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PHARMAKEFTIKI, 30, 2 (2018) 76-87 RESEARCH ARTICLE

«Quality-by-Design» approach to the development of a dosage form for the liposomal delivery system of cytochrome C.

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SUMMARY

Cytochrome C (Cyt C) is a natural catalyst for cellular respiration. Aqueous solutions of Cyt C are used in cardiology, neurology and ophthalmology, where they manifests a high activity towards free radicals and bind aggressive molecules of oxidants.

The aim of the study was to develop and optimize the preparation of freeze-dried cytochrome C-containing liposomes (FD-Cyt-LS) using the Quality by Design (QbD) approach and to compare the stability of aqueous Cyt-LS and FD-Cyt-LS.

In the case of freeze-dried Cyt-LS (FD-Cyt-LS), the influence of cryoprotectants type and concentration, as well as secondary drying temperature, which ensure integrity of liposomal membrane during lyophilization and preservation throughout the shelf life were studied.

Lactose with a concentration of 6% was established to be the optimal type of cryoprotectant. At the lyophilization programme selected the most preferable secondary drying temperature value was 30°C.

The necessity to choose the form of lyophilizate for solution preparation was shown with the example of stability evaluation of aqueous Cyt-LS and FD-Cyt-LS. During the observation period, aqueous Cyt-LS shows the growth of degradation products of free fatty acids and lysophosphatidylcholine (LPC) by 202.5% and 409.7% respectively, compared with the reference values, as well as the decrease of the content of phosphatidylcholine (PC) and dipalmitoylphosphatidylcholine (DPPG- Na) by 1.5 and 1.0%, respectively. At the same time, no significant changes in the composition of FD-Cyt-LS samples were observed.

1. Introduction

Cytochrome C (Cyt C) is a natural catalyst for cellular respiration, which stimulates oxidative reactions and regeneration processes, activates metabolism in tissues, and reduces tissue hypoxia at various pathological states.

Cyt C is used in cardiology, neurology and ophthalmology, where it manifests a high activity towards free radicals, binds aggressive molecules of oxidants, stimulates cerebral circulation in damaged blood vessels, protects the lens and cornea from damage, suppresses and prevents the development of lenticular opacity (cataract), obstructs the degeneration of the retina.

There are the following Cyt C-containing drugs known on the world pharmaceutical market (**Table 1**).

However, use of aqueous solution of Cyt C has a significant drawback. Cyt C insignificantly penetrates through membranes into cells and is quickly removed from the body, which contributes to its low bioavailability. To eliminate this drawback it is expedient to create cytochrome C-containing liposomes (Cyt-LS). It was previously established that use of Cyt-LS for the therapy of corneal injury, contributes to a more rapid process of tissue regeneration and reduction of the inflammatory response in comparison with a non-liposomal form[1] Cyt-LS were shown to be stable superior ophthalmic carriers and were able to markedly retard the onset of cataract development.[2] Cyt-LS show a higher activity than the non-liposomal form of the drug and reduces the arrhythmia period compared to the control non-liposomal Cyt more than 2 times.[3] Also, the infusion of Cyt-LS contributed significantly to the elimination of hemostasis disorders, and prevents the development of decompensated metabolic acidosis, when studying haemostatic activity of the liposomal drugs in acute massive blood loss.[4]

Therefore, when choosing a dosage form, it is worth considering the perspective possibility of using Cyt-LS as a solution for injections and eye drops.

According to the requirements of[5,6,7] for liposomal drugs, the parameters that determine their stability are: the phospholipid composition, particle size and encapsulation efficiency.

When choosing a dosage form, it is necessary to consider the fact that some undesirable phenomena such as aggregation, hydrolysis of phospholipids may occur in aqueous solutions of liposomes. As an alter-

Table 1. Cytochrome C-containing drugs on the world pharmaceutical market.

Trade mark	Manufacturer	Dosage form
Oftan catachrom	Santen, Finland	Eye drops
Vitaphakol Eye Drops	Laboratoires H. Faure, France	Eye drops
Cytochrome C Eye drops	Samson Med, Russia	Eye drops
Cytochrome C	Samson-Med, Russia	Freeze-dried powder for preparation of solution for injections
Cytochrome C	Samson-Med, Russia	Solution for injection
Cytochrome C solution for injection	Pharmstandard-biolik, Ukraine	Solution for injection
Catrix Eye Drops	Incepta Pharmaceuticals Ltd, Bangladesh	Eye drops
Phacovit	Aristopharma, Bangladesh	Eye drops
Ractovit	The IBN SINA, Bangladesh	Eye drops
Vitafol Eye Drops	Popular Pharmaceutical, Bangladesh	Eye drops

native form of liposome aqueous solutions, freeze-dried liposomes can be considered for the preparation of drops or injectable solutions. Lyophilization is usually used to extend the liposome shelf life[8] by storing them in a freeze-dried state. This allows to store the liposomal nanoparticles at temperatures lower than aqueous preparation emulsions, which in turn leads to the minimization of such undesirable phenomena as aggregation and hydrolysis of phospholipids, which are the main components of the liposome membrane.

The purpose of this study was to develop and optimize the preparation of freeze-dried cytochrome C-containing liposomes (FD-Cyt-LS) using the Quality by Design (QbD) approach and to compare the stability of the aqueous Cyt-LS and FD-Cyt-LS.

International Conference on Harmonization (ICH) Q8 (R1) guideline defines QbD as "a systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management"[9]

QbD aims to identify characteristics that are critical for product quality and to determine how critical parameters of the process can be changed in order to sequentially produce a product with the required characteristics. In the case of FD-Cyt-LS it is necessary to select the optimal composition and technological parameters to protect the integrity of the liposomal membrane during lyophilization and to ensure preservation throughout the shelf life.

2. Materials and Methods

2.1 Materials

Cytochrome C (Calzym lab., USA), Egg Phosphatidylcholine, Lipoid E 100 (Lipoid GmbH, Germany) dipalmitoylphosphatidylglycerol (DPPG-Na) (Lipoid GmbH, Germany), phosphatidylcholine standard Lipoid E PC RS (Lipoid GmbH, Germany), dipalmitoylphosphatedylcholine standard DPPG-Na RS (Lipoid GmbH, Germany), lysophosphatidylcholine (Lipoid GmbH, Germany), lactose (Fluka), trehalose (Fluka), chloroform (Sigma Aldrich), methanol (Sigma Aldrich), potassium dihydrogen phosphate (Fluka), sodium dihydrogen phosphate (Sigma Aldrich), disodium hydrogen phosphate (Sigma Aldrich).

2.2 Preparation of liposomes

DPPG-Na and EPC were dissolved in a chloroform:ethanol (4:1) mixture. The resulting mixture was evaporated in a rotary evaporator BUCHI Rotavapor R215 (Switzerland) until a lipid film formed. The lipid film was hydrated with the Cyt C solution until a homogeneous emulsion of multi-lamellar vesicles was formed. The emulsion of multi-lamellar vesicles was homogenized in a high-pressure homogenizer M110P Microfluidizer (USA) until unilamellar LS with an average particle size in the range of 100 to 200 nm were obtained. Lactose and trehalose in concentrations 5%, 6%, 7%, chosen according to design of experiments (DOE) were studied as cryoprotectants. Then, LS were lyophilized in Martin Christ Epsilon 2-6D LSCplus (Germany) Freeze-dryer.

2.3 Assay of Cyt C

The total concentration of Cyt C in the freeze-dried LS-Cyt (Ctotal) was quantified spectrophotometrically on a Shimadzu UV 1800 (Japan) spectrophotometer using the UV absorption spectrum of a diluted emulsion of liposomes with cytochrome C in the range of 400-560 nm.

2.4 Determination of encapsulation efficiency

The encapsulation efficiency was calculated by the formula:

$EE\ \% = [(C_{total} - C_{free})/ Ctotal] \times 10$

Free Cyt C was determined by the gel chromatography method. A Shimadzu LC20 chromatograph was used, a chromatography column Tricorn (GE Healthcare) of 5/200 size filled with a "superose 12" sorbent; mobile phase: 4.515 g/L KH2PO4 pH to 6.0, with 2 M NaOH; flow rate 0.5 ml/min; detection at a wavelength of 409 nm; column temperature 25 °C. The solutions of the Cyt C and Cyt-LS were chromatographed alternately

Table 2. Gradient of mobile phase composition

Time (min)	Flow rate (ml/min)	Phase A (% vol.)	Phase B (% vol.)
0	1.0	95	5
5.0	1.0	80	20
8.5	1.0	60	40
15.0	1.0	0	100
17.5	1.0	0	100
17.6	1.0	95	5
21.0	1.0	95	5
22.0	2.0	95	5
27.0	2.0	95	5
29.0	1.0	95	5

2.5 Assay of phospholipids (lysophosphatidylcholine and free fatty acids)

The content of phospholipids in liposomes was determined by HPLC using a Shimadzu (Japan) chromatograph with evaporative light-scattering detector, under the following conditions: column 0.125 m x 4 mm, 5 μm packing L20, column temperature 55 °C, sample volume 20 μl. Mobile phase: A) 1341.6 g of n-hexane, 334.1 g of 2-propanol, 39.4 g of acetic acid, and 1.45 g of triethylamine (or 2.0 mL of triethylamine). B) 663.5 g of 2-propanol, 140.0 g of water, 15.8 g of acetic acid, and 0.58 g of triethylamine. Gradient composition of mobile phase is presented in **Table 2.**

2.6 Physicochemical characterization of liposomes

The particle size was determined by the dynamic light scattering technique using a Malvern Zetasizer Nano S (UK). The pH of the liposome emulsion with Cyt C was measured using a pH meter by SCHOTT Instruments (Germany).

2.7 Stability studies

The storage of aqueous Cyt-LS and FD-Cyt-LS with the same lipid and cryoprotectant composition were compared.

FD-Cyt-LS were stored for 1 year in a dark place and at temperature - 15 °C (Thermo scientific 7000, USA).

Aqueous Cyt-LS, stored for 1 year in a dark place and at temperature + 5 °C (Memmert ICH256, Germany).

The studies were carried out for a period of 0, 1, 3, 6 and 12 months on the particle size, encapsulation, content of the main components (DPPG-Na, PC, Cyt C), LPC content and free fatty acids, appearance, emulsion formation time, emulsion stability.

2.8 Data processing

The design of the experiments was developed using Statistica software (Statsoft, USA). The experimental data were examined using a statistical module from the Statistica software. The same software was used to calculate statistical parameters.

3. Results and Discussion

As a preliminary QbD step, critical quality attributes (CQAs) were identified. In this study, several CQAs for lyophilized liposomes were selected: particle size, encapsulation efficiency, emulsion formation time, and residual moisture in the lyophilizate. According to the

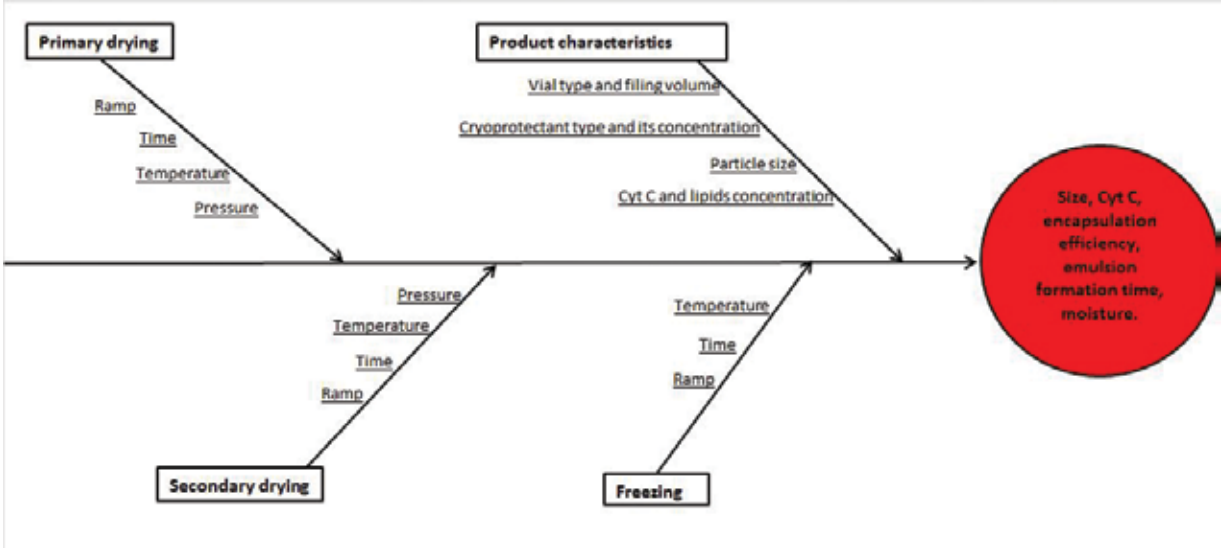

Figure 1. *Ishikawa diagram the risk analysis of the lyophilization process.*

requirements of[6, 7, 9], these properties should be within the appropriate limits to ensure the desired quality of the product.

The optimal size for liposomal vesicles is usually within 10-200 nm due to the fact that the liposome emulsion must be subjected to the sterilizing filtration through the filters with a 0.22 μm pore size. The size of the liposomal nanoparticles within this range can be obtained by homogenization of multilamellar liposomes in a high pressure homogenizer. In our previous studies, the effect of liposome composition on encapsulation efficiency and particle size in the pharmaceutical development of aqueous Cyt-LS was studied[10]

However, the particle size of freshly-prepared liposomes may increase during lyophilization due to the fusion/aggregation of the vesicles and the wrong choice of the freeze-drying regime. In turn, changing the size of Cyt-LS can cause leakage of the encapsulated substance and change of one of the most important CQA of the freeze-drying liposome composition, degree of encapsulation, which can result in a significant change of pharmacological properties of Cyt-LS. Therefore, the change of these parameters after lyophilization should be carefully monitored and prevented[11, 12].

Moisture is one of the key quality indicators of the freeze-dried liposomes' quality. On one hand, a lower moisture content is desirable for the stability improvement, on the other hand, the excessive removal of water from liposomes can lead to a decrease of the encapsulation efficiency[13], change in liposome particles size and a poor ability to form homogeneous emulsions. The most important factors affecting the residual moisture content established from the risk analysis are type and concentration of the cryoprotectant, as well as the selection of the lyophilization process.

Another important factor is the emulsion formation time, during which the lyophilizate forms a homogeneous nanosystem with stable sizes.

The risk factors potential of the lyophilization process, that may affect the product quality, was identified by means of the risk analysis using Ishikawa diagram (**Fig. 1**).

After the risk assessment, three variables were selected for the further study

a) lactose cryoprotectant and its concentration
b) trehalose cryoprotectant and its concentration
c) secondary drying temperature

They are further included into the experimental design of the studies.

To maintain acceptable encapsulation efficiency and to preserve the particle size, it is necessary to optimize concentration and type of the cryoprotectant and lyophilization process. The effect of the secondary drying temperature has a great influence on the moisture and emulsion formation time. In this case it is neces-

Table 3. Independent and dependent variables of the experimental scheme are evaluated for the study of lyophilized liposomes.

Variable	Level used		
	Low (-1)	Medium (0)	High (+1)
Independent variables			
X_1 = lactose concentration, %	5	6	7
X_2 = trehalose concentration, %	5	6	7
X_3 = secondary drying temperature, ^{0}C	20	30	35
Dependent variables (CQAs)			
Y_1 = Liposomal size, nm			
Y_2 = Encapsulation efficiency, %			
Y_3 = Emulsion formation time, min			
Y_4 = Moisture, %			

sary to balance the final temperature and the amount of residual water in the preparation.

The experimental design (DoE) was used in the next stage as a QbD tool to study the risk factors. The variables of the experimental scheme are presented in **Table 3**. As independent variables, the following were chosen: X_1 = lactose cryoprotectant,%; X_2 = trehalose cryoprotectant and its concentration; X_3 = secondary drying temperature, ^{0}C.

As a response to the changes in independent variables, the quality attributes of lyophilized liposomes were chosen: Y_1 = Liposomal size, nm; Y_2 = Encapsulation efficiency,%; Y_3 = Emulsion formation time, min; Y_4 = Moisture,%.

The matrix of experimental design is presented in **Table 4**.

According to the proposed production scheme, a multilamellar Cyt-LS emulsion with a previously established optimal phospholipid composition and lipid-cytochrome C ratio was prepared[1]

The emulsion of multilamellar Cyt-LS was homogenized until unilamelic liposomes were obtained, after which the total volume of the resulting emulsion was divided into 20 samples. Then, according to the experimental design conditions (**Table 4**), the solutions of cryoprotectants were added to the obtained samples up to the selected concentrations. The obtained sam-

Table 4. The matrix of experimental design.

Code	X_1, %	X_2, %	X_3, ^{0}C
1	5	-	20
2	5	-	30
3	5	-	35
4	6	-	20
5	6	-	30
6	6	-	35
7	7	-	20
8	7	-	30
9	7	-	35
10	-	5	20
11	-	5	30
12	-	5	35
13	-	6	20
14	-	6	30
15	-	6	35
16	-	7	20
17	-	7	30
18	-	7	35
19	6	-	30
20	-	6	30

Figure 2. *Freeze-drying program*

ples were filtered through a 0.22 μm membrane filter and then bottled into 10 ml glass vials. Freshly prepared liposomes were characterized by particle size, Cyt C concentration and encapsulation efficiency parameters. The results of measurements are shown in **Table 5**.

All vials with samples were prefrozen at -30 ^{0}C

Then, a lyophilization procedure was carried out. Vials with the frozen product were placed in a freeze-drying chamber with shelf temperature of -35 °C at a chamber pressure of 1000 mBar for 240 minutes. The sublimation of the solvent was initiated by reducing the pressure to 0.1 mBar and raising the temperature to -20 °C during the next 10 hours, after which the selected temperature and pressure were maintained for 330 minutes. Then, secondary drying was carried out to remove the absorbed water from the product. During this process shelf temperature was raised in several stages to the values chosen in the DoE, at the end of secondary drying the pressure was reduced to 0.001 mBar and the product was dried for another 90 minutes at the same temperature. The freeze-drying program is shown in **Fig. 2**

The results of the experimental design studies are shown in **Table 6**.

As it can be seen from the results of the experimental design study, the secondary drying temperature has a significant effect on the particle size after lyophilization. However, the change in secondary drying parameter did not lead to significant changes in encapsulation efficiency. Thus, it was found that at secondary drying temperature of 20°C, the FD-Cyt-LS showed the best results of preservation of the original sizes, while at temperature of 35°C the results of the particle sizes proved to be the worst, regardless of the type of cryoprotectant and its concentration. The same dependence is observed in the effect of the secondary drying temperature on the formation time of stable emulsion. This can probably be due to the excessive removal of water, required to preserve the structure of the lipid bilayer. It is also worth noting that when choosing a secondary drying temperature of 20°C, the average residual moisture in the FD-Cyt-LS was about 4.0%. This obviously can negatively affect the storage of FD-

Table 5. The physico-chemical properties of freshly-prepared liposomes.

Code	Liposomal size	Encapsulation efficiency, %	Cytochrome C, %
1	135 nm – 90% 50 nm – 10%	≥ 95%	100.1
2	144 nm – 100%	≥ 95%	100.2
3	131 nm – 93% 44 nm – 7%	≥ 95%	100.2
4	130 nm – 91% 55 nm – 9%	≥ 95%	100.0
5	133 nm – 93% 46 nm – 7%	≥ 95%	100.3
6	130 nm – 90% 54 nm – 10%	≥ 95%	100.5
7	130 nm – 89% 55 nm – 11%	≥ 95%	100.6
8	135 nm – 90% 51 nm – 10%	≥95%	100.1
9	130 nm – 89% 55 nm – 11%	≥ 95%	100.1
10	133 nm – 94% 57 nm – 6%	≥ 95%	100.0
11	131 nm – 88% 50 nm – 12%	≥ 95%	100.3
12	131 nm – 90% 50 nm – 10%	≥ 95%	100.2
13	137 nm – 95% 45 nm – 5%	≥95%	100.2
14	135 nm – 94% 58 nm – 6%	≥95%	100.3
15	138 nm – 87% 45 nm – 13%	≥95%	100.4
16	131 nm – 95% 58 nm – 5%	≥95%	100.4
17	136 nm – 92% 44 nm – 8%	≥95%	100.2
18	138 nm – 97% 45 nm – 3%	≥95%	100.0
19	135 nm – 94% 44 nm – 6%	≥ 95%	100.0
20	139 nm – 90% 45 nm – 10%	≥95%	100.0

Table 6. The physico-chemical properties of FD-Cyt-LS (the matrix of responses of the experimental design).

Code	Liposomal size, nm	Encapsulation efficiency, %	Emulsion formation time, min	Moisture, %
1	220 nm – 100%	93.3	4	4.0
4	170 nm – 100%	≥ 95%	4	3.9
7	164 nm – 100%	≥ 95%	4	3.9
10	200 nm – 99% 670 nm – 1%	92 %	4	4.0
13	188 nm – 100%	≥95%	4	4.0
16	170 nm – 100%	≥95%	4	3.9
2	215 nm – 95% 1800 nm – 5%	93.5	4	1.5
5	165 nm – 100%	≥ 95%	3	1.5
8	165 nm – 100%	≥95%	4	1.6
11	205 nm – 97% 1750 nm – 3%	91 %	4	1.6
14	194 nm – 100%	≥95%	4	1.5
17	170 nm – 100%	≥95%	4	1.6

Cyt-LS. For this reason, the choice of this value is unacceptable for the use in the technology.

As a result, one can consider 30°C to be the most preferable value of secondary drying temperature.

As it can be seen from the research results, the smaller Low (-1) concentrations of cryoprotectants, regardless of their type, had worse encapsulation efficiency and particle sizes ratios. While in the samples with concentrations of cryoprotectants Medium (0), High (+1), a decrease in the encapsulation efficiency was not observed. In samples of Medium (0) and High (+1) trehalose, particle size increase was slightly larger than in samples with lactose with similar concentrations. The samples with lactose concentration of 6% gave the best results in terms of the emulsion formation time.

Thus, lactose with a concentration of 6% was chosen as the optimum cryoprotectant.

Based on the results of the experiments performed, Cyt-LS emulsion with an optimal phospholipid composition, lipid – Cyt C ratio, type and concentration of the cryoprotectant was prepared. The emulsion of Cyt-LS was bottled in 10 ml glass vials. Filling and capping (under nitrogen) of the

Table 7. Stability study results of aqueous Cyt-LS during storage

Month	0	1	3	6	12
Encapsulation efficiency, %	95.8	95.0	95.5	95.0	95.0
Mean particle size, nm	168	160	166	164	168
pH	6.95	6.81	6.80	6.77	6.65
LPC, %	0.60	0.75	0.88	1.45	2.95
Free fatty acids,%	0.4	0.42	0.5	0.58	0.81
Cyt C, %	100.1	99.8	99.5	99.4	99.2
PC, %	99.8	99.4	99.1	98.5	98.3
DPPG-Na, %	100.2	100.0	99.9	99.4	99.1

Table 8. Stability study results of FD-Cyt-LS during storage.

Month	0	1	3	6	12
Encapsulation efficiency, %	95.8	95.4	95.5	95.0	95.6
Mean particle size, nm	168	163	164	163	169
pH	6.95	6.81	6.80	6.77	6.65
LPC, %	0.60	0.63	0.64	0.64	0.68
Free fatty acids	0.4	0.4	0.43	0.45	0.46
Cyt C, %	100.1	99.8	99.8	99.8	99.6
PC, %	99.8	99.5	99.7	99.4	99.4
DPPG-Na, %	100.2	100.2	100.2	100.2	100.1
Moisture, %	1.5	1.5	1.5	1.6	1.6

vials were performed under aseptic conditions. One part of the vials was left in an aqueous state in a dark place at a temperature of +5°C.

The other part of the vials was lyophilized according to the freeze-drying program, with most preferable value of secondary drying temperature, established as a result of the performed experiments. The FD-Cyt-LS were also stored in a dark place and at a temperature of -15°C

The results of storage of the aqueous Cyt-LS and FD-Cyt-LS with the same composition are shown in **Tables 7 and 8**.

As the stability study results show, it is evident that in the aqueous Cyt-LS during the observation

period there is a growth in degradation products of free fatty acids and lysophosphatidylcholine (LPC) by 202.5% and 409.7% respectively, compared with the original values, as well as the decrease in the content of phosphatidylcholine (PC) and dipalmitoylphosphatidylcholine (DPPG- Na) by 1.5 and 1.0%, respectively.

At the same time, no significant changes in the composition of FD-Cyt-LS samples were observed.

The content of the degradation products - LPC and Free fatty acids is stable during the observation period and is at the initial level, which indicates the preservation of FD-Cyt-LS during 1 year under the chosen storage conditions, in contrast to the aqueous solutions.

In addition, throughout the storage period, the FD-Cyt-LS retained their appearance, formation time and emulsion stability.

4. Conclusion

Current work demonstrates the successful application of the QbD approach in the selection of Cyt-LS dosage form. The influence of such factors as type and concentration of a cryoprotectant, as well as secondary drying temperature on critical quality attributes (particle size, encapsulation efficiency, emulsion formation time and residual moisture) - were determined using DoE.

As a result of the work done, influence of type and concentration of the cryoprotectant as well as the freeze-drying program were established.

The necessity to choose the form of lyophilizate for the preparation of a solution is shown with the example of stability evaluation of a liquid and FD-Cyt-LS.

It can be concluded that QbD is a useful, effective approach to the development of FD-Cyt-LS with a controlled, predictable quality. □

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