In Vivo Microdialysis for Dynamic Monitoring of the Effectiveness of Nano-liposomes as Vehicles for Topical Psoralen Application

Hongyu Zhang, Kai Zhang, Zhe Li, Jihui Zhao, Yongtai Zhang,* and Nianping Feng*

Department of Pharmaceutical Sciences, Shanghai University of Traditional Chinese Medicine; Shanghai 201203, China.

Received April 11, 2017; accepted August 11, 2017

In this study, the skin permeation of liposomes containing psoralen was investigated by in vivo skin microdialysis. Psoralen-loaded nano-sized liposomes were prepared with a mean size of 117.5 nm and a polydispersity index of 0.21, indicating the uniform dispersion of phosphatidylcholine vesicles in the liposomal solution. Based on in vivo microdialysis experiments, the drug concentration in local deep skin of rat increased rapidly and reached a peak concentration \( C_{\text{max}} \) of 319.35 ± 23.72 µg/mL at 180 min, and decreased slowly thereafter. The local area under the concentration–time curve \( AUC_{0-t} \) was 3.81-fold higher than the compared aqueous suspension. The in vivo systemic pharmacokinetics were in agreement with the microdialysis results, in view of the \( C_{\text{max}} \) and \( AUC_{0-t} \), from liposomal group were both significantly higher \((p < 0.05)\) than the compared group. Liposome-associated transdermal psoralen delivery was significantly more effective than delivery via an aqueous suspension. The enhanced skin permeability may be associated with improved skin hydration, lipid exchange and fusion with the stratum corneum (SC), and changes in SC structure, promoting drug permeation into deep skin. After 10 h of treatment with the perfusate, the microstructure of the microdialysis probe exhibited no obvious differences with control probes. The skin surface and the tissue around the probe showed no swelling or inflammation. These findings indicated that liposomes effectively enhanced the skin deposition of psoralen and showed good biocompatibility with skin tissues; additionally, ethanol at a low concentration in ringer’s solution is an alternative perfusate for in vivo skin microdialysis studies.

Key words vesicle; transdermal; nanocarrier; permeability

Liposomes are composed of phospholipid bilayer films. They can be used as carriers to enhance drug permeation into the stratum corneum (SC), leading to the formation of drug reservoirs in the skin and prolonging the duration of action in lesions via sustained drug release.1–3) For the topical application of drugs, liposomes can increase distribution in local tissues and reduce the systemic absorption of drugs, thereby minimizing side effects and improving local bioavailability.4–6)

Psoralen (Fig. 1a) is a photosensitizing ingredient isolated from the herb Fructus Psoraleae.7) It can be applied topically and exposed to sunlight or ultraviolet radiation for the treatment of vitiligo and other skin diseases by affecting melanocytes, increasing tyrosinase activity, and promoting melanin synthesis.8) Liposomes have been employed as carriers of psoralen for topical administration, resulting in effective cutaneous drug absorption.9) However, the precise effects of liposome-mediated psoralen delivery, including drug distribution over time, are unclear.

Microdialysis is a new biological sampling technology based on the permeability and diffusion of small molecule compounds dependent on the mass concentration gradient.10) A semipermeable membrane probe is implanted in a tissue, and a perfusate is pushed through the probe using a micropump. The free small molecule drug can diffuse into the probe according to the mass concentration gradient, but protein-binding drug molecules and other macromolecules are excluded from the probe by the semipermeable membrane.11,12) The level of analytes in the extracellular fluid can be determined by assaying the mass concentration of the drug in the dialysate. In this study, in vivo microdialysis was used to dynamically monitor the psoralen concentration in deep skin and to calculate local pharmacokinetic parameters in order to evaluate the enhanced transdermal drug delivery using liposomes. The in vivo systemic pharmacokinetics of psoralen after transdermal administration were also investigated for evaluation drug absorption by using nanoliposomes as vehicles.

**MATERIALS AND METHODS**

**Materials** Lipoid S 100 containing 95.8% phosphatidylcholine was purchased from Lipoid GmbH (Ludwigshafen, Germany). Psoralen and sophoricoside (Fig. 1b) (purity > 98.0%) was obtained from Ze-lang BioScience (Nanjing, China). The cholesterol and others chemicals were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China) and were of HPLC or analytical grade.

**Preparation of Liposomes and Aqueous Suspensions** The prepared liposome formulation was composed of 6.0% (w/v) Lipoid S 100, 1.5% (w/v) cholesterol, and 0.2% (w/v) psoralen. The drug, Lipoid S 100, and cholesterol were dissolved in ethanol at a low concentration in ringer’s solution and were used as the perfusate in the skin microdialysis experiments. Psoralen-loaded nano-sized liposomes were prepared with a mean size of 117.5 nm and a polydispersity index of 0.21, indicating the uniform dispersion of phosphatidylcholine vesicles in the liposomal solution.

**Microdialysis** Microdialysis was used to determine the concentration of drugs in the extracellular fluid of deep skin. A micropump was used to push the perfusate through the microdialysis probe. The concentration of psoralen in the dialysate was determined by high-performance liquid chromatography (HPLC).

![Fig. 1. Chemical Structures of Psoralen (a) and Sophoricoside (b)](image-url)
solved in 3:1 methanol–chloroform (v/v). The solution was poured into a pear-shaped bottle and the organic solvent was removed using a rotavapor (BUCHI Labortechnik AG, Flawil, Switzerland) in a water bath at 40°C. The vacuum was maintained for 30 min until a uniform lipid film formed on the inner wall of the bottle. The lipid film was hydrated with phosphate-buffered saline (PBS) (pH 7.4) for 1 h at 30°C under a vacuum by rotation at 70 rpm. The preliminary liposome suspension was probe-sonicated for 10 min (power, 900 Hz; quiescent interval, 3 s) in an ice-water bath by using an ultrasonic cell disruption system (Scientz Biotechnology Co., Ltd., Ningbo, People's Republic of China). An aqueous suspension was prepared by adding psoralen at a concentration of 0.2% (w/v) to PBS.

**Liposome Characteristics** The size distribution and polydispersity index (PDI) of the liposomal preparation was measured using dynamic light scattering with a computerized Malvern Autosizer Nano ZS90 Inspection System (Malvern Instruments Ltd., Malvern, U.K.). Liposome appearance was examined using a high-resolution cross-sectional transmission electron microscope (HRTEM) (JEM-2100, JEOL Ltd., Tokyo, Japan). Samples were prepared for negative staining. Briefly, copper nets carrying formvar-supporting films were placed on stencil plates. Liposome solution was dropped gently onto the film, and the film was allowed to dry for about 20 min. A drop of 2% phosphotungstic acid was then added to the film and allowed to dry for 10 min before TEM observations.

The entrapment efficiency of psoralen with liposomes was evaluated by dialysis as described previously. A dialysis bag with a molecular weight cut-off of 10000 Da was used, and dialysis proceeded for 5 h.

**Ultra Performance Liquid Chromatography-Tandem Mass Spectrometry (UPLC-MSn) Analysis** The UPLC-MSn system (Ultimate 3000, Thermo Fisher Scientific Inc., Waltham, MA, U.S.A.) with a Thermo Syncronis C18 reverse phase column (100×2.1 mm, 1.7 µm; Thermo Fisher Scientific Inc.) was used with a gradient elution for the separation and determination of psoralen was used, and the mobile phase sequence with a flow rate of 0.2 mL/min was listed as follows: 0–1 min, 0.1% acetic acid aqueous solution (A)–methanol (B) (70:30, v/v); 1–5 min, A–B (10:90, v/v); 5–8 min, A–B (70:30, v/v). The MS determination conditions were stepwise with alcohol, stained with hematoxylin and eosin, and observed using a scanning electron microscope (SEM) (Quanta FEG250; FEI, Hillsboro, NC, U.S.A.).

**Skin Tissue Irritation** At the end of the microdialysis experiment, rats were sacrificed by anesthesia. The residual liposomes on the tested skin region were removed, and the skin surface was washed with pure water. The skin tissue containing the implanted probe was excised and fixed in 10% buffered formaldehyde solution for 72 h. Skins were embedded in paraffin and sliced transversely. The sections were dehydrated stepwise with alcohol, stained with hematoxylin and eosin, and observed using an optical microscope (BH-2; Olympus Corporation, Hatagaya, Japan).

**In Vivo Systemic Pharmacokinetic Study** The drug administration methods were the same as the above in vivo microdialysis studies. Blood samples were collected, treated, and assayed as described in the above section “2.6.” The pharmacokinetic parameters of psoralen were calculated and analyzed using a noncompartmental analysis implemented in WinNonLin (v.5.2; Pharsight Corporation, Sunnyvale, CA, U.S.A.).

**Statistical Analysis** The results are expressed as the mean±standard deviation. Statistical analyses were performed using one-way ANOVA with p-values of <0.05 considered statistically significant.

In vivo microdialysis studies were performed according to previous methods. Briefly, the fur on the administration region of each rat was manually clipped with a blade. A microdialysis probe was implanted in about 0.22-mm-deep skin tissue and the active dialysis window was placed immediately below the site of topical drug administration. The donor cell was stuck to the skin, just above the probe, and 1 mL of the test formulation was applied to the skin with a contact area of approximately 0.8 cm². Dialysate samples were detected by HPLC following previously described methods, and the drug concentration in deep skin tissue was adjusted by in vivo recovery.

**Effect of Perfusate Treatment on Drug Concentration in Blood and Microdialysis Probe Structure** For evaluation of the perfusate effect on the drug absorption, the in vivo microdialysis experiments of liposomal psoralen were conducted as described in the above section “2.5,” by using 10% (v/v) ethanol ringer’s solution as the perfusate, respectively. Meanwhile blood samples were collected from the ocular vein into heparinized tubes at predetermined time points, and centrifuged at 3000 rpm for 10 min to obtain plasma. Prior to analysis, 50 µL of 50 ng/mL sophoricoside was added as internal standard to 100 µL of the plasma sample and mixed by vortexing. Next, 300 µL acetonitrile was added and the mixture was mixed by vortexing, centrifuged at 5000 rpm for 5 min, and the supernatant was collected and dried under nitrogen, after which 50 µL of methanol was added and the sample was mixed by vortexing for 1 min. The samples were assayed by UPLC-MSn.

After 10 h of perfusion with the perfusate, the dialysis membrane was cut out and washed with pure water, dried using CO₂ by critical point drying, sputtered with platinum, and observed using a scanning electron microscope (SEM) (Quanta FEG250; FEI, Hillsboro, NC, U.S.A.).
RESULTS AND DISCUSSION

Liposomes were spherical (Fig. 2). The mean particle size was 117.5 nm, with a low PDI of 0.21, indicating that the particle size distribution was narrow and the liposomes were homogeneous. The entrapment efficiency of psoralen was 83.37±11.07%. As shown in Fig. 3, after the topical application of drug-loaded liposomes, the psoralen concentration in the microdialysis dialysate increased rapidly over time, reached the peak concentration (C_max) at 180 min, and decreased slowly until the end of the experiment. The final concentration was approximately half of C_max, and the high drug concentration in tissue during the sampling period resulted in a high area under the concentration–time curve (AUC_0→t) (Table 1). However, a much lower concentration of the drug was detected in the perfusate by UPLC-MSn and accordingly the psoralen distribution in the skin after topical administration with the psoralen aqueous suspension, and the C_max as well as AUC_0→t values were both significantly smaller than the liposomal groups, indicating that liposomes significantly improved transdermal drug absorption. Phospholipid amphiphilic molecules and nano-size vesicles can moisturize the SC, strengthen skin surface hydration, and affect SC structure, while increasing the fluidity of SC lipids, thereby promoting transdermal drug absorption.14–16) In addition, drug accumulation in the SC after release from liposomes can result in a high concentration gradient and increase the diffusive force, thereby promoting drug permeation into deep skin.17,18)

Using liposomes as psoralen transdermal carriers, the drug concentration in the skin initially increased and then decreased after administration. As liposoluble ingredients, psoralen molecules are mainly distributed in the liposomal bilayer of phospholipids.19) The drug can be released when the liposomal membrane is destroyed by lipid exchange and fusion with the lipidic SC.20,21) Drug release increased at the skin surface to form a high concentration gradient and enhanced drug permeability, which led to a rapid increase in drug concentration in the skin. In addition, psoralen exhibited sustained diffusion into the deep skin from the drug reservoir that formed in the epidermis, resulting in a high drug concentration in the deep skin and a slow decline after reaching C_max.

The in vivo systemic pharmacokinetic study results were in agreement with the in vivo microdialysis results. After transdermal administration, the psoralen concentration in blood showed much higher by using liposomes as drug carriers than the compared psoralen aqueous suspension (Fig. 4). By encapsulating into the liposomes, the C_max and AUC_0→t of psoralen

- Fig. 2. Appearance of Liposomal Vesicles Observed by High-Resolution Cross-Sectional Transmission Electron Microscopy

- Fig. 3. Ten-Hour Time Course of Psoralen Concentrations after the Application of Drug-Containing Liposomes and Aqueous Suspension to the Abdominal Skin of Sprague–Dawley Rats in Vivo (n=5)

- Table 1. Pharmacokinetic Parameters of Psoralen-Loaded Liposomes or the Aqueous Suspension after in Vivo Application to the Abdominal Skin (n=5), as Determined by Microdialysis

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
<th>Liposomes</th>
<th>Aqueous suspension</th>
</tr>
</thead>
<tbody>
<tr>
<td>T_max</td>
<td>min</td>
<td>180.0±0.0</td>
<td>160.0±96.4</td>
</tr>
<tr>
<td>C_max</td>
<td>ng/mL</td>
<td>319.4±23.7*</td>
<td>90.4±36.5</td>
</tr>
<tr>
<td>AUC_0→t</td>
<td>min/ng/mL</td>
<td>112580.6±1540.2*</td>
<td>29528.1±14349.8</td>
</tr>
</tbody>
</table>

Abbreviations: T_max, time to peak concentration; C_max, peak concentration; AUC, area under the concentration–time curve. Compared with Liposomes group, *p<0.05.

- Fig. 4. Mean Plasma Concentration–Time Curves for Psoralen in Rats after Transdermal Administration of the Psoralen Aqueous Suspension and Psoralen-Loaded Liposomes (n=5)

- Table 2. Mean Plasma Concentration–Time Curves for Psoralen in Rats after Transdermal Administration of the Psoralen Aqueous Suspension and Psoralen-Loaded Liposomes (n=5)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
<th>Liposomes</th>
<th>Aqueous suspension</th>
</tr>
</thead>
<tbody>
<tr>
<td>T_max</td>
<td>h</td>
<td>14.7±8.3*</td>
<td>6.5±4.4</td>
</tr>
<tr>
<td>C_max</td>
<td>ng/mL</td>
<td>57.9±11.0*</td>
<td>24.7±6.9</td>
</tr>
<tr>
<td>AUC_0→t</td>
<td>h/ng/mL</td>
<td>1309.4±327.6*</td>
<td>362.4±259.8</td>
</tr>
<tr>
<td>MRT_0→t</td>
<td>h</td>
<td>15.7±2.8*</td>
<td>8.7±4.1</td>
</tr>
</tbody>
</table>

Abbreviations: T_max, time to peak concentration; C_max, peak concentration; AUC, area under the concentration–time curve; MRT, mean retention time. Compared with Liposomes group, *p<0.05.
were 2.35- and 3.31-fold than the aqueous suspension groups, respectively, indicating the nano vesicles improved drug absorption into blood more effectively than the aqueous suspensions (Table 2). In addition, the drug loaded into liposomes showed more sustained release than the compared formulation after transdermal administration, based on the $T_{\text{max}}$ and mean retention time (MRT) values from liposomal group were both significantly higher than the aqueous suspension group.

As psoralen exhibits poor water solubility, common water-based perfusates, e.g., PBS, normal saline, and ringer's solution, are not effective for drug recovery with a microdialysis probe. Generally, cyclodextrin is added to the perfusate to increase the recovery of lipophilic drugs during microdialysis sampling, but this increases fluid viscosity and makes direct determination by using gas or liquid chromatography difficult.22) In this study, a low concentration ethanol ringer's solution (10%, v/v) as a microdialysis perfusate effectively improved the recovery of psoralen to 40%. Notably, with transdermal administration of liposomal psoralen and meanwhile in vivo cutaneous microdialysis being conducted by using 10% ethanol ringer's solution, the results of plasma psoralen concentration from Fig. 5 exhibited a little higher but no statistical difference ($p<0.05$) in contrast with using simple ringer's solution as the perfusate within the experimental period, indicating the perfusate containing 10% ethanol showed no significant effect on drug permeating into systemic circulation during cutaneous microdialysis. The recovery of psoralen from the probe presented no significant change after 10h of perfusion with 10% ethanol ringer's solution, and the the probe's inner surface and cross-section were intact and were similar to those of the untreated probe, as shown in Fig. 6. In addition, no inflammatory lesions were observed on the rat skin surface and the tissue surrounding the probe (Fig. 7), indicating that the liposomes were not irritating, the probe had good biocompatibility, and 10% ethanol ringer's solution is a suitable microdialysis perfusate.

CONCLUSION

Using in vivo skin microdialysis, we showed that prepared liposomes demonstrated significantly enhanced psoralen permeation into the skin and good safety. The linear probe with a cellulose membrane presented perfect biocompatibility with deep skin tissue. The microdialysis technique is effective for

---

**Fig. 5.** Mean Plasma Psoralen Concentration in Rats after Transdermal Administration of the Psoralen-Loaded Liposomes and Meanwhile in Vivo Cutaneous Microdialysis Being Conducted by Using Ringer's Solution and 10% Ethanol Solution as the Perfusate ($n=5$).

**Fig. 6.** Microstructures of the Microdialysis Probe Membrane (Untreated, Normal Control; Treated with Perfusate, Using 10% [v/v] Ethanol Aqueous Solution as a Perfusate and Perfused for 10h) Obtained by Scanning Electron Microscopy.
the real-time monitoring of drug distribution in skin tissues using nanovesicles as drug vehicles for topical application.

Acknowledgment This work was financially supported by the National Natural Science Fund of China (81673612, 81573619).

Conflict of Interest The authors declare no conflict of interest.

REFERENCES


