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NANOBIOTECHNOLOGICAL OBTAINING OF LIPOSOMAL FORMS OF ANTIOXIDANT PREPARATIONS BASED ON BIOFLAVONOIDS

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Більшість патологічних станів супроводжується перекисним окисненням ліпідів і накопиченням продуктів оксидативного стресу. Відома антиоксидантна дія природних гідрофобних сполук, таких як кверцетин, убіхінон, куркумін, вітамін Е та ін. Крім того відомо, що ці біологічно активні сполуки діють на різні ланки антиоксидантної системи. Однак, їх використання у складі парентеральних препаратів ускладнено, враховуючи їх гідрофобність. Для підвищення біодоступності ліпофільних антиоксидантів і створення їх водорозчинної форми використовують наночастинки, наприклад, ліпосоми.

***Метою роботи** є розробка ліпосомального препарату з соінкапсуляцією двох гідрофобних антиоксидантів, а саме куркуміну та кверцетину.*

***Методи.** При розробці використовувалися технологічні методи отримання ліпосом та аналітичні фізико-хімічні, хроматографічні (ВЕРХ, ТШХ, ГРХ), методи визначення розміру часток, pH.*

***Результати.** В результаті проведеного дослідження запропоновано склад і технологію одержання ліпосомальної форми куркуміну та його композиції з кверцетином. Вивчено вплив жирно-кислотного складу ліпідів, співвідношення «ліпід: діюча речовина» та технологічних умов на утворення ліпосом та ступінь інкапсуляції активного фармацевтичного інгредієнта. Вивчено залежність розмірів наночастинок від значення тиску і кількості циклів гомогенізації. Отримано ліофілізований продукт зі ступенем включення гідрофобних антиоксидантів не менше 85 %. Проведено вивчення фізико-хімічних властивостей отриманих зразків.*

***Висновки.** Запропоновано технологічну схему одержання комплексного препарату, що містить куркумін і кверцетин, що включає отримання ліпідної плівки, гідратацію компонентів, гомогенізацію високого тиску, стерилізуючу фільтрацію і ліофілізацію*

***Ключові слова:** гідрофобні антиоксиданти, біофлаваноїди, куркумін, кверцетин, нанобіотехнологія, ліпосоми, метод одержання ліпосом*

1. Introduction

The creation of nanosized drugs leads to qualitative changes in the physicochemical and pharmacological properties of medicinal substances, that allows to create a new generation of drugs based on liposomal (LS) nanosystems. Liposomes have several advantages: they increase the bioavailability of lipophilic active pharmaceutical ingredients (API), prolong the therapeutic effect of drugs, protect biological membranes from peroxidation, etc. Cardiological, ophthalmic, pulmonological and many other diseases are accompanied by lipid peroxidation of biological membranes. Currently, many studies are focused on creating drugs containing antioxidants of various structures.

Of particular interest are APIs – bioflavonoids (BF), inter alia, quercetin (Quer) and curcumin (Cur), which have high antioxidant activity (AOA) [1]. Both products are plant lipophilic compounds and their use is limited by extremely low bioavailability. An alternative method of creating water-soluble forms of these APIs is to encapsulate them into LS [2, 3]. A significant number of studies have been devoted to the use of LS forms of BFs (Quer, Cur, etc.), confirming their antioxidant, antitumor and anti-inflammatory activity [4, 5]. BFs play an important role in studies of ophthalmic diseases [6]. The possibility of using LS forms of BFs for the prevention and treatment of a number of eye diseases has been established [7]. In our opinion, the use of LS forms of BFs is a promising drug delivery strategy for a number of diseases. The creation of LS forms of APIs based on lipophilic compounds can expand the arsenal of drugs, increase their bioavailability and, as a result, pharmacological action [8]. Our studies have been devoted to the development of LS forms of drugs and the study of their pharmacological properties over a number of years [9].

According to AOA, Quer is one of the known antioxidants that limit the processes of chain reactions of free radical oxidation, preventing excessive oxidation of lipids, proteins and nucleic acids, which protect cell membranes from damage by oxidants. Quer has angioprotective, antioxidant, anti-inflammatory, wound healing and antiviral effects. Currently, the world's first LS form of Quer ("Lipoflavon") is used in clinical practice in Ukraine. “Lipoflavon” is applied in cardiology, oncology, ophthalmology, type 1 and type 2 diabetes, psoriasis, pulmonology, renal failure, nephrotoxicity, dentistry [10].

An antioxidant, successfully used in oncology, ophthalmology, cardiology and other pathologies, is Cur, with respect to which extensive evidence has been accumulated and there is a long-term global experience of observing patients taking *per os* Cur, confirming its safe-

ty and efficacy [11, 12]. Cur is a pleiotropic substance and acts in the body in different directions, exhibiting antioxidant, anti-inflammatory, antitumor properties, etc [13, 14]. It should be noted that the mechanism of action of Cur is now under discussion.

Considering the high AOA of Cur and Quer and their effect on different parts of the antioxidant system, the development of LS complex antioxidant preparation (CAP) and the study of oxidative stress markers on model pathologies is promising [15, 16]. Earlier, researches aimed at creating LS forms of Quer and coenzyme Q_{10}, obtaining their complex preparation with subsequent study of oxidative stress markers on model pathologies have been carried out [17].

The aims of the study were to create LS form of Cur and CAP, containing two antioxidant (Cur and Quer).

2. Planning (methodology) of research

The design of the experiment was conducted taking into account the high AOA of natural BF, the use of which is limited by their low solubility and bioavailability. The encapsulation of BFs into LS membrane allows creating a water-soluble injectable form. The research was carried out according to the following plan:

– study of the conditions of the encapsulation of APIs into LS;

– study of the dependence of the antioxidants encapsulation on the composition of lipid membrane, its charge and technological parameters;

– development of the method for obtaining of LS form of Cur and CAP, containing at least two hydrophobic antioxidants;

– study of the physicochemical properties of monopreparations and CAP.

3. Materials and methods

The researchers used Quer ($C_{15}H1_0O_7$) manufactured by PVP Sociedate Anonima (Brazil) and a highly purified curcuminoid complex obtained according to the developed scheme [18]. Curcuminoids are represented in the used product by diferulomethane (Cur I – $C_{21}H_{20}O_8$) at least 70–75 %, demethoxycurcumin (Cur II – $C_{20}H_{18}O_5$) and bisdemethoxycurcumin (Cur III – $C_{19}H_{16}O_4$) – both fractions 25–30 %. Based on literature data [14], the minor components of curcuminoids can have both antioxidant and anti-inflammatory properties, we used Cur (I, II, III), containing three BF components in the indicated proportions to obtain the LS form. For preparation of LS were purchased phospholipids (PL): phosphatidylcholine of sunflower (SFPC) manufactured by Bioler (Ukraine), egg (EPC), soybean (SPC) and dipalmitoylphosphatidylglycerol (DPHG) – Lipoid (Germany); lactose – Sigma Aldrich; PBS – KH_2PO_4, Na_2HPO_4. The standards of PC and lysophosphatidylcholine (lysoPC) manufactured by Sigma Aldrich were used for analytical studies.

Obtaining of liposomes. Considering the lipophilicity of Cur, LS form of it was obtained by lipid film method followed by the hydration with PBS. The resulting emulsion of multilamellar vesicles was extruded on a Microfluidizer extruder (Microfluidics, USA) to obtain monolamellar LS with a size of 150–-220 nm. The resulting emulsion was subjected to sterile filtration through a cascade of PALL Suppor filters (USA). In developing technology of LS trehalose or lactose were used as cryoprotectants. LS form of Quer was obtained on a previously developed technological platform [19]. The CAP was obtained at ratio of antioxidants 1: 1. The emulsion of the CAP was placed in 20 ml vials and lyophilized (Martin Christ 2-6-D) in the presence of a cryoprotectant.

Analytical studies. The HPLC and TLC methods were used for qualitative and quantitative analysis of Cur and Quer.

HPLC analysis of Cur was performed using a Shimadzu Prominence LC-20 chromatograph with a SPD-M20A diode array detector, a Shim-pack GISS C18 column (5 μm, 250x4.6 mm), a column thermostat CTO-20AC, a column temperature of 30 °C; a mobile phase of water for chromatography: acetonitrile (54:46), glacial acetic acid (pH 3.0 ± 0.05); detection at a wavelength of 427 nm. Sample volumes were 2–10 μl.

HPLC analysis of Quer was performed in isocratic mode on a Shimadzu LC 20 chromatograph with a chromatographic column (250x4.6 mm) filled with a L1 sorbent with particle size of 5 μm Waters Xbridge; a mobile phase of methanol: water: phosphoric acid (100:100:1); a flow rate of the mobile phase 1 ml/min; detection at a wavelength of 255 nm; a column temperature of 30 °C. Sample volume was 20 μl. The average retention time of Quer was 38 ± 0.5 minutes, which corresponds to the retention time of the standard Quer sample. Impurities Quer (kempferol, isoramnetin) were not more than 2.0 %, which corresponds to the data specified by the manufacturer.

TLC was performed on plates on an aluminum substrate manufactured by Sigma Aldrich with chloroform: methanol (98:2) as mobile phase.

The presence of residual solvents in LS was determined by GLC on a Shimadzu GC-2014 ATF/SPL gas chromatograph with AOS-6000 universal autosampler, a column SH-Rtx-624 MS (30 m, 0.32 mm, 5 mkm); an input method – Static Head Space; a sample temperature of 100 °C; a pre-temperature control with shaking for 15 min; a syringe temperature of 120 °C; an injector temperature of 200 °C; a detector temperature of 300 °C; a carrier gas – helium; a gas velocity – 35 cm/sec.; a flow rate – 2.16 ml/min; DMF as a solvent; a volume of the injection – 0.5 ml.

The size of LS was measured on a Malvern Zetasizer Nano ZS nanosizer (UK) using a semiconductor laser at a wavelength of 375 nm and a temperature of 30 °C.

The oxidation index was determined by UV spectrophotometry at a wavelength of 210 and 233 nm.

The determination of the statistical reliability of the experimental results and the suitability of chromatographic systems ware carried out in accordance with the State Pharmacopoeia of Ukraine 2.0.

4. Results

Initially, the conditions for drug encapsulation into LS were studied. Encapsulation of API into LS is one of the most important steps in obtaining LS drugs. To encapsulate Cur into LS we used the lipid film method taking into account its hydrophobicity. The lipid film

method allows one to obtain LS drugs mainly with lipophilic APIs [13]. The principle of the method is to obtain a solution of lipids and lipophilic API in organic solvents, followed by the formation of a film containing lipids and API. Since Cur is a lipophilic compound, an organic solvent is used to dissolve it, which meets the necessary requirements: complete dissolution of Cur and PC, the absence of solvent influence on the structure of the substance, the possibility of use under industrial conditions and the absence of toxic products in the solvent. In this regard, ethanol, chloroform, methanol and mixtures thereof have been studied. A series of experiments showed that the optimal solvent for these active ingredients is ethanol 96 %. We have compared three types of PC in the formation of aqueous emulsions. We used three natural PCs to obtain Ls: SPC, EPC and SFPC. First of all, we took into account the level of oxidation of fatty acids, that were estimated by the value of the oxidation index. EPC, SPC and SFPC had oxidation indices of 0.23–0.25, at least 0.4 and 0.48-0.5, respectively. It should also be noted that SFPC had an extremely low solubility in alcohols, which limits its use in a lipid film obtaining. It was found that EPC has maximum solubility in ethanol and forms a homogeneous emulsion in the aqueous medium, while SPC and SFPC have low solubility in ethanol and form unstable emulsions in the aqueous medium. Apparently, the differences between various PCs are related to their fatty acid composition. Cur was dissolved in ethanol and mixed with ethanol PC solution. The ethanol solution was filtered through 0.2 μm filters. Solutions were prepared in various ratios and concentrations: Cur:lipids (1:10 – 1:40). The solution was concentrated on a Buchi rotary evaporator at a water bath temperature of 41±1.0 °C, at a pressure of 14–15 bar and rotor speed of 45 rpm. The resulting lipid film containing lipids and Cur was treated with nitrogen for 30 minutes and hydrated in a buffer solution containing sugar stabilizers for 2 hours to obtain multilamellar vesicles. In obtained vesicles we investigated the effects of a number of factors affecting their stability and size: temperature (from 30 to 45 °C), pH (from 5.5 to 7.5), as well as the ionic strength of the buffer, lipid concentration and ratio "lipid : API". The size of the resulting vesicles was also determined by the intensity and time of mixing. To prevent lipid oxidation processes, the resulting emulsion was saturated with nitrogen.

As a result of the experiments, it was established that Cur and PLs used for lipid film obtaining are completely soluble in ethanol. In this case, a homogeneous film is formed and suspended in aqueous solvent. The optimal pH value is 6.5–7.0, that enables to obtain a homogeneous emulsion with a vesicle size from 2.5 μm to 3 μm. The use of solutions with other pH values leads to the heterogeneity of the emulsion, making it difficult for dispersion of the emulsion in the homogenizer further. Saturation of the emulsion with an inert gas or nitrogen maintains the lipid oxidation index (0.28–0.32) within the original PC index – about 0.25–0.27. We found that the optimum content of curcumin in the product is about 3 % relative to the PC component. An increase in Cur content in the samples led to its incomplete encapsulation into LS.

LS was obtained by high-pressure homogenization at various pressure value. Initially, homogenization was carried out at 500 bar (3 cycles) and in the next place at 800 bar (5 cycles). When studying the dynamics of the formation of LS and encapsulation of Cur, it was found that at the 1st and 2nd cycle (500 bar) the particle size was more than 350 nm, at the 3rd cycle the main amount of LS was represented by a size of 180–220 nm, with particles larger than 1 μm (2 %). A further transition to 800 bar led to the formation of LS, the bulk of which corresponded to sizes 140–160 nm at 4th and 5th cycles, 130–150 nm – at 6th and 7th cycles. Further homogenization at the indicated conditions (8th cycle) did not lead to a change in LS size. The encapsulation of Cur into LS was 85–90 %. At the same time, it was found that homogenization at 9–10 cycles not only leads to increase the LS size, but also to decrease the encapsulation of Cur to 75–80 %. The next step was sterile filtration through 0.2 μm filter in order to remove non-encapsulated Cur and obtain sterile samples. However, the process of sterile filtration was quite slow. According to literary sources and the data obtained earlier in the development of LS forms of APIs, it was established that encapsulation of anionic PL (cardiolipin, phosphatidylinositol, DPPG) into the LS bilayer significantly increases the filtration efficiency and the level of encapsulation of drug substances into LS [13]. We used DPPG in various concentrations: from 5 to 12 %. Considering the introduction of DPPG into the composition, we changed the solvent: a mixture of ethanol and chloroform. The introduction of DPPG in LS in amount of 10 % led to the sterile filtration efficiency, while the level of encapsulated Cur has not changed. The oxidation index of LS obtained by the developed method has not exceed 0.28–0.33 (the initial oxidation index of PC is not more than 0.25). Considering that organic solvents (hexane, ethanol and chloroform) are used in obtaining PLs, Cur and lipid films, the residual solvents were determined in LS samples in accordance with requirements of the State Pharmacopoeia of Ukraine. Hexane, chloroform and ethanol were absent in LS samples. The suggested technological conditions make it possible to obtain LS form of Cur with standard composition, the bulk of which after sterile filtration through 0.2 μm filter is represented by nanoparticles of 130–140 nm – not less than 75 %, the rest – 20.0–25.0 nm. By HPLC method, it was found that Cur is stable during homogenization of the lipid emulsion as evidenced by the absence of an increase in the impurities and a decrease in the amount of Cur after treatment at a temperature of 37–43 °C and a pressure value of 800 bar. In addition, the amount of PC hydrolysis products in LS, for example, lysoPC, practically have not increased. By TLC method, it was shown that the amount of lysoPC in the samples after the fifth cycle of homogenization at 800 bar did not exceed 0.6-0.65 % (with the content of lysoPC in the initial PC of 0.55–0.6 %). When studying the fractional composition of the obtained LS samples by HPLC, it was demonstrated that the samples contain diferulomethane – at least 70 % (retention time 14.605±0.002), dimethoxycurcumin and bisdemetoxicurcumin – no more than 30.0 % (retention time 13.584±0.002 and 12.630±0.002 respectively).

LS form of Quer was obtained according to the developed technique [19]. To obtain the CAP the lipid film method, high-pressure homogenization and sterilizing filtration were used. The characteristics of the obtained products are shown in Table 1.

The CAP has been used in the study of oxidative stress markers on model pathologies when compared with single APIs in the LS form. Received data will be presented in following reports.

Table 1

The characterization of CAP containing Cur and Quer and their monopreparation

Name of sample	Content of API, mg/ml	Content of PC, mg/ml	Content DPPG, mg/ml	Content of lactose, mg/ml	Encapsulation of API into LS, %	Size of LS before lyophilisation, nm	Size of LS after lyophilisation, nm
LS-Cur	0.785	28.0	2.8	42.0	at least 85	130–150	200–280
LS-Quer	0.75	27.5	-	42.0	at least 90	160–180	180–220
LS-Cur +Quer	0.38/ 0.35	27.5	1.4	42.0	at least 85 and at least 90, respectively	160–200	240–300

5. Discussion

Researches aimed at the creation of LS forms of antioxidants are actively carried out. These works are focused on two directions: the creation of LS preparations containing Cur [5, 12] or Quer [4, 20] and the creation of combination of these compounds with other known natural or synthetic antioxidants [15–17]. We consider the use of LS forms containing a complex of antioxidants allows us to expand the arsenal of drugs due to the synergism, high antioxidant activity and bioavailability of lipophilic compounds for intravenous injection. The LS preparations are produced using various technological methods of obtaining and APIs encapsulation into LS: lipid film method, hydration, sonication, high pressure homogenization. The sizes of LS varied from 80 to 300 nm and the level of encapsulation was from 50 to 80 % depending on the method of obtaining. Therefore, for example, complex LS preparation with Cur and vitamin D3 had higher antioxidant activity, then monopreparation [16]. A number of authors obtained LS with Cur, encapsulated in bilayer, and ascorbic acid and superoxide dismutase, encapsulated in aqueous phase [21]. The resulting product had high pharmacological activity. A lyophilized complex LS preparation, containing Quer and coenzyme Q_{10} was studied [17]. In our opinion, discovered synergistic effect of these two compounds is related to acting on different parts of the antioxidant system. Of interest is the work that shows high antioxidant effect of complex of two polyphenols – quercetin and gallate-epigallocatechin isolated from green tea, encapsulated in LS [15]. Considering that we have not found data on the complex use of Cur and Quer in LS form, the research was focused on the obtaining of composition of these bioflavonoids.

After the research was conducted, the formulation and technology for obtaining of LS form of hydrophobic antioxidants, namely, Cur and its composition with Quer, were offered. The effect of lipid composition on the encapsulation of APIs into LS was investigated. It was found that the maximum encapsulation of APIs into LS is observed when using natural EPC in combination with anionic synthetic PL – DPPG. It was shown that the introduction of charged lipids in lipid membrane, namely, long-chain anionic lipids, leads to increasing the space between the layers of LS. Apparently, an increase the interlamellar space of nanoparticle membrane results in increase in the encapsulation of Cur into LS. In addition, a change in the structure of LS with DPPG leads to stabilization of lipid membrane. The introduction of anionic PL also allowed to increase the efficiency of the technological operation – sterile filtration. As a method for LS obtaining, we used high pressure homogenization. It was found that LS with a particle size of 130-150 nm can be obtained with homogenization a 500 bar (3 cycles) and at 800 bar (4 cycles). At the same time, it was shown that an increase in processing time leads to a change in size and a decrease in the level of encapsulation of the API. The optimal technological parameters were established: pH, temperature, ratio of components, etc. The lyophilized products with encapsulation of APIs at least 85 % were obtained. It was established that the size of LS after lyophilization increased, while remaining in the nanoscale (Tab. 1). High value of IPD (0.505) of CAP attracts attention. In our opinion, a high IPD may be related to the presence of two components (Cur and Quer) in the CAP. In addition, this dispersion value may be because curcuminoids are represented by three compounds: diferulomethane, demethoxycurcumin and bisdemetoxicurcumin. Thus, in the resulting CAP there are four fractions of polyphenols that differ in structure and physicochemical properties. The indicated sizes of nanoparticles both in monopreparations and in CAP allow the use of them for intravenous injection. We consider the presented LS product as a promising delivery of antioxidants.

6. Conclusions

The dependence of the encapsulation of Cur into LS on the fatty acid composition, lipid charge and conditions of homogenization was studied.

The technological scheme for obtaining of CAP containing Cur and Quer, involving the obtaining of lipid film, hydration with PBS, high-pressure homogenization, separation of unencapsulated APIs and lyophilization was proposed.

The physicochemical properties of the obtained products were investigated: size of LS, level of APIs encapsulation, quantitative content of components and their impurities.

Conflict of interest

There is no conflict of interest.

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