Carnosine-Containing Liposomes: Preparation and Properties

N.A. Antonova[1], G.M. Sorokoumova[1,@], T.N. Fedorova[2], S.L. Stvolynsky[2], D.A. Abaimov[2], V.I. Popenko[3], V.I. Shvets[1]

[1]*Moscow Technological University (Institute of Fine Chemical Technologies), Moscow, 119571 Russia*
[2] *Research Center of Neurology, Moscow, 125367, Russia*
[3]*Engelhardt Institute of Molecular Biology RAS, Moscow, 119991, Russia*
[@] *Corresponding author e-mail: galinams@yandex.ru*

*Carnosine is a natural dipeptide antioxidant. It is proved that it protects human's cells from oxidative stress. However, it has a short lifetime in a human organism due to the carnosinase activity. In order to solve this problem we used carnosine encapsulated in liposomes. Thus, the aim of this study was the creation of a new liposomal carnosine drug form. We used two encapsulation methods that show different carnosine beh * avior: a passive and an active one. We took into account that conditions of obtaining liposomes such as lipid composition, pH and temperature are important. In this study the lipid composition providing the maximum encapsulation efficiency was determined. Dipalmitoylphosphotidylcholine (DPPC) and its mixture with cholesterol (Chol) were used as composition lipids. It was shown that the active encapsulation method using the creation of ammonium sulphate pH gradient provided the best results: 41.7% encapsulation efficiency (according to NMR spectroscopy) when using DPPC:Chol (7:3) mixture as lipids. Moreover, the properties of the liposomes were studied. Using the dynamic light scattering and electron microscopy methods carnosine liposomes (DPPC:Chol) were shown to be spherical nanoparticles with an average size of 133 nm. Carnosine release kinetics studied with the use of a France's cell showed that carnosine was released in 24 hours (liposomal composition DPPC:Chol was 7:3). A study of carnosinase action on liposomal carnosine showed that the maximum amount of carnosine remained unchanged in DPPC:Chol liposomes (7:3). The results of the study make it possible to conclude that liposomal carnosine has a better activity in the human organism.*

Keywords: *carnosine, liposomes, dipalmitoylphosphatidylcholine, active carnosine encapsulation methods, carnosinase.*

Introduction

In recent years the interest of researchers in carnosine – a natural dipeptide known for more than 100 years – considerably increased. Carnosine can be found in muscular and nervous tissues of humans and other mammals. It performs the functions of a hydrophilic intracellular antioxidant having anti-ischemic, reparative and anticataract value [1–3]. Nowadays carnosine is generally applied as a biologically active supplement, and only in cataract it is used in the dosage form of eye drops. In Russia and abroad works for the creation [*]of medicines containing carnosine [4–7] are actively conducted. By now attempts of using carnosine in the form of biologically active

[*]*Original Russian Text © N.A. Antonova, G.M. Sorokoumova, T.N. Fedorova, S.L. Stvolynsky, D.A. Abaimov, V.I. Popenko, V.I. Shvets, 2016, published in Tonkie Khimicheskie Tekhnologii /Fine Chemical Technologies, 2016, Vol. 11, No. 6, pp. 55–62.*

supplement Sevitin for increasing the efficiency of basic therapy in patients with dyscirculatory encephalopathy [8] and Parkinson disease have been made [9]. The neuroprotective value of carnosine was shown with the use of experimental models of parkinsonism, cerebral ischemia, Alzheimer disease, aging, and also with the use of pilot clinical studies. However, in order to achieve stable protective effect in patients it is necessary to introduce high daily doses of carnosine: from 1.5 to 2.0 g. This is necessary to compensate its hydrolysis under the effect of tissular and serumal carnosinase. In order to increase the lifetime of carnosine chemical modification with the preparation of its derivatives is carried out, for example, of esters and their salts [10] that are difficult to degrade by this enzyme.

Another approach that allows protecting a medicine from degradation and at the same time prolonging its action in the organism is the creation of the liposomal form of the active substance. Liposomes are nanoparticles having an internal water space surrounded with a lipidic bilayer [11]. It is due to the existence of the lipidic bilayer that liposomes are capable of isolating a medicine, for example, a dipeptide, from the direct influence of the enzyme. Besides, they are capable of changing the pharmacokinetics of medicines increasing their pharmacological efficiency [12].

Work [13] is dedicated to a study on carnosine inclusion in liposomes consisting of various synthetic lipids: dipalmitoylphosphatidylcholine (DPPtdCho), palmitoyloleoylphosphatidylcholine (POPC) and dioleoylphosphatidylcholine (DOPC). It was shown that the efficiency of carnosine inclusion decreased in the following series of liposomes: DPPtdCho < POPC < DOPC and was equal, respectively, to 21.96, 18.86 and 17.12%. In the mentioned work liposomes were obtained by extrusion with the use of passive loading.

The purpose of this work was to optimize the method for obtaining the liposomal forms of carnosine having higher efficiency of its inclusion; to study their morphology and sizes; to determine the kinetics of carnosine release from liposomes; and to determine the kinetics of carnosinase action on liposomal carnosine.

Experimental

We used carnosine (Yonezawa Hamari Chemicals, Ltd, Japan), dipalmitoylphosphatidyl-choline (DPPtdCho), cholesterol (CS) (Sigma–Aldrich, USA), chloroform, ethanol, distilled water, deuterated water, sodium chloride and chemically pure ammonium sulfate (Chimmed, Russia).

We used a Shimadzu UV-1700 spectrophotometer (Japan) to record electronic spectra and a BrukerBioSpin 300 GmbH spectrometer (Germany) to record ^{1}H NMR spectra. Electronic microphotos were obtained by means of a JEOL 100CX (Japan) electronic microscope. The sizes of liposomes were determined by dynamic light scattering with the use of a Delsa NanoC, Beckman CoulterInc device (USA). Carnosine in the reaction with carnosinase was determined by

chromatography-mass spectrometry with the use of a Surveyor LC PumpPlus chromatograph in combination with a LCQ Fleet mass-spectrometric detector (Thermo Scientific, USA). An Ultrasphere ODS analytical column (250 × 4.6 mm; C18, 100A, 5 μm; Hichrom Ltd, Great Britain) was used.

Carnosine inclusion in liposomes was carried out by passive [14] and active [15] loading.

Preparation of liposomes with carnosine by passive loading. Lipids (40 mg, DPPtdCho or DPPtdCho-CS 7:3 mixture) were placed into a round-bottomed flask and dissolved in chloroform. The solvent was evaporated in a rotary evaporator. The obtained lipidic film was dispersed by an aqueous solution of carnosine with a concentration of 20 mg/ml containing 0.9% of NaCl. By this method multilamellar vesicles (MLV) were obtained. Then unilamellar vesicles (ULV) were obtained by extruding the MLVs at repeated squeezing through a filter with 100 nm pores (19 times). The experiments were made at various pH (6.3; 7.1; 8.2).

Preparation of liposomes with carnosine by active loading. Lipids (40 mg, DPPtdCho or DPPtdCho-CS 7:3 mixture) were placed into a round-bottomed flask and dissolved in chloroform. The solvent was evaporated in a rotary evaporator. The obtained lipidic film was dispersed by 1 ml of a 0.3 M solution of ammonium sulfate. After this ULV were obtained by extrusion (as described above). Then the obtained ULV (500 μl) were applied on a column for NAP-5 exclusion chromatography with Sephadex G25 sorbent in order to separate the liposomes from ammonium sulfate. Then 0.75 ml of the obtained liposomes was selected. A solution of carnosine (0.25 ml, C_{start} = 20 mg/ml) was added, and the medicine was kept on a water bath for 30 min. The experiments were made at 50 and 60°C.

Determination of the efficiency of carnosine inclusion in liposomes by ^{1}H NMR. In order to determine the efficiency of carnosine inclusion in the liposomes by ^{1}H NMR, when preparing ULV, deuterated water was used instead of distilled water and isotonic solution. Solutions with various pH were obtained by DCl addition. The obtained ULV (600 μl) were placed in an ampoule for NMR samples. Spectra were recorded with the use of an NMR spectrometer at a frequency of 300 Hz. The number of scans was 11, the number of points was 128000.

Determination of the rate of carnosine release from the liposomes. In order to determine the rate of carnosine release from the liposomes a Franz cell was used. The experiment was made with the DPPtdCho-CS (7:3) liposomes prepared by active loading. For this purpose liposomes with carnosine were placed into a donor cell, and isotonic solution was placed into the recipient cell. A filter with a pore diameter of 60 nm was installed between the cells. During the first 7 h fractions were collected hour by hour, and then every 24 h. Carnosine concentration was determined by the diazo reaction method described in [16].

Determination of the action of carnosinase contained in blood serum on liposomal carnosine was carried out similarly [17].

Preparation of serum containing carnosinase from donors' blood. Blood was taken from the veins of donors on an empty stomach and centrifuged at 1000 g for 15 min. Serum was separated. Then samples from 5–6 donors were combined, divided into 1.5 ml aliquots and frozen at –80°C. All the participants had signed consent to biomaterial draw.

Carnosinase action on liposomal carnosine. After defrosting 200 μl of serum was preliminarily incubated at 37°C for 30 min in an incubatory medium of the following composition: 800 μl of 125 mM Tris-HCl, pH 7.8; 300 μl of 5 mM of $CdCl_2$ prepared from 30 mM Na citrate; 300 μl H_2O. Then the reaction was started by the addition of 400 μl of a dispersion of the carnosine-containing liposomes or a solution of carnosine. The starting concentration of the substrate was 5 mg/ml by carnosine (22 mM). After 0 – 15 – 30 – 60 – 90 – 120 – 150 – 180 min 100 μl tests of the incubatory mixture were selected and mixed with 400 μl of a cooled 20% aqueous solution of trichloracetic acid for proteins sedimentation in an ultracentrifuge at 16000 g for 10 min at +5°C. The obtained supernatant was transferred into a chromatographic vial. The latter was placed into the autosampler of the chromatograph for chromatography-mass-spectrometry analysis.

The content of carnosine in the liposomes subjected to the action of serumal carnosinase was determined by LCMS. The supernatant (10 μl) prepared as described above was introduced into the chromatograph loop. The mobile phase consisted of two solutions: 10 mM ammonium acetate acidified to pH 3.7 by ice acetic acid (solution A); and acetonitrile – 10 mM ammonium acetate mixture (90:10) (solution B). The solutions ratio was 85:15. The mobile phase flow rate was 0.7 ml/min, separation temperature was 35°C. Chromatography time was 10 min. Carnosine retention time was 5.59±0.1 min. The mass spectrometer operated in the mode of registration of ions that had been positively charged by electrospray at a voltage of 5 kV. Carnosine was determined by the total ionic current of daughter ions in the range of masses from 60 to 250 amu formed upon decomposition of carnosine molecular ion (*m/z* 227.1) at a normalized impact energy of 25 eV. The data were processed by means of the Xcalibur 2.1 Foundation 1.0.1 software. The external standard method was applied for quantitative measurement of carnosine concentration. For calibration, the ratio of carnosine chromatographic peaks areas depending on its concentration was measured. Calibration dependence for carnosine was linear and was described by the following formula: $C_{carn} = 0.28784 \cdot S_{carn}$, where C_{carn} is carnosine concentration (mkg/ml), S_{carn} is the area of the carnosine peak in integration units. Correlation coefficient was $R_2 = 0.997$, which corresponds to appropriate analytical approximation. The limit of quantitative determination was 0.39 mkg/ml. The method was metrologically validated. The method error was no more than 10%.

Results and Discussion

Carnosine is a dipeptide (β-alanyl-L-histidine) very soluble in water. It has different ionized forms at differnent pH. For example, at pH 6.3 its zwitterionic form (70.84%) prevails, and at pH 7.1 and 8.2 carnosine is found in the anion form (72.65 and 95.66%, respectively) [18]. Our purpose was to prepare liposomes characterized by a high inclusion efficiency and to reduce the risk of carnosine egress from the water space of the liposomes. On the basis of literature data [13] and physicochemical properties of carnosine we decided to use the following lipids: DPPtdCho – a synthetic phospholipid containing saturated fatty-acid residues, which provides a rigid structure of the liposomes lipidic bilayer; and a mixture of lipids – DPPtdCho-CS (7:3), because cholesterol introduction makes liposomes even stronger by condensing lipids in the bilayer. At the same time we varied the loading method, the medium pH and temperature to achieve the greatest possible content of carnosine in the liposomes.

The efficiency of carnosine inclusion in the liposomes was determined by ^{1}H NMR. This approach was described in [13]. An advantage of this method is that there is no need to carry out preliminary separation of carnosine included and not included in liposomes by various methods, for example, by gel chromatography. This excludes the possibility of carnosine egress from liposomes. The possibility of identifying liposomal and free carnosine in ^{1}H NMR spectra is due to the fact that a carnosine molecule contains two pH-sensitive protons in the imidazole histidine ring. These protons have signals differing in chemical shifts in ^{1}H NMR spectra. Figure 1 shows the region of protons *a* and *b* of the carnosine imidazole ring in ^{1}H NMR spectra of carnosine in aqueous solutions at various pH.

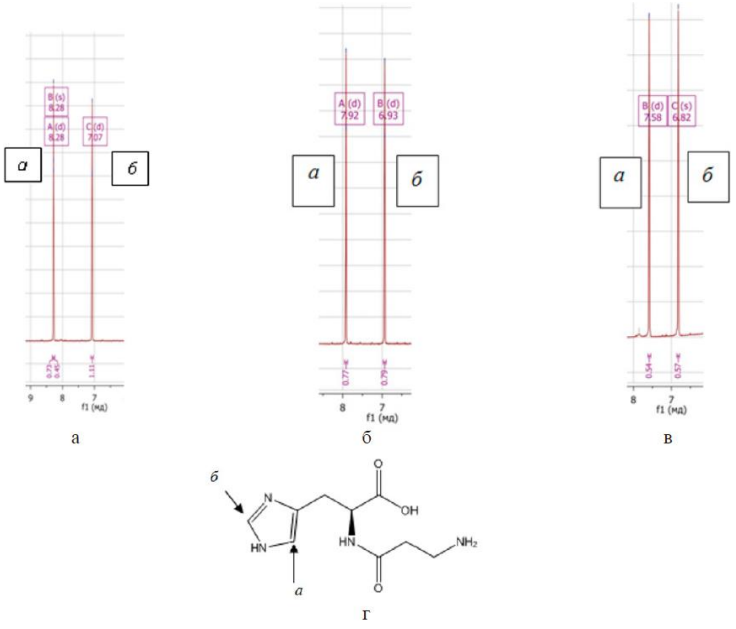

Figure 1. Fragments of ^{1}H NMR spectra of carnosine in aqueous solutions at various pH: a) 6.3; b) 7.1; c) 8.2 in the region of protons *a* and *b* of the imidazole ring of carnosine (d). [а (a) means a (*a*), б (*б*) means b (*b*), в means c, г means d, мд means ppm]

It can be seen from Figure 1 that the chemical shift of proton *a* signal at pH 6.3 is 8.28 ppm, and that of proton *b* – 7.07 ppm. At pH 7.1 the chemical shift of proton *a* signal is 7.92 ppm, and that of proton – 6.93 ppm. At pH 8.2 the chemical shift of proton *a* signal is 7.57 ppm, and that of proton b – 6.81 ppm. Thus, the chemical shift changes depending on pH are most pronounced in case of signals of protons *a* of the histidine imidazole ring: as pH increases, the signal is shifted to the weak field. It is this property of carnosine that we used in further work.

At first carnosine inclusion in the liposomes was carried out by the passive loading method.

^{1}H NMR analysis of the samples of the liposomes containing carnosine obtained at various pH by the method of passive loading revealed that the signals of the imidazole ring proton *a* of carnosine included in the liposomes changed their position depending on pH. Thus, at pH 6.3 a signal in the weak field region at 8.03 ppm (attributed to liposomal carnosine) appeared along with a signal at 8.3 ppm (attributed to free carnosine). At pH 7.1 no second signal was recorded, i.e., there was no signal shift. At pH 8.2 a new (second) high-field signal (attributed to liposomal carnosine) appeared at 7.86 ppm, and the signal at 7.60 ppm indicated the existence of free carnosine (Figure 2). Thus, as shown in work [13] and confirmed by us, apparently, pH close to 7.1 is created in liposomes. Therefore, in the liposomes obtained at pH 7.1 the signals of the imidazole ring protons of liposomal and free carnosine do not differ. In the samples obtained at other pH values carnosine included in liposomes can be determined by the imidazole proton *a* signal: 8.03 ppm at pH 6.3 or 7.86 m at pH 8.2 (Figure 2).

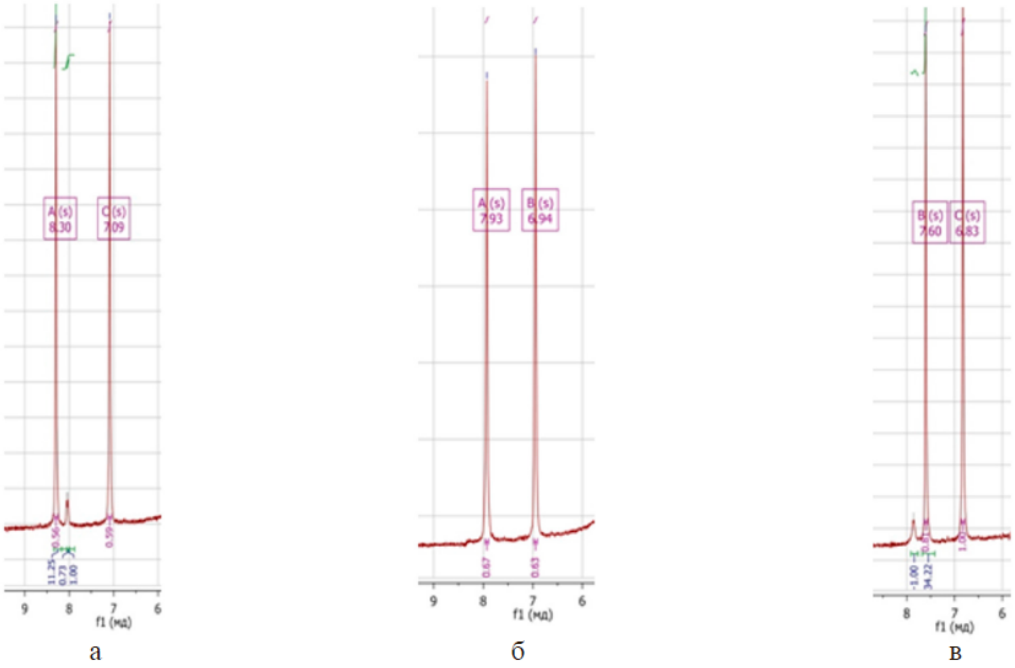

Figure 2. Fragments of ^{1}H NMR spectra in the region of imidazole ring protons of free and liposomal (DPPtdCho-CS (7:3)) carnosine obtained at passive loading at various pH: a) 6.3; b) 7.1; c) 8.2.

[а means a, б means b, в means c, мд means ppm]

The efficiency of carnosine inclusion (IE) in liposomes was calculated by the integration of signals of imidazole protons *a* corresponding to liposomal and free carnosine according to the following formula:

$$EI = (m_{\text{carnosine in liposomes}}/m_{\text{orig. carnosine.}})\times 100\% =$$

$$(I_{\text{carnosine in liposomes}}/(I_{\text{carnosine in liposomes}} + I_{\text{free carnosine}}))\times 100\%$$

The results of experiments with the liposomes obtained by passive loading are presented in Table 1.

Table 1. Dependence of efficiency of carnosine inclusion determined by ^{1}H NMR on lipidic structure of liposomes (C_{lipids} = 40 mg/ml) and medium pH at passive loading

Liposomes composition	Medium pH	Inclusion efficiency, %
DPPtdCho	6.3	8±5
DPPtdCho	7.1	not determined
DPPtdCho	8.2	7±5
DPPtdCho-CS (7:3)	8.2	11.5±5

It can be seen from Table 1 that according to NMR the maximum efficiency of carnosine inclusion in liposomes within the passive loading method (11.5%) was attained at the lipidic composition of liposomes DPPtdCho-CS (7:3) and pH 8.2. This IE value is close to the results of [13].

The following stage of our work aimed at achieving higher efficiency of carnosine inclusion in liposomes was the use of the active loading method by the creation of pH gradient by means of ammonium sulfate. Such approach is described in detail in [14, 15, 19].

In this case, ^{1}H NMR analysis of samples of liposomes with carnosine (pH 8.2) showed that the chemical shift of proton *a* of the imidazole ring of carnosine included in liposomes was equal to 7.96 ppm, and in case of free carnosine, 7.65 ppm. Carnosine IE was calculated by the above formula.

The results of the effect of the lipidic composition of liposomes and temperature on the efficiency of carnosine inclusion in liposomes obtained by active loading are presented in Table 2.

Table 2. Dependence of efficiency of carnosine inclusion on lipidic composition of liposomes (C_{lipids} = 40 mg/ml) and temperature and sizes of liposomes with carnosine at active loading, pH 8.2

Composition of liposomes	Temperature, °C	Inclusion efficiency, %	Average diameter of liposomes, Nm
DPPtdCho	50 AND 60	40.5±5	1941±20
DPPtdCho-CS (7:3)	60	41.7±5	133±20

Changing the active loading temperature did not affect the efficiency of carnosine inclusion in the liposomes. The highest carnosine inclusion in the liposomes determined by ^{1}H NMR was reached at lipidic composition of the liposomes DPPtdCho-CS (7:3) and was equal to 41.7%. The content of carnosine in the DPPtdCho liposomes was slightly lower.

Table 2 shows the average diameter of the liposomes obtained by active loading measured by means of dynamic light scattering. Analysis of the sizes of the obtained liposomes showed that the carnosine-containing DPPtdCho liposomes are unstable and eventually undergo aggregation (the particles reach the sizes of 2 microns). At the same time the carnosine-containing liposomes with the lipidic composition DPPtdCho-CS (7:3) are most stable and maintain their nanosizes for a long time.

An electronic microphoto of carnosine-containing DPPtdCho:CS (7:3) liposomes obtained by the method of active loading is presented in Figure 3. It can be seen that the liposomes have a spherical shape, a smooth surface and are homogeneous in size.

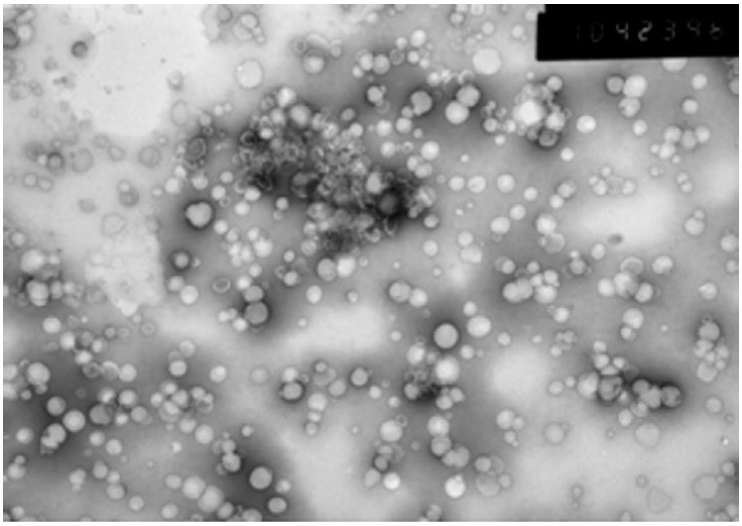

Figure 3. Microphoto of carnosine-containing liposomes of lipidic composition DPPtdCho-CS (7:3) obtained by active loading (10000-fold magnification).

One of the main characteristics of the liposomal form of a medicine is the rate of the medicinal substance release from the liposomes. In order to determine the rate of carnosine release from the liposomes a Franz cell was used. We analyzed liposomes of composition DPPtdCho-CS. The control sample was an aqueous solution of carnosine. The experimental results are presented in Figure 4.

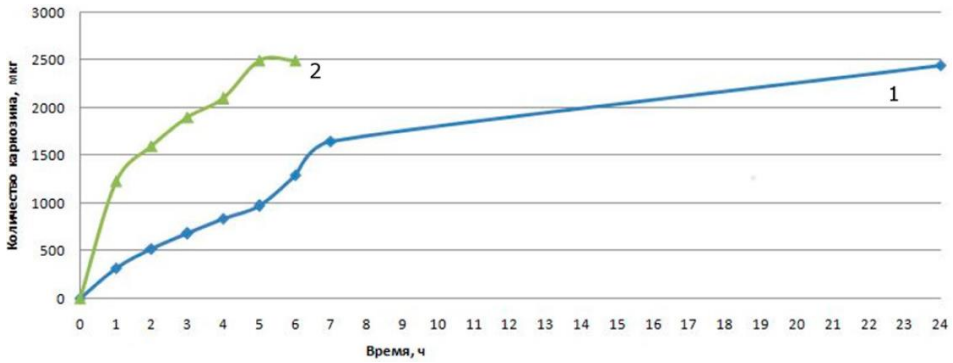

Figure 4. Kinetics of carnosine release from liposomes with lipidic composition DPPtdCho-CS (7:3) (1) and diffusion of carnosine from control solution, $C_{carnosine}$ = 2.5 mg/ml (2) determined with the use of Franz cell.
[Количество карнозина, мкг means Amount of carnosine, μg; Время, ч means Time, h]

It can be seen from the submitted data that carnosine diffusion from solution occurred during 6 h, and carnosine was released from the liposomes during 24 h.

At the final stage of the study the effect of serumal carnosinase on liposomal carnosine was determined. For this purpose samples of carnosine-containing liposomes were incubated with donor serum under the conditions described in the Experimental part. The content of carnosine in the samples after carnosinase action was determined by reverse phase HPLC with the use of a mass-spectrometric detector. The results are presented in Figure 5.

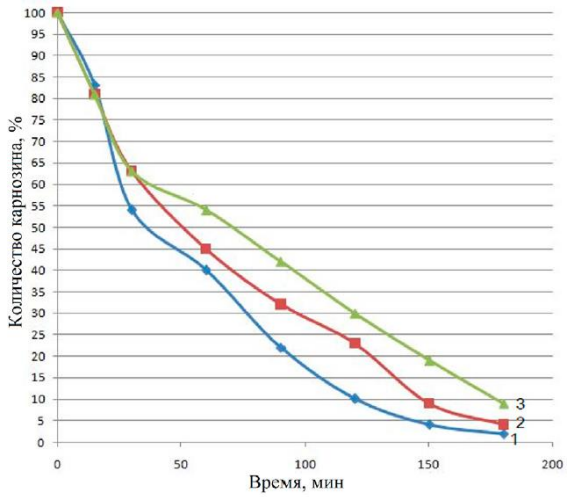

Figure 5. Dynamics of change in carnosine content under the conditions of hydrolysis by serumal carnosinase in solution (1) and in a dispersion of carnosine-containing liposomes of different lipidic structure: DPPtdCho (2), DPPtdCho-CS (7:3) (3).
[Количество карнозина, % means Content of carnosine, %; Время, мин means Time, min]

Analysis of the results showed that carnosine is best protected in the liposomes of lipidic composition DPPtdCho-CS (7:3). Thus, after incubation of these liposomes for 3 h with carnosinase about 12% of native (not hydrolyzed into amino acids) carnosine remained. In contrast, carnosine content in the DPPtdCho liposomes was only 7%.

CONCLUSION

In the course of the study, conditions for preparing liposomes providing the highest possible efficiency of carnosine inclusion were found (composition of liposomes DPPtdCho-CS, 7:3). It was shown that the obtained liposomes are spherical nanoparticles homogeneous in size. Carnosine is released from them gradually, and carnosine encapsulated in these liposomes is better protected from the effect of carnosinase.

The preparation of liposomal forms of carnosine opens the prospects of conducting experimental studies for the purpose of estimating the efficiency of their neuroprotective effect in models of central nervous system diseases and developing a new pharmaceutical composition.

Acknowledgement

The work on the preparation of the liposomal form of carnosine was carried out within the Russian President's grant for the state support of the leading schools of sciences, registration number HIII-7946.2016.11.

References:

1. Boldyrev A.A. Carnosine: Biological significance and application in medicine. Moscow: MSU Publ., 1998. 326 p. (in Russ.).
2. Boldyrev A.A., Aldini G., Derave W. Physiological Reviews. 2013. V. 93. № 4. P. 1803–1845. DOI: 10.1152/physrev.00039.2012
3. Fadeeva D.A., Khalikova M.A., Zhilyakova E.T., Novikov O.O., Novikova M.Yu., Popov N.N., Sorokopudov V.N. Nauchnye vedomosti Belgorodskogo gos. uneversiteta. Ser. Medicina. Farmatsiya (Journal of BGU. Ser. Medicine. Pharmacy). 2010. № 22 (93). Is. 12. P. 179–184. (in Russ.). DOI: 10.1089/rej.2008.0716
4. . Yarigina E.G., Prokop'eva V.D., Bokhan N.A. Uspehi sovremennogo estestvoznaniya (Successes of Modern Natural Sciences). 2015. № 4. P. 106–113. (in Russ.).
5. Cararo J.H., Streck E.L., Schuck P.F., Ferreira G. da C. Aging and Disease. 2015. V. 6. № 5. P. 369–379. DOI: 10.14336/AD.2015.0616
6. Babizhayev M.A., Micans P., Guiotto A., Kasus- Jacobi A. Amer. J. Therapeutics. 2009. V. 16. P. 517–533. DOI: 10.1097/MJT.0b013e318195e327

7. Kaptsov V.V., Boldyrev A.A., Kulebyakin K.Yu., Stvolynsky S.L., Suslina Z.A., Illarioshkin S.N. The use of L-carnosine for the preparation of nanopreparation possessing antihypoxic and antioxidant activity: pat. 2482867 Russian Federation. № 2012110343/15; appl. 20.03.2012; publ. 27.05.2013. (in Russ.).

8. Fedorova T.N., Belyaev M.S., Trunova O.A., Gnezdizkiy V.V., Maximova M.Yu., Boldyrev A.A. Biologicheskie membrany (Biological Membranes). 2008. V. 25. № 6. P. 479–483. (in Russ.).

9. Boldyrev A., Fedorova T., Stepanova M., Dobrotvorskaya I., Kozlova E., Boldanova N., Bagyeva G., Ivanova-Smolenskaya I., Illarioshkin S. Rejuvenation Research. 2008. V. 11. № 4. P. 821–827. DOI: 10.1089/ rej.2008.0716

10. Boldyrev A.A., Nikanorov V.A., Khrebtova S.B., Bulygina E.R., Kramarenko G.G., Leinsoo T.A., Sorokina E.V., Stvolynsky S.L., Tyulina O.V., Fedorova T.N., Yuneva V.O. A method of producing esters of L-carnosine and their salts: pat. 2188204 Russian Federation. № 20011111960; appl. 26.04.2001; publ. 27.08.2002. (in Russ.).

11. Kaplun A.P., Le Bang Sh., Krasnopolskiy Yu.M., Shvets V.I. Voprosy Med. Khimii (Questions of Medical Chemistry). 1999. V. 45. № 1. P. 3–12. (in Russ.).

12. Tarakhovskiy Yu.S. Intelligent lipid nanocontainers in the targeted drugs delivery. Moscow: LKI Publ., 2011. 280 p. (in Russ.).

13. Maherani B., Arab-Tehrany E., Kheirolomoom A., Cleymand F., Linder M. Food Chemistry. 2012. V. 134. P. 632–640. DOI: 10.1016/j.foodchem.2012.02.098

14. Tazina E.V., Kostin K.V., Oborotova N.A. Khimiko-pharmatcevticheskiy zhurnal (Chem.-Pharm. Journal). 2011. V. 45. № 8. P. 30–40 (in Russ.).

15. Baryshnikov A.Yu. Vestnik Rossiyskoi Akademii Meditsinskikh Nauk (Herald of Russian Academy of Medical Sciences). 2012. № 3. P. 23–31 (in Russ.).

16. Severin S.E., Solov'eva G.A. Biochemistry practicum. Moscow: MSU Publ., 1989. 509 p. (in Russ.).

17. Pegova A., Abe H., Boldyrev A. Comp. Biochem. Physiol. 2000. V. 127. Part B. P. 443–446. DOI: 10.1016/S0305-0491(00)00279-0

18. Chemicalize.org byChemAxon [web resource] – access mode: http://www.chemicalize.org/ – (accessed date: 08.08.2016).

19. Sousa I., Gameiro P. J. Pharmaceut. and Drug Delivery Res. 2013. V. 2. Is. 2. P. 1–4. DOI: 10.4172/2325-9604.1000114