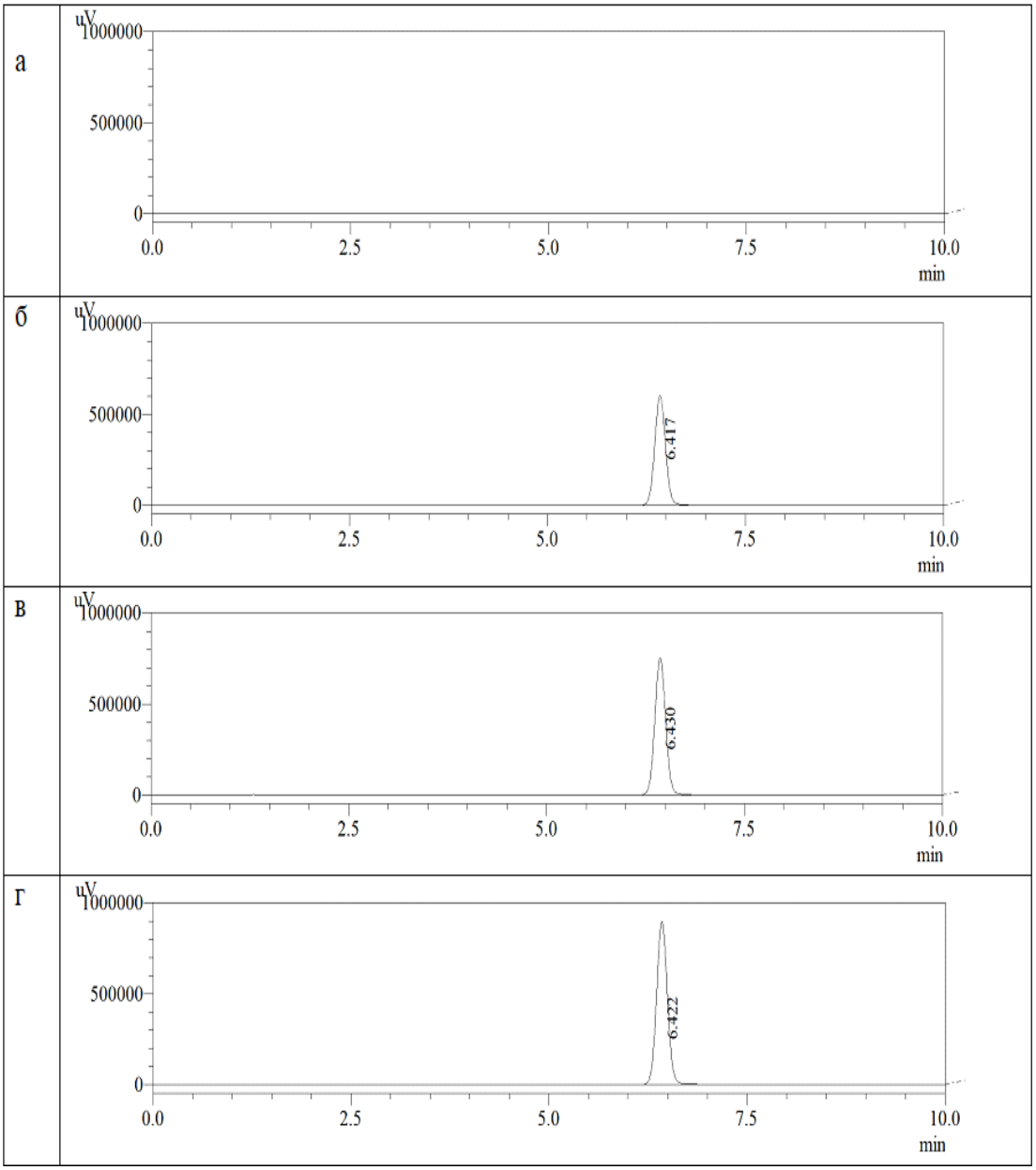
"Shimadzu" liquid chromatograph with UV detector, factory No. C20964330924CS, inventory No. 010466981, calibration certificate No. 3991 dated 06/07/2019



Appendix 2.

Chromatograms of standard solutions

(thiotriazoline concentration 80 (b), 100 (c), 120 (g) μg/nm).



Appendix 3.

CERTIFICATE

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Summary

The dependence of the pressure in the Nucleosil C18 100-5, 250 x4.6 mm column on temperature, the influence of the rate of the liquid phase on the process of chromatography and UV detection of thiotriazoline was investigated. As a result, it was established that the optimal conditions for the chromatographic determination of thiotriazoline using a Nucleosil C18 100-5, 250 x 4.6 mm column are an eluation rate of 1 ml/min and 30^0C. A 0.05 M solution of potassium dihydrogen phosphate, degassed was chosen as the mobile phase. UV detection of thiotriazoline at a wavelength of 220 nm is used.

The results of the research made it possible to develop a optimizided method of quantitative determination of the content of thiotriazoline in drug samples by the HPLC method. Before chromatography of the tablet form, sample preparation was carried out according to the standard method. In optimal conditions of chromatography, excipients of the solid dosage form do not affect the results of the analysis.

In order to determine the linearity of the proposed method, the dependence of the area of the chromatographic peak on the concentration of standard solutions (80, 100 and 120 μg/ml) was investigated and the linear regression equation was calculated. the linear function is meets the requirements of the SPhU.

The content of thiotriazoline according to the results of the chromographic analysis by the optimized method is: in the sample of the liquid dosage form 25.4 ± 0.3 mg/ml; in a sample of a solid dosage form – 199.2 ± 2.1 mg in a tablet.

Verification of correctness indicates sufficient accuracy and reproducibility of the optimized method.