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STANDARDIZATION OF ORIGINAL MEDICINE ANTI-ALCOHOL ACTION ON ASSAY OF GLYCIN

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Мета. *Розробка та валідація доступної методики кількісного визначення гліцину в новому оригінальному лікарському засобі, що застосовується при алкогольній залежності.*
Методи. *Для кількісного визначення гліцину в препараті в формі порошку шипучого для приготування орального розчину була розроблена і валідована спектрофотометрична методика з використанням спектрофотометра Specord 200 фірми «Analytik Jena».*
Результати. *В результаті проведеного дослідження було розроблено модифікований чутливий спосіб кількісного визначення гліцину спектрофотометричним методом. Обрані оптимальні умови проведення реакції гліцин – нінгідрин з метою отримання стабільних результатів аналізу: аналітична довжина хвилі – 568 нм; нагрівання реакційної суміші проводять в киплячій водяній бані протягом 30 хв; об'єм буферного розчину – 4 мл, обраний pH буферного розчину 6.8 і введений відновник – аскорбінова кислота. Встановлено, що в методиці відсутня систематична похибка, відносна невизначеність для ймовірності 95 % не перевищує максимально допустиму невизначеність результатів аналізу (1,77 % ≤ 2,4 %). Для методики кількісного визначення гліцину були вивчені такі валідаційні параметри як специфічність, лінійність, правильність, прецизійність і робасність. Встановлено, що всі розраховані валідаційні параметри відповідають необхідним критеріям прийнятності.*
Висновки. *Розроблена і валідована доступна чутлива спектрофотометрична методика, заснована на здатності продуктів взаємодії гліцину з нінгідрином поглинати у видимій області спектра. Всі валідаційні параметри відповідають критеріям прийнятності*
Ключові слова: *стандартизація, спектрофотометрія, валідація, гліцин, препарат антиалкогольної дії*

1. Introduction

According to WHO data, about 2.3 billion people around the world consume alcohol, and more than 76 million suffer from alcohol dependence (AD). Currently, mortality from alcoholism and directly related diseases is in third place, just after mortality from cardi-

ovascular diseases and malignant neoplasms. Because of alcohol consumption in the world, about 3 million people die annually (among men about 2.3 million deaths and 0.7 million deaths among women), which makes up 5.3 % of all deaths [1, 2].

Official statistics show that in Ukraine the number of people suffering from AD is close to 800 thousand people. This makes up about 1.5 % of the population, with 50–55 thousand new patients being registered annually [3].

From a clinical point of view, AD (alcohol dependence syndrome, F10.2) is a chronic relapsing mental illness. The spectrum of alcohol damage covers a variety of conditions from mild intoxication to persistent toxic damage to many organs and systems of the body. Among the somatic consequences of AD, disorders in the gastrointestinal tract, nervous and cardiovascular systems prevail [4]. The treatment of alcohol intoxication and the state of alcohol withdrawal (hangover) is not specific, it is aimed at maintaining and normalizing vital functions, detoxification. In the pathogenesis of hangover, an important role is given to alcohol metabolism products, especially acetaldehyde. Being present in the body for a long time, acetaldehyde exerts a cumulative effect upon repeated use of alcohol; therefore, not even constant, but regular use of alcohol can lead to the development of alcoholism [5–7].

In recent years, researchers have become increasingly interested in creating agents based on endogenous regulators (ERs) that have the ability to carry out metabolic correction of the metabolic reaction, including in the conditions of development of pathological processes, to increase the stability and adaptive properties of the body due to the activation of internal physiological and biochemical processes. In addition, ER possessing high efficiency and stability of the therapeutic exerted effect are characterized by low toxicity and almost complete absence of side effects. Metabolic therapy activates the internal processes of the body and eliminates pathology in a natural way, by the forces laid down by nature in the body itself. This is, in fact, regulatory therapy [8–10].

In anti-relapse therapy of AD, glycine drugs are widely used as an endogenous regulator (ER), which is involved in metabolic processes and contributes to their normalization in pathological conditions. Glycine preparations are safe, bind acetaldehyde, have a moderate sedative effect and can be used at all stages of alcoholism treatment in isolation or in combination [11, 12].

According to the literature [13–15], 2-aminoacetic acid, called glycine, belongs to the ER. It has the ability to influence the respiratory chain of mitochondria, supports cell bioenergetics and belongs to antihypoxants. An equally important aspect of the metabolic action of glycine is its ability to direct non-specific conjugation of xenobiotics, because of which substances toxic to the cell interact with it and form less dangerous metabolites. The drug, as a detoxifier, binds aldehydes and ketones. In addition to the general metabolic effect, glycine enhances inhibitory neurotransmitter processes arising from endogenous synthesis. Glycine, as an inhibitory neurotransmitter, reduces psychoemotional stress, relieves stress, aggressiveness and conflict, increases social adaptation, improves sleep [6–8, 10].

Therefore, the urgent issue is the creation of new original drugs for the treatment of AD, which include glycine as an active pharmaceutical ingredient (APhI).

The aim of our study was the development and validation of an accessible method for the quantitative determination of glycine in a new original drug used in AD.

2. Planning (methodology) of the research

Amino acids are a complex object of research, since they are volatile, have acidic and basic groups in their structure, and are polar. From the whole variety of methods for the quantitative determination of amino acids in various objects, four main groups can be distinguished: chromatographic, spectrophotometric, titrimetric, and electrochemical methods of analysis [16].

In developing these methods, the main efforts are aimed at increasing the concentration sensitivity of the determination. Since amino acid solutions, as a rule, weakly absorb UV and visible light, do not have strong intrinsic fluorescence, and their molecules do not have electroactive groups, the sensitivity of their determination, respectively, by spectrophotometric and electrochemical methods, is low. To increase the sensitivity of the determination of amino acids, their chemical conversion (derivatization) is carried out. When carrying out a chromatographic analysis of these substances, methods of pre- or post-column derivatization are used [17].

Amino acids are substances whose titration in water is difficult due to weak acid-base properties and / or low solubility. Amino acids are dissolved in glacial acetic acid, and the resulting solution is titrated with a 0.1 M perchloric acid solution. Titration can be carried out with an indicator (crystal violet), or potentiometrically using a glass electrode as an indicator. The analysis technique is characterized by high accuracy of determination, but low sensitivity. This method also has a number of significant drawbacks: the use of aggressive, highly toxic reagents, including for the preparation of titrant (using acetic anhydride); a significant duration of titrant preparation (0.1 M perchloric acid solution – more than 48 hours) [18].

For the analysis of amino acids, methods based on the reaction with ninhydrin are widely used [18, 19]. Ruman first discovered this reaction. It was later established that ninhydrin is specific to aliphatic or alicyclic primary amino groups. Secondary, tertiary and quaternary amines, amides and amino-substituted aromatic compounds give a weak reaction or not at all. An exception is proline (pyrrolidine-α-carboxylic acid), which forms a yellow color with ninhydrin, as some researchers believe, due to the opening of the cycle. Aminoketones initially with ninhydrin form a yellow color, which turns into purple. The reaction of amino acids with ninhydrin is widely used in chromatographic and spectrophotometric methods of analysis.

After analyzing the literature data, we found that it is very important to develop simple and affordable analytical methods for the pharmaceutical industry and analytical laboratories for drug quality control in order to save time and financial resources during routine laboratory tests.

3. Materials and methods

The object of the study in this work was a combined original drug (according to the ATC code:

N07B B – drugs used in AD) in the form of effervescent powder for the preparation of an oral solution, developed on the basis of the State Research Laboratory for Quality Control of Medicines of the National University of Pharmacy.

The preparation is an effervescent powder for oral solution, packet No. 1 and packet No. 2. Based on the studies [20], the following components were proposed that could be combined in the first unit of the dosage form (packet No. 1): glutamic acid, acetylsalicylic acid, ascorbic acid, sorbitol and anhydrous citric acid. Other components that can be combined in the second unit of the dosage form (package No. 2): glycine, sorbitol and sodium bicarbonate.

In package No. 2, the glycine content is 300 mg ± 7.5 %.

To quantify glycine in package No. 2 of this preparation, an accessible spectrophotometric method was developed and validated based on the ability of the products of the interaction of amino acids with ninhydrin to absorb in the visible region of the spectrum using a Specord 200 spectrophotometer from "Analytik Jena".

Glycine (*Sigma Aldrich, certified reference material*, cat. No. PHR1799, s. LRAA8813) was used as a certified reference standard (CRS).

Preparation of the initial solution 1. About 4.00 g of the powder of the preparation from package No. 2 was placed in a volumetric flask with a capacity of 250.0 ml, 150 ml of *water R* were added, dissolved in *water R*, the solvent was brought to the mark and stirred. 2.0 ml of the resulting solution was placed in a volumetric flask with a capacity of 20.0 ml, adjusted to the mark with *water R* and stirred.

Preparation of test solution. 2.0 ml of *the initial solution 1* was placed in a 100 ml conical flask, 4.0 ml of *a buffer solution of pH 6.8 R* , 2.0 ml of a *1 % solution of ninhydrin in ethanol (96 %) R* were added and 2.0 ml 0.05 % aqueous ascorbic acid solution.

Preparation of the initial solution 2. About 0.030 g of *CRS of glycine* was placed in a volumetric flask with a capacity of 100.0 ml, dissolved in 70 ml of *water R*, thereby brought the solvent to the mark and stirred. 10.0 ml of the resulting solution was placed in a volumetric flask with a capacity of 25.0 ml, adjusted to the mark with *water R* and stirred.

Preparation of the reference solution. 2.0 ml of *the initial solution 2* was placed in a 100 ml conical flask, 4.0 ml of *a buffer solution of pH 6.8 R*, 2.0 ml of a *1 % solution of ninhydrin in ethanol (96 %) R* i 2.0 ml 0.05 % aqueous solution of ascorbic acid were added.

The flasks with the contents are heated simultaneously in a boiling water bath for 30 minutes, then quickly cooled under a stream of cold water and the volume of the solution is adjusted to the mark with *water R*.

The optical density of the test solution was measured at a maximum at a wavelength of 568 nm using the following compensation solution: 2.0 ml of *water R* was placed in a volumetric flask with a capacity of 100.0 ml, 4.0 ml of *a buffer solution pH 6.8 R*, 2.0 ml of *a 1 % solution of ninhydrin in ethanol (96 %) R* i 2.0 ml of a 0.05 % aqueous solution of ascorbic acid were added.

In parallel, the optical density of the reference solution was measured.

The glycine content in one package, in grams, was calculated by the formula:

$$X = \frac{A \cdot m_0 \cdot 10 \cdot 2 \cdot 250 \cdot 20 \cdot 100 \cdot P \cdot b}{A_0 \cdot m \cdot 100 \cdot 25 \cdot 100 \cdot 2 \cdot 2 \cdot 100} = \frac{A \cdot m_0 \cdot P \cdot b}{A_0 \cdot m \cdot 10},$$

where A – optical density of the *test solution*;

A_0 – optical density of the *reference solution;*

m_0 – the weight of a sample of *glycine CRS* taken for the preparation of a *the reference solution*, in grams;

m – the weight of the sample powder taken for the preparation of the *test solution*, in grams;

P – the content of the main substance in *glycine CRS* indicated in the quality certificate, in percent;

b – average weight of package contents, in grams.

Validation of the methodology was carried out in accordance with the requirements of the State Pharmacopoeia of Ukraine (SPhU) 5.3.N.2 [21], recommendations of ICH Q 2 (R1) [22] and according to the standardized procedure for validating quantitative determination methods using the standard method [23].

Validation parameters such as specificity, linearity, accuracy, precision and robustness were studied for the glycine quantification procedure.

4. Results of the research

In the qualitative analysis of amino acids, the ninhydrin reaction is widely used [24, 25]. Therefore, we studied the possibility of using it for the quantitative analysis of glycine using the absorption spectrophotometry method in the ultraviolet and visible regions.

Given the low stability of the reaction products of amino acids with ninhydrin, a modified method for the quantitative determination of glycine was developed.

The conditions for the glycine – ninhydrin reaction were optimized in order to obtain stable analysis results: analytical wavelength – 568 nm; heating the reaction mixture was carried out in a boiling water bath for 30 minutes; the volume of the buffer solution was 4 ml, the pH of the buffer solution was 6.8, and a reducing agent, ascorbic acid, was introduced.

Studying the influence of the pH of the medium on the intensity of absorption of the reaction products obtained in the pH range from 6.4 to 7.2, showed that the maximum optical density was achieved at a pH of 6.8. The spectra were characterized by a pronounced maximum at a wavelength of 568 ± 2 nm, the position of which does not depend on the pH of the buffer solution (Fig. 1).

Based on the obtained experimental data, 568 nm was chosen as the analytical wavelength.

Given the low stability of the reaction products of amino acids with ninhydrin, it is necessary to add reducing substances. Small amounts of tin chloride, titanium chloride, hydrazine sulfate, and ascorbic acid increase the sensitivity of the reaction of amines with ninhydrin [24, 25].

When developing the methodology, we chose ascorbic acid, which is the most affordable and non-toxic reagent.

The research results showed that the addition of ascorbic acid to the reaction mixture increases the absorption intensity of the reaction product, that is, increases the sensitivity of the reaction.

The obtained values of the optical density of the studied solutions are presented in Tab. 1.

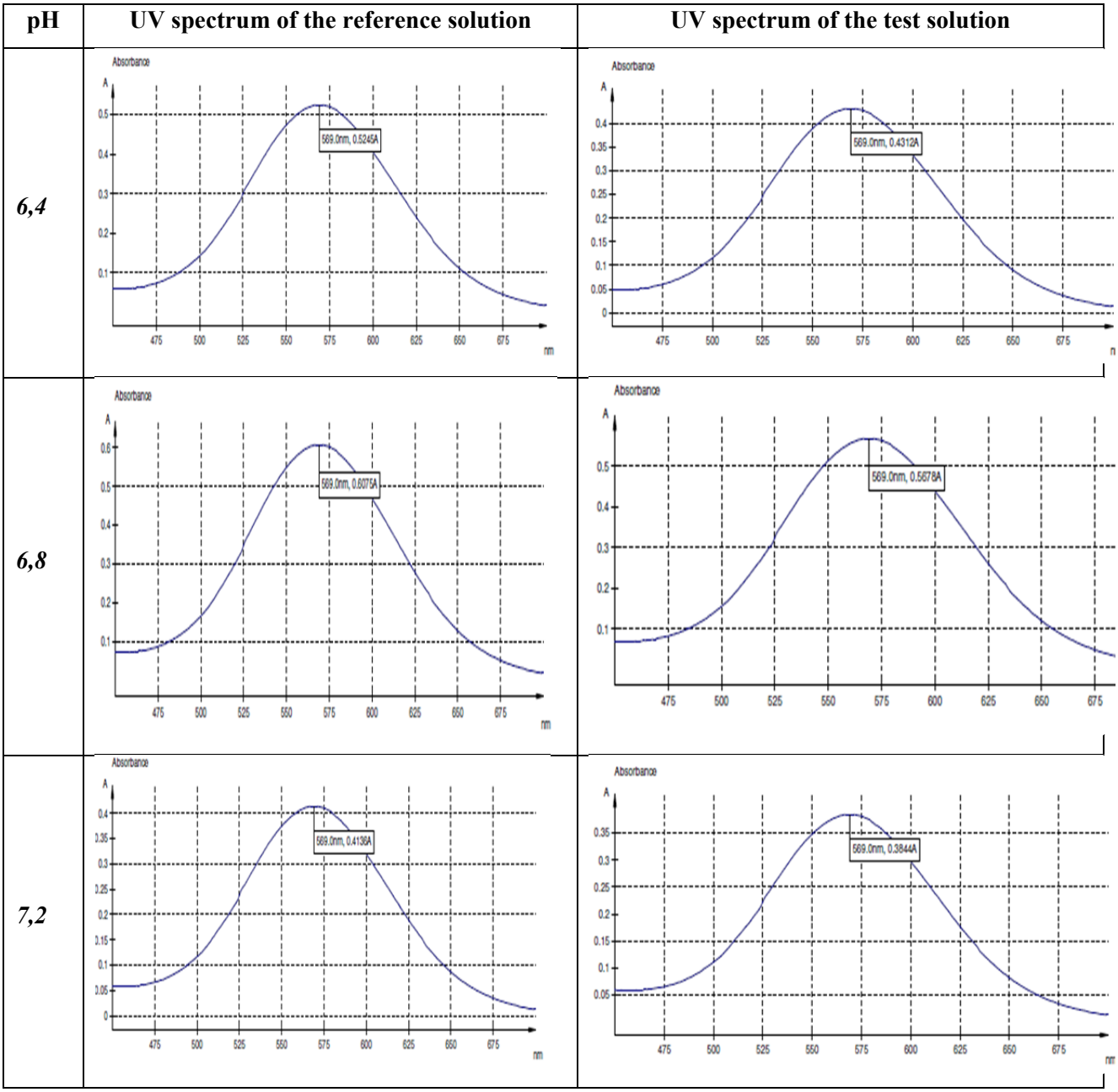

Fig. 1. Spectra of the reference solution and the test solution at different pH

Table 1

The dependence of the optical density of the studied solutions on the addition of 0.05 % aqueous solution of ascorbic acid

Studied solutions	With the addition of a 0.05 % aqueous solution of ascorbic acid			Without the addition of a 0.05 % aqueous solution of ascorbic acid
	pH 6.4	pH 6.8	pH 7.2	
Test solution	0.4314	0.5679	0.3844	0.1644
Reference solution	0.5246	0.6079	0.4138	0.1945

Maximum allowable full uncertainty (*max* Δ_{AS}) of analysis methods associated with the boundaries of the substance in the preparation. In the package, the glycine content is normalized in the range from 0.278 to 0.323 mg, thus the content tolerances are ± 7.5 %.

The maximum permissible total uncertainty of the analysis procedure:

$max\ \Delta_{AS}$, %≤0.32·7.5 %=2.4 %.

Metrological characteristics of the method for determining the quantitative content of glycine in the preparation are given in Table 2.

As can be seen from the above data, there is no systematic error in the methodology, the relative uncertainty for the probability of 95 % does not exceed the maximum allowable uncertainty of the analysis results, that is 1.77 % ≤ 2.4 %.

Table 2

Metrological characteristics of the quantitative content of glycine

m	*v*	X_i, g	X_{cp}, g	S^2	S_{cp}	*P*	*t(P,v)*	*Δx*	*ε*, %
5	4	0.287 0.296 0.295 0.298 0.294	0.294	0.0000175	0.0019	0.95	2.78	0.294 ± 0.005	1.77

The criterion of insignificance of sample preparation compared with the maximum allowable uncertainty of the analysis results ($\Delta_{AS,\ insig}$):

$\Delta_{AS,\ insig}$ %≤$max\ \Delta_{AS}$, %·0.32=2.4 %·0.32=0.77 %.

The results of calculating the total uncertainty for the developed methodology are given in Table 3.

Thus, the calculated total uncertainty of the analysis procedure Δ_{AS} % less than $max\ \Delta_{AS}$ (1.21 %<$max\ \Delta_{AS}$=2.4 %), which meets the requirements for this parameter. To study specificity, a placebo solution (Fig. 2), a reference solution (solutions of glycine CRS) (Fig. 3), and a test solution (Fig. 4) were prepared.

Specificity (placebo effect). To determine the specificity of the method, the average optical density of the placebo solution was found (A_{blank}), due to absorption of excipients: A_{blank}=0,0012; A_s=0.5676.

The placebo contribution to the total absorption of the drug should not exceed the value:

$$\frac{A_{blank}}{A_{st}} \cdot 100 \le 0.77\ \%.$$

Inequalities are fulfilled, i.e. background absorption is insignificant, and the technique is characterized by allowable specificity: 0,21 %≤0,77 %.

Table 3

Uncertainty results of methods of analysis

Parameter	Result, %
Total sample preparation uncertainty, Δ_{SP} %	0.986
*Uncertainty of the final analytical operation, Δ_{FAO} (spectrophotometry)**	0.70
The complete uncertainty of the analysis procedure Δ_{AS}% =$\sqrt{(\Delta_{SP}\%)^2+(\Delta_{FAO}\%)^2}$	1.21

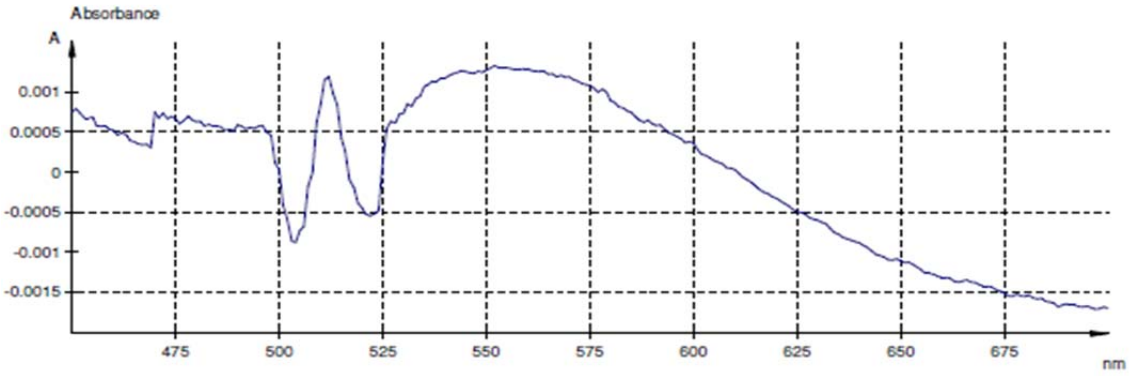

Fig. 2. UV spectrum of a placebo solution

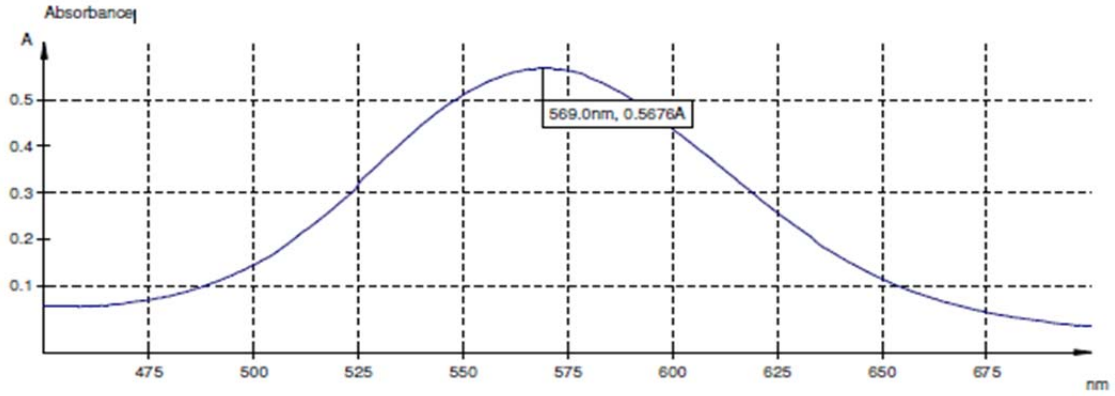

Fig. 3. UV spectrum of the reference solution

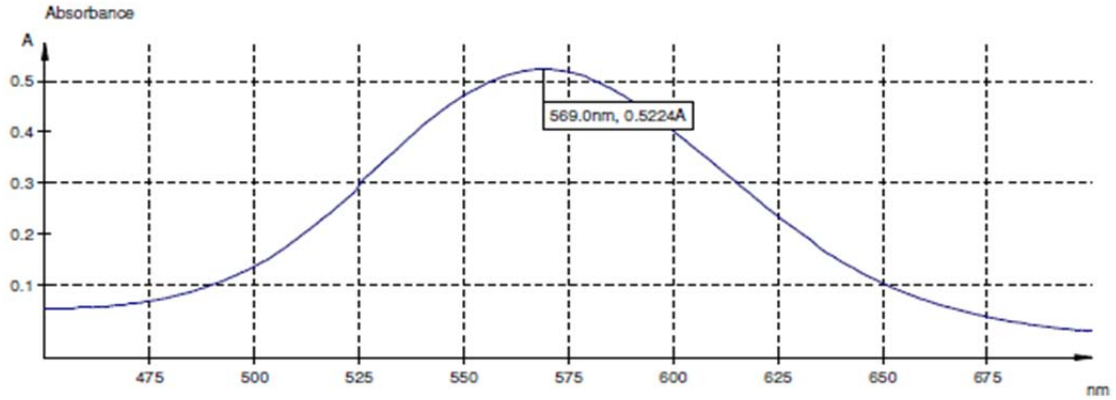

Fig. 4. UV spectrum of the test solution

The quantification procedure should be linear within the range of application, which covers the possible values of the concentration of the APhI. SPhU establish the range of application of quantification methods of 80–120 %. To confirm the linearity of the methodology, 9 model solutions of glycine were prepared, the concentration of which varies uniformly within the application range (step – 5 %).

In Fig. 5 is a graph of the linear dependence of the analytical signal on the actual concentration of the active substance.

For model solutions using the least squares method, the linear dependence parameters were calculated: the absolute term a, the residual standard deviation S_0, and the correlation coefficient r. Acceptance criteria are given in Table 4.

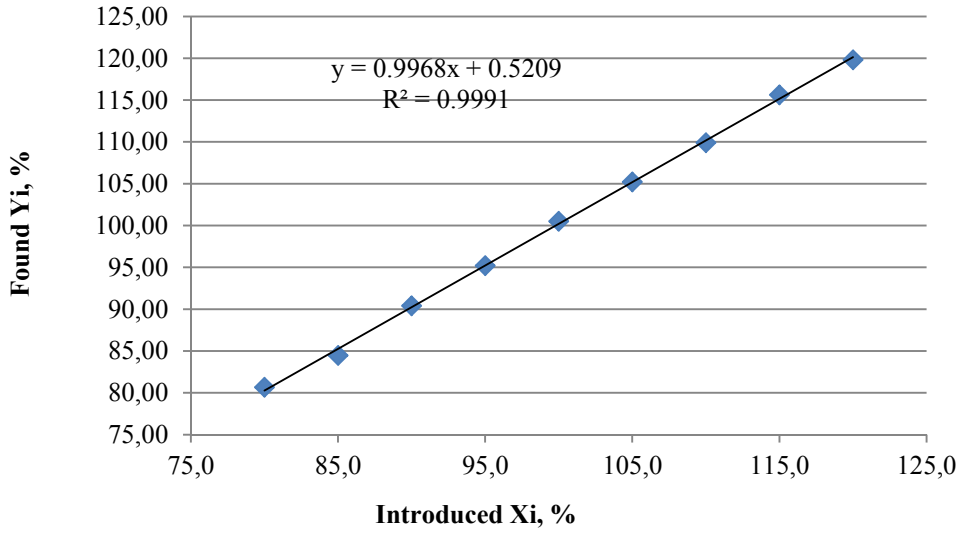

Fig. 5. Linear dependence graph for glycine

Table 4

Linearity check data of quantification methods

Parameter	Requirements	Obtained value	Criteria fulfillment
$\lvert a \rvert$	≤3.8	0.5209	Performed
S_0	≤1.27	0.5734	Performed
r	>0.9957	0.9991	Performed

The obtained results confirm that the method for quantitative determination of glycine in the concentration range from 80 to 120 % is linear.

The fulfillment of the criteria of accuracy and precision for determining glycine in the preparation are given in Table 5.

The method for determining glycine in a preparation satisfies the acceptability criteria of the validity parametrs "Accuracy" and "Precision".

The fulfillment of the criterion of intermediate precision for the determination of glycine in a preparation by the developed method is given in Table 6.

The method for the quantitative determination of glycine in a preparation meets the acceptability criteria of the "Intermediate precision" test.

In the study of robustness, the stability of the solutions was checked during analysis. A study of the stability of glycine solutions was carried out immediately after preparation, after 15, 30, 45 minutes and after 1 hour.

The results are presented in Table 7.

The differences between the obtained glycine values should not exceed the criterion of insignificance compared with the maximum allowable uncertainty of the analysis results ($\Delta_{AS,\ insig}$), that is 0.77 %. The criterion is fulfilled after 15 minutes, 30 minutes, 45 minutes and 1 hour. According to the above data, for quantitative determination it is advisable to use solutions within 1 hour after preparation.

Table 5

The results of the assessment of the accuracy and precision of the methodology

Parameter	Value	Criterion, %		Criteria fulfillment
		Statistical insignificance requirements	Practical insignificance requirements	
$\lvert \bar{Z} - 100 \rvert$	0.21	≤0.27	≤0,77	Performed on two criteria
ΔZ	0.82	≤2.4		Performed

Table 6

Intermediate precision assessment results

Parameter	Criteria requirements, %	Obtained value, %	Criteria fulfillment
Δ_{intra}	≤2.4	0.99	Performed

Table 7

The results of a study of the stability of glycine solutions over time

No.	0 min	After 15 min	Parameter change, %	After 30 min	Parameter change, %	After 45 min	Parameter change, %	After 1 h	Parameter change, %
1	0.4518	0.4511	0.15	0.4508	0.20	0.4502	0.35	0.4499	0.42
2	0.4520	0.4510	0.22	0.4509	0.24	0.4503	0.38	0.4502	0.40
3	0.4523	0.4513	0.22	0.4511	0.27	0.4504	0.42	0.4506	0.38
Δ_{av}			0.20		0.24		0.38		0.40

All validation parameters meet the necessary the acceptability criteria. The results prove that the technique can be correctly reproduced and suitable for further use.

5. Discussion of the results

The methods for quantifying amino acids are of constant interest because of the importance of the biological processes in which these compounds are involved.

Existing methods of analysis, despite the high accuracy of determination, have significant drawbacks: the duration of the preparation of working solutions, their toxicity (potentiometric titration in a non-aqueous medium), the use of expensive equipment, consumables and solvents with the qualification "for chromatography" in chromatographic methods of analysis [16, 17].

Based on this, an affordable sensitive spectrophotometric method has been developed for the quantitative determination of glycine in a new original drug used in AD in the form of an effervescent powder for the preparation of an oral solution.

This method is based on the study of the spectral characteristics of the ninhydrin reaction products and the optimization of its conditions.

When studying the ninhydrin reaction with glycine, it was found that the product spectrum is characterized by a pronounced absorption maximum at a wavelength of 568 ± 2 nm, the position of which does not depend on the pH of the buffer solution and glycine concentration. The reaction of the medium significantly affects the absorption intensity; the optimal pH value

is 6.8. The choice of the wavelength of 568 nm as the analytical one is associated with a higher stability of the reaction product. So, at a wavelength of 568 nm, the maximum optical density is achieved by heating the reaction mixture for 30 minutes. The optimal volume of the buffer solution is 4 ml. The introduction of ascorbic acid into the reaction mixture does not change the nature of the spectrum, however, it increases the absorption intensity of the reaction product, that is, and it increases the sensitivity of the reaction.

When studying such validation parameters as specificity, linearity, accuracy, precision and robustness, it was found that all calculated validation parameters meet the necessary acceptability criteria.

Study limitations. In the course of our study, difficulties arose in choosing a simple and affordable method of analysis. This limitation was associated with the effervescent dosage form of our preparation and the composition of excipients. Titrimetric assays in non-aqueous solvents are most commonly used for titration of amino acids. Nevertheless, this method was not suitable for the quantitative determination of glycine in a new original drug.

This is because sodium bicarbonate is used as auxiliary substance in package No. 2, and when the drug is dissolved in glacial acetic acid, a neutralization reaction occurs with the formation of a large amount of water, which limited the possibility of using the non-aqueous titration method.

Prospective for further research. The research results showed that a sensitive spectrophotometric method based on the reaction of glycine with ninhydrin was developed. Since glutamic acid is used as an APhI in the composition of the preparation, but in package No. 1, studies on the use of the reaction of amino acids with ninhydrin will continue for glutamic acid, and other analysis methods will also be used.

6. Conclusions

1. An accessible sensitive spectrophotometric technique was developed based on the ability of the products of the interaction of glycine with ninhydrin to absorb in the visible region of the spectrum.

2. The conditions for the glycine – ninhydrin reaction were optimized and the spectral characteristics of the ninhydrin reaction products were studied.

3. It was established that the technique does not have a systematic error; the relative uncertainty for a probability of 95 % does not exceed the maximum allowable uncertainty of the analysis results, that is 1.77 % ≤ 2.4 %.

4. The developed methodology was validated; all parameters met the acceptability criteria.

5. This technique can be used in analytical laboratories, eliminates the use of expensive equipment, highly toxic, volatile and explosive reagents.

Conflict of interest

There is no conflict of interest.

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