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Short communication

Optimizing glycerosome formulations *via* an orthogonal experimental design to enhance transdermal triptolide delivery

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Triptolide exerts strong anti-inflammatory and immunomodulatory effects; however, its oral administration might be associated with side effects. Transdermal administration can improve the safety of triptolide. In this study, glycerosomes were prepared as the transdermal vehicle to enhance the transdermal delivery of triptolide. With entrapment efficiency and drug loading as dependent variables, the glycerosome formulation was optimized using an orthogonal experimental design. Phospholipid-to-cholesterol and phospholipid-to-triptolide mass ratios of 30:1 and 5:1, respectively and a glycerol concentration of 20 % (*V*/*V*) were used in the optimization. The glycerosomes prepared with the optimized formulation showed good stability, with an average particle size of 153.10 ± 2.69 nm, a zeta potential of –45.73 ± 0.60 mV and an entrapment greater than 75 %. Glycerosomes significantly increased the transdermal delivery of triptolide compared to conventional liposomes. As efficient carriers for the transdermal delivery of drugs, glycerosomes can potentially be used as an alternative to oral triptolide administration.

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Triptolide (Fig. 1) is an active pharmaceutical ingredient extracted from the herb *Tripterygium wilfordii*. The compound has a melting point of 226–227 °C and it is poorly soluble in water with the apparent solubility in the pure water of 21.38 ± 0.36 μg mL^{-1}. Its apparent oil-water partition coefficient in the *n*-octanol/water system is 0.60 (log *P*) (1). The acidity of triptolide is very weak and the degree of ionization is very small. It shows exceptional therapeutic effects against various immune and inflammatory diseases and is used in the treatment of rheumatoid arthritis (2, 3). However, oral administration of triptolide irritates the gastrointestinal tract and exerts liver toxicity (4, 5). Transdermal administration can reduce side effects and associated toxicity and avoid their first-pass metabolism in the liver (6).

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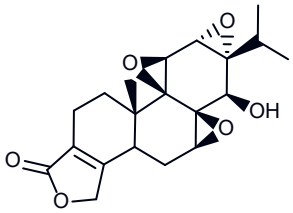

Fig. 1. Chemical structure of triptolide.

Liposomes are commonly used drug carriers for transdermal delivery. Owing to their low deformability, conventional liposomes have poor penetration into the stratum corneum and are generally administered locally for the treatment of skin diseases. To enhance the transdermal permeability of liposomes, some low-chain alcohols are used as edge activators to improve the deformability of liposome vesicles (7). Glycerosomes are formulated using glycerol as the edge activator in liposomes, a finding that was first reported in 2013 (8). Glycerosomes have stronger transdermal permeability than conventional liposomes, thereby increasing transdermal drug delivery. In addition, glycerosomes have excellent stability and biocompatibility and have been used as a vehicle for transdermal and pulmonary drug delivery (9–12).

In the present study, glycerosomes were used as carriers for the transdermal delivery of triptolide. The composition of these glycerosomes was optimized using an orthogonal experimental design. The transdermal permeation of triptolide loaded into glycerosomes was compared to conventional liposomes.

EXPERIMENTAL

Materials and animals

Triptolide (purity ≥ 98 %) was purchased from Chengdu Pufei De Biotech Co., Ltd. (China). Glycerol was obtained from Beijing Dingguo Changsheng Biotech Co., Ltd. (China). Soy lecithin (S80, phosphatidylcholine ≥ 80 %) was provided by Tywei Pharmaceutical Co., Ltd. (China). All other reagents were obtained from Sinopharm Chemical Reagent Co., Ltd. (China).

Fifteen healthy male Sprague Dawley rats, weighing 200 ± 20 g, were provided by the Experimental Animal Center of Shanghai University of Traditional Chinese Medicine. The animal protocol was approved by the Experimental Animal Ethics Committee of Shanghai University of Traditional Chinese Medicine. Animals were kept in an agreeable environment with free access to rodent diet and water and were acclimatized for 1 week before the start of the study.

Analytical method

Triptolide was analyzed using a high-performance liquid chromatography system (HPLC; LC-2010AHT, Shimadzu Corporation, Japan) equipped with a Diamonsil Cl8

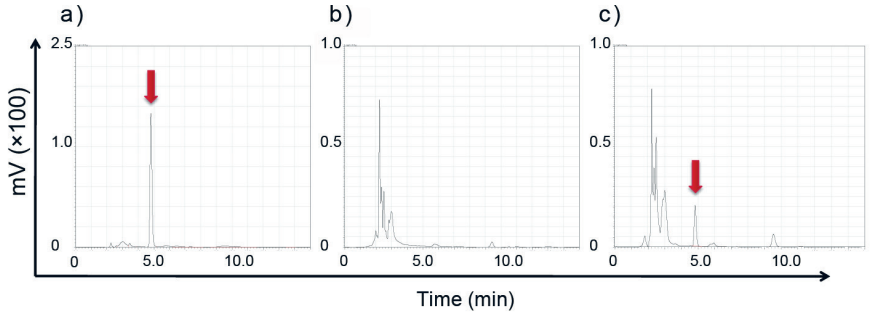

Fig. 2. Chromatograms obtained by high-performance liquid chromatography of: a) triptolide methanol solution, b) fresh receiving solution (without the drug) used for the *ex vivo* transdermal test and c) receiving solution sampled from the triptolide-loaded glycerosomes administered in the *ex vivo* skin permeation experiments. The arrow indicates the chromatographic peak of triptolide.

(250 × 4.60 mm, 5 μm) chromatographic column (Dikma Technology, China). The column was maintained at 35 °C, the mobile phase composition was methanol/water (60:40, *V*/*V*) and the flow rate was 1.0 mL min^{-1}. The detection wavelength was 218 nm, as used in a previous study (13). The retention time of triptolide was 5.0 min and no chromatographic peak appeared at this retention time in the fresh receiving solution (Fig. 2), which did not contain the drug, indicating that there was no interference associated with the quantification of triptolide. The linear relationship between the area of chromatographic peak and triptolide concentration in the receiving solution was good within a concentration range of 0.08–5 μg mL^{-1} (regression equation: $y = 31231x - 1522$, $R^2 = 0.9997$). The precision and accuracy of the method were consistent with the requirements.

Preparation of nanoformulations

Glycerosomes were prepared by the injection method (14). Cholesterol, soy lecithin and triptolide, with the final concentrations of 0.167, 5 and 1 mg mL^{-1} in the glycerosomes, respectively, were dissolved in half the weight of glycerol in the glycerosome formulation and mixed using a magnetic stirrer (IKA®RCT, IKA, Germany) at 600 rpm for 1 h, at 40 °C. The remaining glycerol was slowly injected and stirring continued for 1 h and the final concentration of glycerol was 20 % (*V*/*V*). The size distribution of the glycerosome suspension initially obtained was reduced by ultrasonication for 10 min (900 Hz; interval time, 3 s) with an ultrasonic tissue crusher (SCIENTZ-IID, Ningbo Xinzhi Biotechnology Co., Ltd., China).

For comparison, conventional liposomes were prepared as follows: cholesterol, soy lecithin and triptolide, with the same final concentrations as in the glycerosomes, were dissolved in 1 mL of ethanol, slowly injected into 10 mL of purified water and mixed using a magnetic stirrer at 600 rpm at 50 °C. Thereafter, ethanol was removed by nitrogen sweeping and the dispersion was subjected to ultrasonication for 10 min (900 Hz; interval time, 3 s) to obtain the final liposome preparation.

The aqueous solution of triptolide glycerol (glycerol solution) was prepared by dissolving triptolide (final concentration: 1 mg mL^{-1}) in a 20 % aqueous solution of glycerol and vortexing the mixture for 10 min. All prepared samples were stored at 4 °C.

Characterization of nanoformulations

The nanopreparations were diluted with distilled water and dropped into a sample container. The particle size distribution was determined by dynamic light scattering and the zeta potential was measured using a Zetasizer Nano ZS 90 instrument (UK).

The glycerosome dispersion was placed on a copper mesh with a supporting film and allowed to dry. Negative staining was performed with 2 % phosphomolybdic acid for 2–3 min and the preparation was imaged using a transmission electron microscope (Tecnai G2 Spirit BioTWIN, USA).

Determination of entrapment efficiency and drug loading

A centrifugal ultrafiltration method was used to determine the entrapment efficiency (*EE*) of the nanocarriers. The nanopreparations were placed in an ultrafiltration centrifuge tube (molecular weight cut-off: 100 kDa) and centrifuged at 7853 ×*g* for 20 min to completely filter the dispersion medium. The sediment was washed twice with 0.5 mL of water. The filtrate was analyzed by HPLC to calculate the free drug content. Equations *1* and *2* were used to obtain the *EE* and drug loading (*DL*):

$$EE = \frac{m_2 - m_1}{m_2} \times 100\ \% \tag{1}$$

$$DL = \frac{m_2 - m_1}{m_0 + m_2 - m_1} \times 100\ \% \tag{2}$$

where m_0 is the mass of the empty nanocarrier, m_1 is the mass of free drug and m_2 is the total mass of drug in the preparation.

Optimization of glycerosome formulation using orthogonal experimental design

Glycerol concentration (A), phospholipid to cholesterol mass ratio (B) and phospholipid to triptolide mass ratio (C) were set as independent variables (Table I). The weighted scores of *EE* and *DL* were set as dependent variables. The standard score was 100 points and *DL* and *EE* accounted for 50 % of each score. The glycerosome formulation was arranged according to a four-factor, three-level orthogonal table [L9 (3^4)].

Table I. Factor-level arrangement table

Level	A (%, *V*/*V*)	B (*m*/*m*)	C (*m*/*m*)
1	10	10:1	5:1
2	20	20:1	15:1
3	30	30:1	30:1

A – glycerol concentration, B – phospholipid to cholesterol mass ratio, C – phospholipid to triptolide mass ratio.

Preparation stability

The glycerosomes prepared with the selected formulation were stored at 4 °C and the *EE*, zeta potential and size distribution were measured on the day of preparation and after 30 days of storage.

Ex vivo *transdermal experiment*

The rats were randomly divided into three groups (glycerosomes, conventional liposomes and aqueous glycerol solution; five animals in each group) and anesthetized with ether inhalation. The abdominal hair was carefully shaved with an electric shaver until the length of the hair was less than 2 mm; thereafter, the rats were sacrificed. The abdominal skin was isolated quickly and the subcutaneous fat and adhering materials were carefully removed. The thickness of the final isolated skin was 0.8–1.2 mm. The skin was washed with normal saline, its integrity was examined and it was immediately used for the *ex vivo* transdermal test.

A modified vertical Franz transdermal diffusion cell (with an effective diffusion area of 2.0 cm^2 and a receiving cell volume of 12.5 mL; TT-06, Zhengtong Technology Co., Ltd. (China) was used for performing the transdermal test. The skin was fixed tightly on the donor cell with the stratum corneum facing upward. One mL of the tested triptolide preparation (triptolide concentration: 1 mg mL^{-1}) was applied evenly to the skin surface. A 20 % solution of PEG400 was used as the receiving solution and maintained at 37 °C. A 1 mL aliquot was sampled from the receiving cell at 1, 2, 3, 5, 7, 12 and 24 h and an equal volume of fresh receiving solution, preheated to 37 °C, was added after sampling. The samples were analyzed by HPLC and the cumulative drug penetration per unit area at different time points was calculated.

Statistical methods

The experimental data are expressed as mean ± standard deviation. One-way analysis of variance was used for comparisons between groups. Statistical analyses were performed using the SPSS 18.0 statistical software and a value of $p < 0.05$ was considered to represent a statistically significant difference.

RESULTS AND DISCUSSION

Optimization of glycerosome formulations

Recently, the superiority of glycerosomes as carriers for transdermal drug administration has garnered attention from researchers. Glycerosomes have been used to encapsulate small-molecule compounds, proteins and even some natural extracts with high *EE*, such as *Hypericum scruglii* extract and curcumin (15–20).

The composition of the formulation has a major influence on the *EE* and *DL* of glycerosomes (21). To reduce the number of experiments without affecting the results for optimizing the formulation, various experimental design methods, such as orthogonal design, uniform design and Box-Behnken design, can be used (22–24). Orthogonal design is the

main method of fractional factorial design. When three or more factors are involved with potential interactions among the factors, the workload of the experimenter becomes large and it might be difficult to execute the experiment. Assuming orthogonality in the orthogonal table, representative points are selected and a result equivalent to that obtained after a large number of comprehensive tests can be achieved with a minimum number of tests. Thus, the application of the orthogonal design according to an orthogonal table is an efficient, fast and economical multi-factor experimental design method. For example, for a three-factor three-level experiment, according to the requirements for a comprehensive experiment, $27(3^3)$ experimental combinations should be assessed and the number of repetitions for each combination is not considered. If the experiment is arranged according to the $L_9(3)$ orthogonal table, only nine tests are needed.

Using the $L_9(3^4)$ orthogonal table, the results for the optimization of the glycerosome formulation in the present study are listed in Table II. The *EE* and *DL* of the glycerosomes in each group ranged from 43.25 to 77.40 % and from 3.07 to 16.19 %, respectively. The phospholipid to triptolide mass ratio (C) had a significant effect on the experimental results (Table III). An increase in the phospholipid mass ratio increased the *EE* of the drug and a large amount of carrier material reduced the *DL*; the weighting value of *EE* and *DL* decreased as the proportion of phospholipids increased. Similarly, Pleguezuelos-Villa *et al.* reported stable incorporation of mangiferin in glycerosomes at increasing drug concentrations; however, this pattern was not observed for ethanol-formulated ethosomes (10). The concentration of glycerol (A) and the phospholipid to cholesterol mass ratio (B) had a smaller effect on the experimental results. This is similar to the results obtained in a previous

Table II. Experimental arrangement and results based on the $L_9(3^4)$ orthogonal table

FN	A	B	C	D	*EE* (%)	*DL* (%)	*WS*
1	1	1	1	1	65.67	15.41	90.01
2	1	2	2	2	61.87	5.97	58.41
3	1	3	3	3	55.79	3.12	45.68
4	2	1	2	3	65.56	5.71	60.00
5	2	2	3	1	54.64	3.07	44.78
6	2	3	1	2	77.40	16.19	100.00
7	3	1	3	2	43.25	2.93	37.00
8	3	2	1	3	67.37	15.97	92.84
9	3	3	2	1	54.85	6.06	54.14
K_1	64.700	62.337	94.283	62.977			
K_2	68.260	65.343	57.517	65.137			
K_3	61.327	66.607	42.487	66.173			
Range	69.33	4.270	51.796	3.196			

FN – formulation number, *EE* – entrapment efficiency, *DL* – drug loading of triptolide, *WS* – weighted score, A – glycerol concentration, B – phospholipid to cholesterol mass ratio, C – phospholipid to triptolide mass ratio, D – error term.

Table III. ANOVA results

Factors	DEVSQ	df	F-value	CV
A	72.124	2	4.519	19.000
B	28.869	2	1.809	19.000
C	4260.583	2	266.971*	19.000
D (Errors)	15.96	2		

DEVSQ – the sum of squares of mean deviation, df – degrees of freedom, CV – critical value of the F-test.
* Significant difference compared to CV, based on F-test.

study, wherein it was reported that the *EE* of glycerosomes increased only by 1 %, the *DL* remained almost unchanged when the concentration of glycerol was increased from 20 to 30 % and the phase transition temperature of the corresponding phospholipid membrane from a gel state to a liquid crystal state was almost the same (8). Manca *et al.* also found that when the concentration of glycerol was increased from 10 to 20 %, the amount of transdermal diclofenac sodium in the total drug content of the tested preparation accumulated over 8 h increased from 4.0 to 7.8 %, although it decreased slightly to 7.4 % with a further increase in glycerol concentration to 30 % (8). Based on the results of the orthogonal experimental design, the following optimal composition was selected for the preparation of glycerosomes: the concentration of glycerol was 20 %, phospholipid/cholesterol (*m*/*m*) was 30:1 and phospholipid/triptolide (*m*/*m*) was 5:1 (25).

Three batches of glycerosomes were prepared according to the optimal formulation. The average particle size was 153.10 ± 2.69 nm, the zeta potential was –45.73 ± 0.60 mV, *EE* was 75.97 ± 0.94 % and *DL* was 16.15 ± 0.07 %. The average particle size of the comparable conventional liposomes was 187.25 ± 11.65 nm, which was larger than that of the glycerosomes; this was mainly attributable to the high concentration of glycerol in the glycerosomes, which increased the fluidity of the phospholipid membrane and resulted in a reduction in the particle size (8). Glycerosomes formulated with dipalmitoylphosphatidylcholine are slightly larger but more polydisperse than those formulated without glycerol (26). The zeta potential of conventional liposomes was –19.32 ± 0.80 mV, which was significantly lower than that of the glycerosomes, perhaps owing to the increased negative charge on the surface of glycerosomes conferred by glycerol increasing the zeta potential (27, 28). In addition, the high concentration of glycerol increased the solubility of insoluble triptolide, allowing the drug to be distributed in the bilayer of the huge internal water chamber of glycerosome vesicles, thereby resulting in higher *EE* and *DL* than those for conventional liposomes, which had values of 52.16 ± 1.44 % and 8.75 ± 0.53 %, respectively (29).

Transmission electron microscopy revealed that the glycerosome nanovesicles were spherical, uniformly dispersed and did not show aggregation (Fig. 3). The average particle size distribution was slightly smaller than that determined by dynamic light scattering. This might be because the value determined by dynamic light scattering includes the thickness of the outer water film of the phospholipid membrane and the particle size measured by transmission electron microscopy is based on the morphology of the dried phospholipid vesicles.

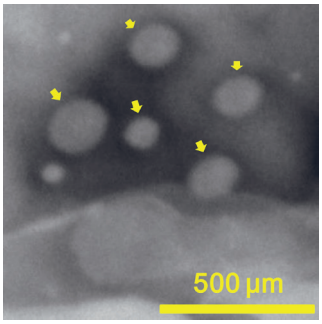

Fig. 3. Micrograph of triptolide-loaded glycerosomes taken by transmission electron microscope (arrows point to glycerosomes).

Glycerol increased the viscosity of the glycerosomes, enhancing the stability of the nanovesicles (30). After storage at 4 °C for 30 days, the particle size distribution, *EE* and *DL* of the glycerosomes were not significantly changed ($p > 0.05$), thus indicating good stability.

Permeation of triptolide in rat skin

Glycerosomes can significantly improve the transdermal delivery of drugs. For example, the transdermal flux of diclofenac sodium mediated by glycerosomes containing 20 % glycerol was reported to be 1.5-times higher than that obtained using conventional liposomes and glycerosome-enhanced lacidipine permeation by 3.65-fold compared with that achieved with lacidipine suspension *ex vivo* (8, 31). In a previous study, we showed that glycerosomes greatly enhanced the transdermal delivery of paeoniflorin, a water-soluble active ingredient (32). Herein, we found that the cumulative transdermal amount of triptolide in the glycerosome group over 24 h was 1.78- and 1.52-times higher than those in the

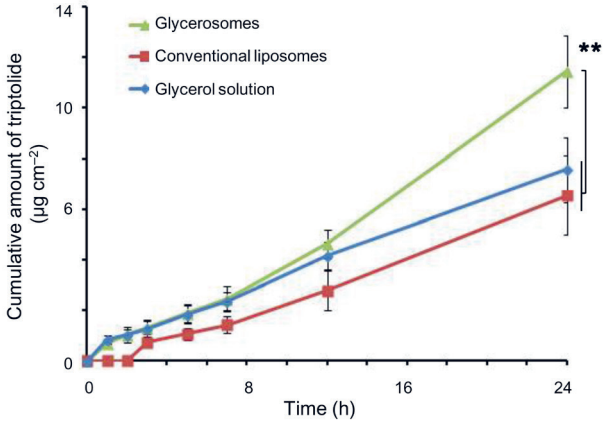

Fig. 4. The *ex vivo* permeation-time curves for permeation of triptolide in glycerosomes, conventional liposomes and 20 % glycerol solution through the isolated skin of rats over 24 h (**$p < 0.01$; $n = 5$).

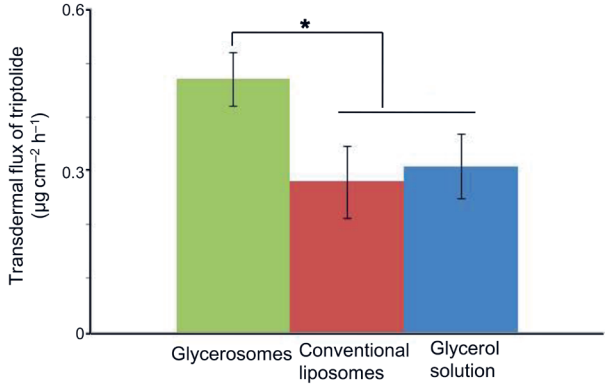

Fig. 5. *Ex vivo* transdermal flux of triptolide in glycerosomes, conventional liposomes and 20 % glycerol solution (*$p < 0.05$; $n = 5$).

conventional liposome group and the triptolide 20 % glycerol solution group, respectively ($p < 0.01$) (Fig. 4). In addition, the transdermal flux in the glycerosome group was significantly greater than that in the conventional liposome and glycerol solution groups ($p < 0.05$) (Fig. 5). The transdermal permeability in the conventional liposome group was lower than that in the glycerol solution group, which indicates that 20 % glycerol had an enhancing effect on the penetration.

Previous reports have shown that the elasticity of glycerosomes increases with an increase in glycerol content (8). At 30 % glycerol concentration, the deformation index of glycerosomes was twice the value of control liposomes, indicating that glycerol could act as an edge activator for phospholipid membranes, though only at high concentrations (> 10 %) (33). The incorporation of certain drugs, such as diclofenac sodium, influences the glycerosome membrane and may interfere with membrane deformability (33). In another study, based on small-angle X-ray scattering, a strong dose-dependent interaction between glycerol and polar portions of phospholipid molecules was observed, whereas encapsulated quercetin did not significantly alter bilayer packing (34). In addition to being dispersed in the phospholipid membrane as an edge activator, the glycerol present in the internal water compartment increases the loading of liposoluble compounds, whereas the glycerol outside the vesicle has a moisturizing and penetration enhancing effect, which helps the drug to penetrate the skin. In summary, glycerol contributes to the deformability of glycerosomes and serves as an enhancer of drug penetration, concomitantly enhancing the plasticity and hydration of the stratum corneum, thereby, resulting in efficient transdermal delivery of drugs (35). In addition, glycerol in the glycerosomes forms more viscous systems than those of conventional liposomes, therefore improving their spreadability on the skin (36).

CONCLUSIONS

In this study, an orthogonal experimental design was used to optimize the composition of glycerosome formulations. The prepared glycerosomes significantly enhanced the percutaneous delivery of triptolide. Glycerosomes had greater transdermal permeability,

stability and biocompatibility than conventional liposomes. Consequently, glycerosomes have great potential as vehicles for dermal and transdermal drug delivery.

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