Tonkie Khimicheskie Tekhnologii = Fine Chemical Technologies.
2024;19(2):127–138 ISSN 2686-7575 (Online)

Chemistry and technology of medicinal compounds
and biologically active substances

Химия и технология лекарственных препаратов
и биологически активных соединений

*UDC 547.466:543.544*
*https://doi.org/10.32362/2410-6593-2024-19-2-127-138*



RESEARCH ARTICLE

# *One-pot* determination of amino acids in drugs by pre-column derivatization with phenyl isothiocyanate

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## Abstract

**Objectives.** To develop a new method to determine amino acids in drugs by means of reverse-phase high-performance chromatography (RP HPLC) with pre-column derivatization using phenyl isothiocyanate (PITC) and *one-pot* sample preparation.

**Methods.** The initial standard solutions of amino acids were prepared by weighing, followed by dissolution in water. Working solutions were then prepared: standard, test, and blank, by dilution in 20 mM hydrochloric acid. Further sample preparation was carried out in Safe-lock polypropylene tubes (*Eppendorf*) in a reaction buffer containing mobile phase A, acetonitrile, and triethylamine in a ratio of 85 : 10 : 5, labeled with a 5% PITC solution in acetonitrile. After thorough mixing for 3–5 min on a vortex, the tubes were kept in a solid-state thermostat with a thermally insulating lid for 2 h. The samples were then cooled for 10 min, centrifuged for 1 min at 13000 rpm, the supernatant was transferred into vials, and the mixture of amino acids was separated by RP HPLC using hydrophobic silica gel with grafted C18 groups as a stationary phase. The quantitative determination of amino acid derivatives was carried out using a diode array detector.

**Results.** A new method for the separation and determination of amino acids in medicinal preparations was developed and validated. Simple *one-pot* sample preparation using available reagents and equipment enabled studies to be carried out without using commercial kits, for example, the AccQ·Tag Ultra Derivatization Kit, USA. Using the analysis of mixtures of histidine and glycine as an example, it was shown that when using two mobile phases, an acceptable separation of amino acid derivatives in a gradient mode can be achieved for 20 min at a flow rate of 1.0 mL/min. The samples prepared according to the new method demonstrated a high level of stability in use and storage. A composition of mobile phases A and B consisting of 10 mM acetate buffer pH 3.5 and 80% acetonitrile solution was proposed. Validation of the method hereby developed in the analysis of the drug Innonafactor®, containing glycine and histidine as excipients, demonstrated high convergence of the results of the quantitative determination of these amino acids.

**Conclusions.** The new method to determine amino acids in medicinal preparations by RP HPLC with PITC pre-column derivatization has a wide range of applications, has a number of advantages when compared to imported commercial kits for the determination of amino acids. These include: lower cost of reagents and materials, high accuracy and repeatability. Thus, it can be recommended for use in quality control laboratories of pharmaceutical enterprises.

**Keywords**

amino acids, glycine, histidine, RP HPLC, pre-column derivatization, PITC (phenyl isothiocyanate), quality control of medicines

**Submitted:** 29.08.2023
**Revised:** 07.11.2023
**Accepted:** 06.03.2024

**For citation**

Kalmykov P.A., Kustova T.P., Kustov S.O., Shestakovskaya P.S., Azmetov T.R., Kalmykova A.A. *One-pot* determination of amino acids in drugs by pre-column derivatization with phenyl isothiocyanate. *Tonk. Khim. Tekhnol. = Fine Chem. Technol.* 2024;19(2):127–138. https://doi.org/10.32362/2410-6593-2024-19-2-127-138

НАУЧНАЯ СТАТЬЯ

# *One-pot* определение аминокислот в лекарственных препаратах методом предколоночной дериватизации с фенилизотиоцианатом

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## Аннотация

**Цели.** Разработать новую методику определения аминокислот в лекарственных препаратах методом обращенно-фазовой высокоэффективной жидкостной хроматографии (ОФ ВЭЖХ) с предколоночной дериватизацией фенилизотиоцианатом (ФИТЦ) с применением *one-pot* пробоподготовки.

**Методы.** Исходные стандартные растворы аминокислот готовили методом навесок с последующим растворением в воде, после чего готовили рабочие растворы: стандартный, испытуемый и холостой, путем разведения в 20 мМ соляной кислоте. Дальнейшая пробоподготовка осуществлялась в полипропиленовых пробирках Safe-lock (*Eppendorf*) в реакционном буфере, содержащем подвижную фазу А, ацетонитрил и триэтиламин в соотношении 85 : 10 : 5 с окрашиванием 5% раствором ФИТЦ в ацетонитриле. После тщательного перемешивания в течение 3–5 мин на вортексе пробирки выдерживали в твердотельном термостате с термоизоляционной крышкой в течение 2 ч. Далее пробы охлаждали в течение 10 мин, центрифугировали в течение 1 мин при 13000 об/мин. Надосадочную жидкость переносили в виалы и проводили разделение смеси аминокислот методом ОФ ВЭЖХ с использованием в качестве неподвижной фазы гидрофобного силикагеля с привитыми группами С18. Количественное определение дериватов аминокислот проводили с применением диодно-матричного детектора.

**Результаты.** Разработана и валидирована новая методика разделения и определения аминокислот в лекарственных препаратах, позволяющая на основе простой *one-pot* пробоподготовки с использованием доступных реактивов и оборудования проводить исследования без использования коммерческих наборов, например AccQ×Tag Ultra Derivatization Kit, США. На примере анализа смесей гистидина и глицина показано, что при использовании двух подвижных фаз удается достичь приемлемого разделения дериватов аминокислот в градиентном режиме в течение 20 мин со скоростью потока 1.0 мл/мин. Приготовленные по новой методике пробы продемонстрировали высокую стабильность в применении и хранении. Предложен состав подвижных фаз А и Б, состоящий из 10 мМ ацетатного буфера с pH 3.5 и 80% раствора ацетонитрила. Валидация разработанной методики при анализе лекарственного препарата Иннонафактор®, содержащего в качестве вспомогательных веществ глицин и гистидин, продемонстрировала высокую сходимость результатов количественного определения данных аминокислот.

**Выводы.** Новая методика определения аминокислот в лекарственных препаратах методом ОФ ВЭЖХ с предколоночной дериватизацией ФИТЦ обладает широким диапазоном применения, имеет ряд преимуществ по сравнению с импортными коммерческими наборами для определения аминокислот: низкую стоимость реактивов и материалов, высокую точность и повторяемость, в связи с чем она может быть рекомендована к использованию в лабораториях контроля качества фармацевтических предприятий.

## Ключевые слова

аминокислоты, глицин, гистидин, ОФ ВЭЖХ, предколоночная дериватизация, ФИТЦ (фенилизотиоцианат), контроль качества лекарственных средств

**Поступила:** 29.08.2023
**Доработана:** 07.11.2023
**Принята в печать:** 06.03.2024

## Для цитирования

Калмыков П.А., Кустова Т.П., Кустов С.О., Шестаковская П.С., Азметов Т.Р., Калмыкова А.А. *One-pot* определение аминокислот в лекарственных препаратах методом предколоночной дериватизации с фенилизотиоцианатом. *Тонкие химические технологии*. 2024;19(2):127–138. https://doi.org/10.32362/2410-6593-2024-19-2-127-138

## INTRODUCTION

In the second half of 2022, a number of foreign pharmaceutical companies announced their suspension of investments in the Russian market. They ceased clinical trials in the country which are required for registration of new drugs. Certain drugs are in short supply due to problems with logistics of finished dosage forms or pharmaceutical substances. Problems with the availability of innovative foreign drugs are most acute in the therapy of orphan (rare) diseases. It should also be noted that certain Russian pharmaceutical companies produce such drugs independently. For example, *Generium*[1] produces drugs for the treatment of hemophilia, cystic fibrosis and Gaucher disease [1]. This is important, since in 2023 Russia introduced expanded neonatal screening of newborns for 36 congenital diseases, including inherited diseases of amino acid, organic acid, and fatty acid metabolism.

Analysis of the Russian pharmaceutical market for 2022[2] showed that the production of pharmaceuticals in the country increased by 8.6% when compared to 2021. According to the Strategy for the Development of the Pharmaceutical Industry until 2030, adopted by the Government of the Russian Federation in June 2023, the share of Russian full-cycle drugs in the market of the Russian Federation is due to increase to almost 70%, while the volume of drug production in monetary terms is expected to double to RUR 1.4 trn.[3]

Drugs containing amino acids and proteins are widely used in medical practice for the treatment of metabolic disorders, cardiovascular, infectious and dermatologic diseases, cancer, gastrointestinal, kidney and central nervous system diseases, as well as pain syndromes [1]. The segment of the pharmaceutical market related to the production of peptide drugs is steadily growing. Therefore, pharmaceutical manufacturers urgently need to improve amino acid analysis for quality control of such drugs. When queried for "amino acid analysis," the *sciencedirect.com* platform currently produces more than 64000 results, 2700 of which are in the last 5 years [2–5].

The Pharmacopoeia of the Eurasian Economic Union (EEU)[4] recommends 12 methods of amino acid analysis. Seven of these methods involve pre-column derivatization of amino acids. Derivatization of amino acids is necessary due to the absence of chromophore groups in the structure of most of their molecules. This does not allow for the use of optical detectors for analysis. In this regard, the professional community is focusing on the functionalization of amino acids, primarily by the *N*-terminal amino group [6–8]. One of the most reliable and well-established methods for amino acid determination is reversed-phase high-performance liquid chromatography (RP-HPLC) with precolumn derivatization with phenyl isothiocyanate (PITC). In amino acid analysis, the HPLC method is used much more often than other methods, since peptides are complex organic compounds which are thermolabile (degrade when heated). This method is characterized by a high level of sensitivity and selectivity. Derivatives with PITC at the *N*-terminal amino group form all amino acids. The reaction proceeds rapidly and quantitatively with the formation of stable amino acid derivatives. No other compounds are formed which interfere with the determination of amino acids. Thus, [9] proposed a method for the quantitative determination of a number of free amino acids (glutamic and asparagic, serine, glycine, histidine, proline, alanine, etc.) in samples of veal meat, laying hens and dried extract of cow brain by SP HPLC with pre-column PITC derivatization. The authors used 3 mobile phases: 2 acetate buffers with pH 4.05 and pH 5.5 and a 1% solution of isopropyl alcohol in acetonitrile. The sample preparation took on average about 20 h. It included acid hydrolysis of proteins, ethanol extraction of free amino acids, selection and drying of aliquots, addition of sodium hydroxide solution, labeling solution (PITC), re-drying and dissolution of the dry residue in bi-distilled water. During amino acid analysis, some analyte loss is known to occur during prolonged sample preparation. Therefore, it is important to limit the number of sample manipulations, since this will increase the analyte detection value and reduce the labor input of the staff.

The objective of this study was the development and validation of a new method using *one-pot* sample preparation with available reagents and equipment without the use of commercial kits of foreign production for the determination of amino acids in drugs by RP-HPLC with pre-column derivatization with PITC. Innonafactor®, a drug belonging to the "Coagulants (including blood coagulation factors), hemostatics" pharmaceutical group was chosen as a model system for the development of the methodology [10]. Recombinant blood coagulation

[1] https://www.generium.ru/products/#. Accessed August 02, 2023.

[2] The pharmaceutical market of Russia for 2022. Analytical review of DSM GROUP. https://dsm.ru/docs/analytics/Annual_report_2023_rus.pdf. Accessed August 02, 2023.

[3] Decree of the Government of the Russian Federation dated June 7, 2023, No. 1495-r "Strategy for the Development of the Pharmaceutical Industry until 2030." http://government.ru/docs/48801/. Accessed August 02, 2023.

[4] https://eec.eaeunion.org/comission/department/deptexreg/formirovanie-obshchikh-rynkov/pharmacopoeia/pharmacopoeia_utv.php. Accessed August 02, 2023.

factor IX, a single-chain glycoprotein with a molecular mass of about 55 kDa, consists of 415 amino acid residues[5]. In order to stabilize the glycoprotein in the preparation, a mixture of amino acids is used in sufficient excess (29-fold for glycine, 2.3-fold for histidine)[6]. Determination of the quantitative content of these amino acids is regulated and must be controlled during release control. In this regard, there is an urgent need for analytical methods to be developed to determine amino acids, which will give reproducible results with simple sample preparation in minimal time.

## EXPERIMENTAL

All the reagents and solvents used in the work were considered chemically pure: histidine (*PanReac AppliChem*, Spain, Cat. No. A1341), glycine (*PanReac AppliChem*, Spain, Cat. No. 143040), sodium acetate (*Sigma-Aldrich*, USA, Cat. No. S2889), concentrated acetic acid (*PanReac AppliChem*, Spain, Cat. No. 141008), hydrochloric acid (*Pallav*, India, Cat. No. 1379B), PITC (*Sigma-Aldrich*, USA, Cat. No. 78780), sodium hydroxide (*PanReac AppliChem*, Spain, Cat. No. 141687), sodium chloride (*Nouryan*, Denmark, Cat. No. 8004337), polysorbate 80 (*PanReac AppliChem*, Spain, Cat. No. 142050), triethylamine (*Sigma-Aldrich*, USA, Cat. No. 471283), acetonitrile (*PanReac AppliChem*, Spain, Cat. No. 221881). Ultrapure water (type I) (Milli-Q, *Merck Millipore*, USA) was used to prepare working solutions.

The labeling solution was prepared in a 2-mL Safe-lock polypropylene tube (*Eppendorf*, Germany) by mixing 950 μL of acetonitrile and 50 μL of PITC.

In order to prepare the mobile phase A (MP A), 0.82 g of sodium acetate was added to a 1-L beaker, and about 800 mL of water was added. Then the pH of the solution was adjusted with acetic acid to 3.5 ± 0.1 using a pH meter (Seven Easy S20, *Mettler Toledo*, Switzerland). The resulting solution was transferred to a 1-L volumetric flask, brought to the mark with water, stirred, filtered through a membrane filter with a pore diameter of 0.45 μm and then degassed using a vacuum pump.

For the preparation of the mobile phase B (MP B), 800 mL of acetonitrile was placed in a chemical beaker with a capacity of 1 L, and 200 mL of water was added. This was then stirred, and filtered through a membrane filter with a pore diameter of 0.45 μm and degassed.

The reaction buffer was prepared in a 15 mL test tube. 8.5 mL of MP A, 1 mL of acetonitrile and 500 μL of triethylamine in the ratio 85 : 10 : 5 were added to the test tube, and the contents were stirred.

The initial test solution of Innonafactor® 500 IU was prepared as follows: 5.0 mL of water for injections was added to the vial with the lyophilizate. It was gently mixed avoiding foaming according to the drug instructions. At such dilution the content of histidine is about 1.55 mg/mL (7.76 mg/flask), and glycine—about 19.52 mg/mL (97.6 mg/flask) which corresponds to the middle of the range specified in the drug instructions.

The initial standard solutions of amino acids were prepared by the method of suspensions with subsequent dissolution in water: the concentration of histidine was 1.55 mg/mL, and glycine—19.52 mg/mL. Then working solutions were then prepared. The initial test solution, initial standard solution, and placebo solution (without histidine and glycine) of 100 μL each were placed in 1.5 mL polypropylene tubes. Then 400 μL of 20 mM hydrochloric acid solution were added and mixed.

Further sample preparation was carried out in 1.5 mL polypropylene tubes in a reaction buffer with staining using labeling solution. After thorough mixing for 3–5 min, the tubes were held in a solid-state thermostat with a Gnome thermo-insulating lid (*DNA-Technology*, Moscow, Russia) for 2 h at 65°C. Then the samples were cooled in a refrigerator for 10 min, and centrifuged for 1 min at 13000 rpm. The supernatant was transferred to vials and the amino acid mixture was separated in a high-pressure liquid chromatograph (Waters e2695 with an Alliance separation module with Waters 2998 PDA detector, USA). Hydrophobic silica gel[7] with grafted C18 groups was used as the stationary phase.

Before commencing amino acid determination, the chromatographic column was equilibrated with a mixture of mobile phases in the ratio 97 : 3 until a stable base line was formed. Then conditioning in gradient mode was carried out at least 2 times.

*Chromatographic conditions:*

- Diaspher-110-C18 stainless steel column, 4.6 × 250 mm (particle size 5 μm) (*BioChemMak ST*, Russia);
- 2 mobile phases: MP A and MP B;
- MP flow rate: 1.0 mL/min;
- column thermostat temperature: 45°C;
- sample temperature: 5 ± 3°C;
- diode array detector (λ = 254.0 ± 4.8 nm);
- injection volume: 5 μL for glycine estimation, 50 μL for histidine estimation;
- chromatogram recording time: 20 min;
- sample injection order: blank solution (1 injection, 5 μL), standard solution (for glycine estimation,

[5] Register of medicines of Russia: *Nonacogum alfa*. https://www.rlsnet.ru/active-substance/nonakog-alfa-2595. Accessed August 02, 2023.

[6] Instructions for the medical use of the drug Innonafaktor®. https://www.generium.ru/upload/preparations/manual/Instruktsiya-Innonafaktor.pdf. Accessed August 02, 2023.

[7] https://bcmst.ru/kolonki/diaspher/. Accessed August 02, 2023.

3–5 injections, 5 μL), test solution (1–3 injections, 5 μL), standard solution (for histidine estimation, 3–5 injections, 50 μL), test solution (1–3 injections, 50 μL).

Elution was carried out in gradient mode. The samples were chromatographed according to the order of sample input. The peaks of the blank solution were not taken into account when processing the chromatograms.

The chromatographic system was considered suitable, if the following acceptance criteria were met:

- the relative standard deviation of retention time and peak area of the derivative no more than 2.0%;
- derivative peak asymmetry factor between 0.8 and 1.5;
- the efficiency of the chromatographic column for the derivative peak at least 50000 theoretical plates.

Amino acid content ($X$) in mg/flask was calculated according to the following formula:

$$X = \frac{S_{test}}{S_{st}} \times C \times 5,$$

wherein $S_{test}$ is the peak area of histidine/glycine derivatives on the chromatogram of the test solution; $S_{st}$ is the peak area of histidine/glycine derivatives on the chromatogram of standard solution; $C$ is the histidine/glycine content in standard solution, mg/mL; 5 is the volume of water added to the vial of the test drug solution during reconstitution of the lyophilizate, mL.

## RESULTS AND DISCUSSION

***Specificity.*** The specificity of the method was evaluated by means of visually comparing chromatograms of blank solution, standard and test solutions. On the chromatogram of the blank solution, there are no peaks with retention times of histidine derivatives (about 8.6 min), or glycine derivatives (about 10.8 min) present on the chromatograms of standard and test solutions (Fig. 1). Thus, the specificity of the technique was proven in relation to the matrix of the test solution (protein, placebo auxiliary components).

Four peaks were found in the retention time domain of amino acid derivatives. One is present on the chromatogram of the blank solution and belongs to the placebo solution. In order to identify the other peaks, the method of single amino acid standards was used. This method showed that the peak with retention time about 10.8 min belongs to the glycine derivative. The peaks with retention times of about 8.6 min and 10.4 min belong to the histidine derivative. According to the data available in literature [2] derivatives of amino acids with several nitrogen atoms in the structure are unstable and may undergo degradation. In order to evaluate these peaks and determine their nature, the absorption spectra of the derivatives in the wavelength range of 200–400 nm were studied using a diode array

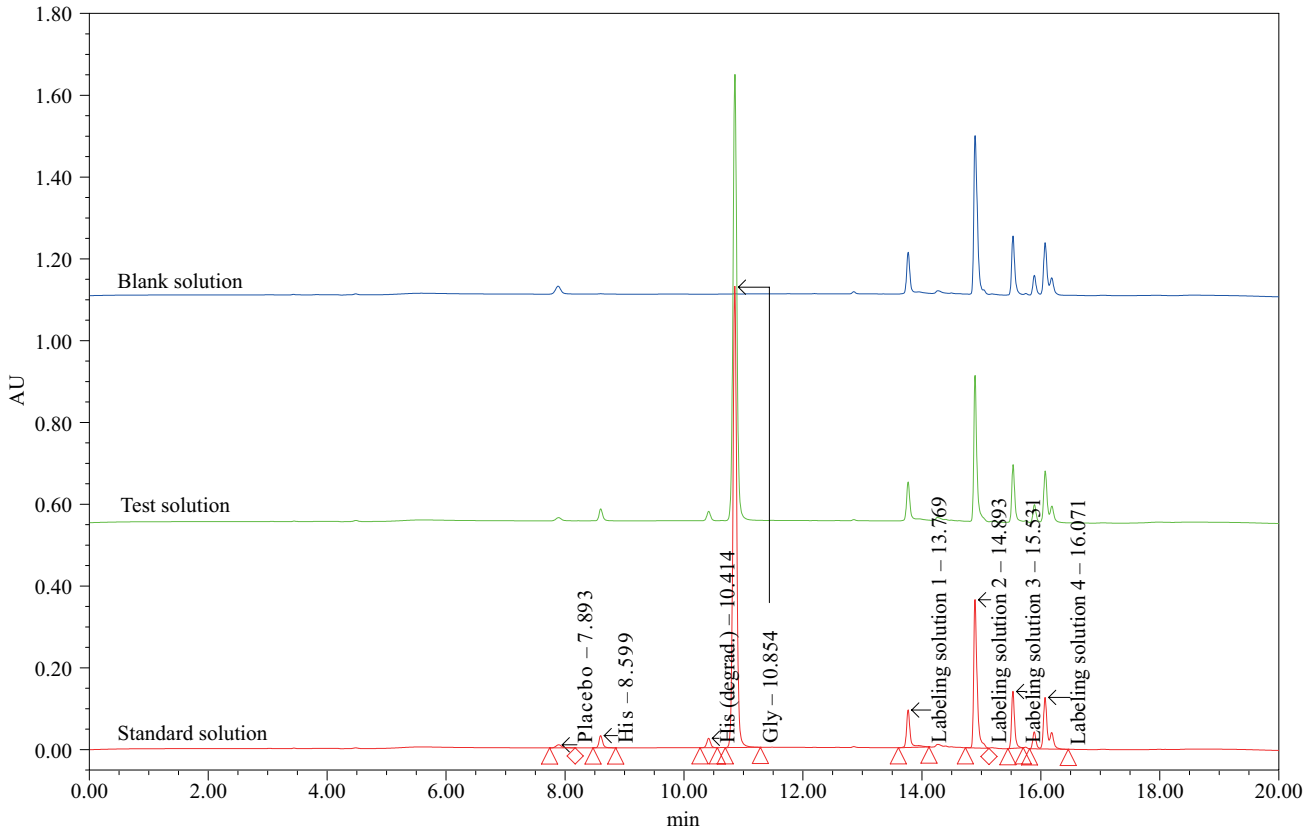


**Fig. 1.** Chromatograms of blank, test, and standard solutions

detector. A slice of peaks in the range of 6–11 min on the 3D spectrum of the chromatogram was established for the standard solution (Fig. 2).

According to the data obtained, the peak of glycine derivative has a spectrum with absorption maximum around 250 nm (Fig. 2d). This is in agreement with the data available in literature (254 nm). A similar spectrum is observed for the peak with retention time of around 8.6 min (Fig. 2b). The peaks with retention times of around 7.9 min and 10.4 min have no absorption maximum around 250 nm. Thus, it can be concluded that the peak of histidine derivative is selected for estimation of quantitative histidine content.

Peaks with a retention time of more than 12 min belong to the PITC dye peaks and system peaks (gradient region of column rewashing).

***Linearity, correctness and analytical range of the method.*** In order to evaluate the linearity, correctness and analytical range of the technique, the method of calibration solutions was used. For this purpose, model solutions (MS) of amino acids in the range of 40–140% of the nominal concentration (the middle of the range of Innonafactor® content) were prepared. The preparation of MS for linearity evaluation is described in Table 1.

Sample preparation was performed according to the method described above; each MR in three repetitions. The results of the linearity evaluation of the methodology on the peak of glycine derivative are summarized in Table 2, and histidine derivative in Table 3 and Fig. 3.

The method is show to have linearity in the selected range with a correlation coefficient of more than 0.98. The technique also shows an acceptable level of correctness in the confidence interval 95–105% for glycine, 90–110% for histidine. This can be associated with a fairly low concentration of histidine in the working test solution.

**Table 1.** Preparation of model solutions (MS) for evaluation of linearity and analytical area

| MS No. | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| Glycine/histidine content, % | 40 | 60 | 80 | 100 | 120 | 140 |
| Glycine content, mg/mL | 7.81 | 11.71 | 15.62 | 19.52 | 23.42 | 27.33 |
| Histidine content, mg/mL | 0.62 | 0.93 | 1.24 | 1.55 | 1.86 | 2.17 |
| Initial standard solution, μL | 40 | 60 | 80 | 100 | 120 | 140 |
| 20 mM hydrochloric solution acids, μL | 460 | 440 | 420 | 400 | 380 | 360 |

**Table 2.** Evaluation of the linearity of the method and the analytical area of the method for determining the content of glycine

| MS No. | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| Calculated concentration, mg/mL | 7.81 | 11.71 | 15.62 | 19.52 | 23.42 | 27.33 |
| Practical concentration, mg/mL | 7.53 | 11.36 | 16.41 | 20.32 | 23.31 | 26.88 |
| Detection percent | 96.4 | 97.0 | 105.0 | 104.0 | 99.5 | 98.4 |

**Table 3.** Evaluation of the linearity of the method and the analytical area of the method for determining the content of histidine

| MS No. | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| Calculated concentration, mg/mL | 0.62 | 0.93 | 1.24 | 1.55 | 1.86 | 2.17 |
| Practical concentration, mg/mL | 0.58 | 0.88 | 1.34 | 1.63 | 1.85 | 2.10 |
| Detection percent | 93.5 | 94.6 | 108.0 | 105.2 | 99.5 | 96.8 |

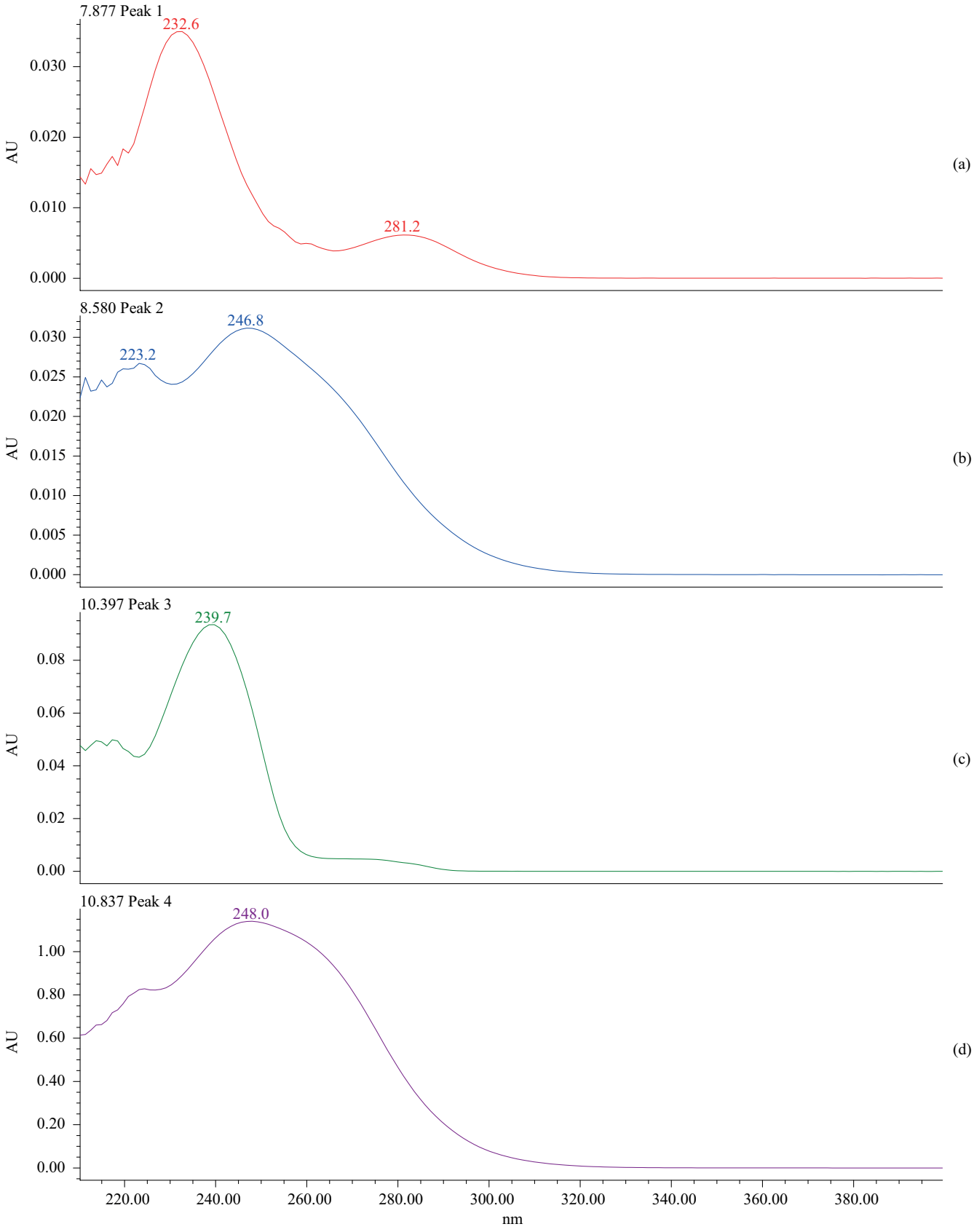


**Fig. 2.** Absorption spectra of peaks on the standard solution chromatogram with a retention time of
(a) 7.9;
(b) 8.6;
(c) 10.4;
(d) 10.8 min

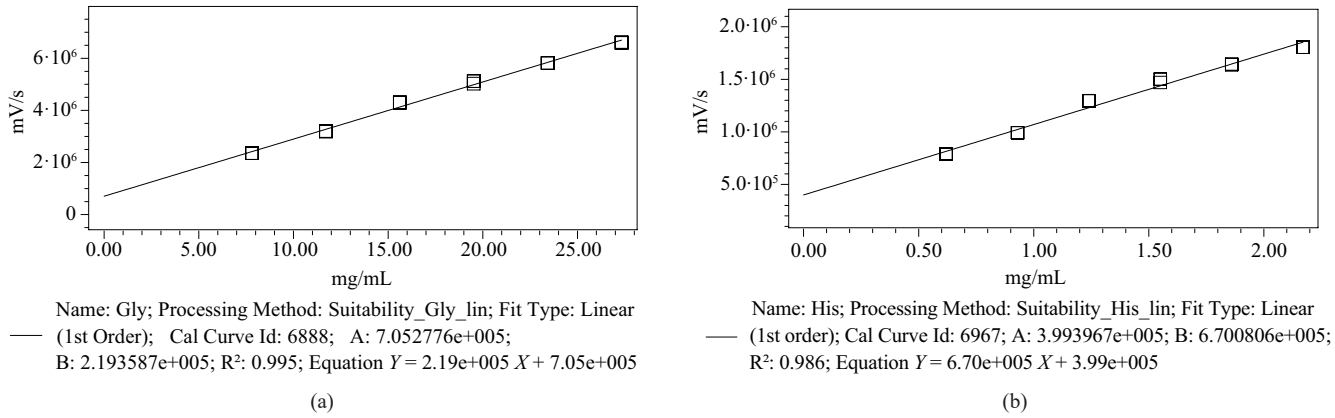


**Fig. 3.** Calibration plots for determining the linearity and analytical area of the method:
(a) glycine content estimate;
(b) histidine content estimate

The analytical technique has an acceptable level of linearity and correctness in the concentration range of 40–140% of nominal or 0.62–2.17 mg/mL for histidine and 7.81–27.33 mg/mL for glycine. Therefore, this concentration range is the analytical domain of the technique.

***Limit of quantification* (*LOQ*).** Based on the test results, the limit of quantification was determined by the calculation method according to the following formula:

$$\mathrm{LOQ} = \frac{X \times 10}{S / n},$$

where $X$ is the concentration of histidine/glycine in the standard solution, mg/mL; $S/n$ is the signal-to-noise ratio for the histidine/glycine derivative peak in

**Table 4.** Assessment of the repeatability of the analytical method

| Injection No. | Amino acid content, mg/flask | | | |
|---|---|---|---|---|
| | Histidine | | Glycine | |
| | (1) | (2) | (1) | (2) |
| 1 | 7.89 | 7.73 | 96.93 | 96.39 |
| 2 | 7.89 | 7.73 | 96.67 | 96.16 |
| 3 | 7.87 | 7.72 | 96.30 | 95.84 |
| 4 | 7.87 | 7.72 | 95.71 | 95.34 |
| 5 | 7.70 | 7.60 | 95.94 | 95.54 |
| 6 | 7.70 | 7.60 | 95.21 | 94.91 |
| 7 | 7.72 | 7.61 | 94.84 | 94.60 |
| 8 | 7.72 | 7.62 | 95.03 | 94.77 |
| 9 | 8.16 | 7.93 | 95.03 | 94.76 |
| 10 | 8.15 | 7.92 | 98.27 | 97.53 |
| 11 | 8.17 | 7.93 | 98.43 | 97.66 |
| 12 | 8.17 | 7.93 | 98.52 | 97.74 |
| Average | 7.9 | 7.8 | 96.6 | 96.1 |
| SD* | 0.2 | 0.1 | 1.4 | 1.2 |
| RSD**, % | 2.5 | 1.8 | 1.5 | 1.3 |

* SD—standard deviation.

** RSD—relative standard deviation.

the chromatogram of the standard solution; 10 is the limit of the signal-to-noise ratio for the estimation of LOQ.

The evaluation was performed for 6 consecutive injections of standard solution. The system acceptance criteria were fulfilled.

According to the data obtained, the analytical technique has high sensitivity. The initial concentration of amino acids in the initial test solution for histidine was about 0.01 mg/mL, for glycine about 0.024 mg/mL, corresponding to the limit of quantification.

***Repeatability.*** In order to assess the repeatability of the methodology, tests were conducted to evaluate the quantitative content of histidine and glycine in Innonafactor®. The test solution was prepared in 6 repetitions and each solution was injected twice. The test results are presented in Table 4. The results were calculated using two methods: (1) by linearity calibration plot and (2) by 6 consecutive injections of standard solution to evaluate the suitability of the system.

According to the data obtained, the technique has a high level of repeatability characteristic of HPLC techniques. Thus, for 6 sample preparations, the relative standard deviation of amino acid determination was about 2–3%. The data obtained for the two methods of calculation of amino acid content coincide. They are in the range of ±10% of the nominal content, according to the preparation instructions. Thus, calculation method (2) can be considered a sufficient condition for the suitability of the system for estimating the amino acid content.

***Stability of standard and test solutions***. The stability of the analytical methodology within the framework of shelf-life evaluation of the standard and test solutions was performed for freshly prepared solutions (A) and after 1 day of storage in a thermostat at 2 to 8°C (B). The results of amino acid content estimation are presented in Tables 5 and 6.

**Table 5.** Evaluation of the stability of the tested solutions

| Injection No. | Amino acid content, mg/flask | | | |
|---|---|---|---|---|
| | Histidine | | Glycine | |
| | (A) | (B) | (A) | (B) |
| 1 | 7.73 | 7.82 | 96.39 | 96.92 |
| 2 | 7.73 | 7.82 | 96.16 | 97.43 |
| 3 | 7.72 | 7.77 | 95.84 | 96.98 |
| 4 | 7.72 | 7.77 | 95.34 | 96.77 |
| 5 | 7.60 | 7.65 | 95.54 | 96.77 |
| 6 | 7.60 | 7.65 | 94.91 | 95.42 |
| 7 | 7.61 | 7.66 | 94.60 | 95.84 |
| 8 | 7.62 | 7.66 | 94.77 | 95.78 |
| 9 | 7.93 | 7.96 | 94.76 | 96.11 |
| 10 | 7.92 | 7.96 | 97.53 | 98.38 |
| 11 | 7.93 | 7.96 | 97.66 | 98.64 |
| 12 | 7.93 | 7.96 | 97.74 | 99.01 |
| Average | 7.8 | 7.8 | 96.1 | 97.1 |
| SD | 0.1 | 0.1 | 1.2 | 1.2 |
| RSD, % | 1.8 | 1.7 | 1.3 | 1.2 |
| Average | 7.8 | | 96.5 | |
| SD | 0.1 | | 1.3 | |
| RSD, % | 1.7 | | 1.3 | |

**Table 6.** Evaluation of the stability of the standard solution

| Injection No. | Amino acid derivative peak area, μV/s | | | |
|---|---|---|---|---|
| | Histidine | | Glycine | |
| | (A) | (B) | (A) | (B) |
| 1 | 1457768 | 1467593 | 5075402 | 5112246 |
| 2 | 1460085 | 1470084 | 5064962 | 5098915 |
| 3 | 1460186 | 1470991 | 4999555 | 5121785 |
| 4 | 1460216 | 1475217 | 4994879 | 5140767 |
| 5 | 1460957 | 1474622 | 4994327 | 5139811 |
| 6 | 1461680 | 1476159 | 4991779 | 5138833 |
| Average | 1460149 | 1472444 | 5020151 | 5125393 |
| SD | 1317 | 3390 | 38975 | 17389 |
| RSD, % | 0.1 | 0.2 | 0.8 | 0.3 |
| Average | 1466297 | | 5072772 | |
| SD | 6873 | | 62037 | |
| RSD, % | 0.5 | | 1.2 | |

The stability of the standard and test solutions was confirmed within 1 day of storage in the thermostat at 2 to 8°C. This can be considered an advantage over similar methods for amino acid determination, such as derivatives with ortho-phthalic aldehyde which have a shelf life of about 2–3 min [11]. The relative standard deviation was less than 2%.

## CONCLUSIONS

The new technique thus developed for the separation and determination of amino acids in pharmaceuticals on the basis of simple *one-pot* sample preparation using available reagents and equipment meets all the required criteria for HPLC quantification methods according to the EEU Pharmacopoeia and the State Pharmacopoeia of the Russian Federation. It can therefore be recommended for use in the laboratories of pharmaceutical companies. The technique is more economical compared to imported commercial kits for amino acid determination in terms of cost of reagents and materials. It also possesses a high level of accuracy and repeatability. Furthermore, the method is universal and has a wide range of application, since the formation of derivatives of amino acids with PITC at the *N*-terminal amino group is characteristic of all amino acids. In the present study, the range of application of this technique was confirmed for the quantification of histidine 0.62–2.17 mg/mL and glycine 7.81–27.33 mg/mL in the initial test solution. The limit of quantification was about 0.01 mg/mL for histidine and about 0.024 mg/mL for glycine. The proposed technique has a high level of repeatability, typical for HPLC techniques which is within the range of 2.0%.

### Authors' contributions

**P.A. Kalmykov**—idea of a new method, concept of the study, analysis and interpretation of experimental data, and writing of the text of the article.

**T.P. Kustova**—concept of the study, analysis of literature on the research topic, analysis and interpretation of experimental data, and writing of the text of the article.

**S.O. Kustov**—experimental studies, and participation in writing of the text of the article.

**P.S. Shestakovskaya**—experimental studies and participation in writing of the text of the article.

**T.R. Azmetov**—experimental studies and participation in writing of the text of the article.

**A.A. Kalmykova**—experimental studies and participation in writing of the text of the article.

*The authors declare no conflicts of interest.*

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*Translated from Russian into English by H. Moshkov*
*Edited for English language and spelling by Dr. David Mossop*