Nanoemulsions as vehicles for transdermal delivery of glycyrrhizin

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The present investigation aims to evaluate an isotropic and thermodynamically stable nanoemulsion formulation for transdermal delivery of glycyrrhizin (GZ), with minimum surfactant and cosurfactant (Smix) concentrations that could improve its solubility, permeation enhancement, and stability. Pseudo-ternary phase diagrams were developed and various nanoemulsion formulations were prepared using soyabean oil as oil, Span 80, Brij 35 as a surfactant and isopropyl alcohol as a cosurfactant. Nanoemulsion formulations that passed the thermodynamic stability tests were characterized for pH, viscosity and droplet size using a transmission electron microscopy. The transdermal ability of glycyrrhizin through human cadaver skin was determined using Franz diffusion cells. The *in vitro* skin permeation profile of the optimized nanoemulsion formulation (NE2) was compared to that of conventional gel. A significant increase in permeability parameters such as steady-state flux (Jss) and permeability coefficient (Kp) was observed in the optimized nanoemulsion formulation (NE2), which consisted of 1% wt/wt of mono ammonium glycyrrhizinate (MAG), 32.4% Span 80, 3.7% Brij 35, 10% isopropyl alcohol, 46.5% soyabean oil and 6.4% distilled water. No obvious skin irritation was observed for the studied nanoemulsion formulation (NE2) or the gel. The results indicated that nanoemulsions are promising vehicles for transdermal delivery of glycyrrhizin through human cadaver skin, without the use of additional permeation enhancers, because excipients of nanoemulsions act as permeation enhancers themselves.

Uniterms: Nanoemulsions/use/*in vitro* skin permeation. Glycyrrhizin/transdermal delivery. Isotropic nanoemulsion formulation/evaluation. Anti-inflammatories/transdermal delivery. Surfactants. Permeation/enhancers/*in vitro* study.

O objetivo da investigação é avaliar uma nanoemulsão isotrópica termodinamicamente estável para a administração transdérmica da glicirrizina (GZ), com concentrações mínimas de tensoativo e co-tensoativo (Smix), que poderiam melhorar a sua solubilidade, a permeação e a estabilidade. Os diagramas pseudo-ternários de fase foram desenvolvidos e diversas nanoemulsões foram preparadas com óleo de soja como óleo, Span 80, Brij 35 como tensoativos e álcool isopropílico como co-tensoativo. As nanoemulsões que passaram por testes de estabilidade termodinâmica foram caracterizadas por pH, viscosidade, tamanho de gota e microscopia eletrônica de transmissão. A capacidade transdérmica da glicirrizina em passar através da pele de cadáver humano foi determinada por células de difusão de Franz. O perfil *in vitro* de permeação cutânea da formulação otimizada (NE2) foi comparada com a de gel convencional. Observou-se aumento significativo nos parâmetros de permeabilidade, como fluxo de equilíbrio (JSS) e coeficiente de permeabilidade (Kp) na formulação otimizado (NE2), que consistiu de 1% wt/wt de monoglicirrizinato de amônio (MAG), 32,4% de Span 80, 3,7% de Brij 35, 10% de álcool isopropílico, 46,5% de óleo de soja e 6,4% de água destilada. Não se observou irritação óbvia da pele para as nanoemulsões estudadas (NE2) ou de gel. Os resultados indicaram que nanoemulsões são promissores veículos para a administração transdérmica de glicirrizina através da pele de cadáveres humanos, sem o uso adicional de promotor de permeação, porque excipientes de nanoemulsões atuam como promotores de permeação.

Unitermos: Nanoemulsão/uso/permeação cutânea *in vitro*. Glicirrizina/administração transdérmica. Nanoemulsão isotrópica/avaliação. Anti-inflamatórios/administração transdérmica. Tensoativos. Permeação/promotores/*in vitro* study.

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INTRODUCTION

Glycyrrhizin (GZ) (Figure 1), a major component of liquorice (*Glycyrrhiza glabra* L.), is used as a remedy for chronic hepatitis, allergies, and other ailments (Koga *et al.*, 2004). It has been reported that liquorice is effective in gastric ulcer treatment (Bennet *et al.*, 1985), and glycyrrhetinic acid — the aglycone of glycyrrhizin — has anti-inflammatory and antiulcer effects (Yano *et al.*, 1989). Liquorice constituents also exhibit anti-arthritic, anti-arrhythmic, antibacterial, antiviral, expectorant and steroid-like anti-inflammatory activity, similar to the action of hydrocortisone. This is due, in part, to inhibition of phospholipase A2 activity, an enzyme critical to numerous inflammatory processes (Okimasu *et al.*, 1983). *In vitro* research has also demonstrated that glycyrrhizic acid inhibits cyclooxygenase activity and prostaglandin formation (specifically, prostaglandin E2), as well as indirectly inhibiting platelet aggregation, all factors involved in inflammatory process (Okimasu, 1983; Ohuchi, Tsurufuji, 1982). It is now known that glycyrrhizic acid and its aglycone glycyrrhetinic acid, present in the root extract, are responsible for biological activities (Gibson, 1978; Norman *et al.*, 1995). It is currently used extensively in the tobacco, food, confectionery, and pharmaceutical industries, throughout the world (Baker, 1995). One of the promising technologies for enhancing transdermal permeation of drugs is nanoemulsion. Nanoemulsions are thermodynamically stable, transparent (or translucent) dispersions of oil and water stabilized by an interfacial film of surfactant molecules with droplet size below 100 nm. Nanoemulsion provides ultra low interfacial tensions and large o/w interfacial areas. Nanoemulsions have higher solubilization capacity than simple micellar solutions and their thermodynamic stability offers advantages over unstable dispersions, such as emulsions and suspensions, because they can be manufactured with little energy input (heat or mixing) and have a long shelf life. The nanosized droplets leading to enormous interfacial areas associated with nanoemulsions appear to influence the transport properties of the drug, an important factor in sustained and targeted drug delivery (Eccleston, 1994; Lawrence, Rees, 2000). The appeal of formulating o/w nanoemulsion systems lies in their ability to incorporate hydrophobic drugs into the oil phase, thereby enhancing solubility (Lawrence, Rees, 2000). Nanoemulsions have been reported to make the plasma concentration profiles and bioavailability of drugs more reproducible (Kommuru *et al.*, 2001; Lawrence, Rees, 2000; Constantinides, 1995; Kawakami *et al.*, 2002). Transdermal delivery of drugs through the skin to the systemic circulation excels by avoiding the hepatic first-pass metabolism, as well as by its potential of long term controlled release, avoiding the typical peak trough plasma-profiles associated with frequent dosage regiments. Transdermal delivery also presents with ease of administration and the possibility of immediate withdrawal of treatment, providing a convenient route of administration for a variety of clinical indications (Kreilgaard, 2002; Benson, 2005). Nanoemulsions can enhance the transdermal permeation of drugs via their finely dispersed oil droplet phase. Additionally, the diffusional barrier of the skin may be modified depending on the composition of the nanoemulsion. Also, an increased thermodynamic activity of the drug may favour its partitioning into the skin (Baroli *et al.*, 2000; Moulik *et al.*, 2001). This article describes the potential of nanoemulsions systems in transdermal delivery of glycyrrhizin through human cadaver skin, without the use of additional permeation enhancers, because excipients of nanoemulsions themselves act as permeation enhancers.
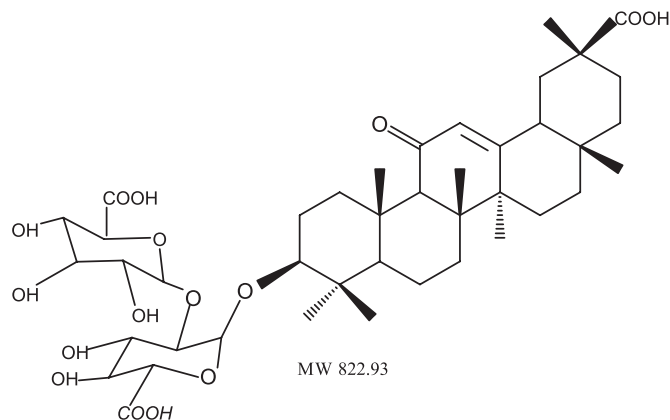

FIGURE 1- Structure of glycyrrhizin.

MATERIAL AND METHODS

Materials

The standard compound mono ammonium glycyrrhizinate (MAG) was purchased from Fisher Scientific, India. Brij 35, Span 80, soyabean oil, isopropyl alcohol, ethanol and Carbopol 940 were purchased from Himedia Chemicals, Delhi, India. Purified water, solvent and other chemicals were of analytical grade.

Construction of pseudo-ternary phase diagrams

In response to finding the concentration range of ingredients for the existing range of nanoemulsions, pseudo-ternary phase diagrams were constructed, using

TABLE I - Compositions of the selected nanoemulsion formulations

Components	NE1	NE2	NE3	NE4	NE5
MAG %	1.0	1.0	1.0	1.0	1.0
Water %	7.40	6.4	6.0	6.0	5.0
Span 80%	29	32.4	36	40.0	43.0
Brij 35%	3.3	3.7	4.0	4.0	5.0
Isopropyl alcohol %	9.0	10.0	11.0	12.0	13.0
Soyabean oil %	50.3	46.5	42.0	37.0	33.0

the water titration method at room temperature (25 °C). Three phase diagrams were chosen, with 3:1, 4:1 and 5:1 weight ratios of Span 80 and Brij 35 to isopropyl alcohol, respectively, and are shown in Figure 2. The ratios of oil to the mixture of S:Cos, for each phase diagram at a specific S:Cos weight ratio, varied: 9.5:0.5, 9:1, 8.5:1.5, 8:2, 7.5:2.5, 7:3, 6.5:3.5, 6:4, 5.5:4.5, 5:5, 4.5: 5.5, 4:6, 3.5:6.5, 3:7, 2.5:7.5, 2:8, 1.5:8.5, 1:9, 0.5:9.5. The mixtures of oil, S:Cos (Smix) at certain weight ratios were diluted by adding water dropwise, under specific magnetic stirring. After being equilibrated, the mixtures were assessed physically and determined as being nanoemulsions.

Preparation of nanoemulsions

Nanoemulsion regions were identified based on phase diagrams, and the nanoemulsion formulations were selected at different ingredients ratios, as described in Table I. Nanoemulsions loaded with MAG, prepared with the mixture of soyabean oil and isopropyl alcohol; a stock solution containing MAG. The clear oily phase containing MAG was obtained by diluting the weighed amount of stock solution with soyabean oil and isopropyl alcohol. Span 80 and Brij 35 were dissolved in distilled water and added to the clear oily phase, drop by drop. Water-in-oil (w/o) nanoemulsions containing MAG were obtained under a magnetic stirring at room temperature. All formulations contained 1% w/w mono ammonium glycyrrhizate.

Characterization of nanoemulsions

Morphology and structure of the nanoemulsions were studied using a transmission electron microscope (TEM) (Hitachi H-7500, Japan). Samples were stained on a carbon-coated copper grid with a 1% aqueous solution of phospho tungustic acid (PTA) and observed under the microscope with an accelerated voltage of 100 kV. The pH values of nanoemulsions were determined using a pH meter (HACH digital pH meter, USA). The viscosity of various nanoemulsions was measured using a Brookfield digital viscometer (Model DV-II, Brookfield, USA). Mean droplet size and polydispersity index of the nanoemulsion were characterized by photon correlation spectroscopy (Malvern Zetasizer Nano ZS-90, Malvern instrument Ltd, UK). Samples were suitably diluted with distilled water to avoid multi-scattering phenomena. Changes in droplet size of the diluted nanoemuslions were not found to be significant. The conductivity of different nanoemulsions was determined using a conductometer (HACH, USA). All parametrs were carried out at 25 °C.

Preparation of conventional glycyrrhizin gel

Conventional glycyrrhizin gel (CG) was prepared by dispersing the Carbopol 940 (1 g) in a sufficient quantity of distilled water. After complete dispersion, the Carbopol 940 solution was kept in the dark for 24 hours for complete swelling. Then mono ammonium glycyrrhizate (1 g) was dissolved in a specified quantity of polyethylene glycol 400. This drug solution was added slowly to the aqueous dispersion of Carbopol 940, with agitation. Then other ingredients like isopropyl alcohol, propylene glycol and triethanolamine were added to obtain a homogeneous dispersion of gel (Shakeel *et al.*, 2007) (Table II).

TABLE II - Formula for preparation of glycyrrhizin gel*

Glycyrrhizin gel	Ingredients (for 100 g of gel)
MAG (% wt/wt)	1
Carbopol 940 (% wt/wt)	1
IPA(% wt/wt)	10
PEG-400 (% wt/wt)	10
PG (% wt/wt)	10
TEA (g)	0.5
Distilled water (qs)	100

*IPA indicates isopropyl alcohol; PEG, polyethylene glycol; PG, propylene glycol; TEA, triethanolamine; qs, quantity sufficient.

Stability of nanoemulsions

The chemical and physical stability of nanoemulsions was studied by observation of clarity and phase separation, determination of droplet size and analysis of glycyrrhizin 251 nm for 6 months, using a UV-double beam spectrophotometer (Shimadzu, 1800). Concentrations of glycyrrhizin in the nanoemulsions were estimated monthly. The centrifuge tests were also performed to assess the phase behavior of nanoemulsions. Nanoemulsions were centrifuged for 30 min at 35,000 rpm in the centrifuge apparatus (Shafiq et al., 2007). Nanoemulsions were kept at 4±1, 25±1 and 45±1 ºC for 6 months. Clarity, phase separation and concentration of glycyrrhizin were then evaluated to find the optimal storage temperature, on a monthly basis.

Calibration curve

Glycyrrhizin10 mg was accurately weighed and dissolved in a sufficient volume of phosphate buffer in a 100 mL volumetric flask. The final volume was made up to the mark with phosphate buffer to obtain a concentration of 100 µg/mL. This stock solution was used to prepare further standard solution of the drug. From the stock solution, aliquots of 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, and 5.0 mL of volume, up to 10 ml were transferred to a series of 10 mL volumetric flask and the volume was made up to the mark with phosphate buffer pH 7.4. Thus, drug solution concentration obtained was in the range of 5-50 µg/mL. All the solution was filtered using Whatman filter paper (# 41). The absorbance of all the resultant solutions was measured against blank using a UV Double-beam spectrophotometer (Shimadzu 1700, Japan) at 251 nm (Aswatha et al., 2010). A calibration curve was obtained: $Y = MX + C$; where $M = 0.0194$, $C = 0.0457$, and $r^2 = 0.9764$.

In vitro permeation studies

In vitro skin permeation of MAG-loaded nanoemulsion formulations was studied using a locally manufactured Franz diffusion cell with an effective permeation area and receptor cell volume of 2.5 cm² and 10 ml, respectively. The temperature was maintained at 37±0.5 °C. The receptor compartment contained 10 ml phosphate buffer (pH 7.4) and was constantly stirred by a magnetic stirrer (Expo India Ltd., Mumbai, India) at 600 rpm. Dermatomed (500 µm thickness) human cadaver skin from abdominal areas was obtained from the Chhattisgarh Institute of Medical Sciences (Bilaspur, India), and stored in a deep freezer at −20 °C until further use. The skin was then carefully checked through a magnifying glass to ensure that

samples were free from any surface irregularity, such as tiny holes or crevices in the portion that was to be used for transdermal permeation studies (Dubey et al., 2007). After verification, the skin was mounted onto a receptor compartment with the stratum corneum side facing the donor compartment. The donor compartment contained 1 g of the sample and sealed with paraffin film to provide occlusive conditions. Samples were withdrawn through the sampling port of the diffusion cell at predetermined time intervals over a period of 24 hours, then analised for drug content using a UV Spectrophotometer at 251 nm (Shimadzu 1700, Japan). The receptor phase was immediately replenished with an equal volume of fresh diffusion buffer. The formulation NE2 provided the highest release, compared to the other nanoemulsion formulations. The formulation NE2 was also converted into nanoemulsion gel formulations, by adding 1% wt/wt Carbopol 940. This was coded as NG2, with the Carbopol 940 used as viscosity modifier and gelling agent. The skin permeation profile of the optimized nanoemulsion formulation was compared to that of the nanoemulsion gel (NG2) and conventional gel (CG), using Dunnett's test of one-way analysis of variance (ANOVA). All experiments were performed in triplicate. Sink conditions were maintained throughout the experiment. The drug release results are shown graphically in Figure 3.

Determination of flux and permeability

The cumulative amount of glycyrrhizin permeating across the cadaver skin was plotted against time. Drug flux J (µg/h/cm²) at steady state was calculated by dividing the slope of the linear portion of the curve by the area of the exposed membrane surface (2.5 cm²). The permeability Kp (cm/h) was calculated by dividing the flux by the initial concentration of glycyrrhizin in the donor phase. The experiment was carried out in triplicate, and results are shown graphically in Figure 4. In-vitro skin permeation and flux of selected gel formulation of nanoemulsion (NG2) in cadaver skin was analysed, and drug release results are graphically shown in Figure 5 and detailed in Table III.

Skin irritation studies

Irritation studies were carried out to determine the localised reaction of nanoemulsion and nanoemulsion-based gel on the skin. For this purpose, a single 10 µL dose of optimized NE, NE (MAG) and NG was applied to the left ear of Swiss albino mice, with the right ear considered as control. The development of erythema was monitored daily for 3 days using the Utelly test (Utelly, 1973). The

animal experiments had been approved by our institutional ethics committee (IAEC/proposal No. 02/2008 Registration no. 994/ac/06/CPCSEA/23/Oct/2006). Extent of erythema development was indicated on the following basis:

0: no erythema development

2: barely visible few blood vessels and no erythema development

4: main blood vessels more obvious and slight erythema development

Irritation potential was calculated using Equation 1:

$$\text{Resultant indices} = \frac{A \times B}{\text{Number of observation days}}$$

where A and B represent erythema value and corresponding day, respectively (Gupta *et al.*, 2005).

Statistical analysis

All skin permeation experiments were repeated three times and data were expressed as mean value ± SEM. Statistical data were analysed by one-way analysis of variance (ANOVA). A multiple comparison test was used to compare different formulations, and a P value of 0.05 was considered to be significant.

RESULTS AND DISCUSSION

Phase studies

The phase diagram consists of safe constituents including soyabean oil, isopropyl alcohol, Span 80 and Brij 35, and water. The concentration ranges of ingredients for the formulation of nanoemulsions were calculated through the construction of phase diagrams. The pseudo-ternary phase diagrams with various weight ratios of Span 80 and Brij 35 to isopropyl alcohol are described in Figure 2.

The translucent nanoemulsion region is shown in the phase diagrams. The w/o nanoemulsions were observed. The gel region is shown to be transparent and translucent. On visual observation, the rest of the region on the phase diagram represents the turbid and conventional emulsions. The isotropic region of the nanoemulsion changed slightly, increasing in surfactant to cosurfactant ratio.

Preparation and characterisation of nanoemulsions

The nanoemulsions were selected from the 5:1 phase diagram. Span 80 and Brij 35 were added into the
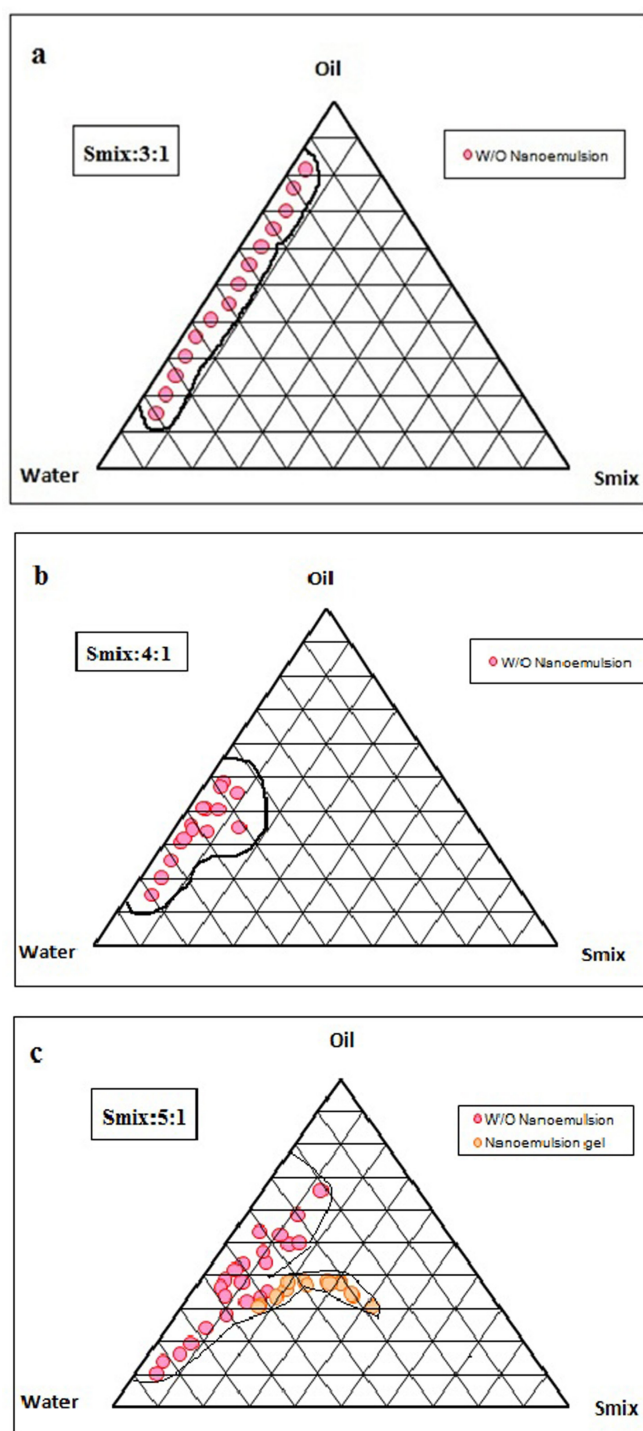
FIGURE 2- Pseudo-ternary phase diagrams indicating water-in-oil nanoemulsion region at different Smix (S:Cos) ratios.

oily phase for the construction of phase diagrams. A time of 1-2 hours was required in order to obtain transparent nanoemulsions under magnetic stirring, with nanoemulsions containing 32.4% Span 80, 3.7% Brij 35, 10% isopropyl alcohol and 46.5% soyabean oil. However, when Span 80 and Brij 35 were solubilised into the aqueous

TABLE III - Permeability parameters for the different formulations

Formulation code	Steady state flux Jss ($\mu g/cm^{-2}/h^{-1}$)	Permeation coefficient (P) Kp ($\times 10^{-3}$ cm h^{-1})
CG	02.50± 0.7	0.25± 0.06
NE1	14.51 ± 0.69	1.45 ± 0.64
NE2	14.93 ± 0.36	1.49 ± 0.30
NE3	13.85 ± 0.35	1.38 ± 0.32
NE4	13.45± 0.50	1.34 ± 0.40
NE5	13.28 ± 0.54	1.32± 0.45
NG2	13.49 ± 0.36	1.34 ± 0.04

phase, and the aqueous phase was then added to the oily phase containing isopropyl alcohol and soyabean oil, clear nanoemulsions were quickly obtained. The order of the addition for Span 80 and Brij 35 did not, however, change the physicochemical properties of the nanoemulsions. Therefore, in order to reduce the equilibrium time, Span 80 and Brij 35 were added to water in preparation of drug-loaded nanoemulsions. Additionally, the influence of the order of the addition of isopropyl alcohol over the preparation of nanoemulsions was also studied. When Span 80 and Brij 35 were added to the aqueous phase, and isopropyl alcohol was added to the oily phase or to the aqueous phase, no change in equilibrium time and in the physicochemical properties was observed. This is not in accordance with the results obtained by Vandamme (Chen *et al.*, 2005; Vandamme, 2002), which stated that the order of the addition of the cosurfactant could influence the time required to achieve equilibrium. This may be due to the quick distribution of isopropyl alcohol between the oily and aqueous phases. It was concluded that the order of the addition of this surfactant should be a very important factor for the preparation of nanoemulsions. The physico-chemical parameters of nanoemulsions are reported in Table IV. The NE2 containing 46.5% oil, 32.4% Span 80, 3.7% Brij 35, 10% isopropyl alcohol had the lowest mean droplet size, viscosity and conductivity. It was obvious that mean

droplet size, viscosity and conductivity of nanoemulsions with less oil and surfactant had increased significantly. In the TEM positive image, the nanoemulsion appeared dark and the surroundings were bright (Figure 6). Some droplet sizes were measured, as the TEM is capable of point-to-point resolution.

These sizes were in agreement with the droplet size distribution measured using photon correlation spectroscopy. The pH values were within the physiological range. The droplet size for all nanoemulsions was in the 10-100 nm range. Mean droplet size of NE2 containing glycyrrhizin was 22 nm. The increase in mean droplet size for the nanoemulsion may be related to embedding of glycyrrhizin and permeation enhancer into the interfacial film.

Stability of nanoemulsions

All nanoemulsion formulations were stable at 45±1 °C in the presence or absence of glycyrrhizin. No changes of droplet size, phase separation and glycyrrhizin degradation were observed during 6 months. The centrifuge tests showed that all nanoemulsions presented with good physical stability. Nanoemulsions stored at 4±1 °C, 25±1 °C and 45±1 °C showed no change in clarity and phase behavior. The concentrations of glycyrrhizin in nanoemulsions were almost constant and no degradation was observed. However, phase separation and turbidity were observed for nanoemulsions at 4 °C. Coagulation of the internal phase might have led to this instability. But these nanoemulsions were easily recovered when heated. Thus, nanoemulsions should be stored at temperatures above 4 °C, at least. Long-term storage of glycyrrhizin in an aqueous environment may result in a potential tendency for hydrolysis. Therefore, it is disadvantageous that the glycyrrhizin was formulated to an aqueous environment. The rate of hydrolysis of glycyrrhizin is also significantly influenced by the pH value. Especially when the pH value is less than 3 or more, hydrolysis of glycyrrhizin could occur. However, when the pH value ranges from 6.5 to 8.5, hydrolysis can be inhibited. All nanoemulsions had

TABLE IV - Physicochemical parameter of the various nanoemulsions

Formulation code	Droplet size (nm)	Polydispersity index	pH	Viscosity (cP)	Conductivity (μS/cm)
NE1	35	0.316	6.75	97.78	16.9
NE2	22	1.000	6.70	98.08	17.9
NE3	34	1.000	6.78	98.02	18.8
NE4	45	1.000	6.85	98.00	20.2
NE5	69	1.000	7.21	98.05	18.5

NE1-5 indicates nanoemulsion formulations of glycyrrhizin

appropriate pH values, and the glycyrrhizin was incorporated into the oily phase of nanoemulsions, avoiding contact with water in the external phase. Hydrolysis of glycyrrhizin in nanoemulsions was not detected during 6 months. It is likely that the nanoemulsion provided an inert circumstance and appropriate pH value for glycyrrhizin. Therefore, glycyrrhizin in nanoemulsions was effectively protected from degradation.

In vitro permeation studies

The nanoemulsion formulations were tested for percutaneous permeation, as shown in Table III. The permeation profiles of glycyrrhizin through human cadaver skin, from various vehicles, are shown in Figures 3-6. A steady increase of glycyrrhizin in the receptor chambers was observed over time. The permeation profiles of nanoemulsions followed zero-order release kinetics. Statistical comparison of the flux throughout 24 hours showed that most of the nanoemulsions provided fluxes ($P < 0.05$) higher than the aqueous solution containing 1% glycyrrhizin. Figure 3 shows that the permeated amounts of glycyrrhizin from the different vehicles – except NE2 – presented no difference before 10 hours. However, significant difference between nanoemulsions and the aqueous solution was observed at the end of experiments. Additionally, the permeation flux from the aqueous solution decreased significantly after 10 hours. This revealed the inability of the aqueous solution to provide prolonged delivery of glycyrrhizin. This phenomenon may have been a result of depletion of driving concentration in the donor chamber.

Formulations NE1 and NE3, containing a lower amount of Span 80, Brij 35 and isopropyl alcohol, provided higher flux ($P < 0.05$) than ME2 and ME4, respectively. The content of surfactant mixture in nanoemulsions significantly affected the skin permeation flux of glycyrrhizin. This may be due to increased thermodynamic activity of the drug in nanoemulsions at the lower concentration of surfactant and cosurfactant (Rhee *et al.*, 2001). The thermodynamic activity of drug in the formulation is a significant driving force for the release and penetration of the drug into the skin (Walters *et al.*, 1998). The thermodynamic driving force for release reflects the relative activities of the drug in different phases (Delgado-Charro *et al.*, 1997). Since the drug can be released from the internal phase to external phase and then from the external phase to the skin, the relative activities may monitor the skin permeation flux. The depletion of glycyrrhizin in the external phase due to permeation into the skin can be supplemented by the release of glycyrrhizin from the internal phase. Then, zero-order release

kinetics and sustained, controlled and prolonged delivery of glycyrrhizin were obtained. This may also be the main mechanism of permeation of glycyrrhizin into the skin from these nanoemulsions. Passive drug permeation across the skin can be increased with a transdermal permeation enhancer that can reversibly remove the barrier resistance. Enhancers can increase the transport through skin by modifying the diffusion or partition coefficient of the drug (Naik *et al.*, 2000). Ideally, an effective permeation enhancer should cause minimal tissue damage and toxicity, especially for chronic applications. In this experiment, significant skin permeation-enhancing effects were observed. *In vitro* skin permeation studies were performed to compare drug release from five different nanoemulsion formulations (NE1-NE5), NG2 and CG all containing the same amount (1% wt/wt) of MAG. *In vitro* skin permeation was highest in formulation NE2 and lowest for CG (Figures 3 and 5).
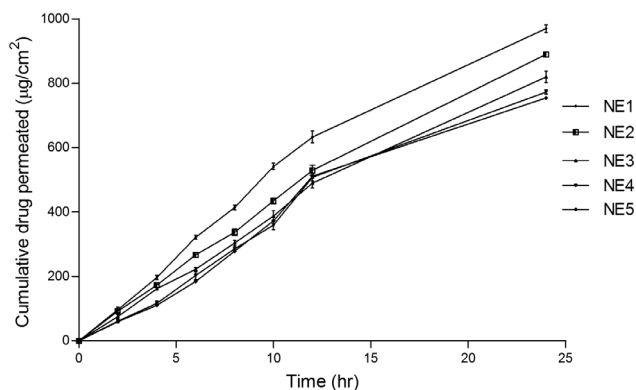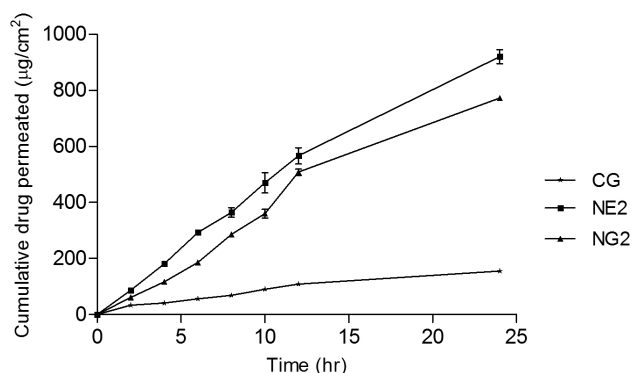

Values represented as mean ± SEM (n = 3); NE stands for nanoemulsion

**FIGURE 3** - Percentage of cumulative drug permeation for the various nanoemulsions through human cadaver skin.
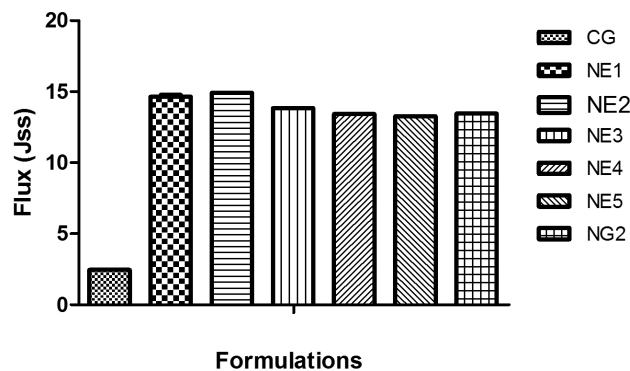
FIGURE 4 - Steady-state transdermal flux of glycyrrhizin across human cadaver skin.

Values represented as mean ± SEM (n = 3), NE2 stands for nanoemulsion 2, NG2 stands for nanoemulsion gel 2, CG stands for conventional gel

FIGURE 5 - Graphic representation of cumulative drug release for CG, NE2 and NG2.
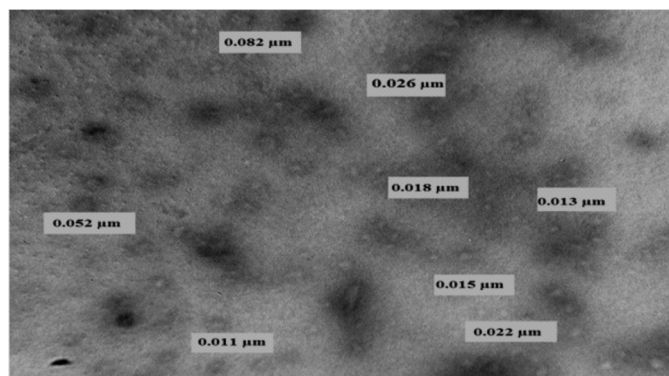
FIGURE 6 - Transmission electron microscopic positive image of glycyrrhizin nanoemulsion showing the size of some droplets.

Formulation NG2 showed an intermediate skin permeation profile. The skin permeation profile for N2 was significantly different when compared with that of CG and NG2 (P < 0.05). The significant difference in glycyrrhizin permeation between nanoemulsion formulations NG2 and CG was probably due to the mean size of the internal phase droplets, which were significantly smaller in the nanoemulsions. The maximum release in NE2 could have been due to this formulation having the lowest droplet size and lowest viscosity of all nanoemulsions. Increase of the loading dose is widely known as an effective method to improve the skin permeation rate of various compounds (Reifenrath *et al.*, 1981). The permeation rate of mono ammonium glycyrrhizate was almost linearly improved as a function of the loading dose and the permeation of nanoemulsions, in accordance with Fick's first law of diffusion. A high loading dose of mono ammonium glycyrrhizate can lead to increased flux. The effect of mono ammonium glycyrrhizate on skin irritation was studied by the Utelly test, and results showed that a hydroalcoholic solution containing 10 µg/mL of MAG could induce obvious erythema and edema on both intact and rat skins (Utelly, 1973). The previous investigation about nanoemulsions with 1% glycyrrhizin validated the anti-inflammatory activity (Ohuchi, Tsurufuji; 1982). Therefore, the suitable concentration of glycyrrhizin in nanoemulsions would be 1%. In a previous work, all the nanoemulsion formulations contained a large amount of surfactants and cosurfactant, with a powerful ability to perturb the stratum corneum. Furthermore, the best formulation, consisting of 46% soyabean oil, 50% Smix/isopropyl alcohol (5:1) and water is a w/o type of nanoemulsion. MAG dissolved in the inner phase of the w/o nanoemulsion might come into direct contact with the surface of the skin. This is suitable for long-term use, from a safety perspective. Therefore, formulation NE2 should be the most valuable for the further exploration.

Skin irritation studies

The irritation studies did not show visible irritation after application of NE2 for 3 days on the skin of rats, with no erythema or edema observed. Only rubefaction occasionally appeared on the skin of some rats on the first or second day, disappearing on the last day. The encapsulation of the nanoemulsion might reduce the skin irritation induced by MAG. The results suggest that this nanoemulsion system for the transdermal delivery of MAG is viable.

CONCLUSIONS

The nanoemulsions containing MAG were studied for transdermal delivery. The different nanoemulsion formulations were selected using the pseudo-ternary phase diagrams. The order of the addition of Smix is a very important factor for the preparation of nanoemulsions. The incorporation of MAG into nanoemulsions led to significant increase in droplet size, due to their location in the interfacial film. *In vitro* permeation studies showed that nanoemulsions with lower content of surfactant mixture could increase transdermal capacity. The MAG-loaded nanoemulsions showed controlled, sustained and prolonged delivery. The permeation rates observed for MAG from nanoemulsions were in accordance with Fick's first law of diffusion. *In vitro* skin permeation studies were performed to compare the release of drug from 5 different nanoemulsion formulations (NE1-NE5), NG2 and CG, all containing the same amount (1% wt/wt) of MAG. *In vitro* skin permeation was highest for formulation NE2 and lowest for CG (Figures 3 and 5). Formulation NG2 showed an intermediate skin permeation profile. The skin permeation

profile for N2 was significantly different when compared to that of CG and NG2 (P < 0.05). The significant difference in glycyrrhizin permeation between nanoemulsion formulations NG2 and CG was probably due to the mean size of internal phase droplets, which were significantly smaller in nanoemulsions. The maximum release in NE2 could be due to this formulation having the lowest droplet size and the lowest viscosity of all nanoemulsions.

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