STUDY OF THE COMPOSITION OF CRYOPROTECTOR AND TECHNOLOGICAL REGIME IN LIOPHILIZATION OF LIPOSOMES WITH OXALIPLATINUM

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Lipophilization is one of the most promising methods for stabilizing the structure of liposomes. Liposomes are known to be an excellent carrier of active pharmaceutical ingredients (API), liposomes possess a unique ability to retain both the native chemical structure of the active and adjuvant components, as well as the three-dimensional structure of liposomal drugs. The most promising, from the technological point of view, is lyophilization method, which allows to preserve both, the native chemical structure of the active substance and preparation's chemical properties. The most promising approach to solving this problem has been initiated and on which the author relies

1. Introduction

Liposomes, as modern drug delivery systems, have been studied for several decades [1]. Over 50 liposomal drugs are licensed, registered and intensively used in various pathological states. It is worth to admit a number of liposomal drugs in the world market with the volume of sales in millions of US dollars, such as AmbiSome (lyophilized liposome form of amphotericin B), Myocet (liposomal form of doxorubicin hydrochloride), Visudine (liposomal form of verteporfin), Mepact (liposomal form of immunomodulator of mifamurtide). Another promising group of preparations, is one that contain platinum, in particular oxaliplatin, as well as preparations of the group of camptothecins, for example, irinotecan, which demonstrate efficacy both in monotherapy and in combination with other antitumor medicine [2]. In addition to being able to act as carriers of active pharmaceutical ingredients (API), liposomes possess a unique ability to reduce the toxic effect of cytostatics, which limits the full realization of their potential [3].

2. Formulation of the problem in a general way, the relevance of the theme and its connection with important scientific and practical issues

However, the chemical instability of some APIs in their aqueous solutions, the propensity of the phospholipid bilayer liposomes to oxidation, loss of encapsulation during storage make it difficult to develop liposomal drugs, limiting the launching of these drugs to the pharmaceutical market [4].

3. An analysis of the latest research and publications in which the solution of this problem has been initiated and on which the author relies

There is exists a number of methods for stabilization of liposomal drugs. The most promising, from technological point of view, is lyophilization method, which allows to preserve both, the native chemical structure of active and additional components, as well as the three-dimensional structure of liposomes, even after rehydration process [5].

The lyophilization process is the sublimation of water from the preparation, transformation of water into vapor and its removal, excluding a liquid phase. The main components of the freeze drying system are a sample chamber, a condenser, a vacuum pump, a shelf temperature control system, and a condenser cooling system. The product to be lyophilized is placed in the sample chamber. With the help of the shelf cooling unit, the product is frozen to a temperature of “–40” °C. Condenser shelves, must be cooled to a temperature from “–75” °C to “–80” °C. The chamber is sealed and with a vacuum pump, through the condenser, a pressure of about 0.03 mm Hg is created. Below the triple point (for pure water: 6.1 mbar at 0 °C) there are only a solid and gas phases for water. This physical principle is a base for the process of sublimation drying. Ice from the preparation, is sublimated at the temperature of the shelves, and then into water vapor. At the same time, take place its mass transfer to the condenser chamber, where, at a lower
temperature, it precipitates as ice on the condenser’s shelves [6].

The sublimation process can be divided into two physical stages – sublimation of ice from the production and its capture by condenser shelves. The degree of water transfer, as well as the speed of the process is regulated by the temperature program of the shelves in product chamber and the total process time [7].

In Fig. 1 showed the type of vial with the product during the primary drying process. Part of the ice, at the top of the vial, sublimated, opening the 3-D structure of the preparation, and in the same time, the process of evaporation in the lower part of the product takes place.

Three main technological stages of the lyophilization process can be distinguished: freezing, primary drying, and secondary drying, when the bound and occlusal water is removed.

4. The selection of previously unsolved parts of the general problem, which is devoted to the article

The lyophilization process is characterized by a number of critical points - such as the total drying time, the secondary drying temperature, which should be higher than the melting point of the solvent being removed, but at the same time not destroy the matrix of nanoparticles. [8]. The most important stage in the development of liposomal drugs is the selection of a cryoprotectant, which in a dried preparation preserves the 3-D structure and prevents the collapse of the phospholipid bilayer.

5. Formulating the goals (objectives) of the article
The aims of the study are to obtain liposomes with oxaliplatin; to determine the type of cryoprotectant and its concentration; to study lyophilization parameters to manufacture the product with maximum encapsulation parameters, along with remaining the size of liposomes in the nanoscale and with optimum residual moisture content.

6. Statement of the main research material (methods and objects) with justification of the results
For preparation of liposomes was purchased egg phosphatidylcholine from Lipoid, Germany. Cholesterol, citric acid monohydrate, trehalose dihydrate, solvents were purchased from Sigma-Aldrich, USA. The lipid film was prepared on a Buchi 210 rotary evaporator with a vacuum controller, at a residual pressure of 0.02 atm. For homogenization we used a high pressure extrusion method. The extrusion was carried out using a Microfluidiser M-110P model from Microfluidics, USA at a pressure of 1500 atm. The size of the liposomes was determined at a temperature of 20°С on a Zetasizer Nano ZS, Malvern Instruments, UK. Ultrafiltration was carried out on a Minim2 model, FALL, USA. Lyophilization was carried out in the Quarco model, P.R.C. The residual water content was determined by K. Fisher’s method, on a V-20 Mettler Toledo, USA. The encapsulation degree was determined by HPLC on a LC-20 instrument Shimadzu, Japan, according to a method developed earlier [9]. The preparation was sterile filtered and bottled in aseptic conditions in sterile vials VAT050-2C, 50 ml capacity, manufactured by Schott, Germany.

Liposomal oxaliplatin was prepared by the method of “passive” encapsulation in combination with the method of ion sorption. Lipids: PC/Chol/DPPC in a ratio of 50/20/30 (by weight) was placed in a round-bottomed flask, dissolved in a chloroform-anhydrous ethanol mixture with short-term exposure to ultrasound (at 35 kHz) until the opalescence disappeared. The resulting lipid film was hydrated with a solution of oxaliplatin at a concentration of 4 mg/ml for 60 minutes at 20 °C.

Homogenization was carried out on a high pressure homogenizer until a liposomes with size of 80120 nm was obtained. The concentration of oxaliplatin in the obtained liposomes was adjusted by water for injection. In the final product, the concentration of oxaliplatin was 2 mg/ml, the total lipid concentration was 20 mg/ml.

A screening experiment was conducted to select the most prospective cryoprotectant for liposomal oxaliplatin. A 52-hour drying program was used. Encapsulation of oxaliplatin in liposomes before lyophilization was 62 %, the size of liposomes was 112 nm. The results of the experiment are presented in Table 1.

<table>
<thead>
<tr>
<th>No.</th>
<th>Cryoprotectant</th>
<th>The oxaliplatin concentration, mg/ml</th>
<th>Size of liposomes, nm</th>
<th>Oxaliplatin encapsulation, %</th>
<th>Water content, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Lactose monohydrate, 5 %</td>
<td>2.0</td>
<td>116</td>
<td>42</td>
<td>0.8</td>
</tr>
<tr>
<td>2</td>
<td>Sucrose, 5 %</td>
<td>2.0</td>
<td>110</td>
<td>37</td>
<td>0.7</td>
</tr>
<tr>
<td>3</td>
<td>Trehalose dihydrate, 5 %</td>
<td>2.0</td>
<td>114</td>
<td>46</td>
<td>0.8</td>
</tr>
<tr>
<td>4</td>
<td>Maltose, 5 %</td>
<td>2.0</td>
<td>112</td>
<td>35</td>
<td>0.5</td>
</tr>
</tbody>
</table>
It can be seen from Table 1 that after use of different cryoprotectants and a 52-hour drying program, the encapsulation is reduced from 62% to 35–46%, while the size of the liposomes remains practically unchanged. A low variance of the encapsulation meaning is explained by the loss of encapsulated oxaliplatin, while the ion-sorption capture mechanism operates with any type of cryoprotector [10]. The low content of residual water is explained by the high duration of the lyophilization process – 52 hours (3120 min).

The best result for the encapsulation of oxaliplatin into liposomes after lyophilization was observed with trehalose dihydrate as cryoprotectant. To study the effect of different cryoprotectant content on the degree of encapsulation and the size of liposomes, an experiment was carried out. The cryoprotectant was studied in concentrations of 4%, 6%, 8%, 10%. Liposomes were prepared according to the previously described technology, a cryoprotectant solution filtered through a polyethersulfone filter with a pore size of 0.22 μm was added in the last step, with a final concentration of oxaliplatin of 2 mg/ml and a lipid concentration of 20 mg/ml. The results of the experiment are shown in Fig. 2.

As can be seen from Fig. 2, the optimum concentration of the cryoprotectant is 80 mg/ml or 8%. Also, was carried out an additional experiment to optimize the time of freeze-drying process. The 52-hour program, used in the screening experiments, showed residual water content of less than 0.8%, what can be evidence for excessive duration of the process. So, on the next step was studied the process of lyophilization with duration of 29 hours (1740 minutes), similar to one that used in the lyophilization of liposomes with irinotecan [article in press].

In Fig. 3 are shown the results obtained during the process of lyophilization within 29 hours.

After the use of the drying process of 29 hours it has been found that the residual water content is 7.4%, what is out of specification limits. It can be seen from Fig. 3 that the temperature of the product in the vicinity of the 1300th minute do not reach the temperature of the shelves, which is evidence of the non-complete primary drying stage, and indicating the presence of residual water in the sample. After the end of the process, it was noted that the lyophilized mass had a "foamy" appearance, indicating that there was a significant amount of moisture in the stage of secondary drying. Differences in the technological parameters in compare to liposomal irinotecan can be explained by the presence in the lipid bilayer of liposomal oxaliplatin polar DPPG modifier, which effectively retains water, in comparison with the neutral membrane of liposomal irinotecan, and requires a longer drying process. Given that the primary drying time was not enough, the process of the program was
Liposome size, residual water content and encapsulation were measured. The size of liposomes was 112 nm, the degree of encapsulation was 57%, while taking into account that analogical values before lyophilization was 65%, the loss was 8%.

This is sufficiently high value, indicating an effectiveness of cryoprotectant and a rational program of lyophilization. The residual water content was 2.3%.

7. Conclusion

A technology for obtaining liposomal oxaliplatin has been proposed and screening studies have been conducted to determine the cryoprotectant. It is proposed to use as a cryoprotector trehalose dihydrate as the most prospective. The effect of different concentrations of trehalose dihydrate on the degree of encapsulation of oxaliplatin in liposomes was studied. It was found that the optimum concentration of the cryoprotectant in the preparation is 8% (by mass).

2. The technological parameters for the process of liposomal oxaliplatin lyophilization were developed: the temperature of secondary drying, the time of secondary drying, the total time of the process.

The decrease in encapsulation of oxaliplatin in liposomes during lyophilization did not exceed 8.0% with a residual water content of about 2.3%.

The size of the liposomes after lyophilization was 112 nm.

References


Introduction
Diabetes mellitus (DM) is the most widespread endocrinological disease, and its complications, especially diabetic cardiomyopathy (DC) decreases quality of patients’ lives and often results in fatal outcome. According to the WHO’s data, 2.2 million deaths induced by cardiovascular complications in case of excessive hyperglycemia were registered at the beginning of 2012 [1, 2].

Formulation of the problem in a general way, the relevance of the theme and its connection with important scientific and practical issues
One of the basic mechanisms of DC development is hyperglycemia-induced oxidative stress (OS) that includes excessive formation of reactive oxygen species (ROS) – superoxide radicals (SRs) as well as antioxidant system defense depletion. SRs have significant implication in the intracellular signaling and mediate various cellular functions, including activation of transcription factors, kinases and ion channels. Furthermore, increased generation of SRs initiates activation of 5 basic signaling ways including in the pathogenesis of diabetic complications, such as: polyol pathway, increased formation of advanced glycation end products (AGEs), elevated expression of the receptor for AGEs and its activating ligands; activation of protein kinase C (PKC) isoforms; hyperactivity of the hexamine pathway [3, 4].

Additionally, overproduction of SR initiate formation of the most aggressive hydroxyl radicals (HO·) and reactive nitrogen species (RNS) in reaction with NO in form of toxic peroxynitrite ONOO−, especially under hypoxia condition [5].

Analysis of recent studies and publications in which a solution of the problem and which draws on the author
It has been proved that hyperglycemia-induced accumulation of mitochondrial ROS is associated with disturbance of transmembrane potential that directly induces opening of the mitochondrial pore and results in mitochondrial dysfunction and DC development [6]. Therefore, the search for new pharmacological schemes in DC treatment which minimize oxidative damage of cells has become an actual problem.

Lately, the effectiveness of synthetic antioxidant N-acetylcysteine (NAC) for correction of cardiovascular