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Approaches to the assessment of quantitative composition of drugs based on natural peptides containing glycosaminoglycan-peptide complex

©2020. N.G. Vengerovich[1,2*], N.V. Efimov[3], N.I. Rogozhina[4], V.I. Stepchenkov[4]

[1] Saint Petersburg State Chemical and Pharmaceutical University, Saint Petersburg, Russia
[2] Institute of Military Medicine, Russian Federation of Ministry of Defense, Saint Petersburg, Russia
[3] NGHCI Railway Clinical Hospital JSCo "RZD", Saint Petersburg, Russia
[4] Peoples' Friendship University of Russia, Moscow, Russia

* e-mail: nickolai.vengerovich@pharminnotech.com
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The preparations isolated from the biomaterial of farm animals and fish were studied. These preparations (rumalon, alflutop) cannot be obtained in equal proportions and concentrations during their production. An algorithm for quantitative determination of components of peptides of natural origin based on the study of model drugs containing a glycosaminoglycan-peptide complex has been developed. Using infrared spectroscopy and high performance liquid chromatography, the concentrations of chondroitin sodium sulfate, hyaluronic acid, glucosamine, protein, free amino acids and amino acids that make up the peptides (alflutop and rumalon) were established. In comparison with the majority of monocomponent drugs registered in the territory of the Russian Federation and containing chondroitin sodium sulfate at a concentration of 100 mg/ml, glucosamine drugs (200 mg/ml), hyaluronic acid drugs (10 mg/ml) it was found that the main active components of the studied drugs were more than 50, 100 and 7 times smaller. At the same time, the preparations under study contained a large percentage of free amino acids or short-chain peptides. It indirectly suggests their participation in the clinical efficacy of drugs based on natural peptides.

The developed algorithm for quantitative determination of components of peptide preparations of natural origin, including common and generally available methods sequence (identification of samples by infrared spectroscopy in comparison with standard samples, quantitative determination of chondroitin sodium sulfate, hyaluronic acid, glucosamine, protein and amino acids by infrared spectroscopy and highly efficient liquid chromatography) is advisable to use when determining minimum specific values of contents (concentrations) of active components and adjuvants of peptide origin in studies and registration of drugs based on them, as well as to justify ways in order to search and explain the mechanisms of action of such compounds.

KEYWORDS: peptides; glycosaminoglycan; chondroitin sulfate; hyaluronic acid; rumalon; alflutop; infrared spectroscopy; high performance liquid chromatography; amino acids; short-chain peptides

ABBREVIATIONS:

HPLC - high performance liquid chromatography

INTRODUCTION

Peptide-based drugs have been known in pharmacology for a long time, and peptides of natural origin were used even before the formation of pharmacology as a science [1]. Despite the fact that peptide drugs make up a relatively small part of the drug market (more than 60 peptides approved by the Food and Drug Administration are registered in the United States of America), about 140 peptide drugs are currently undergoing clinical trials while more than 500 peptide drugs are undergoing preclinical trials [2].

At present, one group of such drugs is composed of glycosaminoglycan-peptide complexes. Some of these drugs are designed for regeneration of cartilaginous and bone tissues. Moreover, the mechanisms of their action are not fully understood. There is a hypothesis that, when entering cells, low molecular weight peptides of animal origin act as structural elements of the matrix, participate in the formation of collagen and proteoglycan fibrils, make up for the missing components and affect tissue formation [3].

S. Camarero-Espinosa and J. J. Cooper-White [4] suggested that drugs containing glycosaminoglycans serve as a trigger for starting the processes of directed differentiation of multipotent mesenchymal stromal cells. Clinical effectiveness of these drugs was proved only at the level of uncontrolled studies. The only published randomized controlled five-year trial of rumalon did not reveal evidence of its effect on progression of osteoarthrosis of hip and knee joints [5]. Glycosaminoglycans are also used as systems for the delivery of growth factors to damaged bone tissue [6, 7] and as carrier matrices for controlled delivery to nerve tissue [8].

Unlike synthetic short-chain peptides [3], preparations isolated from material of farm animals and fish cannot be obtained in equal proportions and concentrations. It significantly limits the study of their effectiveness and also impedes registration due to the inability to reproduce the composition with sufficient accuracy. Pan Y, et al. [9] attempted to decompose such mixtures into components, which made it possible to determine the most active components: chondroitin sulfate (18–49 kDa), heparan sulfate, and fibroblast growth factor associated with chondroitin sulfate, which is likely to account for tissue repair and damage regeneration.

To date, studies on the development of methods for identification of qualitative composition [10] and the assessment of quantitative composition of peptides of natural origin [11] form the basis for the development of this particular research area as a whole.

AIM OF STUDY

The aim of the present study is to develop an algorithm for the quantitative determination of the components of peptides of natural origin based on the study of model drugs containing a glycosaminoglycan-peptide complex.

MATERIALS AND METHODS

The following drugs were used in the study:

- alflutop, injection solution , 1 ml ampoule № 10, series 3381017, expiry date 09.2022 (K. O. Bioteknos S. A., Romania);
- alflutop, injection solution, 2 ml ampoule № 5, series 3310418, expiry date 03.2021 (K. O. Bioteknos S. A., Romania);
- rumalon, solution for intramuscular injection, 1 ml ampoule № 25, series 1717751, expiry date 11.2022 (K.O. Rompharm Company S. R. L., Romania).

All drugs were purchased in a retail pharmacy network. They are registered in the Russian Federation as drugs and contain glycosaminoglycan-peptide complexes (from small sea fish - alflutop or cartilage and bone marrow of young calves - rumalon), which made it possible to choose them for research.

As reference standards the following substances were used:

- chondroitin sodium sulfate (EP RS, 250 mg, code Y0000280, series 2.0, identification number 000mM8);
- sodium hyaluronate (USP RS, 250 mg, FOM296 series);
- glucosamine hydrochloride (USP RS, 200 mg, GOM183 series);
- amino acid RS (NCl0180, Pierce, USA).

Quantitative determination of the composition of drugs based on the peptides of natural origin was carried out using infrared spectrometry techniques and high performance liquid chromatography. Identification of drug components by IR spectroscopy and their comparison with reference standard substances was carried out using a Cary 630 Agilent Fourier transform IR spectrometer equipped with a module for impaired total internal reflection in the region of 4000–650 cm -1. After recording the IR spectra, they were processed using MicroLabFTIR Software. According to the results of overlapping the obtained spectra and their processing, tables were formed indicating the percentage of coincidence of the spectrum of a reference standard with the spectrum of a test sample. Tested sample preparation was carried out in accordance with the requirements of the general pharmacopoeial article 1.2.1.1.0002.15 "Spectrometry in the infrared region". [12]

Chondroitin sodium sulfate was quantitatively determined by turbidimetric titration with cetylpyridinium chloride according to the requirements of the method of quantitative determination of sodium chondroitin sulfate [13] using an automatic T 50 titrator complete with a DP5 phototrode, Mettler Toledo (Switzerland).

The content of chondroitin sodium sulfate in test preparations was calculated by the formula:

$$X=\frac{C\times 50}{V\times 1000}=\frac{C}{V\times 20},,$$

where V is the volume of the test drug, ml; C is the concentration of chondroitin sodium sulfate, determined according to the calibration graph, mg/ml

Quantitative determination of hyaluronic acid was carried out in accordance with the requirements of the method of quantitative determination of sodium hyaluronate [13] spectrophotometrically by reaction with carbazole.

Using the calibration curve constructed from the optical densities of each of the standard solutions the average concentrations of glucuronic acid D in test solutions were determined by the formula:

$$\frac{c_g}{c_s}\times Z\times\frac{100}{100-h}\times\frac{401,3}{194,1},$$

where cg is the average concentration of D-glucuronic acid in test solutions, mg/ml; cs is the average concentration of test substance in test solutions, mg/ml; Z - a certain percentage of C6H10O7 in D-glucuronic acid; h is loss in mass upon drying, %; 401,3 is a relative molecular weight of a disaccharide fragment; 194,1 is a relative molecular weight of glucuronic acid.

Quantitative determination of glucosamine content was carried out by high performance liquid chromatography (HPLC), in accordance with methods presented in the literature [14, 15].

Chromatographic conditions: column Waters μ Bondapak NH2 30 cm, column temperature: 26 ± 1°C, detector: ultraviolet spectrophotometric, wavelength 195 nm, flow rate: 1.3 ml/min. Mobile phase: a mixture of acetonitrile and 0,026 M of phosphate buffer KH2PO4 with the addition of 0,25 ml of NH4OH pH = 7,5 (75:25).

The determination of total protein was carried out in accordance with the requirements [12, 13]. The Lowry colorimetric method was used, based on the reaction of proteins with copper (II) salts in an alkaline solution and the reduction of phosphoromolybdenum-tungsten reagent (Folin's reagent) with the formation of colored products, the color intensity of which was determined by optical density at a wavelength of 750 nm.

To calculate the protein content (X, mg) in 1 ml of the drug, taking into account its dilution, the following formula was used:

$$X = \frac{C \times 25 \times 6{,}5}{V \times 1},$$

where C is the protein concentration found by the calibration curve, mg/ml; V is a volume of the drug taken for analysis, ml.

The determination of total amount of amino acids was carried out according to the requirements of the European Pharmacopoeia [13].

The content in 1 ml of the drug in μmol (X) was calculated by the formula:

$$X = \frac{1{,}67 \times \sum S_{ак.пр}}{2 \times 0{,}67 \times \sum S_{ак.ст}},$$

where ∑S.ac.pr is the sum of proteins in the sample sample, μmol/ml; AkSac.st - the sum of proteins in a protein standard, μmol / ml.

Descriptive statistics is presented as M - arithmetic mean and SD - standard deviation.

RESULTS AND ITS DISCUSSION

It was found that the spectra of the drug alflutop coincide with RS of chondroitin sodium sulfate and sodium hyaluroant (73,8 ± 3.4 and 77,5 ± 4,1%), Fig. 1, 2. In so doing, the coincidence of the spectra of the drug rumalon was 92,2 ± 3,7 and 88,3 ± 2,9%, respectively (Fig. 3, 4). Due to the low concentration of chondroitin sodium sulfate in the drug alflutop, the isolation of the active substance from this dosage form by standard methods was difficult.

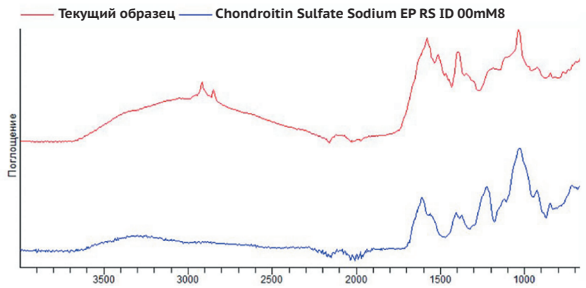

Fig. 1. IR spectrum of the drug alflutop s. 3381017 in comparison with the standard spectrum of chondroitin sodium sulfate

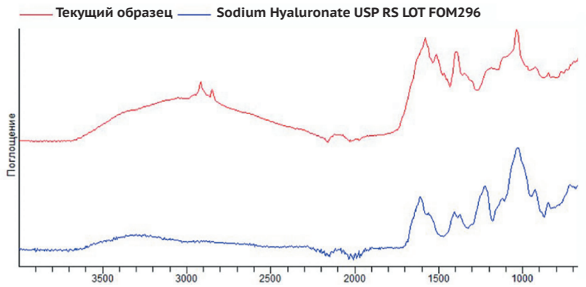

Fig. 2. IR spectrum of the drug alflutop s. 3381017 compared to the standard spectrum of sodium hyaluronate

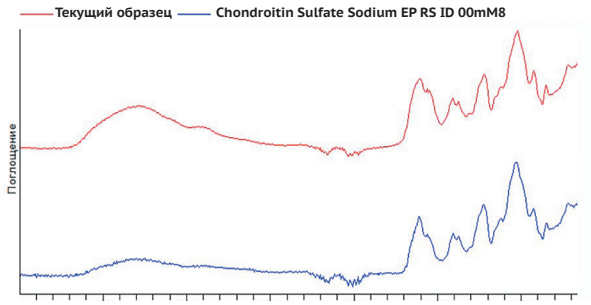

Fig. 3. IR spectrum of the drug rumalon s. 1717761 in comparison with the standard spectrum of chondroitin sodium sulfate

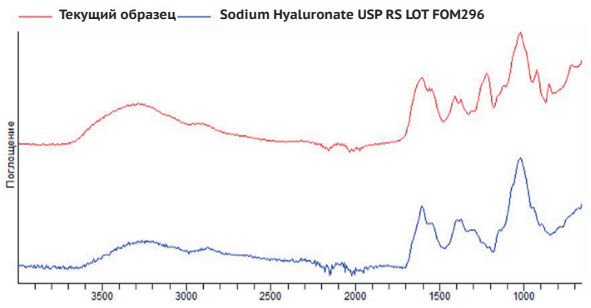

Fig. 4. IR spectrum of the drug rumalon s. 1717761 in comparison with the standard spectrum of sodium hyaluronate

The content of chondroitin sodium sulfate in the drug alflutop is almost 5 times lower in comparison with the drug rumalon (Table 1).

The content of hyaluronic acid in the preparation of rumalon s.1717751 was 1.42 ± 0.08 mg/ml, in the drug alflutop

s.3381017 it was 0.27 ± 0.08 mg/ml. It should be noted that the concentration of hyaluronic acid in the preparation of rumalon was quite low, while the optical density of the test solutions did not exceed 0.05.

In a series of preliminary studies it was found that amino acids, presented in model preparations, make it difficult to determine glucosamine by spectrophotometric method. In this regard, HPLC was used to determine real glucosamine content in drugs under study. Fig. 5 shows the chromatogram of the initial standard glucosamine hydrochloride solution, which allowed us to construct a calibration graph of the peak area versus the concentration of this component based on the obtained peak area results.

The analysis of chromatograms of rumalon and alflutop preparations (Fig.6, 7) made it possible to determine the content of glucosamine in them. So, in the drug rumalon the content was 3.6 ± 0.05 mg / ml, in the drug alflutop – 1.51 ± 0.04 mg/ml, respectively.

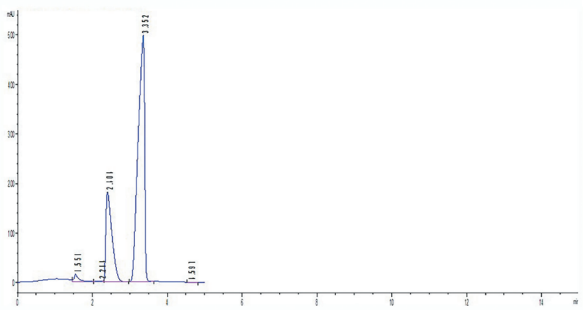

Fig. 5. Chromatogram of the initial standard glucosamine hydrochloride standard solution with a retention time of 3.352 min

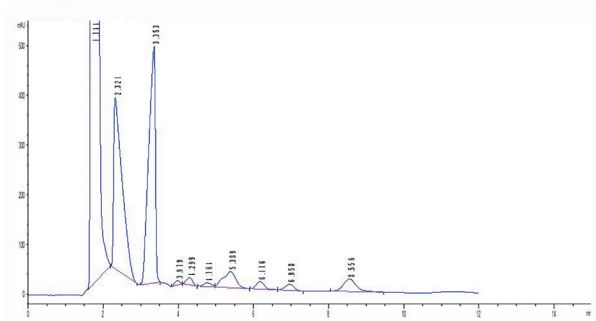

Fig. 6. Chromatogram of the drug rumalon s. 1717761. Glucosamine retention time 3.353 min

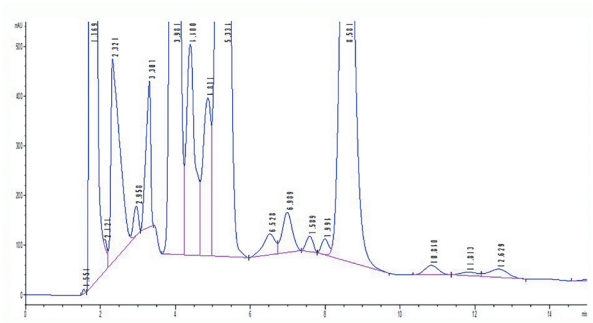

Fig. 7. Chromatogram of the drug alflutop s. 3310418. Glucosamine hydrochloride retention time 3.307 min

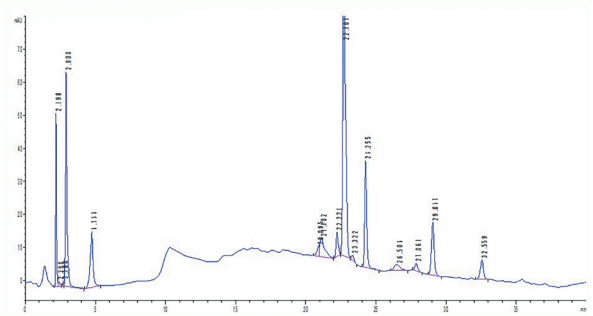

Fig. 8. Chromatogram of the amino acid standard

When 0.5 ml of trichloroacetic acid was added to the drug alflutop, the solution did not cloud. This indicated the absence of protein in the drug. The concentration of protein in the preparation of rumalon was in the range of 0.36–0.45 mg/ml.

The HPLC method revealed the presence of free amino acids in both preparations (Fig. 8–13).

It has been found that following acid hydrolysis of the samples under study the total number of amino acids has increased from 1,22 ± 0,11 to 3,3 ± 0,24 μmol/L for rumalon and from 10,98 ± 0,47 to 17,6 ± 0,72 for alflutop. This finding allows the conclusion that there is a rather large representation of short-chain peptides and/or free amino acids in initial samples of drugs under study (37-62%).

Quantitative composition of model drugs containing glycosaminoglycan-peptide complex is presented in Table 2.

From Table 2 it follows that the content of the main components of the studied peptides of natural origin is presented in fairly small quantities.

Table 1.

The results of turbidimetric titration for determination of the content of chondroitin sodium sulfate (cholesterol) in the studied model preparations, M±SD

Rumalon s. 1717751		Rumalon s. 1717761		Alflutop s. 3381017	
The average volume of titrant, ml	The content of ChS, mg/ml	The average volume of titrant, ml	The content of ChS, mg/ml	The average volume of titrant, ml	The content of ChS, mg/ml
5,59±0,21	1,904±0,024	5,62±0,36	1,914±0,041	2,44±0,13	0,402±0,029

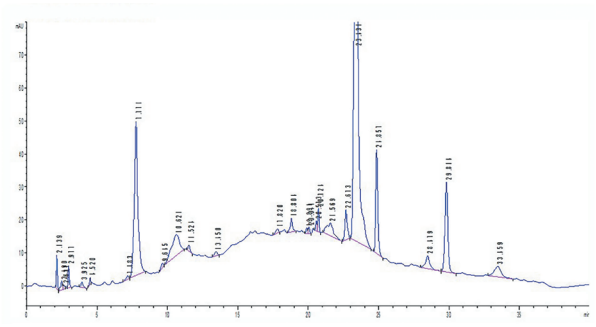

Fig. 9. Chromatogram of the initial drug rumalon s.1717761 following derivatization

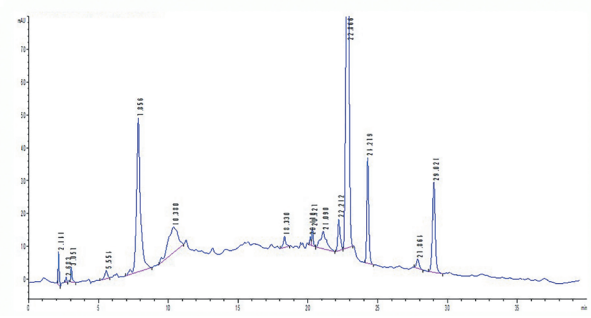

Fig. 10. Chromatogram of the starting drug rumalon s. 1717761 following derivatization

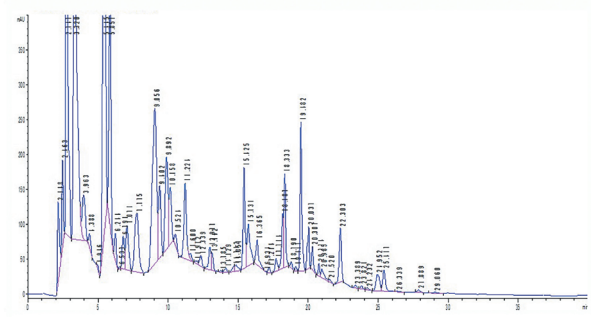

Fig. 11. Chromatogram of the initial drug alflutop s. 3310418 following derivatization

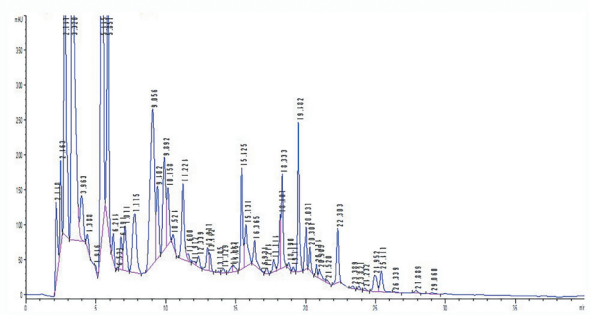

Fig. 12. Chromatogram of the hydrolyzed drug rumalon s.1717761 following derivatization

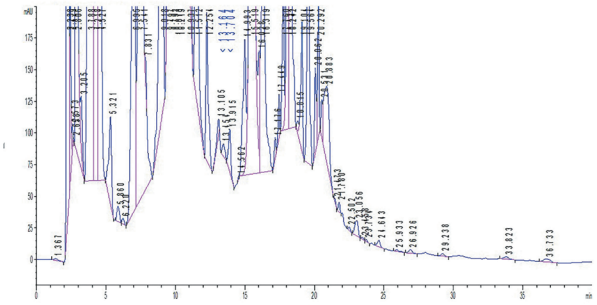

Fig. 13. Chromatogram of the hydrolyzed drug alflutop s. 3310418 following derivatization

It is known that the majority of monocomponent medicines registered in the Russian Federation contain chondroitin sodium sulfate at a concentration of 100 mg/ml (arthogistan, injectran, honsat, artravir icampharm, drastop, chondroxide, chondrogard, mucosate, chondromed-lefpharm, etc.). Monocomponent drugs glucosamine as a solution for intramuscular administration contain an active principle in concentrations of 200 mg/ml (sustagard arthro, elbona, dona). Monocomponent drugs contain hyaluronic acid at a concentration of 10 mg/ml (hyalgan phidium).

Thus, the results obtained indicate that the amount of active components in the studied preparations (chondroitin sulfate, glucose and hyaluronic acid) is 50, 100 and 7 times less than their values in monopreparations.

CONCLUSION

The assessment of the interaction of the active components in the composition of the combined drugs should be considered as one of the possible hypotheses explaining the effectiveness of such compounds. In this case the type of interaction of the active components is most likely to be synergistic. Another possible explanation for the effectiveness of such drugs can be the presence of growth factors and/or components in their composition that initiate necessary differentiation of mesenchymal stromal cells in the damaged zone. This assumption can indirectly confirm the presence of a large number of free amino acids and, possibly, short- chain peptides in the composition of drugs under study based on peptides of natural origin.

The algorithm proposed for the quantitative determination of the components of the peptide preparations of natural origin, including a sequence of common and generally available methods (identification of samples by IR spectroscopy in comparison with standard samples, quantitative determination of chondroitin sodium sulfate, hyaluronic acid, glucosamine, protein and amino acids by IR spectroscopy and HPLC), may be used for the determination of minimum values of the active components and adjuvants of natural peptides in their study and registration as medicinal preparations.

Table 2.

The results of determining the amount of amino acids in the tested drugs, M±SD

Drug	The amount of amino acids, mmol/ml		
	Free amino acids	After hydrolysis	The amount attributable to the peptides
Rumalon s. 1717761	1,22±0,11	3,3±0,24	2,08±0,16
Alflutop s. 3381017	10,98±0,47	17,6±0,72	6,62±0,31

Table 3.

The results of drug studies, M±SD

Indicator	Rumalon	Alflutop
Chondroitin Sodium Sulfate, mg/ml	1,904±0,024 (c.1717751) 1,914±0,041 (c.1717761)	0,402±0,029 (c.3381017)
Hyaluronic acid, mg/ml	1,42±0,08 (c.1717751)	0,27±0,08 (c.3310418)
Glucosamine, mg/ml	3,60±0,05 (c.1717761)	1,51±0,04 (c.3310418)
Protein, mg/ml	0,36±0,01 (c.1717751) 0,45±0,03 (c.1717761)	Not determined
Free amino acids, mmol/ml	1,22 (c.1717761)	10,98 (c.3310418)
Amino acids that make up the peptides	2,06 (c.1717761)	6,62 (c.3310418)

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ADDITIONAL INFORMATION ABOUT AUTHORS

Nikolai G. Vengerovich, Doctor of Medicine (MD), Professor of the Industrial Ecology Department, Saint Petersburg State Chemical and Pharmaceutical University, Saint Petersburg, Russia; Deputy Head of Department State Research Institute of Military Medicine, Russian Federation of Ministry of Defense, Saint Petersburg, Russia; e-mail: nickolai.vengerovich@pharminnotech.com

Nikolai V. Efimov, Doctor of Medicine (MD), Professor, Head of Clinical Research Department, Railway Clinical Hospital JSCo "RZD", Saint Petersburg, Russia; e-mail:nvef@rambler.ru

Natalia I. Rogozhina, master student, Peoples' Friendship University of Russia, Moscow, Russia; e-mail: rogozhina26@gmail.com

Vladimir I. Stepchenkov, master student, Peoples' Friendship University of Russia, Moscow, Russia; e-mail: vstepchenkov@icloud.com

ДОПОЛНИТЕЛЬНЫЕ СВЕДЕНИЯ ОБ АВТОРАХ

Венгерович Николай Григорьевич, д-р мед. наук, профессор кафедры промышленной экологии Санкт-Петербургского государственного химико-фармацевтического университета Министерства здравоохранения Российской Федерации, Санкт-Петербург, Россия; заместитель начальника научно-исследовательского отдела Государственного научно-исследовательского испытательного института военной медицины Министерства обороны Российской Федерации, Санкт-Петербург, Россия; e-mail: nickolai.vengerovich@pharminnotech.com

Ефимов Николай Владимирович, д-р мед. наук, профессор, заведующий отделением клинических исследований Дорожной клинической больницы открытого акционерного общества «Российские железные дороги», Санкт-Петербург, Россия; e-mail: nvef@rambler.ru

Рогожина Наталья Ивановна, магистрант Российского университета дружбы народов, Москва, Россия; e-mail: rogozhina26@gmail.com

Степченков Владимир Иванович, магистрант Российского университета дружбы народов, Москва, Россия; e-mail: vstepchenkov@icloud.com

Авторы заявляют, что у них нет конфликта интересов.

Подходы к оценке количественного состава лекарственных средств на основе пептидов природного происхождения, содержащих гликозаминогликан-пептидный комплекс

©2020. Н.Г. Венгерович[1,2*], Н.В. Ефимов[3], Н.И. Рогожина[4], В.И. Степченков[4]

[1] Санкт-Петербургский государственный химико-фармацевтический университет Министерства здравоохранения Российской Федерации, Санкт-Петербург, Россия
[2] Государственный научно-исследовательский испытательный институт военной медицины Министерства обороны Российской Федерации, Санкт-Петербург, Россия
[3] Дорожная клиническая больница открытого акционерного общества «Российские железные дороги», Санкт-Петербург, Россия
[4] Российский университет дружбы народов, Москва, Россия

* e-mail: nickolai.vengerovich@pharminnotech.com
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Исследованы препараты, выделенные из биоматериала сельскохозяйственных животных и рыб. Эти препараты (румалон, алфлутоп) при производстве не могут быть получены в равных соотношениях и концентрациях. Разработан алгоритм количественного определения компонентов пептидов природного происхождения на основе изучения модельных лекарственных препаратов, содержащих гликозаминогликан-пептидный комплекс. Методами инфракрасной спектроскопии и высокоэффективной жидкостной хроматографии установлены концентрации хондроитина сульфата натрия, гиалуроновой кислоты, глюкозамина, белка, свободных аминокислот и аминокислот, входящих в состав пептидов (алфлутопа и румалона). При сравнении с большинством зарегистрированных на территории Российской Федерации монокомпонентных лекарственных средств, содержащих хондроитина сульфата натрия в концентрации 100 мг/мл, лекарственных препаратов глюкозамина (200 мг/мл), лекарственных препаратов гиалуроновой кислоты (10 мг/мл) было установлено, что основные активные компоненты исследованных препаратов оказались более чем в 50, 100 и 7 раз меньше соответствующих значений для монопрепаратов. При этом процентное содержание свободных аминокислот или короткоцепочечных пептидов в исследованных препаратах велико (37–62%). Это позволяет предположить, что они оказывают влияние на клиническую эффективность лекарственных средств на основе природных пептидов.

Разработанный алгоритм количественного определения компонентов пептидных препаратов природного происхождения, включающий в себя последовательность распространенных и общедоступных методик (идентификация образцов методом инфракрасной спектроскопии в сравнении со стандартными образцами, количественное определение хондроитина сульфата натрия, гиалуроновой кислоты, глюкозамина, белка и аминокислот методами инфракрасной спектроскопии и высокоэффективной жидкостной хроматографии), целесообразно использовать при определении минимальных значений концентрации активных компонентов и адъювантов пептидного происхождения при исследованиях и регистрации лекарственных препаратов на их основе, а также для обоснования путей поиска и объяснения механизмов действия подобных соединений.

КЛЮЧЕВЫЕ СЛОВА: пептиды; гликозаминогликан; хондроитина сульфат; гиалуроновая кислота; румалон; алфлутоп; инфракрасная спектроскопия; высокоэффективная жидкостная хроматография; аминокислоты; короткоцепочечные пептиды