

Formulation of Liposome for Topical Delivery of Arbutin

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The aims of this study were to encapsulate arbutin (AR) in liposome to enhance the skin-whitening activity, and to investigate the effect of liposome formulation on the entrapment efficiency (EE%), skin permeation rate and skin deposition. The liposomes were prepared by a film dispersion method with several different formulations and were separated from the solution by using the gel-filtration method. The physical (size distribution, morphology) and chemical (drug entrapment efficiency, hairless mouse skin permeation and deposition) properties of liposomes were characterized. The entrapment efficiency in all liposome formulations varied between 4.35% and 17.63%, and was dependent on the lipid content. The particle sizes of liposomes were in the range of 179.9~212.8 nm in all liposome formulations. Although the permeation rate of AR in the liposome formulations decreased compared with AR solution, the deposition amount of AR in the epidermis/dermis layers increased in AR liposomal formulation. These results suggest that liposomal formulation could enhance the skin deposition of hydrophilic skin-whitening agents, thereby enhancing their activities.

Key words: Arbutin, Liposome, Entrapment efficiency, Skin permeation, Skin deposition

INTRODUCTION

In recent years, researchers have focused their attention on skin as a target site for the application of biological response modifiers. Topical delivery of bioactive substances is indeed a powerful strategy to reduce their systemic toxicity and, at the same time, to restrict the therapeutic effects to specific tissues (i.e. skin or internal organs) (Bonina *et al.*, 1995). However, the main obstacle to an efficient delivery of bioactive substances through the skin is indeed constituted by the stratum corneum barrier properties that prevent the active compound from reaching the deeper skin strata (Abraham and Downing, 1989).

Liposomes have been widely investigated for their properties as a biomembrane model and potential drug delivery systems (Manosroi *et al.*, 2002). They have become valuable experimental and commercially important drug delivery systems due to their biodegradability, biocompatibility, low toxicity and ability to entrap both lipophilic and

hydrophilic drugs. The application of liposomes has been reported to have the advantages to enhance the drug efficacy and potency through the reduction of the toxicity of encapsulated drugs, targeting to specific tissue sites, controlling the timing and the amount of drug released (Manosroi, 2002).

Thus, liposome is an excellent formulation as drug and cosmetic carriers. Liposomes can be explored as slow release vehicles for drug delivery in extravascular regions such as skin by applying directly at the site of action (Gesztos and Mezei, 1988). Possible use of liposomes as carrier *via* the transdermal route has been reviewed and well documented (Egabaria and Weiner, 1984). Despite of the reports that liposomes can not carry drugs across skin (Ganesan *et al.*, 1984; Ho *et al.*, 1985), the use of liposomes for enhancing drug absorption has been extensively reviewed (Mezei and Gulasekharam, 1980; Mezei and Gulasekharam, 1982; Vermorken *et al.*, 1983; Rowe *et al.*, 1984).

Arbutin (hydroquinone-D-glucopyranside, AR) is a glycosylated hydroquinone (Fig. 1) that has been found at high concentrations in the leaves of several plant species, such as *Vaccinium spp.* (Hincha *et al.*, 1999). It has been widely known as a skin-whitening agent, and effectively restrains the activity of tyrosinase and the formation of the

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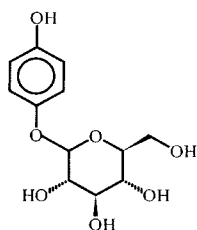


Fig. 1. Chemical structure of AR

melanin in the skin. However, the formidable barrier property of the stratum corneum and the high hydrophilicity of AR (logP value, -1.49) make it difficult to permeate through the skin and reach to its site of action (i.e., melanocytes). In many cases, traditional formulations, such as ointment and lotion, failed to achieve a sufficient skin deposition of AR. Thus, the present study reports the development of the liposomal formulations for AR, which were prepared by a chloroform film method with sonication. The characteristics of the AR liposomes, and their *in vitro* skin permeation and skin deposition behavior were systematically observed and were compared with aqueous solutions as a control.

MATERIALS AND METHODS

Materials

Soybean phosphatidylcholine (S_{75} PC, Lot No. 776075-4) was a gift from Lipoid Co. (Ludwigshafen, Germany). Cholesterol (CH) was purchased from Kasei Co. (Tokyo, Japan). AR was purchased from Sigma Chemical Co. (St. Louis, MO). All the reagents were of analytical reagent grade, and were used as received from commercial sources.

Preparation of hairless mouse skin

The animals used for the skin permeation and the deposition studies were male ICR hairless mouse (5 weeks) obtained from Orient Co. (Seoul, Korea). The animals received standard laboratory chow and had free access to water before the experiments. They were sacrificed by ether right before the experiments, and full-thickness skin was surgically removed. The skin specimens were cut into appropriate size after carefully removing subcutaneous fat and washing with normal saline.

Preparation of liposomes

The liposomes made of Soybean-PC (S_{75} PC) and AR were prepared in a similar way as described elsewhere (Manosroi *et al.*, 2002; Nastruzzi *et al.*, 1990). Briefly, liposome dispersion samples were prepared by a chloroform film method with sonication. Nine different liposome formulations composed of PC/CH = 10:1 (w/w)

with different AR concentrations (2, 3, and 4%, w/v) and lipid contents (2, 5, and 8%, w/v) were prepared. The PC and CH were dissolved in 5 mL of chloroform and were dried in a 500 mL round bottom flask using a rotary evaporator (BÜCHI Labortechnik AG CH-9230, Type R-200, Flawil, Switzerland) under an aspirate vacuum and a water bath (BÜCHI B-490, Flawil, Switzerland) with temperature maintained at 40°C. A formed thin film layer was further dried by vacuum-desiccator for 15 min and then flushed with nitrogen gas for 1 min (Manosroi *et al.*, 2004). The thin film was re-suspended with 5 mL of AR solution in phosphate buffered saline (PBS), and swelled by swirling for 30 min at 100 rpm. The liposome dispersions were sonicated with a Sonics (Model VC 750, SONIC & MATERIALS, Inc. Newtown, CT) for 15 sec (amplitude 30% of 750 Watts). Then, the liposome suspensions were serially passed through 3, 1.2, 0.6, 0.4 and finally 0.2 μm pore size filters (Isorpore™ membrane filters, County Cork, Ireland) under the nitrogen gas using a high pressure extruder (Northern Lipids Inc., Vancouver, BC, Canada).

Scanning electron microscopy investigations (SEM) of liposomes

An aliquot of liposome suspension (200 μL) was diluted into 5 times with distilled water. A drop of the suspension was transferred onto a clean and dry microscope slide, and the sample was air-dried at room temperature. Then, the dried sample was coated with gold sputter. The morphology of AR liposome sample was observed using a Scanning Electron Microscope (S-4200, Hitach, Japan).

Determination of particle size of liposomes

The particle size distribution of liposome was determined using a NICOMP 370 Submicron Particle Sizer (Particle Sizing Systems, Santa Barbara, CA). The liposome samples were diluted into appropriate multiple ($\times 200\sim 500$) and were put into the transparent cell in the system. The particle size distribution was processed with computerized inspection system (Imura *et al.*, 2001).

Determination of AR entrapment efficiency in liposomes

The entrapment efficiency (EE%) of AR liposomes was determined using a gel sephadex column chromatography (Sephadex G-25, Amersham Biosciences, Baied'Urfe, Quebec, Canada) (Bian *et al.*, 2003; Nastruzzi *et al.*, 1990). An aliquot of liposomes (100 μL) was loaded and the fractions were collected every 5 min, then optical absorption in each tube was determined with UV-spectrometry at 280 nm wavelength (Gene Spec III, Naka Instruments, Japan). Drug concentration in free drug fraction was directly analyzed by HPLC. Liposomal fractions were dissolved in 0.5% triton X-100 solution before analyzed by

HPLC. The entrapment efficiency was calculated by the following equation:

$$EE(\%) = \frac{C_{\text{lipo}} \times V_{\text{lipo}}}{C_{\text{lipo}} \times V_{\text{lipo}} + C_{\text{free}} \times V_{\text{free}}} \times 100$$

where, C_{lipo} and C_{free} represent AR concentration in liposome and free solution, respectively, while V_{lipo} and V_{free} represents the volume of liposome and free solution, respectively.

In vitro skin permeation studies of AR solution and liposomal formulation

In vitro hairless mouse skin permeation study was conducted in the Keshary-Chien diffusion cells with a permeation area of 2.14 cm² (Bian *et al.*, 2003). The temperature of the diffusion assembly was maintained at 37°C by circulating water jacket to maintain 32°C on the surface of the skin. The fluid in the receptor cells were constantly stirred by Teflon coated magnetic bars at 600 rpm. The receptor cells were filled with 12 mL of receptor solution [PBS containing 0.01% (w/v) of gentamicin as an antibiotic to keep AR stable during the permeation process].

Freshly excised hairless mouse skin was mounted between the donor and receptor cells (3 mL). Liposome with 8% of lipid and 4% of AR was applied to the donor cells on the stratum corneum side of the skin and was occluded with parafilm in order to prevent the evaporation. AR in PBS (4%) was also studied as a control. At each predetermined time intervals, an aliquot (1 mL) of receptor solution was taken to determine the amount of drug permeated, and refilled with the same volume of the fresh receptor solution. Samples were kept in the freezer until analyzed by HPLC.

In vitro skin deposition study of AR solution and liposomal formulation

Deposition amount of AR in the stratum corneum and the epidermis/dermis layers was determined at 4, 8 and 12 h of permeation experiment, as described previously (Bian *et al.*, 2003). Donor solution was removed at 4, 8, 12 h of permeation experiment, and the skin that was in contact with AR solution or the liposome (2.14 cm²) was taken and was carefully rinsed 5 times with distilled water to remove AR on the surface. The stratum corneum of the permeation-treated site was collected using the tape-stripping method. The Scotch tapes (1.8x1.8 cm², 3 M Company) were utilized while each piece of tape was used in two successive strips, thus the total strip number was 20 times. Then, the all pieces of tape were extracted with 10 mL of PBS by overnight. The stripped skin was surgically peeled and was cut into small pieces, and homogenated with 2 mL of PBS for 2 min at 10,000 rpm.

Then, the suspension was centrifuged for 10 min at 4,000 rpm. The supernatant was filtered through the micro-pore syringe filters (0.2 μm, Sartorius Co., Germany). Concentration of AR in all samples was analyzed by HPLC.

HPLC analysis of AR

Concentration of AR was determined using a HPLC system equipped with a binary pump (Waters 515 HPLC Pump) and auto-injector (Waters 717 plus Auto-sampler) and a UV-VIS detector (Waters 2487 Dual λ Absorbance Detector). A Merck RP-8 column (LiChroCART, 5 μm, 250 x 4 mm, Merck, Darmstadt, Germany) was used at ambient temperature. The mobile phase was a mixture of methanol and water (15:85, v/v) at a flow rate of 0.8 mL/min. The wavelength of UV detector was set at 280 nm, and 20 μL of samples were injected. The retention time of AR was about 5.1 min.

Data analysis

All data are expressed as mean±standard deviation. The Student’s unpaired t-test was used to test the difference between the control solution and liposomal formulation. In all cases, p<0.05 was accepted as representing a statistical difference.

RESULTS AND DISCUSSION

Particle size and morphology of liposome

The mean particle sizes of nine liposome formulations are presented in Table I. It showed that the range of particle size of liposomes in all formulations was between 179.9 nm (±34.03) and 212.8 nm (±43.37). A representative particle size distribution of the liposomal formulation is shown in Fig. 2. The liposomal particle size distribution was mono-modal (Gaussian) distribution. The Scanning Electron Microscope (SEM) image of liposome is shown

Table I. The characteristics of liposomal formulations

Formulations		Entrapment efficiency (%)	AR concentration (mg/mL)	Particle size (nm)
Lipid (% w/v)	AR (% w/v)			
2	2	4.76±0.67	0.95±0.13	199.6±18.63
	3	5.00±0.30	1.50±0.08	212.8±43.37
	4	4.35±0.21	1.74±0.08	205.8±7.99
5	2	11.99±0.99	2.40±0.20	199.4±3.50
	3	11.83±2.22	3.55±0.67	198.0±8.90
	4	11.47±2.78	4.60±1.11	189.7±2.52
8	2	17.63±1.38	3.53±0.27	196.7±28.24
	3	15.70±0.38	4.71±0.12	179.9±34.03
	4	15.41±1.10	6.17±0.44	197.3±38.95

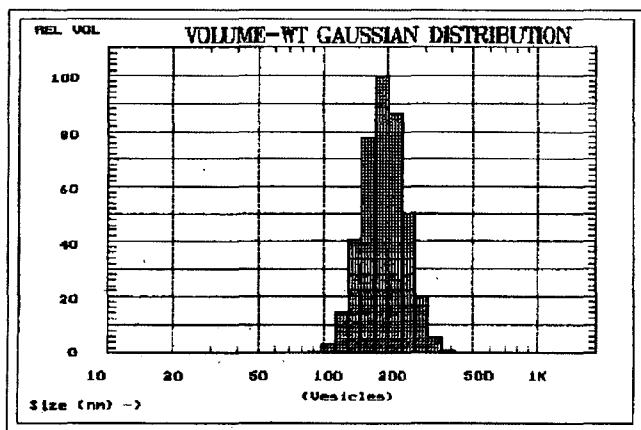


Fig. 2. Typical liposome size distribution by a laser light scattered system

in Fig. 3. The image from SEM showed that liposomes were discrete particles with sharp boundaries.

Determination of AR entrapment efficiency (EE%) in liposomes

The gel sephadex column chromatography elution profile of AR liposome using sephadex G 25 chromatography was shown in Fig. 4. The profile between time and absorption showed two peaks; the first and smaller peak was AR entrapped in the liposomes. The second and bigger peak in the chromatogram represents the elution of free AR. This indicated that a low yield in AR entrapment was obtained under these experimental conditions.

The entrapment efficiency was dependent on the lipid content in the formulation (Table I). That is, as the lipid content increased from 2% to 8%, the entrapment efficiency increased from 4% to 15%. However, the increase of the AR content from 2% to 4% did not significantly change the entrapment efficiency of AR. As shown from Fig. 4 and Table I, the entrapment efficiency of AR was generally low

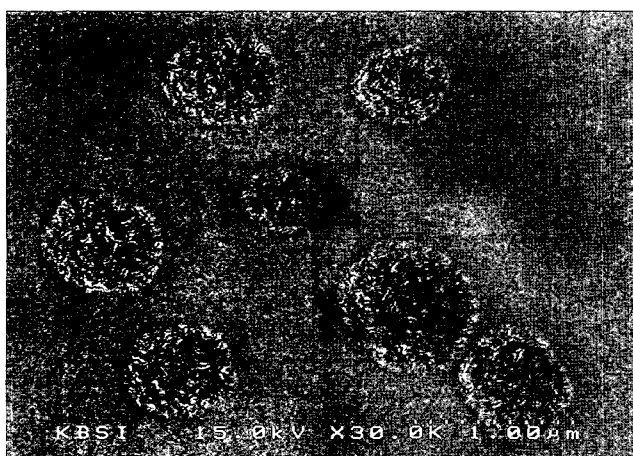


Fig. 3. The scanning electron microscopy (SEM) image of the liposome

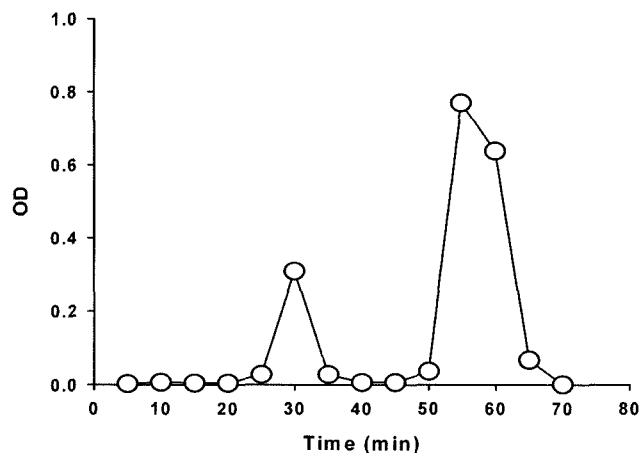


Fig. 4. Separation the AR entrapped in liposome from free AR using a Gel filtration method. The profile between time and absorption showed two peaks; the first and smaller peak was AR entrapped in the liposomes. The second and bigger peak in the chromatogram represents the elution of free AR.

in all the formulations tested in present study. Because AR is a highly hydrophilic compound, it seemed that it is difficult to obtain high entrapment efficiency. Nevertheless, the concentration of AR in the liposomal formulation increased as the content of AR increased. As a result, liposomal formulation composed of 4% AR and 8% lipid showed the highest AR concentration among the tested formulations. Therefore, liposomal formulation composed of 4% AR and 8% lipid was selected for skin permeation and skin deposition study, using 4% AR in PBS solution as a control.

In vitro skin permeation of AR solution and liposomal formulation

As shown in Table II, the permeation rate of AR solution in the steady state was $0.49 (\pm 0.29)$ mg/cm²/hr, which was significantly different from that of AR liposomal formulation ($0.13 (\pm 0.03)$ mg/cm²/h) ($p < 0.05$). As a result, cumulative amount of AR permeated in the case of the AR solution was significantly higher than that of the AR liposomal formulation (Fig. 5). There was, however, no difference in lag time between the two formulations.

The underlying mechanism for the lower permeation rate shown in the AR liposomal formulation has still been

Table II. Hairless mouse skin permeation parameters of AR solution and AR liposomal formulation in 12 h at 37°C. Each formulation contained 4% AR

Formulations	Permeation rate (μg/h/cm ²)	Lag time (h)	Permeation coefficient (cm/h × 10 ⁵)
Solution	0.49 ± 0.29	1.43 ± 0.77	1.20 ± 0.73
Liposome	0.13 ± 0.03*	0.87 ± 1.11	1.70 ± 1.50

* $p < 0.05$ compared with solution.

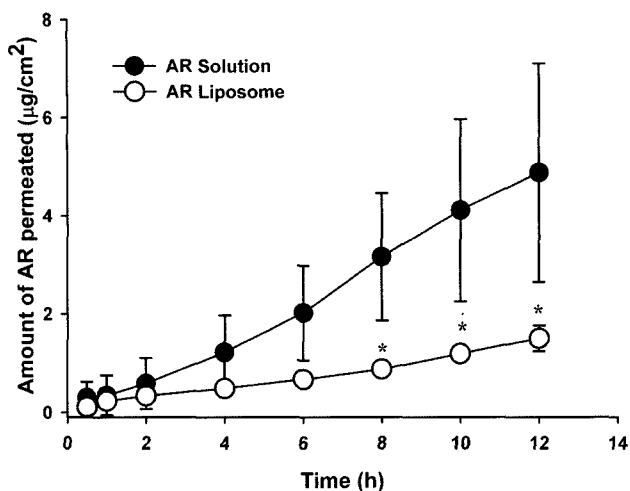


Fig. 5. Cumulative permeation profile of AR solution and liposomal formulation at 37°C. Cumulative amount of AR permeated in AR solution (●) and AR liposomal formulation (○) was measured by HPLC. Each data point represents the mean±S.D. *: Significantly different from AR solution (p<0.05).

unknown. It is a possibility that the release of AR, highly hydrophilic compound, from the bilayers of the liposomes takes time, which resulted in the sustained release of the AR. In addition, stratum corneum, a barrier for hydrophilic compound to penetrate, was fully hydrated by occluded condition, which might enhance the penetration of free AR in the solution.

In vitro skin deposition of AR solution and liposomal formulation

Fig. 6 shows the deposition amounts of AR for the control solution and liposomal formulation in the skin for 4, 8 and 12 h in the stratum corneum and in the epidermis/dermis of skin, respectively. The deposition amount of AR in the stratum corneum generally increased in AR control solution than in AR liposomal formulation (Fig. 6A). However, the deposition amount of AR in epidermis/dermis in AR liposome was comparable to that in AR solution at 4, 8 h, and significantly increased at 12 h (Fig. 6B) (p<0.05). The skin permeation and deposition results showed that the application of the AR liposomal formulation resulted in the enhanced AR concentration in, and a lower systemic absorption than the AR solution. These results might imply that liposomes work as a drug reservoir in the epidermis/dermis layers, as previously reported by Wester *et al.* (Wester *et al.*, 1984).

Other researcher groups have reported similar results (Mezei and Gulasekharan, 1980, Planas *et al.*, 1992). Considering that the depigmentation effect of AR works through an inhibition of the melanosomal tyrosinase activity and melanocytes locate mainly between epidermis and dermis layers, it is important for topically delivered AR

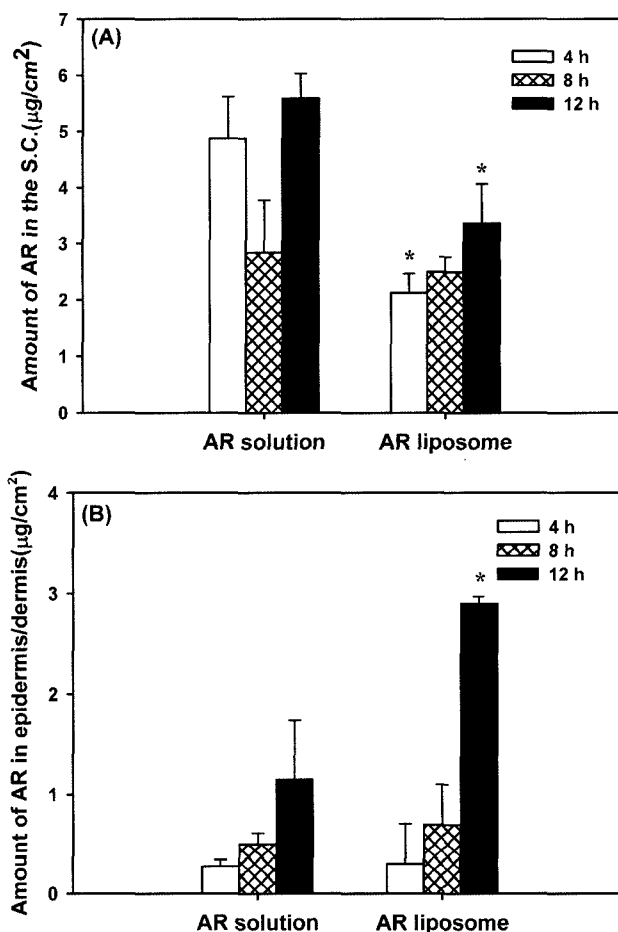


Fig. 6. (A) Deposition of AR to the stratum corneum layer from AR solution and AR liposomal formulation. Deposition amount of AR in the stratum corneum layer was determined at 4, 8 and 12 h of permeation experiment. Each data point represents the mean±S.D. *: Significantly different from AR solution (p<0.05). (B) Deposition of AR to the epidermis/dermis layers from AR solution and AR liposomal formulation. Deposition amount of AR in the epidermis/dermis layer was determined at 4, 8 and 12 h of permeation experiment. Each data point represents the mean±S.D. *: Significantly different from AR solution (p<0.05).

to penetrate through the stratum corneum and to maintain an effective concentration near the melanocytes for a certain period of time. In the point of view above mentioned, our present results provide a good rationale in the development of the skin whitening formulation containing AR.

In conclusion, liposomes containing AR, a skin-whitening agent, was successfully prepared by using the film dispersion method. When these liposomes were applied occlusively to the mouse skin, the amount of AR permeated into the skin decreased when AR was encapsulated into liposome, while the enhanced accumulation of AR in the epidermis/dermis layers was found in the liposomal formulation. These findings might help to optimize targeting

of AR, creating new opportunities for topical application of AR.

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