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FIATURES OF LIOPHILIZATION OF NANOLIPOSOMAL DRUG*

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The conditions of lyophilization of nanoliposomes with encapsulated gentamicin (liposomal gentamicin) were studied. A mode of its safe lyophilization was suggested. A program for eliminating adverse factors was developed. It includes smooth increase of temperature from –70°C (freezing temperature) to room temperature (22±2)°C and gradual reduction of vacuum from 30 to 10 Pa without jumps and drops. The characteristics of the labile nanoliposomal drugs were not lost. The suggested program consisted of 10 steps allowing computer control to attain a slow and uniform increase in the temperature of the frozen product. The precise control of pressure in the chamber was provided by a system of automatic regulation of vacuum supplied with the equipment. After the rehydration the lyophilized preparations completely restored their original shape and properties.

Keywords*: lyophilization, nanoliposomes, drugs, cryoprotectants, temperature, vacuum regulation.*

Introduction

The discovery of pharmaceutical forms based on liposomes opened new opportunities of using pharmacological means capable of accumulating in effector tissues due to their physical and chemical properties. Liposomal preparations were registered in the 13th edition of the State Pharmacopoeia that came into effect since January 1, 2016 according to the Order of the Ministry of Health of the Russian Federation No. 771.

Therapeutic use of liposomes facilitates selectively delivering pharmaceuticals to the centers of infections, as well as protecting encapsulated preparations against the effect of detoxication enzymes. This allows increasing their half-life, decreasing their daily dose and increasing the antibiotic tolerance. Thus, it becomes possible to considerably increase drugs efficiency and to adapt the terms of patient hospitalization [1]. Among methods of patients' treatment widely used so far, antibiotic treatment remains one of the most demanded ones. Besides, the improvement of pharmaceutical forms of antibiotics is an urgent problem of Russian health care [2, 3].

This work is devoted to studying the conditions of lyophilization of a nanoliposomal preparation containing encapsulated gentamycin sulfate (liposomal gentamycin). It is known that gentamycin sulfate is classified as an aminoglycoside antibiotic with a wide spectrum. It is in demand so far in case of many infections including nosocomial ones. In our opinion, using gentamycin in the liposomal form is of special interest. At the same time, the task of producing the

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nanoliposomal form of dry gentamycin sulfate allowing both to stabilize the drug properties and to increase many times its storage life without cooling becomes as pressing as ever [4].

Lyophilization is a technological process of substances drying in a special way: from the frozen state without transition to the liquid state. This allows keeping almost completely all the main properties and activity of drugs even after their long-term storage [5].

In order to obtain high-quality lyophilized liposomal forms it is important to pay attention to the method of preparing liposomes, their structure, cryoprotector type and concentration, as well as to the mode of drying itself [6].

Selecting lyophilization parameters in each case is a complex task, because the process is associated with a change of aggregate state and directly affects the drug activity and the possibility of using it after rehydration. It is known that liposomes are spherical vesicles separated from the external environment by one or several bilayer phospholipidic membranes with an internal aqueous phase. The latter may contain biologically active substances. Their reorganization upon lyophilic drying due to crystallization and evaporation of water from spherules is closely related to the necessity of ensuring the preservation of the liquid-crystal state of phospholipids comprising bilayer membranes [7].

The purpose of this work is improving the technology for obtaining nanoliposomal gentamycin sulfate at the final stage of production associated with the selection of a cryoprotector and lyophilization parameters for the maximum possible preservation of the drug properties after drying and rehydration.

Experimental

In order to prepare liposomal gentamycin we used chromatographically pure phosphatidylcholine (the Kharkiv plant of bacterial preparations, No. 18.3.1097-0500) and cholesterol ("Serva", Germany) in molar ratio 7:3. Liposomes were obtained by evaporation and inversion of phases. For this purpose 56.7 mg of a mixture of lipids (39.7 mg of phosphatidylcholine and 17 mg of cholesterol) was dissolved in 3.8 ml of chloroform. 1.5 ml of gentamycin sulfate (JSC “Sintez”, Kurgan, Russia) was dissolved in 0.01 M phosphate buffer (pH 7.2) was added to the solution of lipids in the organic phase. (The final concentration of the antibiotic was 150 units). The mixture was treated by ultrasound (200 W, 20 kHz, 5 min) until a water-in-oil emulsion was formed. The obtained suspension was transferred into the flask of a rotary evaporator. The organic solvent was completely evaporated in vacuum until a gel was formed. (The mixture temperature was maintained higher than the transition temperature of the phospholipids.) Then 5 ml of the 0.01 M phosphate buffer (pH 7.2) was added, and the mixture was shaken until the formation of a homogeneous suspension.

Non-encapsulated antibiotic was separated by centrifugation at 40000 g for 1 h with the use of a “Ja-21” centrifuge (Beckman, USA).

The quantity of liposomes in 1 ml of the preparation and their sizes were determined with the use of an Nanotrac Wave device (USA) and of a JEM-100-SX electronic microscope (JEOL, Japan).

Klein peroxidation index (the degree of lipids oxidation in the liposomal membrane) was determined by spectrophotometry [9]. For this purpose 3 ml of the liposomal preparation (equivalent to 2 mm of lecithin) was emulsified in a 0.1 M solution of potassium chloride. Absolute ethanol was added. Samples were tested by spectrophotometry at $\lambda = 215$ and 233 nm, and A_{233}/A_{215} ratio was calculated.

The preparation was frozen and kept in a freezer at –70° C for 18 h. A 40% solution of saccharose, as well as a preservative containing 40% of saccharose and 10% of gelatin were used as cryoprotectors.

The preparation was lyophilized with the use of a COOLSAFE-100-9 installation ("SCANLAF", Denmark). Its package contains an RZ-1 vacuum pump (VACUUBRAND company), as well as a computer and a color printer for programming and graphic visualization of drying parameters. An additional order was made to supply the lyophilization equipment with a block of automatic vacuum control ("SCANLAF", Denmark).

The preparation was photographed in the course of microscopy by means of a "JEM-100SX" electron beam microscope (JEOL, Japan) at 10 000× magnification.

Results and Discussion

It is known that lyophilization efficiency is closely related both to the features of products subjected to sublimation and to the choice of a cryoprotector, as well as to the technological features of lyophilization: the temperature conditions of drying, its rate, vacuum depth and the duration of various sublimation stages [10].

Liposomal gentamycin was used as an object of lyophilization. It is rather labile because of its nanostructure: it has a thin lipidic membrane. At the same time the size of vesicles did not exceed 100 nm, which allows considering them as nanoparticles [11]. Their Klein peroxidation index was (0.9±0.5), while its value for unoxidized lipids should not exceed 1.0 [12]. The initial quantity of liposomes containing gentamycin sulfate in 1 ml of the preparation was $1.0 \cdot 10^5$; the suspension pH was 7.3.

However, it is known that product freezing directly preceding lyophilization is inevitably followed by the growth of formed ice crystals and, as a result, a mechanic damage of the preparation nanoparticles. According to literature, the freezing temperature of a substance before lyophilization should not exceed its eutectic temperature, i.e., it should not be higher on the average than –40÷–60°C [13]. In our study a low-temperature freezer with temperature range –50÷–86°C

was used (Ultra-Low Temperature Freezer, MDF-U3286S, "SANYO", Japan). The nanoliposomal preparation was cooled by means of it to –70°C.

In order to structure bioobjects thus preventing their damage in the process of cryoconservation, cryoprotectors were added into the drying medium. Usually, both substances interacting with water molecules due to hydrogen bonds, so-called penetrating substances (glycerin, dimethyl sulfoxide, etc.) and nonpenetrating ones (oligosaccharides: sucrose, trehalose, etc., as well as high-molecular compounds: polyvinyl pyrrolidone, gelatin, etc.) are used. It is believed that the protective effect of oligosaccharides, as well as that of high-molecular nonpenetrating compounds, is caused by their capability of acting as an intermediate matrix between separate vesicles. It prevents the latter from coming close together. This is especially important for liposomes, the close contact of bilayers of which is a prerequisite to membrane merge [14].

In our study we subjected to lyophilization nanoliposomes with the addition of a cryoprotector, namely:

1) 40% saccharose in the ratio with liposomes 3:7;

2) 40% saccharose and 10% gelatin in the ratio with liposomes 3:7.

We used native nanoliposomes as control without cryoprotector addition.

It was found that the most part of the liposomes that were subjected to sublimation without cryoprotector were destroyed in the control after rehydration.

The addition of gelatin into the preservative resulted in the preparation solubility falloff after drying. Apparently, this can be due to gelatin polymerization: its separate molecules are enormously larger than liposomal nanoparticles. Therefore, gelatin does not lose the capability of forming intermolecular cross links (cross-linking polymerization).

Lyophilization in the drying medium containing saccharose without gelatin, allowed keeping the preparation native properties almost completely (Table 1).

Table 1. Effect of different cryoprotectors on lyophilization efficiency

No	Cryoprotector	Quantity of liposomes in 1 ml of preparation:		Preparation solubility	Entirety of liposomes structure
		Before lyophilization	After lyophilization		
1.	Saccharose 40%	$1 \cdot 10^5$	$1 \cdot 10^5$	+++	+++
2.	Saccharose 40% + gelatin 10%	$1 \cdot 10^5$	$3 \cdot 10^4$	–	+
3.	Control	$1 \cdot 10^5$	–	–	–

Thus, using saccharose as a cryoprotector provided efficient protection of liposomal gentamycin in the course of its lyophilization.

When carrying out lyophilization, such an important property of the substance as eutectic temperature depending on the nature, structure, concentration and other characteristics of the product is of particular importance. The more components in the system that is subjected to drying, the lower the eutectic temperature of the mixture. Generally, the eutectic temperature of immunobiological preparations is in the range 25±5°C. There is an opinion that in case of nanoliposomes – fine biologically active structures – it is especially important not to exceed this temperature limit in the course of lyophilization until all free moisture is removed from the preparation [8].

At the beginning of lyophilization, when there is a large amount of free moisture, temperature is determined not only by the extent of the agent prefreezing, but also by vacuum in the camera. Evaporation of water in the frozen state (sublimation of ice crystals) in vacuum is accompanied by even larger decrease in the substance temperature, and the deeper vacuum, the stronger the preparation is cooled. Note that the conditions of lyophilization in the modern equipment differ from the conventional modes applied in sublimation installations of the previous generation: modern equipment enables using deep vacuum (less than 0.1 mmHg, ≈ 13 Pa). New technologies enable accelerating the process many times. Nevertheless, the accelerated sublimation of free moisture after its full evaporation can cause a sharp jump in temperature, which leads to considerable or total loss of the dried preparations properties [6, 7].

In our study we had an opportunity of observing partial foaming of the preparation in the first hours of drying due to deep vacuum (more than 0.15 GPa). The sharp rise in temperature presented in the graphics of drying (Figure 1) was followed by loss in the preparation activity and destruction of the liposomes after lyophilization and rehydration.

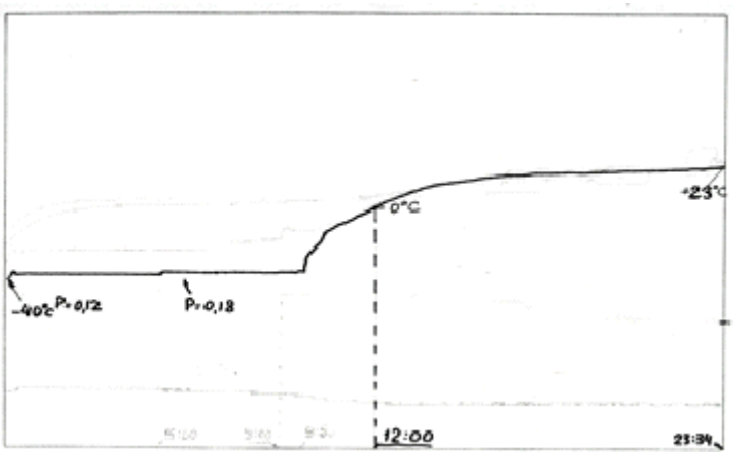

Figure 1. Diagram of drying (time dependence of preparation temperature) accompanied by a sharp rise in preparation temperature.

Thus, sharp jumps in temperature arising at attempt to reduce drying time can result in the loss of native properties of the lyophilized preparation after its rehydration.

In order to eliminate adverse factors we developed a program providing a smooth temperature increase from –70°C (freezing temperature) to room temperature (22±2)°C and gradual vacuum decrease from 30 to 10 Pa without jumps and drops. As a result, labile nanoliposomal preparations did not lose the initial characteristics.

The suggested program included 10 steps allowing to attain slow and uniform increase in the frozen product temperature (Table 2) under computer control. Exact control of pressure in the camera was provided by a system of automatic vacuum control included in the lyophilic equipment package.

Table 2. Mode of nanoliposomal preparations lyophilization

Step number	Duration (h)	Temperature (°C)	Pressure (GPa)
1	4.0	–70	0.30
2	2.0	–20	0.25
3	2.0	–15	0.20
4	2.0	–10	0.20
5	2.0	–5	0.20
6	2.0	0	0.20
7	2.0	5	0.15
8	2.0	10	0.15
9	2.0	20	0.15
10	4.0-6.0	25	0.10

It follows from the presented data that lyophilization duration was 24 – 26 h. Simultaneous change of temperature and vacuum allowed providing a mode of smooth temperature change and passing through 0°C over a period of about 12 h.

In order to compensate the temperature decrease in the process of drying we supplied additional heat to the shelves in the lyophilic installation camera. However, heating the shelves at the initial stage can also cause passing the sublimation limit and boiling of moisture even at low temperatures in the operating camera. So, temperature control of lyophilization is obviously important.

During numerous experiments we noted that the preset shelf temperature considerably differed from the temperature of the preparation itself. Apparently, this was due to evaporation of ice crystals, especially at the first stages of lyophilization, when the substance still contains a large amount of free moisture.

In order to obtain the real lyophilization pattern the temperature sensor was placed directly into the ampoule with the preparation. This allowed monitoring the lyophilization process with maximal accuracy.

Figure 2 shows a lyophilization diagram obtained with the use of the suggested program.

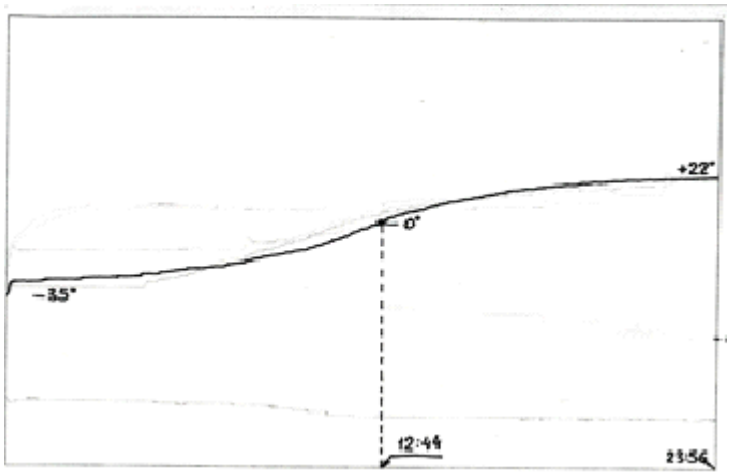

Figure 2. Lyophilization diagram (time dependence of temperature) of liposomal gentamicin with the use of the suggested program.

The presented drying mode allowed obtaining lyophilized nanoliposomal preparations with the preserved initial characteristics. In all the samples Klein peroxidation index was 0.9±0.5. Gentamycin concentration corresponded to 0.08 g/ml of liposomes. Particle size did not exceed 100 nm, and their shape did not change (Figure 3).

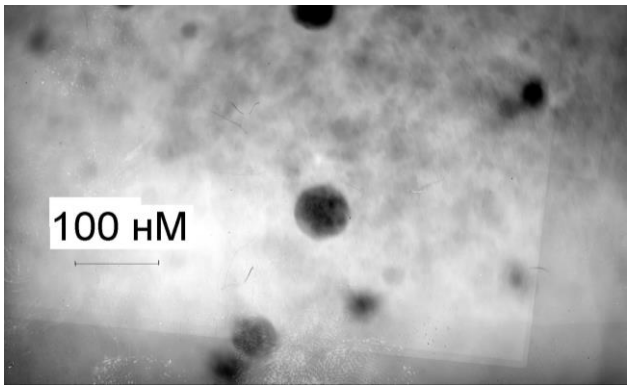

Figure 3. Photomicrogram of liposomal gentamycin after lyophilization and a rehydration (electron microscope JEM-101-SX), 10 000-fold magnification.

Thus, decreasing sublimation intensity enables to avoid completely the loss of nanoliposomal preparations activity. This is attained by reducing vacuum depth to 25–30 Pa at the initial stage of drying (until temperature passes the eutectic threshold). Then vacuum can be gradually increased. Automation of lyophilization stages allows monitoring all the process parameters and providing their smooth control.

Conclusions

1. We developed a mode for the lyophilization of nanoliposomes with encapsulated antibiotic gentamycin (liposomal gentamycin) using modern sublimation equipment with a built-in vacuum control system for longer storage life of samples and convenience of using them.

2. In order to preserve the initial activity of the preparation lyophilizate outlet time at 0°C should not be less than 12 h. The optimum sublimation mode for nanoliposomes is smooth temperature increase from –70°C (freezing point) to room temperature (22±2°C) without jumps and drops with gradual vacuum decrease from 30 to 10 Pa.

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