Lyophilization Process of Hydroxypropyl Tetrahydropyrantriol Liposomes

Shengqian TIAN△, Enlong WANG△, Xin ZHANG, Lili HE*

College of Pharmacy, Southwest Minzu University, Chengdu 610041, China

Abstract [**Objectives**] To enhance the skin permeability of hydroxypropyl tetrahydropyrantriol and provide a reference for the subsequent prevention or treatment of skin aging. [**Methods**] The lyophilization process of hydroxypropyl tetrahydropyrantriol liposomes was investigated using a single factor method, and a quality evaluation system was established based on the appearance, particle size, PDI, and re-dispersibility of the lyophilized samples. [**Results**] The lyophilization process of hydroxypropyl tetrahydropyrantriol liposomes was determined by single factor experiments. The pre-freezing period was 16 h at −80 ℃, the total drying time was 36 h, and the addition of 10% mannitol-sucrose was used as the lyoprotectant. [**Conclusions**] The product prepared by the lyophilization method exhibits a fluffy and full appearance, with minimal shrinkage and collapse. The volume remains consistent before and after lyophilization, and the re-dispersibility is satisfactory. The re-dissolution process is rapid, and the particle size and polydispersity index (PDI) remain largely unchanged before and after lyophilization.

Key words Hydroxypropyl tetrahydropyrantriol, Liposome, Lyoprotectant, Lyophilization process

1 Introduction

Hydroxypropyl tetrahydropyrantriol, a C-xylopyranoside derivative, has been demonstrated to induce the expression of key skin constituents, including glycosaminoglycans (GAGs) and proteoglycans (PGs)[1-4]. This process indirectly modulates growth factors[2,5], induces protein deposition in basement membranes and dermal-epidermal junctions (DEJs), and promotes the expression of collagen, thereby improving the condition of the skin[2,6]. Nevertheless, hydroxypropyl tetrahydropyrantriol is subject to the disadvantage of poor skin permeability, which significantly constrains its efficacy. Liposome, a popular drug-carrying system in recent years, possess a distinctive bilayer vesicle structure that enables them to exhibit good biocompatibility while also prolonging the drug release time to a certain extent, thus allowing for the potential of a slow-release or long-lasting effect. This has the potential for significant future development. However, liposomes themselves are inherently unstable and prone to aggregation, sedimentation, degradation, fusion, and other complications during storage. In order to enhance the stability of the formulation, this study examined the lyophilization process of hydroxypropyl tetrahydropyrantriol liposomes in terms of the pre-freezing temperature, pre-freezing time, drying time, type and dosage of single lyoprotectants and combined lyoprotectants. The optimal lyophilization conditions were identified in order to provide a reference for the subsequent prevention or treatment of skin aging.

Received: February 20, 2024 Accepted: July 8, 2024

Supported by Youth Science Foundation Project of Sichuan Provincial Department of Science and Technology (2022NSFSC1437); Special Project of Scientific and Technological Research of Sichuan Provincial Administration of Traditional Chinese Medicine (2021MS121); Fundamental Research Funds for the Central Universities of Southwest Minzu University (ZYN2022040).

△These authors contributed equally to this work.

* Corresponding author. E-mail: 871268031@qq.com

2 Materials and methods

2.1 Materials

2.1.1 Instruments. SCIENTZ-12N freeze dryer (Ningbo Scientz Biotechnology Co., Ltd.); ultrasonic cell crusher (Ningbo Scientz Biotechnology Co., Ltd.); JA2003 electronic balance (Shanghai Shunyu Hengping Scientific Instrument Co., Ltd.); LT224D one-ten-thousandth electronic analytical balance (Jiangsu Changshu Tianliang Instrument Co., Ltd.); R-201 rotary evaporator (Shanghai Shenshun Biotechnology Co., Ltd.); Milli-Q Integral water purifier (Millipore, USA); ultrasonic cleaner (Tianjin Automatic Science Instrument Co., Ltd.); digital constant temperature water bath (Changzhou Guohua Electric Co., Ltd.); Zetasizer Nano ZS 90 laser particle size analyzer (Malvern Panalytical, UK).

2.1.2 Reagents. Hydroxypropyl tetrahydropyrantriol (Chengdu Yunxi Chemical Co., Ltd., Batch No.: YS2101BSY5); soy lecithin, cholesterol, trichloromethane, and PBS dry powder (Chengdu Kelon Chemical Co., Ltd.); mannitol, sucrose, and glucose (Chengdu Ponstar Biotech Co., Ltd.).

2.2 Methods

2.2.1 Preparation of hydroxypropyl tetrahydropyrantriol liposomes. Hydroxypropyl tetrahydropyrantriol was prepared via the reverse evaporation method, as previously described in the experiment [7]. The appropriate quantities of soy lecithin and cholesterol were weighed and the appropriate quantity of chloroform was added to fully dissolve the materials as the organic phase. The appropriate quantity of hydroxypropyl tetrahydropyrantriol was dissolved in a phosphate buffer solution, which served as the aqueous phase. Subsequently, the aqueous phase was added to the organic phase by stirring and sonication, thus forming a suspension. The suspension was transferred to a round-bottom flask and evaporated under reduced pressure in order to remove the organic solvent and form a lipid film. The appropriate amount of phosphate buffer should be added and the evaporation process continued under reduced pressure until the film was fully hydrated, thus obtaining a suspension

of hydroxypropyl tetrahydropyrantriol liposomes. The liposome suspension was subjected to sonication in an ice water bath for a period of 10 min, with intermittent sonication for 5 sec and a subsequent pause of 5 sec. Thereafter, the resulting liposomes were extruded through a membrane.

2.2.2 Optimization of lyophilization process of hydroxypropyl tetrahydropyrantriol liposomes. (i) Screening of pre-freezing temperature. Prior to lyophilization, it is essential to pre-freeze the samples. The temperature at which this is done has a significant impact on the appearance and particle size of the lyophilized preparations. The pre-freezing temperature should be set at a temperature 10 – 20 ℃ below the eutectic point of the material and maintained for a period of time. If the pre-freezing temperature is not sufficiently low, the samples will exhibit the phenomenon of bottle spraying in the process of lyophilization, resulting in an uneven surface finish. Therefore, it is essential to screen the pre-freezing temperature to ensure optimal results. In this experiment, two temperatures were selected for the pre-freezing stage: – 20 ℃ and – 80 ℃. The suitability of these temperatures was evaluated based on two criteria: product appearance and re-dispersibility.

(ii) Screening of pre-freezing time. The optimal pre-freezing duration exerts a profound impact on the lyophilization process. A suboptimal pre-freezing time may result in the bottle spraying of samples during the lyophilization process. In this experiment, samples were pre-frozen at – 80 ℃ for 8, 16, and 24 h. The appearance and re-dispersibility of the products were used as indicators to screen for the optimal pre-freezing time.

2.2.3 Screening of drying time. The drying stage can be divided into two distinct categories: sublimation drying and desorption drying. Sublimation drying is the predominant method employed to remove the majority of free water, accounting for approximately 80% of the total drying time. Desorption drying is a process that primarily removes water of crystallization and bound water combined with hydrogen bonds, which can reduce the water content of the sample to 0.5% – 4.0%. This process ensures the storage stability of the product. In this experiment, we selected a pre-freezing temperature of – 80 ℃ for 16 h, followed by drying for 24, 36, and 48 h, respectively. This was done to identify the optimal drying time, with the appearance and re-dispersibility of the product serving as the criteria for evaluation.

2.2.4 Screening of the type and dosage of single lyoprotectant. Commonly utilized lyoprotectants include sucrose, glucose, lactose, alginate, mannitol, and sorbitol. In this study, sucrose, glucose, and mannitol were employed as lyoprotectants, which were incorporated in an additive manner. Samples devoid of any lyoprotectant constituted the control group, and the status of the products in each group was observed to assess the impact of distinct lyoprotectants on the products.

(i) Mannitol dosage. Mannitol was employed as a lyoprotectant, with the quantity of mannitol adjusted to 1%, 2%, 3%, 4%, 5%, and 10%, respectively. The appearance of the product, particle size, and polydispersity index (PDI) were utilized as evaluation criteria for screening the quantity of mannitol.

(ii) Sucrose dosage. Sucrose was employed as a lyoprotectant, with the quantity of sucrose adjusted to 1%, 2%, 3%, 4%, 5%, and 10%, respectively. The appearance of the product, particle size, and PDI were utilized as evaluation criteria for screening the quantity of sucrose.

(iii) Glucose dosage. Glucose was employed as a lyoprotectant, with the quantity of glucose adjusted to 1%, 2%, 3%, 4%, 5%, and 10%, respectively. The appearance of the product, particle size, and PDI were employed as evaluation criteria for screening the quantity of glucose.

2.2.5 Screening of the type and dosage of combined lyoprotectant. Mannitol-sucrose and mannitol-glucose were employed as lyoprotectants. The amount of lyoprotectant was fixed at 5%, 6%, 7%, 8%, 9%, and 10%, with a mass ratio of the two lyoprotectants of 1 ∶ 1. The appearance, particle size, PDI, and re-dispersibility of the lyophilized samples were employed as evaluation indicators to investigate the protective effect of mannitol on the lyophilized samples when it was utilized as a lyoprotectant in combination with sucrose and glucose, respectively.

2.3 Evaluation criteria for lyophilized preparations A lyoprotectant sample of optimal quality should exhibit the following characteristics: a white, loose, solid, smooth surface without wrinkles or collapse, and a maintained lyophilized volume. Additionally, it should demonstrate good re-dispersibility, rapid re-solubilization, uniform clarification after re-solubilization, and a particle size and PDI that do not significantly change.

3 Results and analysis

3.1 Pre-freezing temperature The temperatures of – 20 ℃ and – 80 ℃ were selected as the pre-freezing temperatures. The product appearance and re-dispersibility were used as the indicators to screen the suitable pre-freezing temperatures, and the results are shown in Table 1. If the pre-freezing temperature is not sufficiently low, the bottle may be sprayed during the lyophilization process, which could affect the appearance of the product. Furthermore, the re-dispersibility may be compromised. Therefore, – 80 ℃ was selected as the pre-freezing temperature in this experiment.

Table 1 Effects of pre-freezing temperature on the product

Pre-freezing temperature // ℃	Appearance	Re-dispersibility
– 20	Collapsed spray bottle	Rather poor
– 80	Flat and smooth	Comparatively good

3.2 Pre-freezing time The product was subjected to pre-freezing at – 80 ℃ for 8, 16, and 24 h, respectively. The appearance and re-dispersibility of the product were employed as indicators to identify the optimal pre-freezing duration. The results are presented in Table 2. Insufficient pre-freezing time may result in bottle spraying during the lyophilization process, which can affect the appearance of the sample and lead to poor re-dispersibility. There was no discernible difference between the products that had been pre-frozen for 16 h and those that had been pre-frozen for 24 h. The products were full and loose, and it has been demonstrated

that pre-freezing for too long may also negatively affect the structure and function of the samples. Therefore, 16 h was chosen as the pre-freezing time for this experiment.

Table 2 Effects of pre-freezing time on the product

Pre-freezing time //h	Appearance	Re-dispersibility
8	Collapsed spray bottle	Rather poor
16	Full and loose	Rather poor
24	Full and loose	Rather poor

3.3 Drying time The product was pre-frozen at −80 ℃ for 16 h, and then dried for 24, 36, or 48 h, with the appearance and re-dispersibility of the product serving as the indicators to screen for the appropriate drying time. The results of this process are shown in Table 3. When the drying time was 24 h, a portion of the bound water was not resolved due to insufficient drying time, resulting in a slight collapse of the product surface. There was no discernible difference between the two groups of products with the drying time of 36 and 48 h. Therefore, 36 h was selected as the drying time.

Table 3 Effects of drying time on the product

Drying time//h	Appearance	Re-dispersibility
24	Slightly collapsed	Rather poor
36	Flat, full and loose	Comparatively good
48	Flat, full and loose	Comparatively good

3.4 Type and dosage of single lyoprotectant Commonly utilized lyoprotectants include sucrose, glucose, lactose, alginate, mannitol, and sorbitol. In this study, sucrose, glucose, and mannitol were employed as lyoprotectants, which were incorporated in an additive manner. Samples devoid of any lyoprotectant constituted the control group, and the status of the products in each group was observed to assess the impact of distinct lyoprotectants on the products.

3.4.1 Screening of mannitol dosage. Mannitol was employed as a lyoprotectant, with the quantity of mannitol adjusted to 1%, 2%, 3%, 4%, 5%, and 10%, respectively. The results are presented in Table 4. As the dosage of mannitol increased, the appearance of the product exhibited a gradual improvement. Nevertheless, the particle size and PDI exhibited a tendency to increase in parallel with the aforementioned phenomenon. This phenomenon may be attributed to the propensity of mannitol to form crystals during the lyophilization process, which can enhance the capacity for support. However, the crystals also disrupt the structure of the liposomes, which leads to fusion of the liposomes and an increase in the size of the particles.

Table 4 Screening of mannitol dosage

Dosage %	Appearance	Particle size//nm	PDI
1	Significant shrinkage with wall climbing phenomenon	376.18	0.486
2	Significant shrinkage with wall climbing phenomenon	383.21	0.617
3	Significant shrinkage with wall climbing phenomenon	461.01	0.484
4	Powdery with wall climbing phenomenon	398.11	0.533
5	Powdery, with a slight rise in products	433.08	0.773
10	Powdery, with wall climbing and rise in products	545.26	0.656

3.4.2 Screening of sucrose dosage. Sucrose was employed as a lyoprotectant, with the quantity of sucrose adjusted to 1%, 2%, 3%, 4%, 5%, and 10%, respectively. The results are presented in Table 5. The appearance of the products obtained with different dosages of sucrose as a lyoprotectant was characterized by obvious shrinkage and wall climbing phenomena. However, with the increase of sucrose, the particle size and PDI of the products after re-dissolution gradually decreased.

Table 5 Screening of sucrose dosage

Dosage %	Appearance	Particle size//nm	PDI
1	Powdery, shrinkage, with wall climbing phenomenon	593.26	0.588
2	Significant shrinkage with wall climbing phenomenon	488.03	0.572
3	Significant shrinkage with wall climbing phenomenon	279.35	0.383
4	Significant shrinkage with wall climbing phenomenon	217.79	0.311
5	Significant shrinkage with wall climbing phenomenon	201.13	0.229
10	Significant shrinkage with wall climbing phenomenon	173.33	0.177

3.4.3 Screening of glucose dosage. Glucose was employed as a lyoprotectant, with the quantity of glucose adjusted to 1%, 2%, 3%, 4%, 5%, and 10%, respectively. The results are presented in Table 6. The appearance of the products obtained with different dosages of glucose as a lyoprotectant exhibited shrinkage and wall climbing phenomena that were analogous to those observed with sucrose. However, with the increase in glucose dosage, the particle size and PDI of the products after re-dissolution gradually decreased.

Table 6 Screening of glucose dosage

Dosage %	Appearance	Particle size//nm	PDI
1	Powdery, shrinkage, with wall climbing phenomenon	413.66	0.733
2	Significant shrinkage with wall climbing phenomenon	293.33	0.513
3	Significant shrinkage with wall climbing phenomenon	227.87	0.379
4	Significant shrinkage with wall climbing phenomenon	153.01	0.188
5	Significant shrinkage with wall climbing phenomenon	223.18	0.350
10	Significant shrinkage with wall climbing phenomenon	181.11	0.211

The preceding experimental data demonstrated that the use of a single lyoprotectant, mannitol, can result in a greater support capacity due to its tendency to form crystals during the lyophilization process. Furthermore, the appearance of the lyophilized samples gradually improved as the dosage of mannitol increased. However, the crystals also disrupted the structure of the liposomes, which resulted in fusion of the liposomes and an increase in the size of the particles. Although the products of glucose and sucrose exhibited clear shrinkage, wall climbing phenomena and a poor appearance in all groups, the particle size and PDI values measured after the lyophilized samples were redissolved gradually decreased with the increase in the dosage of sucrose and glucose. Consequently, the experiment considered the combination of mannitol and sucrose or mannitol and glucose as a potential lyoprotectant.

3.5 Screening of the type and dosage of combined lyoprotectants The amount of lyoprotectant was fixed at 5%, 6%,

7%, 8%, 9%, and 10%, with a mass ratio of the two lyoprotectants of 1∶1. The appearance, particle size, PDI, and re-dispersibility of the lyophilized samples were employed as evaluation indicators to investigate the protective effect of mannitol on the lyophilized samples when it was utilized as a lyoprotectant in combination with sucrose and glucose, respectively. The results are shown in Table 7 and Fig. 1.

Table 7 Screening of the type and dosage of combined lyoprotectants

Dosage and type	Appearance	Re-dissolution time//sec	Particle size before lyophilization //nm	Particle size after re-dissolution //nm	PDI before lyophilization	PDI after re-dissolution
5% Mannitol-sucrose	Shrinkage and wall climbing	19.9	102.5	102.0	0.242	0.221
5% Mannitol-glucose	Shrinkage and wall climbing	40.15	103.3	97.85	0.236	0.211
6% Mannitol-sucrose	Rising	26.1	104.3	104.7	0.250	0.224
6% Mannitol-glucose	Full and wall climbing	37.3	104.5	98.31	0.221	0.224
7% Mannitol-sucrose	Rising	33.6	129.4	136.9	0.181	0.338
7% Mannitol-glucose	Partial shrinkage	26.3	132.6	112.8	0.212	0.196
8% Mannitol-sucrose	Partial shrinkage	39.4	134.5	144.8	0.218	0.335
8% Mannitol-glucose	Rising	37.9	134.2	121.4	0.221	0.206
9% Mannitol-sucrose	Partial shrinkage	40.13	118.9	109.9	0.239	0.196
9% Mannitol-glucose	Partial shrinkage	42.1	120.2	133.0	0.234	0.363
10% Mannitol-sucrose	Loose and full	32.3	92.85	99.96	0.208	0.169
10% Mannitol-glucose	Partial shrinkage	49.7	93.49	113.8	0.214	0.346

Fig. 1 Lyophilized samples using different combined lyoprotectants

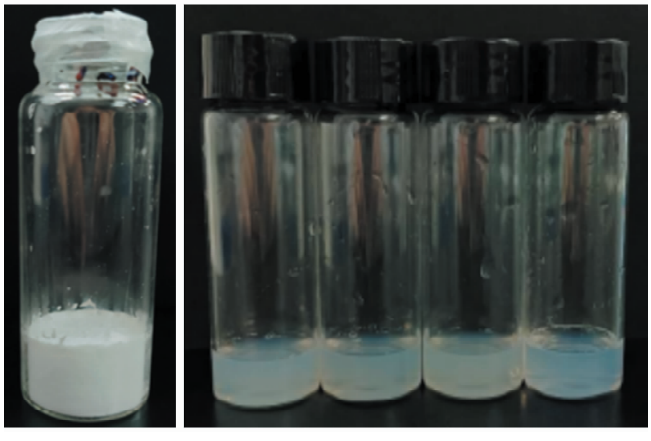

Fig. 2 10% mannitol-sucrose lyophilized liposomes and samples after re-dissolution

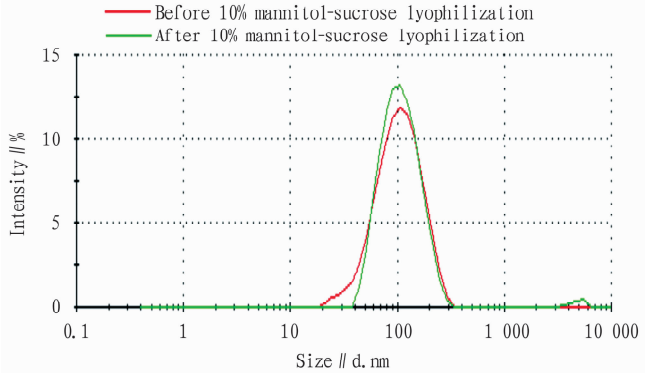

Fig. 3 Particle size distribution diagram of samples before and after lyophilization

A comprehensive weighted scoring method was employed for a more intuitive analysis with a comprehensive score. The relative importance of each indicator was determined based on the appearance and morphology scores, which were assigned a value of 1 – 5. The weighting coefficient for the appearance (A) of the lyophilized liposomes was found to be 0.5, while the weighting coefficient for the increase in particle size before and after lyophilization (B) was 0.3. The weighting coefficients for the time of re-dissolution (C) and the increase in the PDI before and after lyophilization (D) were both 0.1. The results are presented in Table 8.

Table 8 Comprehensive score of combined lyoprotectants

Dosage and type	Appearance score	Particle size increase//nm	Re-dissolution time//sec	PDI increase	Comprehensive score
5% Mannitol-sucrose	2	0	19.9	0	0.160 0
5% Mannitol-glucose	1	0	40.15	0	0.019 2
6% Mannitol-sucrose	3	0.4	26.1	0	0.241 6
6% Mannitol-glucose	4	0	37.3	0.003	0.323 0
7% Mannitol-sucrose	3	7.5	33.6	0.157	0.021 6
7% Mannitol-glucose	3	0	26.3	0	0.247 1
8% Mannitol-sucrose	3	10.3	39.4	0.117	−0.005 9
8% Mannitol-glucose	4	0	37.9	0	0.323 7
9% Mannitol-sucrose	4	0	40.13	0	0.319 3
9% Mannitol-glucose	4	12.8	42.1	0.129	0.044 1
10% Mannitol-sucrose	5	7.11	32.3	0	0.330 0
10% Mannitol-glucose	4	20.31	49.7	0.132	−0.084 1

The results demonstrated that the product obtained with a 10% mannitol-sucrose combination as the lyoprotectant exhibited the highest overall score. This was evidenced by its superior appearance, rapid re-dissolution, and the solution's enhanced clarity after re-dissolution, which exhibited a light blue milky hue. Additionally, the particle size and PDI after re-dissolution were comparable to those observed before lyophilization, as illustrated in Figs. 2 – 3.

Comprehensive score $= 0.5A/A_{max} - 0.3B/B_{max} - 0.1C/C_{max} - 0.1D/D_{max}$

4 Discussion

In order to enhance the stability of hydroxypropyl tetrahydropyrantriol liposomes, a preliminary study of their lyophilization process was carried out. The lyophilization process, type, and dosage of single lyoprotectant and combined lyoprotectants of hydroxypropyl tetrahydropyrantriol liposomes were evaluated using the appearance, re-dispersibility, particle size, and PDI of the products as indicators.

The use of a single lyoprotectant resulted in a gradual improvement in the appearance of the product with an increase in the dosage of mannitol. However, this was accompanied by an increase in particle size and PDI, a reduction in re-dispersability, and a slow re-dissolution rate. The increase in sucrose and glucose concentration did not result in an improvement in the appearance of the product. However, there was a significant reduction in particle size and PDI, and the re-dispersability was enhanced. The results of the screening for the type and dosage of single lyoprotectant were used to inform the examination of the type and dosage of combined lyoprotectants. The final lyophilization process was determined to be as follows: pre-freezing for 16 h at −80 ℃, with a total drying time of 36 h; hydroxypropyl tetrahydropyrantriol lyophilized liposomes were prepared by using additional 10% mannitol-sucrose as the lyoprotectant, and the resulting product had a fluffy and full appearance without shrinkage and collapse. The resulting products exhibited a fluffy and full appearance, with no shrinkage or collapse. There was no change in volume before and after lyophilization, and the products demonstrated good re-dispersibility, rapid re-dissolution, and minimal change in particle size and PDI before and after lyophilization.

References

[1] PINEAU N, BERNERD F, CAVEZZA A, *et al*. A new c-xylopyranoside derivative induces skin expression of glycosaminoglycans and heparan sulphate proteoglycans[J]. European Journal of Dermatology, 2008, 18(1): 36 – 40.

[2] SOK J, PINEAU N, DALKO-CSIBA M, *et al*. Improvement of the dermal epidermal junction in human reconstructed skin by a new c-xylopyranoside derivative[J]. European Journal of Dermatology, 2008, 18(3): 297 – 302.

[3] PINEAU N, CARRINO DA, CAPLAN AI, *et al*. Biological evaluation of a new c-xylopyranoside derivative (c-xyloside) and its role in glycosaminoglycan biosynthesis[J]. European Journal of Dermatology, 2011, 21(3): 359 – 370.

[4] MARIA D, LIONEL B. C-glycoside compounds for stimulating the synthesis of glycosaminoglycans: US7049300B2[P]. 2006 – 3 – 23.

[5] SHUTE J. Glycosaminoglycan and chemokine/growth factor interactions [J]. Handbook of Experimental Pharmacology1, 2012(207): 307 – 324.

[6] DELOCHE C, MINONDO AM, Bernard BA, *et al*. Effect of c-xyloside on morphogenesis of the dermal epidermal junction in aged female skin. An ultrastuctural pilot study[J]. European Journal of Dermatology, 2011, 21(2): 191 – 196.

[7] CHUN B, ZHANG X, DING JY, *et al*. Preparation process of hydroxypropyl tetrahydropyrantriol liposomes[J]. Medicinal Plant, 2023, 14(4): 51 – 57.

(From page 34)

[3] Collaborative Writing Group of Sichuan Chinese Medicine Record. Sichuan Chinese medicine (Volume 2)[M]. Chengdu: Sichuan People's Publishing House, 1982: 224. (in Chinese).

[4] State Administration of Traditional Chinese Medicine Chinese Materia Medica Editorial Committee. Chinese Materia Medica (Volume III)[M]. Shanghai: Shanghai Science and Technology Press, 1999: 703 – 704. (in Chinese).

[5] Editorial Committee of Flora of China, Chinese Academy of Sciences. Flora of China[M]. Beijing: Science Press, 1987(33): 194, 337. (in Chinese).

[6] JIA MR, ZHANG Y. Dictionary of Chinese Ethnic Medicine[M]. Beijing: China Medical Science Press, 2016: 1194 – 1195. (in Chinese).

[7] HOU CC, XU S. Analysis on the key influence factors of paraffin section's quality[J]. Chinese Agricultural Science Bulletin, 2009, 25(23): 94 – 98. (in Chinese).

[8] YANG JP. Improvement of traditional paraffin section preparation methods [J]. Journal of Biology, 2006, 23(1): 45 – 46. (in Chinese).

[9] National Pharmacopoeia Commission. Pharmacopoeia of the People's Republic of China (Volume I)[S]. Beijing: China Pharmaceutical Science and Technology Press, 2020. (in Chinese).