J Nanostruct 11(2): 398-408, Spring 2021

RESEARCH PAPER

High Performance Liquid Chromatography Method for Determination of Carnosine and Taurine and Composition them with Fe_3O_4 Nanoparticles

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ARTICLE INFO

Article History:
Received 16 January 2020
Accepted 26 March 2021
Published 01 April 2021

Keywords:
Carnosine
Eye drops
HPLC
Nanocomposite
Taurine

ABSTRACT

The present study reports the determination of the Fe_3O_4 nanoparticle based carnosine and taurine enzymes. The aim of the study was to develop a methodology that allows the determination of amino acids by HPLC in their joint presence in eye drops. In the result a method has been developed for the simultaneous quantitative determination of the components of eye drops (carnosine and taurine) by HPLC with preliminary preparation of dinitrophenyl (DNP) derivatives. This method has been validated for specificity, accuracy, precision, linearity, and analytical range, ranging from 80% to 120% of nominal.

How to cite this article
Fadeeva D. A, Zhilyakova E. T , Malyutina A. Y, Kazakova V. S, Avtina N. V, Timoshenko E. V. High Performance Liquid Chromatography Method for Determination of Carnosine and Taurine and Composition them with Fe_3O_4 Nanoparticles. J Nanostruct, 2021; 11(2): 398-408. DOI: 10.22052/JNS.2021.02.019

INTRODUCTION

Nanotechnology is one of the quick rising areas of progressing technology, thus being a source of prospect for medicine and pharmacy. It is approved that, exactly metal oxides designed as nanoparticles will be used to soak medical devices and clothes, to fight viruses and bacteria, in new drug delivery systems or cancer therapy [1]. For the medicinal science to completely profit from the new nanotechnology accomplishments, it is serious to determine the nanoparticles penetration mechanisms into the cells, their action inside the cells, the accumulation degree in various organs of living organisms. It has been cleared that numerous efforts at elucidating the biocompatibility of nano alumina for animals and humans [2]. One of the promising substances with a wide spectrum of pharmacological activity is the dipeptide carnosine [3-5]. The antioxidant activity of this compound has been proven during peroxidation both in the hydrophobic and in the hydrophilic layer of the membrane, and carnosine provides protective properties regardless of the type of inducer of the oxidative process [6]. It has been found that carnosine activates the functions of the immune system and also has a neuroprotective effect [7,8]. Carnosine is widely used as a component of biologically active additives. However, researchers try to confirm the pharmacological activity of this natural compound [9]. Using carnosine in ophthalmology, including in the composition of multicomponent dosage forms, is determined by its proven antioxidant activity [7]. Liquid chromatography is useful for

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quality control of amino acids. Currently, there is highly specialized equipment - amino acid analyzers [10,11]. Therefore, the simplest and most accessible method of analysis in this regard is high performance liquid chromatography with precolumn derivatization [12]. This is a modern analytical method used for the analysis of substances of amino acid nature due to its high sensitivity and selectivity [13]. Precolumn derivatization is carried out using various reagents. Since the simplest and most frequently used method of fixing the analytical signal is spectrophotometric detection, and the objects of study do not have significant intrinsic absorption in the ultraviolet region of the spectrum, it was decided to use their dinitrophenyl derivatives (DNP derivatives) for analysis [14 – 17]. These compounds were obtained by reacting amino acids with 1-fluoro-2,4-dinitrobenzene, which has been successfully used for the analysis of amino acids for a long period of time [18, 19]. The aim of the present work is determining Carnosine and Taurine, compositing with a certain metal oxide nanoparticle.

MATERIALS AND METHODS

The objects of the study are a standard sample of carnosine (Sigma), a standard sample of taurine (Supelco), API of taurine (FS.2.1.0039.15, Russia), API of carnosine (USPh, China), a model mixture of eye drops with carnosine and taurine. To develop the method, the following reagents were used: sodium tetraborate 10-aqueous, 1-fluoro-2,4-dinitrobenzene, methanol, lithium dihydrogen phosphate, dioxane, orthophosphoric acid, water for chromatography. The study was carried out on a high-performance liquid chromatograph Shimadzu LC-20AD, a Reprosil-Pur C18-AQ 150x4 mm chromatographic column (sorbent particle size 5 µm).

Determination of the enzymes

In a typical experiment for preliminary derivatization of amino acid standards, 0.1 g (accurately weighed) of a standard sample of an amino acid is placed in a volumetric flask with a capacity of 50 ml, 40 ml of 0.05 M sodium tetraborate solution are added, stirred until the substance is completely dissolved, and the volume of the solution is adjusted with the same solvent to labels and mix. 50 ml of the resulting solution is transferred into a flask with a capacity of 50 ml, add 25 ml of 0.05 M sodium tetraborate solution, 2.0 ml of 10% solution of 1-fluoro-2,4-dinitrobenzene, incubate for 40 minutes at 40 °C and cooled to room temperature. The resulting solution is quantitatively transferred into a volumetric flask with a capacity of 50 ml, the volume of the solution is brought to the mark with 50% methanol, stirred and filtered through a fluoroplastic filter with a pore size of no more than 0.5 µm.

In another experiment for preliminary derivatization of a mixture of amino acids, 0.1 g of the substance of carnosine and 0.1 g of taurine (exact weighed portions) are placed in a volumetric flask with a capacity of 50 ml, 40 ml of 0.05 M sodium tetraborate solution are added, stirred until the substances are completely dissolved, the volume of the solution in a volumetric flask, bring to the mark with the same solvent, mix. 10 ml of the resulting solution of a mixture of amino acids is transferred into a flask with a capacity of 100 ml, 50 ml of 0.05 M sodium tetraborate solution, 4 ml of a 10% solution of 1-fluoro-2,4-dinitrobenzene are added, incubated for 40 minutes at a temperature of 40 °C and cooled to room temperature. The resulting solution is quantitatively transferred into a volumetric flask with a capacity of 100 ml, the volume of the solution is brought to the mark with 50% methanol, mixed and filtered through a fluoroplastic filter with a pore size of no more than 0.5 µm. The dinitrofluoride derivative of the eye drop solution is prepared in the same way, taking 5 ml of the drug for analysis. Chromatography is performed by reversed-phase high performance chromatography. Column temperature - 40 °C, analysis time - 30 minutes. The below mixtures of solvents are used as a mobile phase:

phase A: 8.5% methanol solution in phosphate buffer solution (pH 2.0),

phase B: 85% methanol solution in phosphate buffer solution (pH 2.0).

Elution process is carried out with a phase gradient by the below procedure:

From 0.0 to 2.0 min - 100% of phase “A”; from 2.0 to 20.0 min - increase in the content of phase “B” from 0% to 100%; from 20.0 to 25.0 min - 100% of phase “B”; from 25.0 to 27.0 min - decrease in the content of phase “B” from 100% to 0%; from 27.0 to 30.0 min - 100% of phase “A”. The volume of the injected sample is 5 µl, the flow rate of the mobile phase is 0.75 ml / min. Detection - spectrophotometric at a wavelength of 375 nm.

The identification of carnosine and taurine

is carried out by comparing the peak times of dinitrophenyl derivatives of taurine and carnosine with the retention times of the peaks of dinitrophenyl derivatives of standard samples. The content of taurine or carnosine (Xi) in the preparation, in milligrams per ml, was calculated by the formula 1:

$$Xi = \frac{S_i \cdot m_{oi} \cdot P_i}{S_{oi} \cdot 500}, \quad (1)$$

where: S_i - the average value of the peak areas of the dinitrophenyl derivative of taurine or carnosine, calculated from the chromatograms of the test solution; S_{oi} - is the average value of the peak areas of dinitrophenyl derivative of taurine or carnosine, calculated from the chromatograms of the reference solution of taurine and carnosine; m_{oi} - the weight of a sample of a standard sample of taurine or a standard sample of carnosine, g; P_i - the content of the basic substance in a standard sample of taurine or a standard sample of carnosine, %.

The content of taurine in 1 ml of the preparation should be from 0.018 g to 0.022 g. The content of carnosine in 1 ml of the preparation should be from 0.018 g to 0.022 g.

Preparation of a 10% solution of 1-fluoro-2,4-dinitrobenzene: 1.0 g of 1-fluoro-2,4-dinitrobenzene was placed in a 10 ml volumetric flask, 7 ml of dioxane was added, stirred until complete dissolution, the volume of the solution was brought to the mark with the same solvent and mixed. Preparation of phosphate buffer solution pH 2.0: 5.2 g of lithium dihydrogen phosphate was dissolved in 900 ml of water for chromatography, and phosphoric acid was added with continuous stirring until the pH of the solution was 2.00 ± 0.05. The resulting solution was transferred into a volumetric flask with a capacity of 1000 ml, the volume of the solution was brought to the mark with water for chromatography, and stirred.

Preparation of mobile phase "A": 400 ml of phosphate buffer solution pH 2.0 was placed in a volumetric flask with a capacity of 500 ml, 42.5 ml of methanol for chromatography was added, mixed, the volume of the solution was brought to the mark with phosphate buffer solution pH 2.0 and stirred. Preparation of mobile phase "B": 75 ml of phosphate buffer solution pH 2.0 was placed in a volumetric flask with a capacity of 500 ml, the volume of the solution was brought to the mark with methanol for chromatography and stirred. Before use, the solutions were filtered through a membrane filter with a pore size of no more than 0.5 µm.

RESULTS AND DISCUSSIONS

Characterization

Fig. 1 presents XRPD pattern of the carnosine/Fe_3O_4 nanocomposite. It can be seen that the sites and intensity of the diffraction peaks are consistent with the standard pattern for JCPDS Card No. (79 - 0417). The sample show very broad peaks, indicating the ultra-fine nature and small crystallite size of the particles. Fig. 2 presents the structure of enzyme and magnetic material

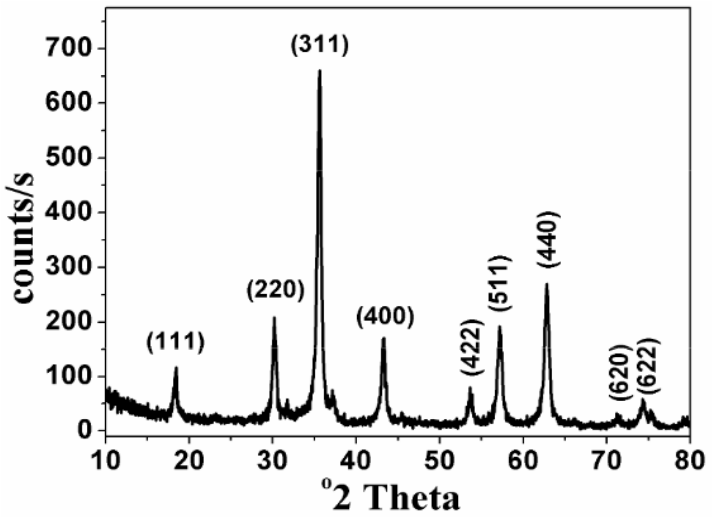

Fig. 1. XRPD pattern of the carnosine-Fe_3O_4 nanocomposite.

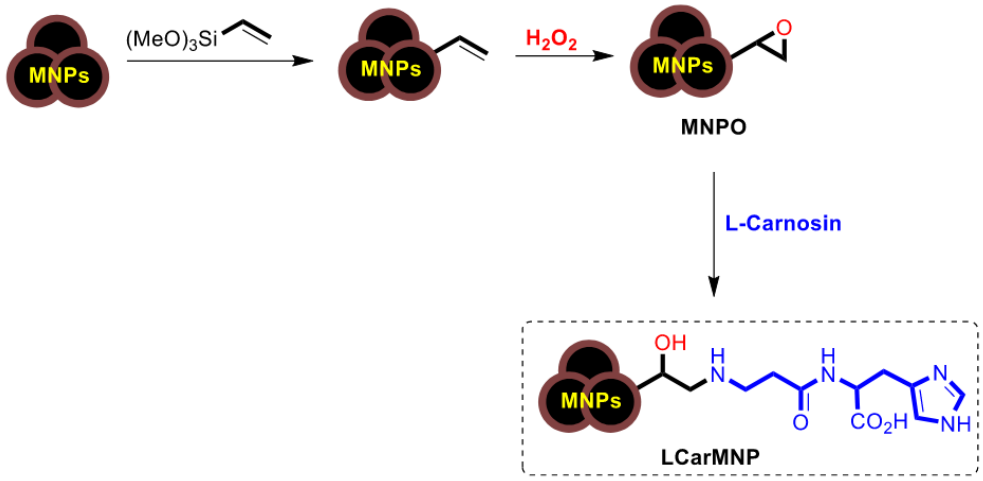

Fig. 2. Chemical structure of carnosine based nanomagnetic composite.

nanocomposite. The enzyme is attached to Fe_3O_4 as linker and make a shell around the nanoparticle surface.

SEM image of the as-prepared carnosine-Fe_3O_4 nanocomposite is shown in Fig. 3. It is found that the particle sizes of the nanocomposites are in the range of 20 to 30 nm.

The experiment to develop a method for the quantitative determination of carnosine and taurine was carried out in several stages, allowing to fully support the pharmaceutical development process. At the first stage of the experiment, to confirm the selectivity of the method, we obtained chromatograms of the reagent - 1-fluoro-2,4-dinitrobenzene (Fig. 4), as well as dinitrophenyl derivatives of standard amino acid samples (Figs. 5 and 6). The reagent in this system is characterized by the presence of two peaks with retention times of 16.4 and 16.7 minutes, which are also observed on the chromatograms of carnosine and taurine derivatives. Derivatization of carnosine gave two derivatives of the compound with retention times of 13.8 min and 20.1 min, and the retention time of dinitrophenyl-taurine derivative was 14.3 min.

Was studied the possibility of applying the technique to determine the authenticity of amino acids in the joint presence. The experiment was carried out on a model mixture containing carnosine and taurine. The chromatogram (Fig. 7) shows the peaks coinciding in retention time with the peaks of carnosine and taurine derivatives, the coefficients divisions met the requirements

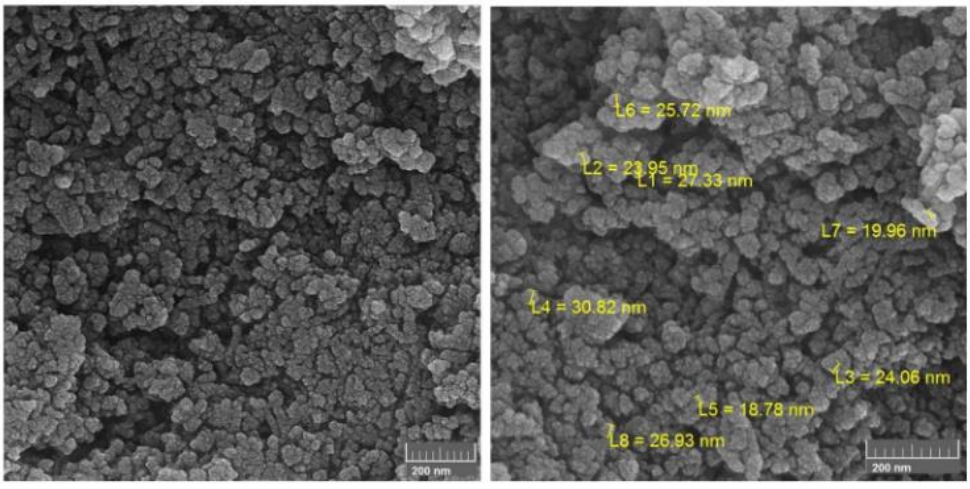

Fig. 3. SEM images of carnosine-magnetic nanocomposite at different magnifications.

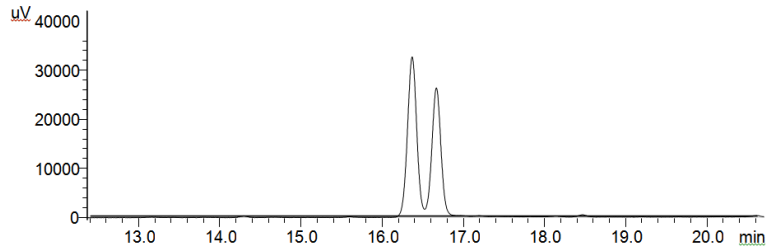

Fig. 4. Chromatogram of 1-fluoro-2,4-dinitrobenzene

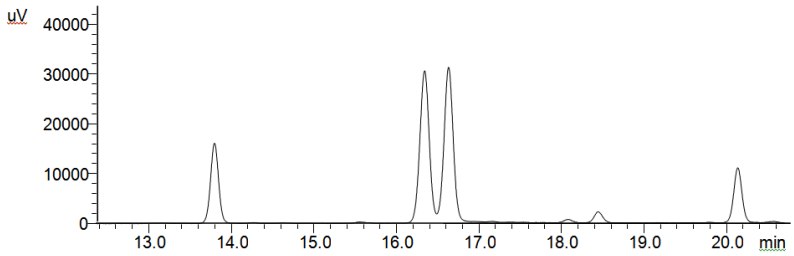

Fig. 5. Chromatogram of DNP-derived standard sample of carnosine.

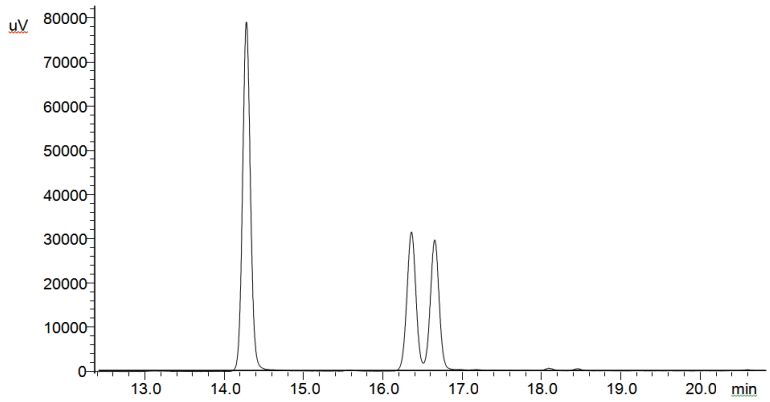

Fig. 6. Chromatogram of DNP-derived Taurine reference.

of regulatory documentsto the methods of determining the authenticity. Thus, this technique can also be used to determine the authenticity of amino acids when present together.

The final stage in the development of the technique was testing on a model mixture of eye drops, which contained 20 mg / ml of the studied amino acids. The results of quantitative determination and their statistical processing are presented in table 1. Studies have shown that the relative error does not exceed 3% (carnosine - 2.56%, taurine - 2.75%).

The absence of a systematic measurement error was confirmed by the experiment with the addition of standard samples of amino acids (Table 2). This experiment confirmed that the relative error does not exceed the error of a single determination, and also has deviations towards both positive and negative values, thus, there is no systematic error.

The most important stage in the development of a methodology is its validation - the main condition for ensuring the quality and safety of manufactured products, which is also a guarantor of the reliability of the results of analytical studies at all stages of the drug product life cycle [19]. According to modern requirements, methods of quantitative determination are subject to validation, including methods for determining the limit of impurity content. Authentication

techniques are validated if necessary to confirm their specificity. The developed method for determining the authenticity and quantitative determination of carnosine and taurine was validated in accordance with the requirements of the Russian State Pharmacopoeia, 14th edition [19, 20]. During validation, the analytical method was assessed according to the following characteristics: specificity, analytical area, linearity, right, precision. The specificity of the method as its

Table 1. Results of quantitative determination of carnosine and taurine in a model mixture by HPLC.

Defined substance	Found (X), mg / ml	S	$\Delta\overline{X}$	ε,%
Carnosine	20.31 19.85 19.78 20.14 20.07 19.91 $\overline{X}$ = 20.01	0.20	0.51	2.56
Taurine	20.07 20.18 19.82 20.02 19.91 19.87 $\overline{X}$ = 19.98	0.14	0.35	2.75

Table 2. Results of experiments with the addition of standard samples of carnosine and taurine for the method of quantitative determination of carnosine and taurine in a model mixture.

The substance to be determined	Taken, g	Added **CO**, g	Must be g	Found, g	Mistake	
					Absolute, g	Relative,%
Taurine	0.0202	0.0051	0.0253	0.0256	+0.0003	1.1742
	0.0202	0.0101	0.0303	0.0307	+0.0004	1.3029
	0.0202	0.0152	0.0354	0.0348	-0.0006	1.5805
	0.0202	0.0202	0.0404	0.0400	-0.0004	1.0000
Carnosine	0.0198	0.0050	0.0248	0.0250	+0.0003	1.0000
	0.0198	0.0099	0.0297	0.0302	+0.0005	1.6556
	0.0198	0.0149	0.0347	0.0342	-0.0004	1.3158
	0.0198	0.0198	0.0396	0.0390	-0.0006	1.5385

Table 3. The results of the analysis of model mixtures of taurine and their statistical processing.

Solution No.	Introduced taurine in % of the concentration of the reference solution (X,%)	Found taurine in % to the concentration of the reference solution (Y,%)	Found in % to the entered Z = 100x (Y/X)
1	81.00	80.00	98.77
2	84.50	85.50	101.18
3	90.50	91.50	101.10
4	96.00	95.50	99.48
5	99.00	99.50	100.51
6	104.50	105.00	100.48
7	111.60	111.00	99.46
8	115.00	115.50	100.43
9	122.00	121.00	99.18
	Average Z,%		100.07
	Relative standard deviation, Sz, %		0.87
	Relative confidence interval Δ, % = t (95%, 7) x Sz		2.04
	Critical value for the convergence of results, max Δ As, %		6.4
	Systematic error, δ = Zcp -100		0.07
	The criterion of insignificance of systematic error 1) δ≤Δ / 3		done
	2) if not satisfied 1), then δ≤2,0		done
	General conclusion about the technique		correct

ability to unambiguously evaluate the determined substances in the presence of accompanying components was determined by comparing the retention times of the standard sample of carnosine and taurine and working solutions at the same concentrations. The average retention time of the peaks of the determined substances on the chromatograms of standard sample solutions should not differ from the average retention time of the peak of carnosine and taurine derivatives on the chromatograms of the test sample by more than 2.0%. The chromatogram of the mobile phase should not show peaks that coincide in retention time with the peaks of the compounds to be determined and the reagent 1-fluoro-2,4-dinitrobenzene.

Thus, the specificity of the method was proved by the coincidence, with an accuracy of 0.5%, of the retention times of the peaks of carnosine and taurine on the chromatograms of the test solution with the retention times of the corresponding peaks on the chromatograms of standard sample solutions. The characteristics of correctness and precision were studied on model solutions with concentrations of active substances in the range from 80% to 120% of the content in relation to the nominal value (range of application of the method). The research results are shown in tables 3 and 4.

For both active ingredients of the eye drops, the technique is characterized by sufficient accuracy and precision in the entire range of investigated concentrations and is correct. In the range of carnosine and taurine concentrations from 80% to

Table 4. Results of analysis of model mixtures of carnosine, and their statistical processing.

Solution No.	Introduced carnosine in % of the concentration of the reference solution (X,%)	Found carnosine in % to the concentration of the reference solution (Y,%)	Found in % to the entered Z = 100x (Y/X)
1	79.50	80.05	100.69
2	85.50	85.00	99.42
3	91.50	92.00	100.55
4	95.50	96.00	100.52
5	100.50	100.00	99.50
6	105.50	106.00	100.47
7	110.50	112.00	101.36
8	115.50	116.50	100.87
9	119.50	120.50	100.84
	Average Z,%		100.47
	Relative standard deviation, Sz, %		0.63
	Relative confidence interval Δ,% = t (95%, 8) xSZ		1.48
	Critical value for the convergence of results, maxΔAs,%		6.4
	Systematic error, δ = Zcp -100		0.47
	The criterion of insignificance of systematic error 1) δ≤Δ / 3		done
	2) if not satisfied 1), then δ≤2,0		done
	General conclusion about the technique		correct

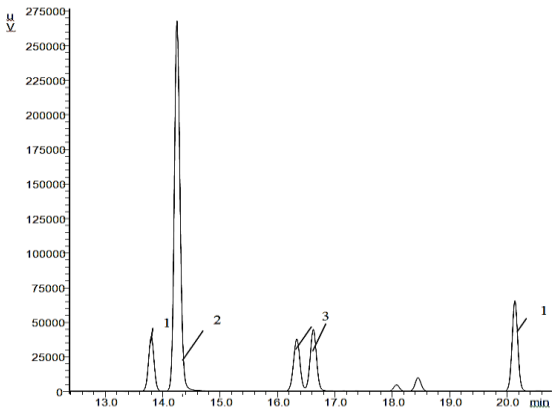

Fig. 7. Chromatogram of carnosine and taurine derivatives in the joint presence of: 1 - peaks of DNP-derivatives of carnosine; 2 - peak of DNP-derivative of taurine; 3 - peak of 1-fluoro-2,4-dinitro-benzene.

Table 5. Metrological characteristics of the linear dependence of the peak area on the amount of carnosine and taurine.

Paremeters	Values		Requirements 1		Requirements 2		Conclusion	
	carnosine	taurine	carnosine	taurine	carnosine	taurine	carnosine	taurine
b	1.0276	0.9848						
Sb	0.0055	0.0023						
a	-2.2671	1.5727	≤1.895x Sa = |4.0|	≤1.895x Sa = |4.0|	≤ |2.6|	≤ |2.6|	met by 1 criterion	met by 1 criterion
Sa	1.238	0.945						
RSD0	0.756	0.356						
RSD0/b	0.7357	0.3615	≤ |3.38|	≤ |3.38|			done	done
r	0.9992	0.9983	>|0.99809|	>|0.99809|			done	done

120% in relation to the nominal concentration, the method has no significant systematic error.

The linearity of the method was investigated in the range of carnosine and taurine concentrations from 80% to 120% with respect to the nominal value. For the study area, the correlation coefficient (r) must be at least 0.99 and the square of the correlation coefficient (r2) must be at least 0.98. The results of the determination confirmed that the requirements for the parameters of the linear dependence are met and the linearity is determined in the concentration range from 80% to 120% of the nominal value. Linear relationships were calculated using the least squares method as shown in Fig. 8. The metrological characteristics presented in tables 5 confirm that the linearity requirements for the method for quantifying carnosine and taurine are met. The analytical area of the technique was established by the range of experimental data that satisfy the linear model and meets the requirements of the State Pharmacopoeia of the Russian Federation 14th edition and is applicable in the range from 80 to 120%.

This technique was used in the quality control of eye drops containing carnosine and taurine as API, and excipients: an ingredient to increase the viscosity of the solution - hydroxypropyl methylcellulose and a preservative benzalkonium chloride. The proposed technique was used to control the quality of the produced eye drops according to the “Authenticity” and “Quantitative determination” indicators. It has been established that this technique is applicable in the presence of “interfering” components - excipients that are part of this dosage form. To confirm the “Authenticity” indicator, chromatograms obtained during the “Quantification” test were used. The retention times of the peaks of dinitrophenyl derivatives of taurine and carnosine in the studied eye drops coincided with the retention times of the peaks of DNP derivatives of amino acids in the chromatogram of comparison solutions of taurine and carnosine (Fig. 9).

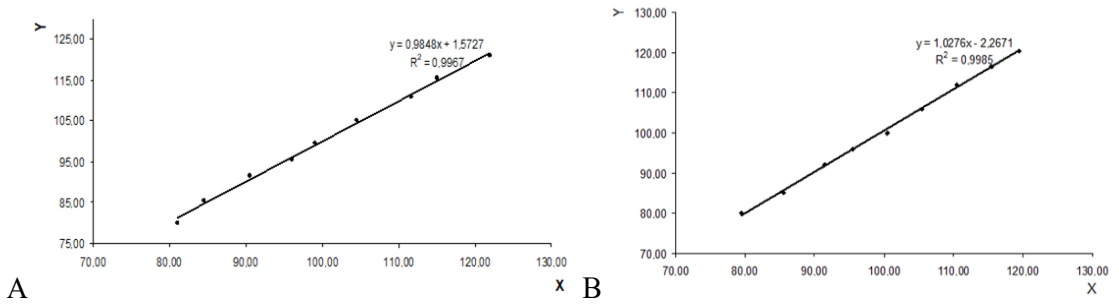

Fig. 8. Linear dependence of the peak area on the amount of taurine (A) and carnosine (B) in normalized coordinates.

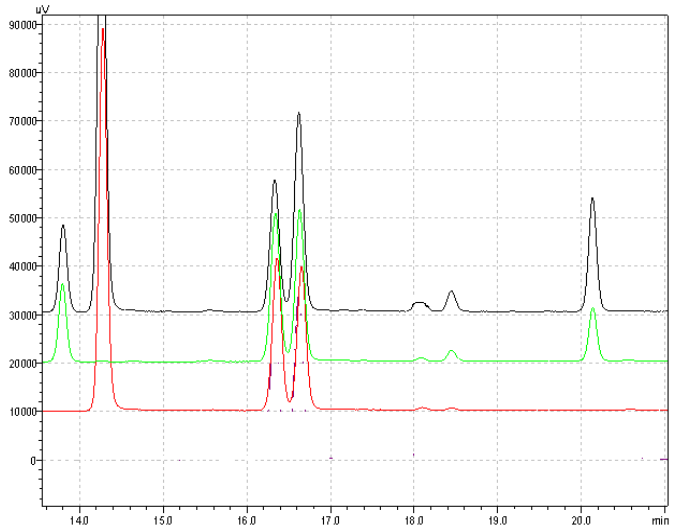

Fig. 9. Chromatograms of dinitrophenyl derivatives of 0.2% aqueous solution of a standard sample of taurine (1), 0.2% solution of a standard sample of carnosine (2) and eye drops (3).

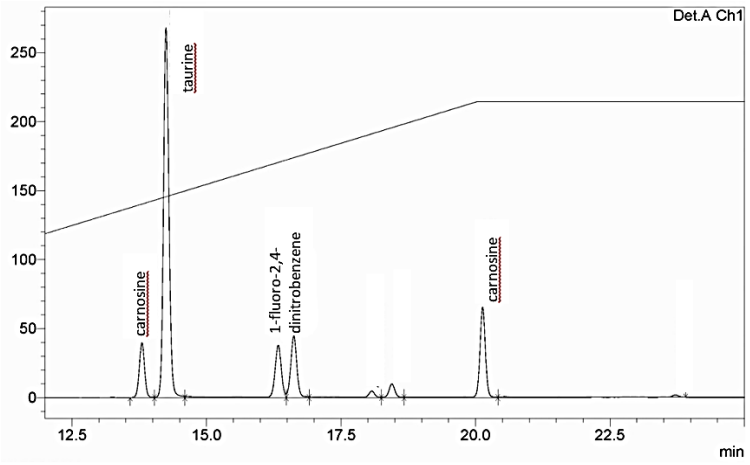

Fig. 10. Typical chromatogram of eye drops after precolumn derivatization 1-fluoro-2,4-dinitrobenzene.

During the determination of the "Quantification" indicator, a number of chromatograms were obtained. A typical chromatogram of DNP-derived components of eye drops is shown in Fig. 10. The content of taurine in 1 ml of eye drops in accordance with the draft regulatory documentation is regulated in the range from 0.018 g before 0.022 g, carnosine - from 0.018 g to 0.022 g. Studies have shown that the content of taurine in eye drops was 0.0200 ± 0.0006 g / ml, carnosine - 0.0201 ± 0.0006 g / ml.

CONCLUSION

At the present work, preparation of a magnetic carnosine/Fe_3O_4 nanocomposite is reported successfully. XRD and SEM images were used to study the characterization and physical properties of the fabricated samples. Besides, a method for the quantitative determination of carnosine and taurine in eye drops was developed by the method of high-performance liquid chromatography with diode-matrix detection. This method has been validated for specificity, accuracy, precision,

linearity, and analytical range. The method for the quantitative determination of carnosine and taurine in eye drops has been validated in the range from 80% to 120% of the nominal value.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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