

Menthosomes, Novel Ultradefordable Vesicles for Transdermal Drug Delivery: Optimization and Characterization

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Menthosomes, novel deformable carriers for the enhancement of transdermal delivery are introduced in this study. Meloxicam (MX)-loaded mentosomes were formulated, and their physicochemical characteristics and skin permeability were evaluated. A two-factor spherical and second-order composite experimental design was used to prepare the formulation of the mentosomes. Ten formulations of mentosomes composed of a phospholipid as the lipid bilayer carrier, cholesterol (Chol) as a stabilizer and cetylpyridinium chloride (CPC) and L-menthol as penetration enhancers were prepared. The amounts of Chol and CPC were selected as causal factors. Physicochemical characteristics (particle size, size distribution, zeta potential, elasticity and drug content) and an *in vitro* skin-permeation study of meloxicam-loaded mentosomes were evaluated. The concentrations of MX that permeated the skin at 2–12 h and the flux were selected as response variables. The optimal formulation was estimated using a nonlinear response-surface method incorporating thin-plate spline interpolation. The experimental values were very close to the values predicted by the computer programs in this study. A Bayesian network analysis was applied to gain a mechanistic understanding of the relationships between causal factors and response variables.

Key words mentosome; liposome; optimization; skin permeation; meloxicam; menthol

For the past few decades, the use of liposomal vesicles in drug-delivery systems for skin permeation has evoked considerable interest and attracted increasing attention. Many reports focus on the use of liposomes for enhancing skin permeation of hydrophilic and lipophilic compounds, proteins and macromolecules. However, recent studies indicate that in most cases, classic liposomes are of little or no value as transdermal drug-delivery carriers because they do not penetrate skin deeply, but rather remain confined to the upper layers of the *stratum corneum*. Confocal microscopic studies showed that intact fluorescent labeled liposomes were not able to penetrate into the granular layers of the epidermis.¹⁾

Since the first paper to report the effectiveness of deformable liposomes which can be used for skin delivery of drug into deep skin region was published by Cevc and Blume,²⁾ new categories of vesicles with high elasticity or flexibility, such as transfersomes,³⁾ ethosomes,⁴⁾ flexosomes⁵⁾ and invasomes⁶⁾ have been introduced and developed. These vesicles mainly consist of phospholipids and an edge activator or penetration enhancer in which only a specially designed vesicle was shown to be able to allow transdermal drug delivery. Mentosomes, novel deformable carriers consist of phospholipids, surfactant and menthol were also introduced in this study.

Several intensive studies suggested that the permeability of drug in liposomes and their analogues depends on their physicochemical characteristics (*e.g.*, particle size, size distribution, zeta potential, lamellarity, elasticity, drug content, *etc.*), and these characteristics were directly affected by lipid composition and/or formulation factors. However, liposomes can vary with respect to lipid composition (*e.g.*, phospholipid, cholesterol, edge activator, penetration enhancer, *etc.*) and method of preparation. Furthermore, whether the skin model

used, human or animal (*e.g.*, pig, rat, mice, rabbit, snake, *etc.*) may be the factor that determines the effectiveness of drug in liposomes and analogues remains a much debated question and must be designed and tested on a case-by-case basis. In this context, the type and lipid composition are still needed to define the effect of the formulation factors on physicochemical characteristics and skin permeability of drug in liposomes.

In this study, meloxicam (MX), a nonsteroidal anti-inflammatory drug (NSAID) as a preferential cyclooxygenase-1 (COX-1) inhibitor, was used as the model drug.⁷⁾ Because oral and injectable administrations of MX are not appropriate for peptic ulcers and patient compliance, MX is suitable for development as a transdermal delivery candidate. In this study, the possibility of developing a transdermal liposomal carrier containing MX was evaluated.

In the development of a transdermal drug-delivery system, it is important to design the optimized pharmaceutical formulations having appropriate skin permeation. For this objective, it is very important to determine the optimized formulation of MX in mentosomes vesicles. A nonlinear response-surface method incorporating thin-plate spline interpolation (RSM-S) was employed. Using RSM-S, complicated relationships between causal factors and response variables can be easily understood, and a stable and reproducible simultaneous optimal solution is obtained.⁸⁾ A bootstrap (BS) resampling method and a Kohonen self-organizing map (SOM) were used to evaluate the reliability of the optimal solution estimated by RSM-S. These statistical approaches are helpful in formulating an appropriate transdermal delivery system for MX. Moreover, a Bayesian network (BN) was used to construct a probabilistic graphical model of the latent structure and elucidate the relationships within the latent structure by estimating conditional probability distributions.

The authors declare no conflict of interest.

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MATERIALS AND METHODS

Materials Phosphatidylcholine (PC) from soy was generously supplied by LIPOID GmbH (Cologne, Germany). Cholesterol (Chol) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Cetylpyridinium chloride (CPC) was purchased from MP Biomedicals (Illkirch, France). L-Menthol (MEN) was purchased from Tokyo Chemical Industry (Tokyo, Japan). Meloxicam (MX) was supplied from Fluka (Buchs, Switzerland). All other chemicals used were of reagent grade and purchased from Wako Pure Chemical Industries, Ltd.

Preparation of MX-Loaded Mentosomes Mentosomes were prepared according to formulations obtained from a two-factor spherical second-order composite experimental design.⁹⁾ As shown in Table 1, 10 formulations of MX-loaded mentosomes composed of a controlled amount of PC, MEN and MX, and various amounts of Chol as membrane stabilizer and CPC as penetration enhancer were prepared. MEN has been reported to improve the skin permeation of various drugs by increasing drug partition and diffusion.^{10,11)} The concentration of Chol (X_1) and CPC (X_2) were selected as causal factors. The concentration of PC, MEN and MX were fixed at 0.773, 0.077 and 0.077% (w/v), respectively. Mentosomes were prepared by the sonication method. Briefly, lipid mixtures of PC, Chol, CPC, MEN and MX were dissolved in chloroform-methanol (2:1 v/v ratio). The solvent was evaporated under nitrogen gas stream. The lipid film was placed in a desiccator for 6h to remove the remaining solvent. The dried lipid film was hydrated with acetate buffer solution (pH 5.5). Vesicles were subsequently sonicated for two cycles of 15 min using a bath-type sonicator (5510J-DTH Branson Ultrasonics, Danbury, U.S.A.). The vesicle formulations were freshly prepared or stored in airtight containers at 4°C prior to use.

Measurement of Vesicle Size, Size Distribution, Zeta Potential and Morphology Average diameters, size distribution and zeta potentials of the mentosomes were measured by photon correlation spectroscopy (PCS) (Zetasizer Nano series, Malvern Instruments, U.K.). All measurements were performed at room temperature, after diluting the vesicle suspension with deionized water. Twenty μL of the mentosomes formulation were diluted with 1480 μL of deionized water. At least three independent samples were taken, and the particle size was measured at least three times.

Measurement of Elasticity Value The elasticity value of the lipid bilayer of the vesicles was directly proportional to $J_{\text{Flux}} \times (r_v/r_p)^2$:

$$\text{Elasticity value (mg} \cdot \text{s}^{-1} \cdot \text{cm}^{-2}) = J_{\text{Flux}} \times (r_v/r_p)^2$$

where J_{Flux} is the rate of penetration through a permeable barrier ($\text{mg} \cdot \text{s}^{-1} \cdot \text{cm}^{-2}$), r_v is the size of the vesicles after extrusion (nm) and r_p is the pore size of the barrier (nm). To measure J_{Flux} , the vesicles were extruded through a polycarbonate membrane (Nuclepore, Whatman Inc., MA, U.S.A.) with a pore diameter of 50 nm (r_p), at a pressure of 0.5 MPa. After 5 min of extrusion, the extrudate was weighed (J_{Flux}), and the average vesicle diameter after extrusion (r_v) was measured by PCS.

Determination of MX-Entrapment Efficiency The concentration of MX in the formulation was determined by HPLC analysis after disruption of the vesicles with Triton[®] X-100 (0.1% w/v) at a 1:1 volume ratio and appropriate dilution with phosphate buffer solution (pH 7.4). The vesicle/Triton[®] X-100 solution was centrifuged at 10000 rpm at 4°C for 10 min. The supernatant was filtered with a 0.45 μm nylon syringe filter. The entrapment efficiencies of MX loaded in the formulation were calculated according to the following equation:

$$\% \text{ entrapment efficiency} = (C_L/C_i) \times 100$$

where C_L is the concentration of MX loaded in the formulation as described in the above methods and C_i is the initial concentration of MX added to the formulation.

In vitro Skin-Permeation Study The excised skin of hairless mice (Laboskin[®], HOS: HR-1 Male, 7 weeks, Sankyo Labo Service Corporation, Inc., Tokyo, Japan) was used as a permeation membrane for the *in vitro* study. A side-by-side diffusion cell with an available diffusion area of 0.95 cm^2 was employed. The receiver chamber was filled with 3 mL of phosphate buffer solution (pH 7.4, 32°C) and the donor chamber was filled with 3 mL of MX loaded mentosome formulation. The amount of MX in each formulation applied was 122.52 to 733.32 $\mu\text{g}/\text{mL}$. At appropriate times, an aliquot of the receiver fluid was withdrawn, and the same volume of fresh buffer solution was placed in the receiver chamber. The concentration of MX in the aliquot was analyzed using HPLC.

After the skin-permeation study, the amount of MX remaining in the skin was measured. The skin was isolated from the diffusion cell, and the residual suspension was removed

Table 1. Composite Spherical Experimental Design for Two Factors and Model Formulation of Meloxicam Loaded Mentosomes

Formulation	X_1	Chol (%)	X_2	CPC (%)
1	-1	14.4	-1	14.4
2	-1	14.4	1	35.6
3	1	35.6	-1	14.4
4	1	35.6	1	35.6
5	$-\sqrt{2}$	10.0	0	25.0
6	$\sqrt{2}$	40.0	0	25.0
7	0	25.0	$\sqrt{2}$	10.0
8	0	25.0	$-\sqrt{2}$	40.0
9	0	25.0	0	25.0
10	0	25.0	0	25.0

The concentration of PC, MEN, and MX were fixed at 0.773, 0.077 and 0.077% (w/v), respectively. The total amount of each vesicle formulation was adjusted to 100 mL by addition of buffer.

from the skin surface with a cotton swab with distilled water. The full-thickness skin was cut into small pieces with scissors and fine forceps, and sonicated for 2 h with 1 mL of phosphate buffer solution to extract the MX. After centrifugation at 10000 rpm for 10 min, the clear supernatant was analyzed by HPLC.

HPLC Analysis of Meloxicam The HPLC system consisted of a SIL-20A autosampler, LC-20AT liquid chromatograph and SPD-20AUV detector (Shimadzu Corporation, Kyoto, Japan). The analytical column was YMC-Pack ODS-A (150 mm × 4.6 mm i.d., S-5, YMC Co., Ltd., Kyoto, Japan), and the mobile phase consisted of acetate buffer solution (pH 4.6)–methanol (50:50, v/v). The flow rate was set at 0.8 mL/min, and the wavelength used in this determination was 272 nm.

Determination of Optimal Formulation An optimization study of the formulation based on RSM-S was performed with the data set obtained for the model formulations. Details of the simultaneous optimization methods with RSM-S have been fully given previously.^{8,12–14} The optimal formulation was defined as a sufficient concentration of MX-permeated skin at 2, 4, 6, 8, 10 and 12 h and the flux of MX. The best vesicle formulation should have the maximum value of flux and concentration of MX-permeated skin at 2–12 h. Once the RSM-S-estimated optimal formulation was obtained, its reliability was evaluated using BS resampling, which has been fully described previously.^{15–17} The number of BS replications was fixed at 2300. The Kohonen SOM was then applied to the set of BS solutions to separate the global optimal and some local optimal clusters.^{13,14}

Latent Structure Analysis To elucidate the latent structure underlying the mentosomes, a BN analysis was applied. BN was used to construct a probabilistic graphical model of the latent structure and elucidate the relationships within the latent structure by estimating conditional probability distributions that could clarify the relationships between formulation factors (causal factors), the basic characteristics (latent variables) and skin-permeability response variables, as a path diagram.

Computer Programs dataNESIA, Version 3.2 (Yamatake Corp., Fujisawa, Japan) was used for drawing the response surfaces for each variable and predicting the latent variables and response variables (skin permeation) for the various formulations. SOM clustering was performed using Viscovery SOMine, (Version 5.0, Eudaptics Software GmbH, Vienna, Austria). BayoNet (Version 5.0, Mathematical Systems Inc., Tokyo, Japan), was used to construct the probabilistic graphical model among the formulation factors, the latent variables and the response variables, and to estimate conditional independencies.

Ethics in the Animal Study This animal study was performed at Hoshi University and complied with the regulations of the committee on Ethics in the Care and Use of Laboratory Animals.

RESULTS

Identification of the Response Surface by RSM-S Ten formulations of mentosomes were formulated and prepared. The concentrations of PC, MEN and MX were fixed at 0.773, 0.077 and 0.077% (w/v), respectively. The concentrations of

Chol (liposome stabilizer) and CPC (penetration enhancer) were varied from 10 to 40% mole ratio according to the formulation obtained from the two-factor spherical second-order composite experimental design (Table 1). The amounts of Chol and CPC were selected as causal factors. The physicochemical characteristics of mentosomes (size, polydispersity index (PDI), zeta potential, elasticity and MX content in the formulations) were selected as basic characteristics (latent variables). The concentration of MX-permeated skin at 2, 4, 6, 8, 10 and 12 h, and the steady-state flux were selected as response variables. The response surfaces estimated by RSM-S show the relationship between causal factors and latent variables, and the relationship between causal factors and response variables.¹⁸⁾

The effect of incorporation of Chol and CPC on physicochemical characteristics of MX-loaded mentosomes is shown in Fig. 1. Figure 1A shows the response surfaces of latent variables (size, PDI, zeta potential, elasticity and drug content) determined by RSM-S. The response surfaces indicated that an increase of Chol resulted in a significant increase in size, a decrease in elasticity and a slight increase in MX content in the formulation. An increase in CPC resulted in a significant decrease in size, an increase in zeta potential, an increase in elasticity, an increase in MX content in the formulation and a slight decrease in PDI. The accuracy and reliability of the response surfaces were determined by a leave-one-out-cross-validation (LOOCV).¹⁹⁾ The results are shown in Fig. 1B. The LOOCV results for size, zeta potential, elasticity and drug content were relatively high, and some of them were very high. However, the LOOCV result for PDI was quite low. In the cases that the correlation coefficients were not extremely high, the latent variables were not only dependent on formulation factors, but also on the method of preparation. This result suggested that RSM-S successfully estimated the relationship between the causal factors and some latent variables attributed to the mentosomes.²⁰⁾

The skin permeability of the model formulations was evaluated using an *in vitro* skin-permeation study. The effect of incorporating Chol and CPC on the skin permeation of MX-loaded mentosomes using the response surfaces estimated by RSM-S is shown in Fig. 2. The response variables were the concentration of MX-permeated skin at 2, 4, 6, 8, 10 and 12 h and the steady-state flux. The similar patterns of response surface of the response variables suggested that the values of concentration of MX-permeated skin at 2–12 h and flux increased as the concentrations of Chol and CPC increased. Although in the early phase of skin permeation, a slight difference in the response surfaces was observed, for the most part the response surfaces showed a similar pattern. The accuracy and reliability of the response surfaces were determined with a LOOCV, and are shown in Fig. 3. The result (correlation coefficients) of the estimated and experimental values for the concentration at 6 h was quite low, which makes it a little difficult to specifically indicate all of the factors that affected the concentration of MX permeated at 6 h. However, the results for 2, 4, 8, 10 and 12 h and the flux were fairly high, suggesting that RSM-S successfully estimated the relationship between the causal factors and output response variables.

Formulation Optimization Using RSM-S The formulation of MX-loaded mentosomes was optimized based on the original data set using RSM-S. The search directions for the

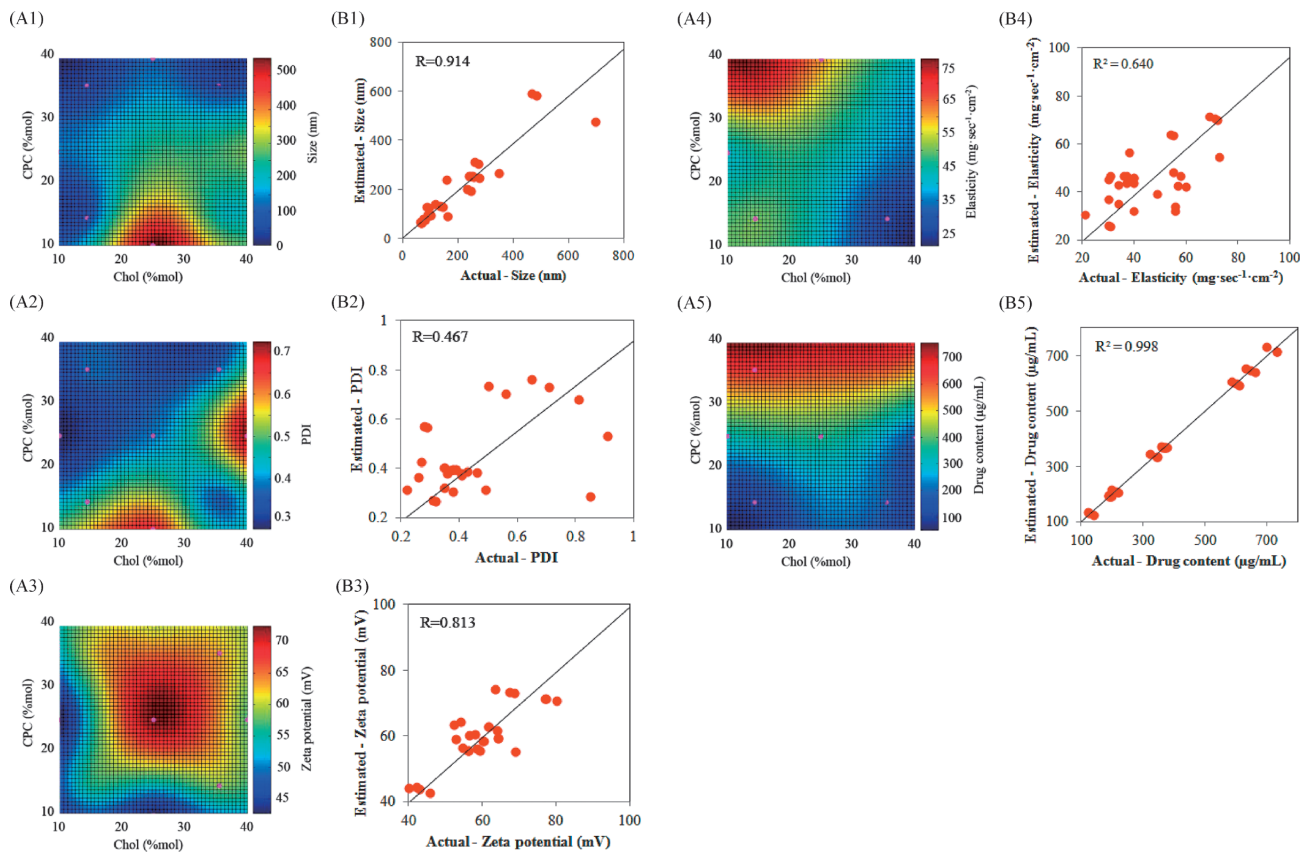


Fig. 1. The Response Surface (A) and LOOCV Results (B) for the Model Formulation of Size (1), Size Distribution (PDI) (2), Zeta Potential (3), Elasticity (4) and Drug Content in the Formulation (5)

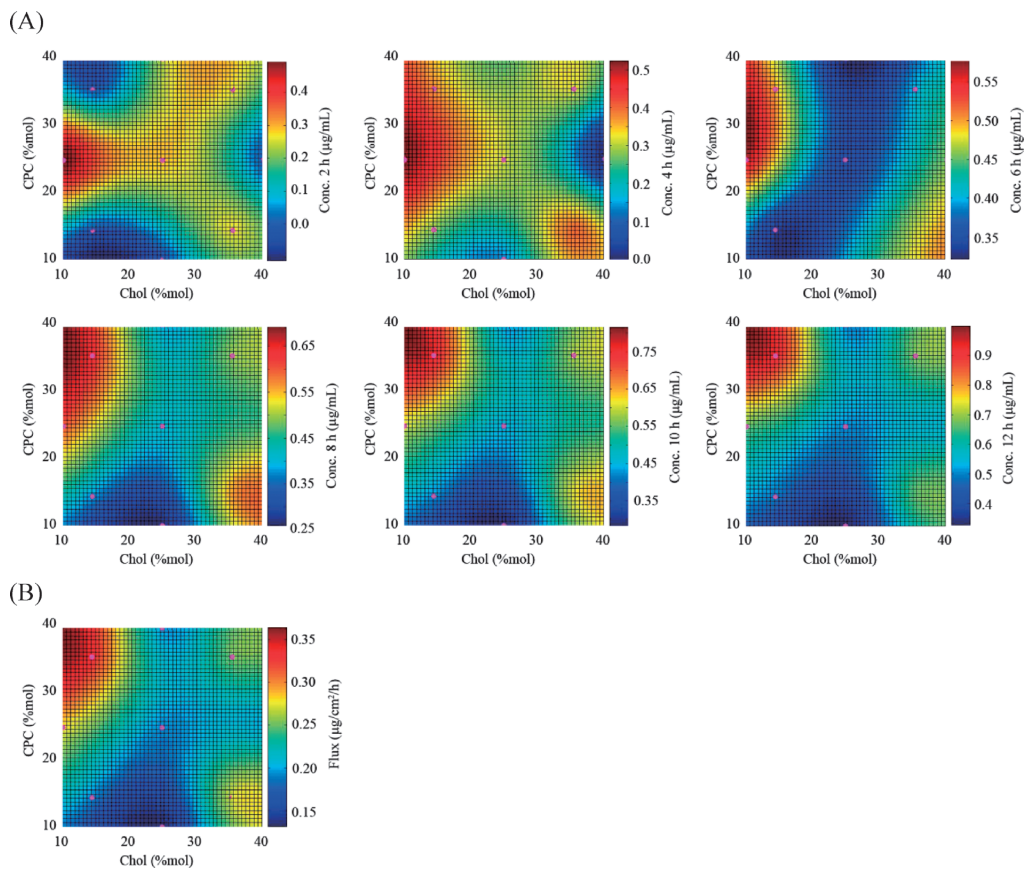


Fig. 2. The Response Surface for the Model Formulation of the Concentration of MX-Permeated Skin at 2, 4, 6, 8, 10 and 12h (A) and the Flux (B)

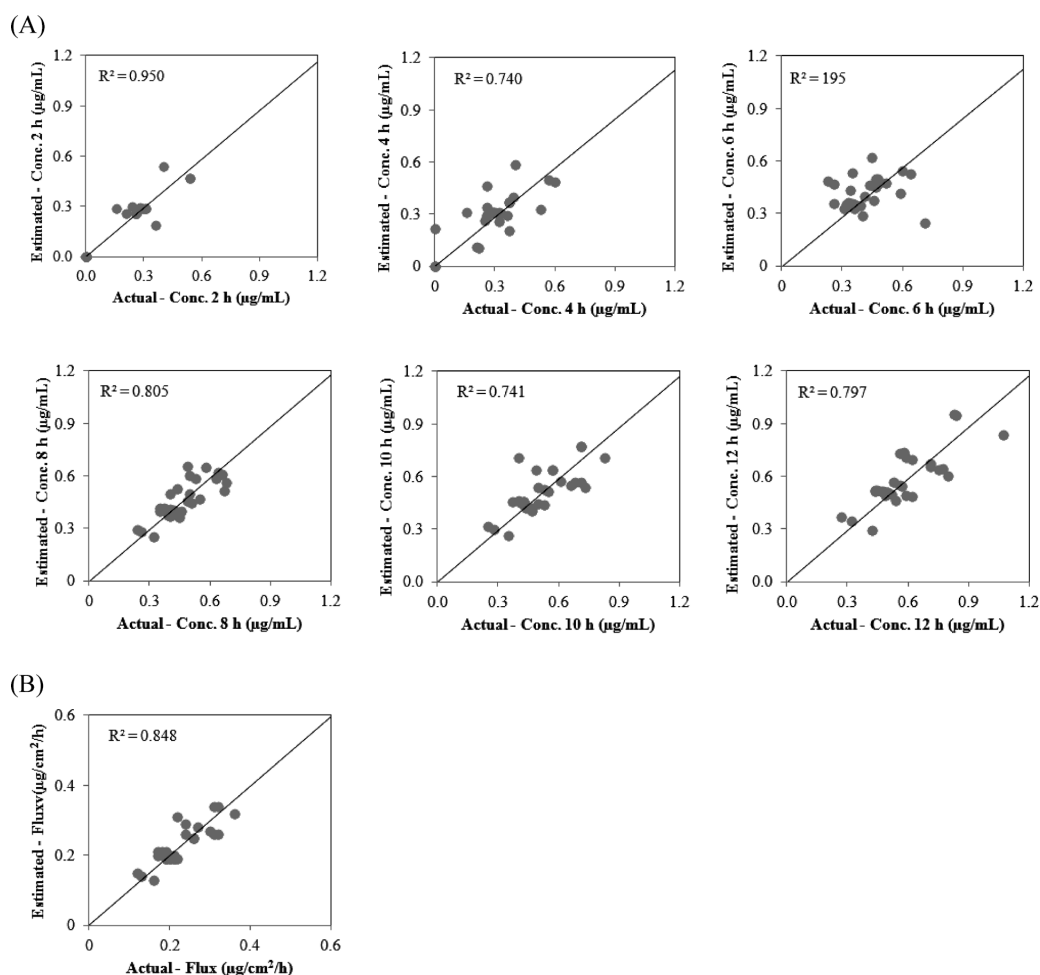


Fig. 3. The LOOCV Results of the Model Formulation of the Concentration of MX-Permeated Skin at 2, 4, 6, 8, 10 and 12h (A) and the Flux (B)

response variables were set to produce a high concentration of MX-permeated skin at 2, 4, 6, 8, 10 and 12h and also a high steady-state flux. $X_1=10.55$ (% mole ratio) and $X_2=29.02$ were estimated as the optimal formulation. The following variables were estimated to be the optimal response variables: conc. 2h= $0.36 \mu\text{g/mL}$, conc. 4h= $0.50 \mu\text{g/mL}$, conc. 6h= $0.57 \mu\text{g/mL}$, conc. 8h= $0.64 \mu\text{g/mL}$, conc. 10h= $0.68 \mu\text{g/mL}$, conc. 12h= $0.79 \mu\text{g/mL}$ and steady state flux= $0.31 \mu\text{g/cm}^2/\text{h}$ (Table 2).

BS-resampling and SOM-clustering methods were applied to determine the set of BS-optimal solutions in the global optimal cluster.²¹ The 95% confidence intervals (CIs) for the optimal formulation were calculated using the data in the global optimal cluster. Results are shown in Table 2. The experimental values, concentration of MX-permeated skin at 2–12h and flux were all in the 95% CI range. To evaluate the robustness of the optimal formulation, histograms of the BS optimal solution of causal factors, Chol and CPC, and the response variables, conc. at 2–12h and flux were calculated based on BS re-sampling method, are shown in Fig. 4. The shape of the histogram constructed from the arithmetic means of the BS samples follows a normal distribution. Some skew distributions, such as conc. at 2h and conc. at 4h, were observed; however, most of the histograms were close to a normal distribution.

Estimation of Quantitative Latent Structure Model Using BN To analyze the causal relationships between

formulation factors, the basic characteristics (latent variables) and skin-permeability response variables, the latent structure model was estimated using a BN analysis. All variables were discretized to five levels for the inference of the conditional probability distribution. The K2 algorithm based on Akaike information criterion was employed to seek an appropriate BN path diagram. As shown in Fig. 5, the concentration of Chol and CPC correlated with all latent variables (size, PDI, zeta potential, elasticity and drug content). The elasticity correlated with all skin-permeability response variables, whereas the size correlated with the flux and phase III (terminal phase of skin permeation; 10–12h). PDI correlated with phase II (middle phase of skin permeation; 6–8h) and phase I (early phase of skin permeation; 2–4h). The zeta potential correlated with phase II of skin permeation, and the MX content only correlated with phase I of skin permeation. In addition, the path diagram indicated that the size and elasticity significantly correlated with phase III, whereas the PDI significantly correlated with phase II and phase I.

DISCUSSION

Several studies have reported that the lipid compositions (formulation factors) affect the physicochemical characteristics of liposomes. However, in the past, the effect of the formulation factors was not fully clarified as there were both

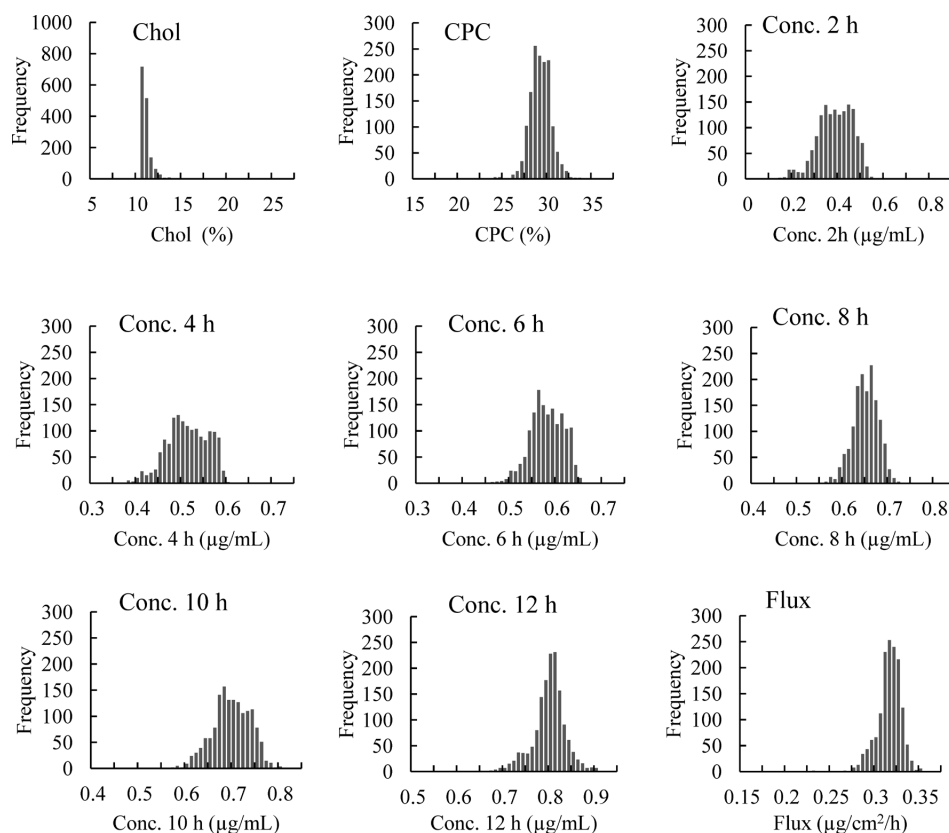


Fig. 4. Histogram of Optimal Causal Factors (Chol, CPC) and Response Variables (Conc. at 2–12 h and Flux) Estimated by BS Resampling and SOM Clustering

BS resampling was repeated 2300 times. These histograms are composed of 1488 optimal solutions classified in cluster 1 in Fig. 4.

Table 2. Predicted and Experimental Response Variables for the Optimal Formulation

Response	Predicted	95% Confidence intervals (Lower–Upper)	Experimental ^{a)}
Conc. 2 h (µg/mL)	0.36	0.20–0.50	0.40 ± 0.13
Conc. 4 h (µg/mL)	0.50	0.41–0.58	0.50 ± 0.14
Conc. 6 h (µg/mL)	0.57	0.50–0.63	0.58 ± 0.14
Conc. 8 h (µg/mL)	0.64	0.58–0.69	0.68 ± 0.15
Conc. 10 h (µg/mL)	0.68	0.61–0.76	0.73 ± 0.11
Conc. 12 h (µg/mL)	0.79	0.71–0.86	0.82 ± 0.11
Flux (µg/cm ² /h)	0.31	0.28–0.33	0.31 ± 0.06

a) The mean ± S.D. of 4 determinations.

positive and negative results. Moreover, most reports studied under different conditions (*in vitro*, *in vivo*, *ex vivo*), different lipid composition (type, amount), different type of drug (hydrophilic, lipophilic) and different skin model (human, animal); therefore, the obtained results cannot be compared and used to fully understand the behavior. For example, Chol affects the physicochemical properties of liposomes, e.g., size, zeta potential and electrostatic repulsion.²²⁾ A previous study reported that incorporated Chol led to a decrease in the size of liposomes;²³⁾ however, several studies reported that incorporated Chol led to an increase in the size of liposomes.²⁴⁾ On the other hand, Chol slightly affected drug entrapment, but did not affect the size and zeta potential of liposomes.²⁰⁾ Moreover, numerous studies have reported contradictory effects of

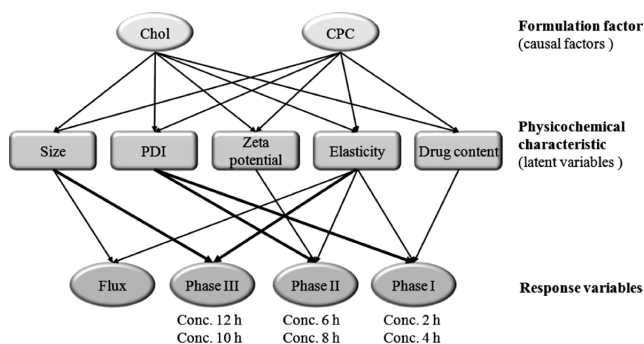


Fig. 5. BN Model of the Latent Structure between Formulation Factors, Latent Variables and Skin-Permeability Response Variables Estimated by the K2 Algorithm Based on Akaike’s Information Criterion

The nodes in the graph correspond to formulation factors, latent variables and skin-permeability response variables, respectively, and directed arcs represent dependencies between them (gentle arcs represent normal correlation and dense arcs represent strong correlation). Chol, cholesterol; CPC, cetylpyridinium chloride; Flux, steady-state flux; Phases III, II and I, concentration of MX-permeated skin at terminal phase (10–12 h), middle phase (6–8 h) and early phase (2–4 h), respectively.

Chol on size, zeta potential, drug entrapment, *etc.* Because of this problem, the effect of formulation factors on the physicochemical characteristics and skin permeability of MX in menthosomes was studied by reliable statistical techniques.

The response surface of the basic characteristics (Fig. 1A) suggested that formulation factors (Chol, CPC) were closely related to physicochemical properties (size, PDI, zeta potential, elasticity and drug content) of the liposome, and the reliability of this result was confirmed by LOOCV (Fig. 1B).

Moreover, the causal relationships between formulation factors and basic characteristics were estimated by BN, and also confirmed in a path diagram (Fig. 5).

In the case of the size of the liposome, the presence of Chol in the formulation resulted in two trends in the size. First, the size increased when the concentration of Chol in the formulation was low (10–25% mole ratio). It has been reported that 11 mol% Chol can reduce the van der Waals attraction force and increase the net repulsion force between PC bilayers,²²⁾ whereas the size decreased as the concentration of Chol in the formulation was high (30–40% mole ratio). This might be attributed to the decrease in surface energy with increase in hydrophobicity.²³⁾ These results indicate that the amount of Chol affects the size of the vesicles. It has been reported that the size of vesicles loaded with a positively charged drug was smaller for anionic vesicles because of neutralization of their negative charge.²⁵⁾ Correspondingly, the vesicles with our negatively charged drug, MX, were also smaller when incorporated in a positively charged (cationic) surfactant because of neutralization.

The presence of Chol also affected the PDI. As CPC was increased, PDI decreased. However, the role of the formulation factors is not the only aspect affecting the PDI; there are also other factors such as the method of preparation. It is a little difficult to explain all of the influences on the complicated relationship between the formulation factors and latent variables at present. The presence of Chol had a slight effect on zeta potential.²²⁾ However, in our study, the addition of CPC cationic surfactants resulted in an increase in positive charges on the vesicle surface. Those results indicate that the addition of CPC, and CPC content, significantly affect the zeta potential of the vesicles. The presence of Chol within the formulation affected the elasticity of the vesicles. A previous study²⁶⁾ indicated that inclusion of Chol results in an increased packing density of PC molecules, which led to increased rigidity of the PC bilayers and sustained a greater shear stress after Chol was incorporated into the formulation. Moreover, the roles of CPC within the formulation also had a significant effect in increasing the elasticity because of edge activators. An edge activator is often a single-chain surfactant, having a high radius of curvature that destabilizes lipid bilayers of the vesicles and increases deformability of the bilayers.²⁶⁾ These results indicate that the fluidity of the vesicles was also changed, depending on Chol and CPC contents. MEN increased elasticity of lipid bilayer, while Chol decreased elasticity and increased rigidity and stability of lipid bilayer by changing transition temperature of total lipid component.²²⁾ Our previous study suggested that MEN enables to interact with Chol in liposomes.²⁷⁾ Thus, the effect of Chol might arise from change in the fluidity of vesicles due to the interaction between MEN and Chol. The optimal ratio of these compositions in the formulation was significant to investigate to improve the fluidity and stability of the vesicles bilayer at the same time.

In the case of MX content, the presence of Chol within the formulation affected the MX content in two trends related to particle size. The incorporation of Chol had a slight effect on increasing MX content when low Chol (10–25% mole ratio) was used in the formulation. However, formulations containing high Chol (30–40% mole ratio) resulted in no significant increase or a slight decrease in MX content. The effect of Chol content on size and MX content has been correlated

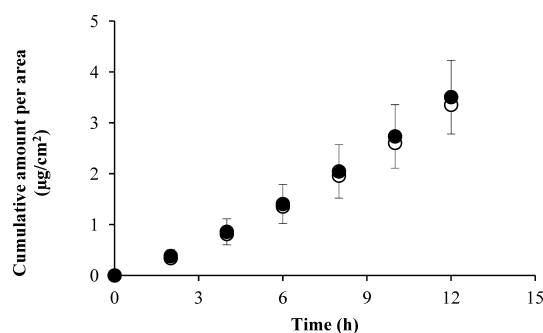


Fig. 6. The Accumulated Skin-Permeation Profile of MX from the Optimal Formulation

● experimental values; ○ predicted values. Each experimental value is a mean \pm S.D. ($n=4$).

in a previous study,²⁰⁾ which suggested that when high Chol content (30–50%) is incorporated in the vesicle formulation, the hydrophobicity in the interfacial region of the vesicle bilayer can increase, and this factor could influence MX content within the lipid bilayer. However, our results indicate that in the case of this hydrophobic drug (meloxicam; MX), a minor increase in MX content occurred in the formulations containing low Chol and a minor decrease in MX content in the formulations containing high Chol may be the result of two contradictory factors. On the one hand, increase in hydrophobicity of the bilayer with increasing Chol content (at low Chol) may efficiently trap the MX within the vesicle bilayer. On the other hand, high Chol content may compete with MX for packing space within the vesicle bilayer, thereby excluding MX as the amphiphiles assemble into vesicles. Moreover, the presence of CPC within the formulation also makes a significant difference in increasing MX content because of the intrinsic properties of CPC, in which the beneficial role of surfactant within lipid bilayers is well recognized as leading to solubility enhancement of MX in the vesicle bilayer.

The formulation factor was the most important factor affecting the physicochemical characteristics of vesicles, and also affected the efficiency of transdermal delivery of MX vesicles, as shown in Fig. 2. The response surface of the response variables suggested that skin permeability of MX in mentosomes formulation was related to the causal factors (Chol and CPC content), and the reliability of this result was confirmed by the LOOCV (Fig. 3).

To confirm the accuracy and reliability of the optimal formulation estimated using RSM-S, the optimal formulation was confirmed by experiment. Studies of the physicochemical properties and *in vitro* skin permeation were also performed with the experimental optimal formulation. The composition of the optimal formulation was PC:Chol:CPC=100:10.55:29.02 mol ratio. The concentration of MX-permeated skin at 2–12 h and the flux values predicted by the RSM-S were very close to the experimental values (Table 2, Fig. 6). Moreover, all experimental values were also in the 95% CI range. The previous study indicated that the flux values of ondansetron hydrogels predicted by RSM-S coincided well with experimental value evaluated by *in vitro* skin permeation study.¹⁸⁾ In addition, predicted value of diltiazem hydrochloride release profile estimated by RSM-S also coincided well with the release profile of optimal formulation measured by the experiment.¹³⁾ The results were sufficiently

high reliability (Table 2, Fig. 6) suggesting that RSM-S successfully estimated the optimal formulation of MX loaded menthosomes.

To evaluate the robustness of the optimal formulation, histograms of the optimal factors and responses were estimated based on the BS-resampling method. The BS resampling and SOM clustering exhibited a normal distribution, suggesting that CIs of the formulation factors and the response variables were satisfactorily estimated (Table 2).

To elucidate quantitatively the relationships between the formulation factors, latent variables and skin-permeability response variables, the probabilistic inference model was performed using a BN. The distinctive probabilistic model was estimated by the arcs, which represent conditional dependencies, and between the nodes, which represent the variables. In this model (Fig. 5), the nodes of Chol and CPC were connected by arcs to the latent variables and response variables, respectively. The results suggest that the formulation factors, latent variables and response variables were conditionally dependent on each other. Dependencies between each of Chol and CPC on physicochemical properties (latent variables) and skin permeability as response variables were clarified. The size and elasticity significantly correlated with the skin permeability as the small size and high elasticity may improve the skin permeability of MX in vesicles. PDI also correlated well with skin permeability. A similar mean size particle but different PDI may result in a different skin permeability. Our data are the first results that obviously demonstrate the causal relationships between formulation factors, latent variables and skin-permeability response variables. We also suggest that the BN model constructed in this study is highly robust, and the relationships between the variables are appropriately expressed as probability variables.

CONCLUSION

The optimal formulation of menthosomes, defined as the formulation with an appropriate penetration of MX, was estimated using RSM-S and was also determined by experiment. The experimental value agreed well with the predicted value. Considering the concentration of MX-permeated skin and steady-state flux value of this formulation, we were successful in showing the feasibility of transdermal delivery of MX using menthosomes. BS resampling together with SOM clustering was applied to estimating 95% CIs of the optimal solution. Moreover, BN was used to construct a probabilistic graphical model and elucidated the relationships within the latent structure by estimating conditional probability distributions. Further study is required to confirm the potential of menthosomes compared with classic liposomes.

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