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Elastic liposomes for skin delivery of dipotassium glycyrrhizinate

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Abstract

The aim of this study was to evaluate the possibility of using liposomes for skin delivery of dipotassium glycyrrhizinate (KG), an anti-inflammatory agent employed in treating acute and chronic dermatitis, and of formulating such liposomes in an oil-in-water emulsion (O/W). KG had emulsifying properties and the possibility of producing elastic liposomes was verified. Liposomes containing soya lecithin (PC) or hydrogenated soya lecithin (HPC) mixed with KG in w/w ratios of 2:1, 4:1 or 8:1 were prepared by the solvent evaporation method and then passed through a high pressure homogeniser. Liposome size and entrapment efficiency were determined and the interaction between KG and HPC was investigated using differential scanning calorimetry (DSC). Transepidermal permeation through intact pig skin and skin deposition of KG from liposomes and O/W emulsion containing liposomes were assessed and compared with values for aqueous control solutions. No marked differences were observed between PC and HPC liposomes. Liposome sizes ranged from 90 to 120 nm. Entrapment efficiency depended on the lipid:KG ratio; the maximum efficiency was obtained at 4:1 w/w. KG interacted with liposomes disrupting and fluidising the lipid bilayer, forming elastic liposomes able to penetrate through membrane pores of diameter much smaller than their own diameter. The liposome structure was maintained when dispersed in an O/W emulsion. The skin fluxes were less than the HPLC detection limit for all systems, while skin deposition increased 4.5-fold compared with aqueous solutions when KG was formulated in liposomes. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Elastic liposomes; Dipotassium glycyrrhizinate; Skin delivery

1. Introduction

The percutaneous route for drug administration has many advantages over other pathways including avoiding the hepatic first pass effect, continuous drug delivery, fewer side effects and improved patient compliance (Barry, 1983).

A major obstacle to percutaneous drug delivery is the low penetration of drugs through the skin. The stratum corneum provides the principle barrier to the percutaneous permeation of topically applied substance and consists of corneocytes embedded in a lipid matrix. This lipid matrix is composed of highly organised bilayers, containing

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mainly free fatty acids, ceramides, cholesterol and cholesterol sulphate (Elias and Friend, 1977). Lipid material between the corneocytes is not only simple but also highly organised, thus, acting as extra intercellular glue sealing the spaces between the cells in the skin.

In order to cross intact skin, drug carriers must pass through a series of very fine pores with an average diameter typically around 30 nm or less, under the influence of a suitable transdermal gradient (Wertz and Downing, 1989). Several approaches have been developed to either destroy or fluidise the lipid bilayers, thereby, enhancing the penetration of drugs (Barry, 1987; Hadgraft and Walters, 1992).

One of the possibilities for increasing the penetration of drugs through the skin is the use of vesicular systems such as liposomes. Due to their biocompatibility and capability of incorporating both hydrophilic and lipophilic drugs, liposomes have been investigated as parenteral drug carrier systems and more recently as transdermal drug delivery systems (Egbaria and Weiner, 1990; Scheier and Bouwstra, 1994). The strategy of using liposomes is of interest, but remains controversial owing to their large minimum size. To date, no consensus exists on whether or not the administration of liposomes can lead to vescicle penetration into or through the intact mammalian skin, but many agree that most liposomes do not reach deep into the intact skin.

A novel type of lipid aggregates has been recorded to penetrate intact skin if applied non-occlusively in vivo, by virtue of their very high and self-optimising deformability (Cevc et al., 1996).

Depending on the composition, bilayers of vescicles are in either a liquid–crystalline or a rigid gel-state. Several studies have investigated whether the physical state of vescicles is essential for their mode of action. When comparing the interactions of elastic and rigid vescicles with hairless mouse skin in vivo, only the elastic liquid-state vescicles affected the ultrastructure of the viable tissue. No changes in the ultrastructure were observed with any of the classical liposomes (van den Bergh et al., 1999).

Such aggregates can, therefore, squeeze themselves between the cells in the stratum corneum inspite of their large size, probably under the influence of the transepidermal water activity gradient. In contrast to liposomes, the deformable vescicles, when applied non-occlusively, may bring a remarkable amount of the applied lipid mass into the normal horny layer, reaching a depth of at least 30 μm (Aguiella et al., 1994). The precise reach, as well as the kinetics, of elastic liposome penetration through the skin depends on the carrier type, total mass applied, and the detailed application conditions. In order to achieve an optimum carrier efficacy of these systems and the best possible therapeutic results, a number of the carrier system properties must, therefore, be simultaneously considered and perfected. Such multiple interdependencies bring many formulation difficulties but also offer the means for achieving special, and frequently quite desiderable, drug distribution.

These elastic aggregates contain phosphatidylcholine and a surfactant, and consist of at least one inner aqueous compartment surrounded by a lipid bilayer; they resemble liposomes in morphology but not in function. Sodium cholate (Cevc et al., 1996), Span 80 or Tween 80 (El Maghraby et al., 2000) were employed as surfactants.

Dipotassium glycyrrhizinate (KG) is a compound obtained by extraction with water from liquorice root. Many clinical reports deal with the application of this product to medicines for external use in the field of dermatology; it is apparently effective in treating acute and chronic dermatitis (Yano, 1958; Kerube, 1970). Moreover, continuous application is almost without side effects, and because of its chemical stability, good solubility and emulsifying properties, it is widely used in cosmetics. The critical micelle concentration is reported to be 0.75% w/w (Yonezawa et al., 1976). It is also used in internal and external drugs, as well as in sweeteners.

The present study aimed to evaluate the ability of KG to produce elastic liposomes with commercial soya lecithin or commercial hydrogenated lecithin and to study the in vitro skin delivery. The possibility of formulating such liposomes in an oil-in-water (O/W) emulsion was also verified.

2. Materials and methods

2.1. Materials

Phosphatidylcholine (PC, Phospholipon® 100) from soya bean (purity > 98%) and hydrogenated lecithin (HPC, Phospholipon® 100 H) were obtained from Nattermann Phospholipid (Koln, Germany) and used without further purification. KG was purchased from Maruzen Pharmaceuticals (Hiroshima, Japan).

Arachidyl behenyl alcohol–arachidylglucoside (Montanov® 202) was from Seppic Inc. (Fairfield, NJ, USA) and octyl benzoate (Finsolv® EB) from Prodotti Gianni (Milan, Italy).

All other chemicals were reagent grade and used as received. Water was freshly bidistilled.

2.2. Preparation of liposomes

Lipid vescicles containing PC or HPC alone or mixed with different amounts of KG were prepared by a conventional rotary evaporation method. Appropriate amounts of PC or HPC (0.2–1.0 g) were dissolved in a minimum amount of methanol/chloroform (1:2). The organic solvent was evaporated under a stream of nitrogen and solvent traces were removed by maintaining the lipid film under vacuum overnight. The films were hydrated with water (25 ml) by vortexing for 10 min at 30 °C for those containing PC and at 55 °C for those containing HPC. For elasticity determinations, the hydrated vescicles were sonicated for a sufficient time to obtain a liposome mean size of 280–310 nm. PC liposomes of about 200 and 150 nm were also prepared. For the other experiments, the liposome suspensions were passed through a high pressure homogeniser for three cycles at 250 bar pressure (Panda, Niro Soavi, Parma, Italy), and then maintained for 24 h at room temperature. When KG was used the weight ratio of lipid and surfactant was 2:1, 4:1 or 8:1. The liposome suspensions were diluted with water to obtain a final concentration of KG of 0.25% w/w.

2.3. Preparation of oil-in-water emulsion

An O/W emulsion was prepared as follows: the mixture of oil (Finsolv) and emulsifier (Montanov 202) was heated to 65 °C and then added to water at 70 °C using an Ultra Turrax. The mixture was then cooled at room temperature under gentle stirring. Emulsion containing KG was prepared by adding KG, dissolved in the minimum amount of water, to the O/W emulsion at room temperature; it was maintained for 24 h at room temperature before testing. The final composition (w/w) of the emulsion was: 0.25% KG, 74.75% water, 20% oil, 5% Montanov 202.

2.4. Preparation of liposomes in oil-in-water emulsion

Liposomes in O/W emulsion were prepared by adding the liposome suspension to the O/W emulsion at room temperature under moderate stirring and it was maintained for 24 h at room temperature before testing. The final concentration of KG was 0.25% w/w.

2.5. Liposome size determination

Dynamic light scattering measurements (DLS) were done to determine the mean size of the liposome suspensions using a Brookeven Instrument (Holtville, NY, USA). Liposome suspensions were suitably diluted with bidistilled water and filtered through 0.4 μm membrane to minimise interference particulate matter before sizing. The experiments were performed in triplicate and each sample was analysed three times.

2.6. Differential scanning calorimetry (DSC)

DSC was performed with a Perkin Elmer differential calorimeter (DSC7, Perkin Elmer, Nortwalk, CT, USA). Liposome suspension containing 1% w/w HPC with or without 0.25% w/w KG, an O/W emulsion and a formulation of liposomes containing KG dispersed in O/W emulsion (final concentration 1% HPC) were placed in conventional aluminium pan and a scan speed of 2 °C min^{-1} was employed. The weight of each sample was 12–15 μg.

2.7. Entrapment efficiency

The entrapment efficiency (percentage of initial KG incorporated into liposomes) was measured by determining the amount of non-entrapped KG using the non-equilibrium dialysis method. A cell consisting of a donor and a receptor compartment of equal volume and separated by a dialysis membrane of 3.14 cm^2 area (cut-off 12000 Da) was used. Receiving medium was bidistilled water. The cell was thermostatted at 37 °C, and two magnetic stirrers eliminated the boundary layer effects in each compartment. At appropriate intervals an aliquot of the receiving solution was withdrawn for HPLC analysis, and the cell refilled with fresh water. Aqueous solution containing 0.25% KG was used as reference solution. The percentage of KG entrapment was calculated from the decrease of the slope of the straight line obtained by plotting the amount of KG diffused from the formulation versus time compared with that obtained from the reference solution. Each experiment was repeated three times.

2.8. Measurements of liposome elasticity

Liposome elasticity was determined by measuring the size of the liposomes before and after filtration through a microporous filter with pore diameter of 100 nm (Isopore, Millipore, Bedford, MA, USA) using a stainless steel pressure filter holder for 47 mm diameter filters, with 200 ml capacity barrel (Pall Gelman Laboratory, Ann Arbor, MI, USA) connected to 0.5 MPa. pressure source. The experiments were performed in triplicate and each sample was analysed two times.

2.9. In vitro permeation and skin deposition studies

Full-thickness pig ear skin was used for permeation experiments using a vertical cell, as proposed by Franz (1975). The skin was rinsed with normal saline and, to maintain an in vivo transepidermal hydration gradient (Warner et al., 1988), the skin was pre-hydrated by floating with the stratum corneum upward on 0.002% w/v aqueous sodium azide. The skin was then sandwiched between two areas of ground glass with the stratum corneum side up. The receptor chamber was filled with 6 ml of 0.002% w/v aqueous sodium azide. The test formulations (100 μg) were applied to the skin surface, which had an available diffusion area of 2.05 cm^2, and left to dry out. The content of the receptor cell, continuously stirred at 37 °C, was removed at appropriate intervals for HPLC determination and the cell was immediately refilled with fresh receptor solution.

At the end of the permeation experiments (12 h), the surface of the skin was washed five times with 50% ethanol, then with water to remove excess drug on the surface. This washing protocol was verified and and was found to remove more than 99% of the applied dose at zero time. The skin was then cut into small pieces. The tissue was further homogenised with 50% ethanol (10 ml) and left for 4 h at room temperature. After shaking for 5 min and centrifuging for 5 min at 5000 rpm, the KG content in the upper phase was determined by HPLC. About 0.25 and 1% KG aqueous solutions were used as control formulations. Each experiment was repeated at least in triplicate from two different batches of the formulation.

2.10. HPLC assay

The concentration of KG was determined by HPLC. The HPLC system consisted of a pump (LC10-AD), an UV detector (SPD-10, $\lambda = 247$ nm), a data station (Shimadzu, Kyoto, Japan), and a 5 cm-C18 column (LiChrospher, Merck, Darmstadt, Germany). The mobile phase comprised methanol, acetonitrile, 0.1 N phosphoric acid (60,30,10 v/v) and was delivered at a flow rate of 1 ml min^{-1}. The injection volume was 20 μl and the relative retention time was found to be 4.3 min.

2.11. Statistical analysis

The observed effects were tested for significance by analysis of variance or linear regression and considered significant when $P < 0.01$.

3. Results and discussion

3.1. Liposome size

The mean sizes ($\pm$S.D.) of the liposome suspensions obtained by high pressure homogenisation were: PC:KG (2:1) 87 ± 14 nm, PC:KG (4:1) 102 ± 21 nm, PC:KG (8:1) 120 ± 24 nm, HPC:KG (2:1) 92 ± 18 nm, HPC:KG (4:1) 108 ± 20 nm, HPC:PC (8:1) 114 ± 26 nm.

No marked differences were observed between liposomes prepared using PC and HPC, while an increase in size was observed on increasing the PC:KG ratio. A possible explanation for the size profile obtained with increasing surfactant concentrations is that, at high concentrations, vescicles and mixed micelles coexist. This was in agreement with the data reported for the lecithin sodium cholate systems, where vescicles and mixed micelles were found to coexist at 20% sodium cholate, only mixed micelles being found at 30% (11).

3.2. Differential scanning calorimetry

DSC was used to evaluate the interactions between KG and liposomes. HPC was used for DSC measurements because its transition temperature (Tm) can be measured easily. Fig. 1 a and b shows DSC traces of HPC liposomes and HPC liposomes containing KG (lipid:surfactant 4:1 w/w, 4.5:1 molar ratio). The DSC trace of HPC liposomes showed a peak transition at 49.8 ± 0.2 °C and an enthalpy of 7.8 ± 0.4 Kcal mol^{-1} in accordance with the values of 41.4 °C and 8.2 kcal mol^{-1} reported in literature (Small, 1986) for the pure dipalmitoylphosphatidyl choline (DPPC) liposomes and 54.5 °C and 10.2 kcal mol^{-1} for pure distearoylphosphatidylcholine (DSPC).

Compared with pure DPPC and DSPC liposomes, where a pretransition peak was present, no pretransition peak was detected by this technique with HPC liposomes. The HPC used in this research is a product obtained by hydrogenation of commercial soya lecithin and contains a mixture of hydrogenated lipids. Thus, the highly ordered gel state with the hydrocarbon chains in all-*trans* configuration, present in the pure DPPC and DSPC liposomes, no longer exists and the pretransition peak was lost.

Incorporation of KG into liposomes reduced the T_m value to 48.6 ± 0.2 °C and the enthalpy to 7.0 ± 0.6 kcal mol^{-1}. The temperature ranges at half peak height were 1.3 ± 0.2 °C for HPC liposomes containing KG compared with 0.9 ± 0.1 °C for pure HPC liposomes. The presence of KG reduced the cooperativity of the transition which reflected the presence of a system having different degrees of disruption in the packing characteristics. The decrease in T_m value may indicate that the surfactant perturbs the packing characteristics and, thus, fluidises the lipid bilayer.

A reduction of Tm values of DPPC in the presence of Tween, Span or sodium cholate has been reported in the literature (El Maghraby et al., 2000). To explain the differences in the interaction of the different surfactants with the lipid bilayers, the molar ratio of individual surfactants in the liposomes and the HLB, which gives a measure of the physicochemical properties of surfactants in terms of their affinity for water or lipids, was considered: the affinity for lipids was found to be in the order Span 80 > Tween 80 > sodium cholate. Sodium cholate (molar ratio 3.6:1 lipid:surfactant) produced a ΔT_m of 0.79 °C and a temperature range at half peak height of 0.7 °C. Considering the similar HLB values of sodium cholate and KG and the similar lipid:surfactant molar ratios, the ΔT_m of 1.2 °C and a temperature range at half peak height of 1.25 °C obtained in the presence of KG might indicate a perturbation of the packing characteristics and a fluidisation of the lipid bilayer similar to that of sodium cholate.

In Fig. 1 the DSC traces of the emulsion and of the HPC:KG (4:1 w/w) liposomes containing emulsion are also reported. A peak transition at 64.7 ± 1.2 °C (Fig. 1c and d) was obtained for both systems, indicating the temperature transition of the emulsifier, while the broad peak at 49.6 ± 0.5 °C (Fig. 1d) with a temperature range at half peak height of 3.6 ± 0.8 °C indicated the presence of liposome structures even with a certain degree of destruction.

3.3. Entrapment efficiency

Fig. 2 reports the amounts of KG diffused through a dialysis membrane from KG aqueous solution and from liposome suspensions at different lipid:surfactant ratios, as a function of time.

The results of the permeation studies indicate that the in vitro diffusion of KG from liposome suspensions containing 0.25% KG and different amounts of PC or HPC were lower than that from 0.25% aqueous solution.

The diffusion of an entrapped molecule from disperse systems (oil in O/W emulsion or inner aqueous compartment and lipid bilayer in lipo-

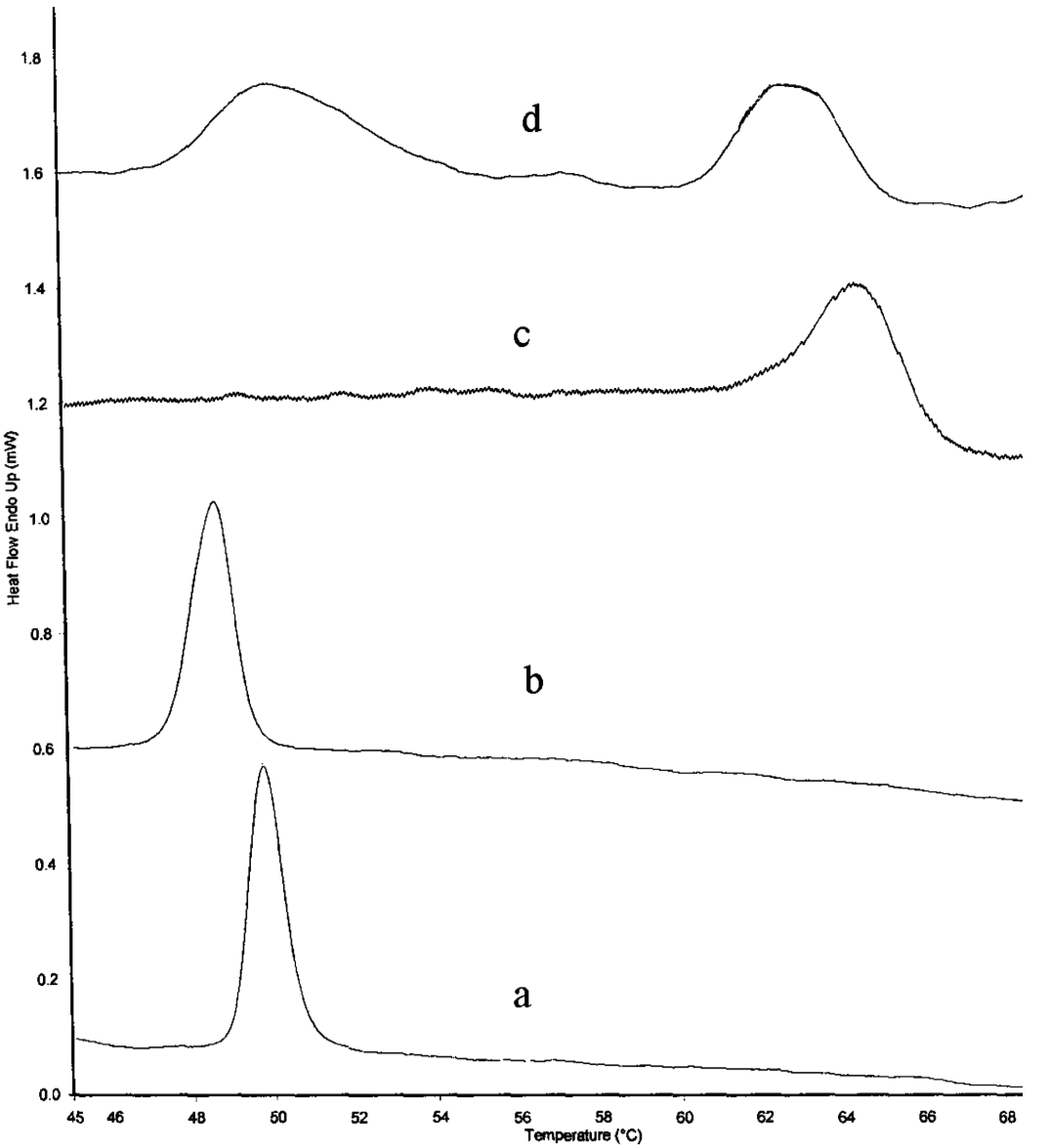

Fig. 1. Differential scanning calorimetric traces of HPC liposomes (a), HPC:KG liposomes (b), O/W emulsion (c) and HPC:KG liposomes dispersed in O/W emulsion (d).

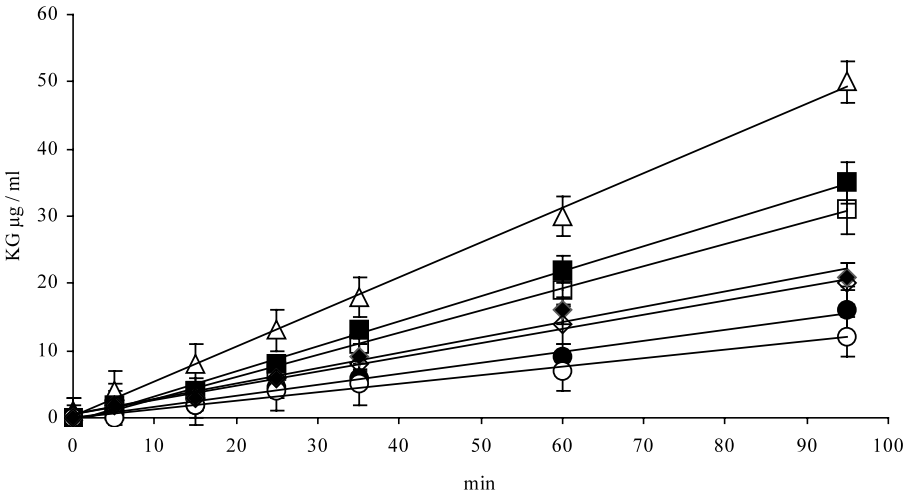

Fig. 2. Cumulative amount of KG diffused through a dialysis membrane from 0.25% KG aqueous solution (r), PC:KG (8:1) liposomes (■), HPC:KG (8:1) liposomes (£), PC:KG (4:1) liposomes (˜), HPC:KG (4:1) liposomes (™), PC:KG (2:1) liposomes (¿) and HPC:KG (2:1) liposomes (¯).

some systems) is governed by two main processes: transfer of the molecule from the disperse systems to the aqueous external phase, and diffusion of the molecule through the dialysis membrane from the external phase to the sink solution. Only molecules present in the external aqueous phase are able to permeate through the membrane. Thus, the permeation of molecules through the membrane will initially be governed by their concentration in the external aqueous phase of the disperse system (Trotta et al., 1989). In our case, the different diffusion rates from solution and liposomes can, thus, be attributed to the entrapment of KG in liposomes (inner aqueous compartment and lipid bilayer). From the slopes of the lines obtained plotting the KG amounts as a function of time, an entrapment of KG of 30% (38%), 68% (76%) and 58% (60%) was found for liposome at 2:1, 4:1 and 8:1 w/w PC:KG ratio, respectively (data for HPC:KG liposomes in brackets).

The maximum entrapment efficiency was obtained at 4:1 w/w lipid:KG ratio. The increase in permeation rate for the system at 2:1 ratio might be due to the formation of lipid:KG mixed micelles of small size able to permeate through the pores of the dialysis membrane. Another possibility for the increased diffusion at low lipid:surfactant ratio may be due to an increased disruption of the lipid membrane so that it becomes more permeable to the entrapped drug.

3.4. Liposome elasticity

In Table 1 the mean diameters of the liposomes in the absence and in the presence of KG at different lecithin:KG ratios are reported. The size of liposomes containing KG before and after pore passage is nearly the same, unless their size is

Table 1
DLS measurements of liposomes just before and after filtration through a microporous filter with pore diameter of 100 nm

	Before filtration (nm)	After filtration (nm)
PC	284 ± 30	–
PC	206 ± 22	–
PC	159 ± 14	147 ± 18
HPC	276 ± 33	–
PC–KG (2:1)	288 ± 28	292 ± 38
HPC–KG (2:1)	290 ± 26	276 ± 41
PC–KG (4:1)	301 ± 25	310 ± 36
HPC–KG (4:1)	309 ± 33	307 ± 41
PC–KG (8:1)	312 ± 24	305 ± 44
HPC–KG (8:1)	320 ± 35	331 ± 42

quite large (280–310 nm). The passage of these liposomes through pores that are too narrow is nearly complete, even if their size exceeds the pore diameter by a factor of about three.

In contrast to this, simple PC and HPC liposomes of about the same size are far less deformable. Consequently, they are also less capable of passing through barriers with pores smaller than their own diameters. When liposomes are slightly larger than the pore diameter, sieving, therefore, sets in. PC liposomes of different sizes were also prepared and filtered. Only the liposomes of about 150 nm are able to pass through the pores: exclusion from the flowing material is complete when this mismatch exceeds a factor of about 1.5.

The reason for this difference is that KG is able to accomodate to particle shape changes (and, thus, deformation) of the liposomes under stress. Owing to the higher propensity of KG for highly-curved structures (e.g. micelles) this rearrangement diminishes the energy required for particle deformation, consequently they can penetrate relatively easily even through pores that are much smaller than they are.

3.5. In vitro permeation and skin deposition

The pig skin permeation parameters of KG from liposome systems and from aqueous solutions are summarised in Table 2. All systems showed a negligible flux, below the UV–HPLC detection limit. The results suggest that pure and elastic liposomes are not beneficial in delivery of glycyrrhizinate through the skin and also indicate that the surfactant, whether as monomer (0.25% w/w) or as micelles (1.0% w/w), is not able to cross through intact skin.

Table 2 also shows the residual amount of KG in the skin after administration of different preparations. PC and HPC liposomes with KG incorporated promote the transfer of KG into the pig skin, while the KG solutions, below and above the CMC, failed to achieve transport. There were no significant differences between liposomes containing PC or HPC. The skin deposition increased by 5 fold compared with 0.25% KG control solution and compared with KG micelles, both vesicles resulted in fairly large amounts of drug deposited on the skin. The enhancement effect was, thus, not as a result of the extraction of membrane protein due to the presence of surfactant.

Table 2
KG skin permeation and skin accumulation from different formulations containing 0.25% w/w KG and from aqueous control solutions

	Flux	KG in the skin ($\mu g\ cm^{-2}$)
KG water solution (0.25%)	Negligible	12 ± 3
KG water micelle solution (1%)	Negligible	19 ± 5
Liposomes (2:1w/w PC:KG)	Negligible	60 ± 8
Liposomes (4:1w/w PC:KG)	Negligible	71 ± 10
Liposomes (8:1w/w PC:KG)	Negligible	62 ± 8
Liposomes (2:1 w/w HPC:KG)	Negligible	63 ± 7
Liposomes (4:1 w/w HPC:KG)	Negligible	66 ± 8
Liposomes (8:1 w/w HPC:KG)	Negligible	65 ± 6
O/W emulsion	Negligible	13 ± 4
O/W emulsion-liposomes (2:1 w/w HPC:KG)	Negligible	52 ± 5
O/W emulsion-liposomes (4:1 w/w HPC:KG)	Negligible	49 ± 5
O/W emulsion-liposomes (8:1 w/w HPC:KG)	Negligible	47 ± 6

In accordance with what has been reported for lecithin:cholate vescicles (Kirjavainen et al., 1996), two main reasons for the function of flexible liposomes may be hypothesised to explain the synergetic effect of lecithin and KG on the penetration of the liposomes into the skin. The first is that liposomes containing KG are able to penetrate through the interstices of the stratum corneum under the influence of transcutaneous hydration force caused by the water concentration difference between the skin surface and skin interior. On the otherhand, the enhancement effect could be due to fusion of the vescicles with the skin, facilitated by an increase in the fluidity of the phospholipid bilayers containing KG.

The data of KG skin deposition from O/W emulsion and O/W emulsion containing HPC liposomes are also reported in Table 2. The KG fluxes were in all cases negligible and the skin deposition from liposomes formulated in emulsion was lower than those obtained from liposome suspensions. This reduction may probably be ascribed to the structural changes of the lipid bilayer by the presence of Montanov and Finsolv, as can be seen from the DSC data. However, an increase in KG deposition of about 4 fold in comparison with a simple O/W emulsion or aqueous water solution was observed.

Considering the amount of KG applied to the skin surface, the relative amount of KG transported into the skin after 12 h by means of elastic liposomes, was of about 40–50% of the dose.

4. Conclusion

Deformable liposomes, when applied non-occlusively, significantly improve the in vitro skin delivery of KG compared with aqueous solution. The fact that the efficacy of the topical preparation containing KG was observed in an eczema-dermatitis group, a major pediatric dermal disease, suggests the therapeutic significance of this anti-inflammatory agent, it was supposed that the use of liposomes containing KG creates new opportunity for the well controlled and modern topical medication.

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