The study of liophilization parameters in the liposomal irinotecan development

The creation of the liposomal irinotecan is one of the main ways to reduce toxicity and increase the effectiveness of chemotherapy. Lyophilization makes it possible to obtain a product with a guaranteed stability of the size and encapsulation efficiency.

Aim. To optimize the content of the cryoprotector in the liposomal irinotecan, and develop lyophilization parameters to produce liposomes with the maximum encapsulation of irinotecan in them, alongside while maintaining the nanosize.

Materials and methods. Egg phosphatidylcholine from Lipoid (Germany) was used for preparation of liposomes. Lyophilization was carried out in a Quarco device (PR China). The encapsulation degree was determined on a Shimadzu LC-20 instrument (Japan) by HPLC method developed earlier.

Results and discussion. The optimal content of the cryoprotector – trehalose dihydrate has been studied. It has been found that the optimal content of trehalose dihydrate is 8 % (w/w). The modes of the product lyophilization have been studied. The secondary drying temperature in the range of 10-20 °C has been determined. At the secondary drying temperature of 10 °C the residual moisture content was 5-8 %, which was beyond the target range. At 20 °C the water content in the lyophilized was 0.5-0.8 %, the loss of encapsulation was up to 20 %. The mode of drying at 15 °C was optimal, while the residual water content in the lyophilized was 1.5-2.8 %, the loss of encapsulation was 13 %. The size of the liposomes after lyophilization and rehydration did not change significantly compared to the initial one.

Conclusions. As a result of the studies, liposomes with irinotecan have been obtained. The content of trehalose dihydrate as a cryoprotector in the range of 4-10 % has been studied. It has been shown that the optimum content of trehalose dihydrate is 8 % (w/w); moreover, the encapsulation decrease in lyophilization is 13 %. The modes of the liposomal irinotecan lyophilization have been studied at the final drying temperature of 10, 15 and 20 °C. It has been found that the optimum final drying temperature is 15 °C.

Key words: liposomes; chemical gradient; high pressure extrusion; cryoprotector; freeze drying
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Исследование параметров лиофилизации при получении липосомальной формы иринотекана

Создание липосомальной формы иринотекана — один из основных путей уменьшения токсичности и повышения эффективности при проведении химиотерапии. Лиофилизация позволяет получать продукт с гарантированным сохранением размеров и параметров инкапсуляции, поэтому исследование параметров данного процесса является актуальным.

Целью исследования являются оптимизация содержания криопротектора в липосомальной форме иринотекана, разработка процесса лиофилизации для получения ЛС с максимальной инкапсуляцией в них иринотекана при сохранении наноразмеров.

Материалы и методы. Для изготовления ЛС использовали яичный фосфатидилхолин фирмы Lipoid, лиофилизацию проводили в аппарате Quarco производства КНР. Определение степени инкапсуляции проводили методом ВЭЖХ на приборе Shimadzu LC-20.

Результаты и их обсуждение. Проведено исследование содержания криопротектора — трегалозы дигидрата. Установлено, что оптимальным является содержание трегалозы дигидрата 8 % (масс). Проведено изучение режимов лиофилизации продукта. Изучена температура вторичного высыхания от 10 до 20 °С. При температуре вторичной сушки 10 °С содержание остаточной влаги составляло 5-8 %, что выходило за целевые рамки. При 20 °С содержание воды в лиофилизате составляло 0,5-0,8 %, потеря инкапсуляции — до 20 %. Оптимальным являлся режим при досушивании при 15 °С, при этом содержание остаточной воды в лиофилизате составило 1,5-2,8 %, потеря инкапсуляции составила 13 %, размер липосом после лиофилизации и регидратации существенно не изменился в сравнении с исходным.

Выводы. В результате проведенных исследований получены лекарственные средства с иринотеканом. Исследовано содержание трегалозы дигидрата в качестве криопротектора в диапазоне от 4 до 10 %. Показано, что оптимальным является содержание трегалозы дигидрата 8 % (мас), при этом снижение инкапсуляции при лиофилизации составляет 13 %. Исследованы режимы лиофилизации ЛС с иринотеканом при температуре досушивания 10, 15, 20 °С. Установлено, что оптимальной является температура досушивания 15 °С.

Ключевые слова: липосомы; химический градиент; экструзия высокого давления; криопротектор; лиофильная сушка

In the middle of the last century the basic principles of lyophilization for water-containing foods were discovered [1]. Over the following years, lyophilization has found wide application not only in food technology, but also in the manufacture of medicines [2]. At present, lyophilization is the main way to preserve labile drug products, including nanostructured forms and biologically active substances [3, 4]. At the same time, along with preservation of the structure and the biological activity, the solubilization properties of the product, as well as its ability to rapidly change from the state of the solid lyophilized mass to the form of a solution or emulsion when the solvent is added improve.

Liposomal forms of cytostatics are one of the research objects for nanobiotechnology [5]. The main drawback of conventional cytotoxic drugs such as solutions and concentrates is their high toxicity on the body as a whole. Liposomal carriers selectively act on the organ that needs therapy due to EPR effect (enhanced permeability and retention effect) and regeneration of damaged biological membranes in the body by components of the phospholipid membrane [6]. This leads to decrease of toxicity in the cytostatic action, and can be used both to improve the quality of life of the patient, and to increase the dosage and improve the effectiveness of chemotherapy. Very promising for chemotherapy are drugs of the camptothecin group, in particular irinotecan, and drugs of the group of platinum-containing compounds, preferably oxaliplatin [7]. Liposomal forms of these drugs will reduce toxicity and increase the effectiveness of therapy.

Liposomal preparations are one of the objects of scientific and practical research when developing the lyophilization technology [8, 9]. In this case, the most important technological criteria for lyophilization are the type of a cryoprotector, the temperature of the secondary drying, and duration of the whole process [10].

Fig. 1 is a schematic representation of a modern freezing drying apparatus. The main blocks are a chamber, a condenser, a vacuum pump, a shelf refrigeration system and a condenser refrigeration system. The product to be lyophilized is placed in the chamber of the device. With the help of the shelf cooling system the product is frozen to (-40) °C. The condenser shelves are cooled...
to a temperature of (-70) °C (-80) °C. The chamber is sealed, and with the help of a vacuum pump through a condenser a vacuum of about 0.03 mm Hg is created. Based on the phase diagram of the water state below the triple point (for pure water: 6.1 mbar at 0 °C, below the eutectic point) there is only solid and gaseous states exist [11]. The sublimation process is based on this physical principle. When the shelves of the chamber cool down, and the pressure decreases, sublimation of ice in the product from the solid state to the gaseous state takes place. At the same pressure the temperature of the condenser shelves is lower than the shelves with the product, and based on the water state diagram, water at this temperature exists as ice. It causes crystallization of water vapor on the shelves of the condenser. The degree of the mass transfer of water and the speed of the lyophilization process are regulated by the temperature program of the shelves of the lyophilizer chamber.

The aim of the study is to optimize the content of the cryoprotector in the liposomal irinotecan, and develop lyophilization parameters to produce liposomes with the maximum encapsulation of irinotecan in them, alongside while maintaining the nanosizes.

**Materials and methods**

Egg phosphatidylcholine from Lipoid (Germany) was used for preparation of liposomes. 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 the residual pressure of 0.02 atm. For homogenization the high pressure extrusion method was used. The extrusion was carried out using a Microfluidiser M-110P model from Microfluidics (USA) at the pressure of 1500 atm. The size of liposomes was determined on a Shimadzu LC-20 instrument in a Quarco device (PR China). The encapsulation degree from PALL (USA). Lyophilization was carried out for “chemical gradient” was carried out on a Minim2 Nano ZS from Malvern Instruments (UK). Ultrafiltration was determined at the temperature of 20 °C on a Zetasizer (Japan) by HPLC method developed earlier [11]. The preparation was sterile filtered and bottled in aseptic conditions in 50 ml sterile vials VAT050-2C manufactured by Schott (Germany).

Liposomes with irinotecan were obtained using the “chemical gradient” approach in its “pH gradient” modification. Lipids were placed in a round bottom flask in the ratio of egg phosphatidylcholine/cholesterol – 80 : 20. The sample was dissolved in a minimum volume of the chloroform-anhydrous ethyl alcohol mixture until opalescence disappeared. The lipid film was prepared using a rotary evaporator. The vacuum treatment was carried out until a porous mass was obtained, and the odor of chloroform was disappeared. As an internal buffer, 0.2 M solution of citric acid monohydrate with pH 1.9 was used. Homogenization was carried out until the size of liposomes of 80-120 nm was reached. To obtain the “chemical gradient” ultrafiltration cassettes with the upper cut-off limit of 30 kD were used. As an external solution, 0.01 M phosphate buffer with the pH of 5.0 was used. After ultrafiltration anhydrous irinotecan hydrochloride was loaded up to its concentration of 2 mg/ml in a liposomal emulsion. The liposome emulsion was thermostated for 12 hours at room temperature [12, 13].

**Results and discussion**

Our preliminary screening experiments confirmed by the literature data showed that trehalose was the optimal cryoprotector in lyophilization of liposomes [14, 15]. For the study the cryoprotector range was selected from 4 to 10 %. Four samples of liposomes with irinotecan with the total content of trehalose dihydrate of 4 %, 6 %, 8 %, 10 % (w/w) were prepared. The selection criterion was preservation of the liposome sizes and the degree of encapsulation of irinotecan in them after carrying out the lyophilization process. The data obtained are presented in Table and Fig. 2.

From Table and Fig. 2 it is seen that the lowest loss of encapsulation is observed when the content of trehalose dihydrate is 8 %. At the same time, the loss of the encapsulation degree is 13 %. The smallest change in size is observed at 8 % of the trehalose dihydrate cryoprotector. The size increases from 110 nm before lyophilization to 115 nm after rehydration. Based on this

| The amount of trehalose dihydrate, % | The degree of encapsulation before lyophilization, % | The liposome size before encapsulation, nm | The degree of encapsulation after lyophilization, % | The size of liposomes after encapsulation, nm |
|-------------------------------------|-------------------------------------------------|----------------------------------------|-----------------------------------------------|---------------------------------------------|
| 4                                   | 88                                              | 109                                    | 42                                            | 132                                         |
| 6                                   | 95                                              | 112                                    | 72                                            | 119                                         |
| 8                                   | 98                                              | 110                                    | 85                                            | 115                                         |
| 10                                   | 93                                              | 115                                    | 68                                            | 137                                         |

*Fig. 2. The loss of encapsulation with different content of trehalose dihydrate. The upper graph is encapsulation prior to lyophilization; the lower graph is the encapsulation after lyophilization*
the content of 8.0 % trehalose dihydrate was used in further experiments.

One of the main factors affecting the lyophilization mode of the preparation is the duration of the primary drying stage at which the bulk of water is removed from the lyophilizate. In addition, the temperature of secondary drying, the so-called “final-drying” has a great impact. In this case it is necessary to balance the final temperature and the amount of residual water in the preparation. It has been found that the water content in the finished lyophilizate from 1 to 3% is optimal for the liposomal irinotecan.

The effect of the secondary drying temperature on the amount of residual moisture was studied. The experiment was carried out at the temperatures of 10, 15 and 20 °C. It was found that at the secondary drying temperature of 10 °C the residual moisture content was 5-8 %. It makes this mode unacceptable for use in the technology. In the case of the secondary drying temperature of 15 °C, the residual water content was from 1.5 to 2.8 %, while the loss of encapsulation was 13 %. In the case of the secondary drying at temperature of 20 °C, the amount of residual moisture was from 0.5 to 0.8 % with the loss of the encapsulation degree more than 20 %. It may be due to “overdrying” of the product, and the removal of water required for preservation of the lipid bilayer structure. As a result, the mode with the final drying at 15 °C was most preferable.

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CONCLUSIONS

1. As a result of the studies, liposomes with irinotecan have been obtained. The content of trehalose dihydrate as a cryoprotector in the range of 4-10 % has been studied. It has been shown that the optimum content of trehalose dihydrate is 8 % (by wt); moreover, the encapsulation decrease in lyophilization is 13 %.

2. The modes of the liposomal irinotecan lyophilization have been studied at the final drying temperature of 10, 15 and 20 °C. It has been found that the optimum final drying temperature is 15 °C.

Conflict of Interests: authors have no conflict of interests to declare.

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