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Deformable liposomes for dermal administration of methotrexate

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Abstract

Deformable liposomes were prepared to investigate the effectiveness of dermal administration of methotrexate (MTX). The phospholipids used to prepare the liposomes were soybean lecithin (PC) or hydrogenated lecithin (HPC) and dipotassium glycyrrhizinate (KG) as surfactant. The lipid/KG ratio (w/w) was 2:1 and 4:1. Liposomes size, entrapment efficiency and MTX release through dialysis membrane were determined and the interaction between MTX and liposomes was investigated using differential scanning calorimetry. The MTX amount permeated through pig skin were three- to four-fold higher using liposomes containing KG compared to those from water solution or normal liposomes. No significant differences were observed between PC-KG liposomes and HPC-KG liposomes. At the end of the skin permeation assay using deformable liposomes, up to 50% of the administered dose was found in the skin. This capability depends on the self-regulating carrier deformability. These results suggest that liposomes containing KG may be of value for the topical administration of MTX in the treatment of psoriasis.

Keywords: Deformable liposomes; Dipotassium glycyrrhizinate; Methotrexate; Dermal administration

1. Introduction

Methotrexate is a folic acid antagonist with antineoplastic activity. It is also effective in controlling recalcitrant psoriasis when administered by the oral or parenteral route long-term. It has been shown to selectively inhibit DNA synthesis in psoriatic epidermal cells, thus decreasing mitotic activity (Flaxman et al., 1977). However, the systematic use of this drug may provoke any of a number of side effects, notably hepatotoxic effects (Van Dooren-Greebe et al., 1994).

To reduce these effects, clinical studies have been done with topical methotrexate (Condit, 1961; Hwang et al., 1995). A major problem in topical administration of methotrexate is that the drug is hydrosoluble and is mostly in the dissociated form at physiological pH: its capacity for passive diffusion is thus limited.

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 recently been investigated as transdermal drug delivery systems (Egbaria and Weiner, 1990). 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 administration of liposomes can lead to penetration into or through intact skin, but agreement is general that most liposomes do not reach deep into intact skin.

A novel type of liposome has been reported to penetrate the skin if applied non-occlusively by virtue of the very high and self-optimizing deformability (Cevc et al., 1996; El Maghraby et al., 2000). These de-

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formable liposomes 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.

In contrast to liposomes, deformable liposomes may carry a remarkable amount of the applied lipid mass into the skin (Auguiella et al., 1994).

In a previous study, we prepared deformable liposomes containing soya lecithin or hydrogenated soya lecithin mixed with dipotassium glycyrrhizinate (KG), a compound with emulsifying properties (Yonezawa et al., 1976) extracted from liquorice root. Skin fluxes of KG from deformable liposomes were negligible, while skin deposition increased 4.5-fold compared with aqueous solution.

A rational way to alter the biodistribution pattern of a water-soluble drug is to encapsulate it in deformable liposomes. The present study evaluates the possibility of producing deformable liposomes containing methotrexate and examines in vitro skin delivery.

2. Materials and methods

2.1. Materials

Epikuron 200 (PC, containing 95% phosphatidylcholine) was a kind gift from Lucas Meyer (Hamburg, Germany). Hydrogenated lecithin (HPC, Phospholipon 100 H) was from Nattermann Phospholipid (Koln, Germany). Dipotassium glycyrrhizinate (KG) was from Maruzen Pharmaceuticals (Hiroshima, Japan). Methotrexate was a kind gift from Wyeth Lederle (Aprilia, Italy).

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

2.2. Preparation of liposomes

Lipid vesicles containing PC or HPC, alone or in the presence of different amounts of KG, were prepared by a reverse-phase evaporation technique. Appropriate amounts of PC or HPC were dissolved in a minimum amount of diethyl ether and chloroform (1:1). Phosphate buffer (pH 7.4), together with KG and MTX, was added to the solution.

The biphasic system was shaken to obtain a stable and homogeneous emulsion and evaporated under nitrogen stream; solvent traces were removed by maintaining the lipid film under vacuum overnight.

The films were hydrated with buffer solution (pH 7.4) by vortexing for 10 min at 30 °C, for those containing PC, and at 55 °C for those containing HPC. The liposome suspensions were then sonicated for 8 min at 32 W. For elasticity determinations, the hydrated vesicles were sonicated at 16 W to obtain a liposome mean size of 280–310 nm.

When KG was used the total weight of lipid and KG was maintained constant; w/w ratio of lipid to KG was 2:1 (PC:KG 2:1, HPC:KG 2:1) or 4:1 (PC:KG 4:1, HPC:KG 4:1). Lipid vesicles containing only PC (PC) or HPC (HPC) were also prepared as controls. The w/w lipid (KG):MTX ratio was 50:1. Free MTX was removed by ultracentrifugation for three cycles at 25,000 × g for 30 min. The final MTX concentration was 650 ± 50 μg MTX/ml liposome suspension (PC:MTX, HPC:MTX, PC:KG 2(4):1-MTX, HPC:KG 2(4):1-MTX).

2.3. Liposome size determination

The average diameter and size distribution of liposome suspensions were determined by photocorrelation spectroscopy using a 90 Plus Particle Size Analyzer (Brookhaven Instrument, New York, USA) at a fixed angle of 90° and at 25 °C.

Liposome suspensions were suitability diluted with phosphate buffer (pH 7.4) and filtered through a 1 μm polycarbonate membrane to minimize interference particulate matter before sizing. Each measurement was in triplicate.

2.4. Differential scanning calorimetry (DSC)

DSC was performed with a Perkin-Elmer differential calorimeter (DSC7, Perkin-Elmer, Nortwalk, Connecticut, USA). HPC liposome and HPC:KG liposome (4:1 w/w) suspensions with or without MTX were placed in conventional aluminum pan and a scan speed of 2 °C/min was employed. The weight of each sample was 12–15 mg.

2.5. Entrapment efficiency

The entrapment efficiency, calculated as percentage of initial MTX incorporated into liposomes, was deter-

mined after removing free MTX. Subsequently, liposomes were redispersed in phosphate buffer (pH 7.4) containing 2% Triton X-100. The final clear solution was analyzed by HPLC for MTX content.

2.6. Measurement 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 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 Laboraty, Arbor, MI, USA) connected to 0.5 MPa pressure source. The experiment was performed in triplicate.

2.7. Determination of MTX release

The in vitro release of MTX was determined using the non-equilibrium dialysis method. A multicompartmental rotating cell system consisting of a donor and a receptor compartment of equal volume (1.5 ml) separated by a dialysis membrane (cut-off 12,000 Da) was used. Receiving medium was phosphate buffer (pH 7.4) and the cell was thermostatted at 37 °C.

MTX aqueous solution and liposome suspensions were used as donor formulations. At fixed times, the receptor solution was tipped out and used for HPLC analysis and the cell was refilled with fresh phosphate buffer. The drug concentration was determined by HPLC.

The results were evaluated as apparent permeability constant of MTX (K_{dapp} cm min^{-1}) calculated from the slope of the straight line obtained by plotting the amount of MTX diffused from the donor formulation versus time, assuming pseudo zero-order kinetics.

2.8. 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). A preliminary wash of the ears was done with cold water followed by drying between filter paper. The hair of the outer skin surface was removed with dissecting scissors brought as close as possible to the skin without damaging it. The skin was carefully dissected with a scalpel and forceps. The skin was rinsed with normal saline and pre-hydrated by floating it with the stratum corneum upward on 0.002% w/v aqueous sodium azide, to maintain an in vivo transepidermal hydration gradient (Warner et al., 1988). The skin was then sandwiched between two areas of ground glass with the stratum corneum side upwards. The receptor chamber was filled with 6 ml of buffer solution pH 7.4. The test formulations (200 μl) were applied to the skin surface, which had an available diffusion area of 1.7 cm^2, and left to dry. MTX aqueous solution was used a control formulation. 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 (24 h), the skin surface was washed five times with ethanol:buffer solution pH 7.4 (1:1), then with water to remove excess drug from the surface. The skin was then cut into small pieces. The tissue was further homogenized with ethanol:buffer solution pH 7.4 (1:1) and left for 6 h at room temperature. After shaking for 5 min and centrifuging for 5 min at 5000 rpm, the MTX content was determined by HPLC. Each experiment was repeated at least in triplicate from two different batches of the formulation.

2.9. HPLC assay

The concentration of MTX was determined by HPLC. The HPLC system consisted of a pump (LC 10-AD), an UV detector (RF-551, $\lambda = 302$ nm), a data station (Shimadzu, Kyoto, Japan), and a 5 cm-C18 column (LiChrospher, Merck, Darmstadt, Germany). The mobile phase comprised methanol/acetonitrile/pH 5.4 buffer solution (8.5/6.5/85 v/v) and was delivered at a flow rate of 0.6 ml min^{-1}. The injection volume was 20 μl and the relative retention time was found to be 9.8 min.

3. Results and discussion

After preliminary studies, the reverse-phase evaporation technique was used to prepare the liposomes, because it has higher drug encapsulation efficiency than other methods, and sonication was applied to obtain smaller and more uniform vesicles.

Table 1
Average particle size (±S.D.) of empty and methotrexate loaded liposomes and percentage of methotrexate (percentage MTX) incorporated in liposomes with or without potassium glycyrrhizinate (KG)

Liposomes	Particle size (nm)		Percentage MTX
	Empty	Loaded	
PC	212 ± 25	247 ± 21	31 ± 3
HPC	235 ± 32	240 ± 30	33 ± 2
PC:KG 2:1	148 ± 18	159 ± 21	20 ± 1
HPC:KG 2:1	155 ± 20	153 ± 22	22 ± 2
PC:KG 4:1	174 ± 22	180 ± 17	27 ± 3
HPC:KG 4:1	188 ± 24	186 ± 21	28 ± 3

3.1. Liposome size and MTX entrapment efficiency

Table 1 gives average particle size and MTX entrapment efficiency of liposomes at different lipid/KG ratios.

Liposomes prepared using PC did not markedly differ from those using HPC, whereas size increased on increasing the lipid/KG ratio. This is in agreement with data from a previous study and is related to the emulsifying properties of KG (Trotta et al., 2002). The presence of MTX did not significantly alter the size of liposomes, while decreasing the lipid/KG ratio reduced drug entrapment. There was no significant difference between PC liposomes and HPC liposomes.

3.2. Differential scanning calorimetry

Differential scanning calorimetry was used to evaluate the interactions between MTX and liposomes with or without KG; Fig. 1 reports the thermograms.

HPC was used for DSC measurements because its transition temperature (T_m) can easily be measured. The DSC trace of HPC liposomes showed a peak transition at 49.7 ± 0.2 °C and an enthalpy of 37.7 ± 0.5 J g^{-1}. Incorporation of KG into liposomes reduced the T_m value to 48.6 ± 0.2 °C and the enthalpy to 35.7 ± 0.5 J g^{-1}. The decrease in T_m value may indicate that the surfactant perturbs the packing characteristics and, thus, fluidizes the lipid bilayer. The presence of MTX did not change either T_m or the enthalpy values, indicating that this molecule is entrapped in the hydrophilic core of the liposomes.

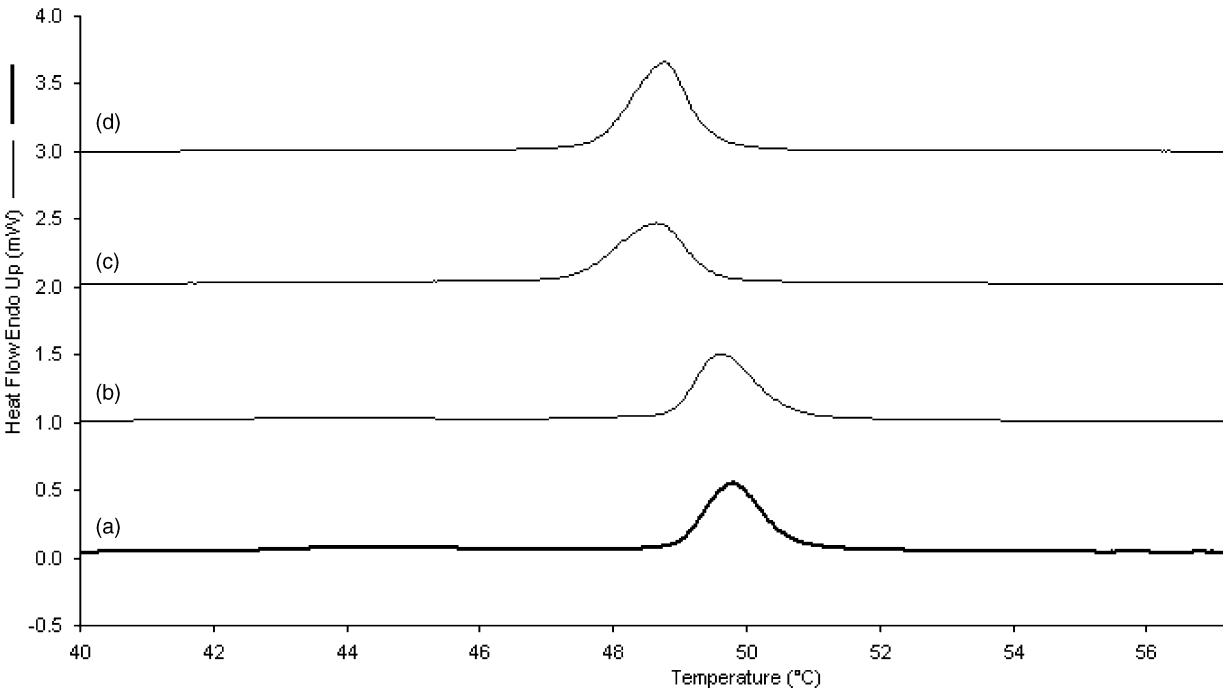

Fig. 1. Differential scanning calorimetry traces of HPC liposomes (a), HPC-MTX liposomes (b), HPC:KG 4:1 liposomes (c) and HPC:KG 4:1-MTX liposomes (d).

Table 2
Mean diameter (±S.D.) of different liposome formulations, before and after filtration through a microporous filter with pore diameter of 100 nm

	Before filtration (nm)	After filtration (nm)
PC-MTX	297 ± 30	–
HPC-MTX	280 ± 25	–
HPC:KG 2:1-MTX	332 ± 34	328 ± 25
PC:KG 2:1-MTX	357 ± 35	341 ± 15
PC:KG 4:1-MTX	352 ± 28	345 ± 20
HPC:KG 4:1-MTX	325 ± 33	305 ± 25

3.3. Liposome elasticity

Table 2 shows the mean liposome diameters in the absence and in the presence of KG at different lipid:KG ratios. The size of liposomes containing KG and MTX before and after pore passage (100 nm) is very similar unless they are quite large (280–310 nm). These liposomes pass almost completely through narrow pores, even if their size exceeds the pore diameter by a factor of about 3.

In contrast, 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 they are therefore withheld by the membrane.

The reason for this difference in deformability is probably linked to KG's propensity for highly curved structures (e.g. micelles), thus diminishing the energy required for particle deformation (Trotta et al., 2002).

3.4. MTX release

Table 3 reports the apparent permeability constants (K_{dapp}) of MTX from PC or HPC liposomes containing KG compared with drug solution. The diffusion of an entrapped molecule from disperse systems is governed by the transfer of the molecule from the disperse system to the external aqueous 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. The K_{dapp} of MTX entrapped in liposomes were lower than that of MTX solution. According to these results, the diffusion of the drug from the bilayers of the vesicles is the main rate-determining step for the release of MTX and the different K_{dapp} from solution and liposomes can, thus, be attributed to entrapment of MTX in liposomes. In addition, encapsulation provides sustained release of the drug.

Table 3
Apparent permeability constants (K_{dapp}) of methotrexate from different liposome formulations and from aqueous control solution

	K_{dapp} ($\times 10^3$ cm min^{-1})
Aqueous control solution	3.00 ± 0.2
PC:KG 2:1-MTX	0.50 ± 0.2
HPC:KG 2:1-MTX	0.45 ± 0.2
PC:KG 4:1-MTX	0.61 ± 0.1
HPC:KG 4:1-MTX	0.56 ± 0.2

3.5. In vitro permeation and skin deposition

Table 4 reports the cumulative amount of MTX permeated after 24 h trough the pig skin from liposome systems and from aqueous solution at the same drug concentration. The results indicate that aqueous solution and pure liposomes are quite similar in term of MTX delivery through the skin, while deformable liposomes produce an increase in the amount of MTX permeated: the values moderately increased from liposomes containing the lipid/KG ratio of 4:1 to 2:1.

In accordance with reports about lecithin:cholate liposomes (Kirjavainen et al., 1996), the reason for the function may be that deformable liposomes can penetrate through the interstices of the stratum corneum under the influence of the transcutaneous hydration force caused by the different water concentration between skin surface and skin interior. On the other

Table 4
Cumulative amount of methotrexate permeated after 24 h (microgram MTX) and skin deposition (percentage MTX) from different liposome formulations and from aqueous control solution

	Microgram MTX	Percentage MTX
Aqueous control solution	5.7 ± 2.0	15 ± 4
PC-MTX	3.5 ± 1.5	14 ± 3
HPC-MTX	5.7 ± 1.9	16 ± 3
PC:KG 2:1-MTX	29.6 ± 5.1	49 ± 6
HPC:KG 2:1-MTX	33.7 ± 5.3	51 ± 6
PC:KG 4:1-MTX	16.8 ± 4.0	40 ± 7
HPC:KG 4:1-MTX	23.55 ± 4.3	43 ± 8

hand, the enhancement effect could be due to fusion of MTX-bearing liposomes with the skin, facilitated by the increase in the fluidity of the phospolipid bilayers containing increasing KG percentages.

Several studies have compared transdermal MTX transport from different vehicles, but the data are quite controversial; this probably depends on the influence of the components on the skin barrier properties, on the different species and types of skin and on different experimental procedures. In particular, Vaidyanathan et al. (1985) used 50% propylene glycol in aqueous medium at different pH and found, a drug permeation parameters through human skin similar to that obtained from elastic liposomes, while Weintein et al. (1989) and Alvarez-Figueroa et al. (2001) reported very low MTX permeation from aqueous solutions. Other studies report MTX fluxes from microemulsion vehicles (Trotta et al., 1996); the amount permeated through mouse skin, calculated from the fluxes reported from W/O microemulsions containing benzyl alcohol as cosurfactant, is similar to that now obtained from KG liposomes, while very low permeation values have been obtained from other microemulsion formulations (Alvarez-Figueroa and Blanco-Mèndez, 2001). However, to compare these last data, the possible enhancing effect of alcohol must be considered.

Table 4 also shows the residual amount of MTX in the skin after 24 h administration of different preparations. PC and HPC liposomes with KG incorporated promoted the transfer of MTX into pig skin. Skin deposition increased by a factor of 3 compared with either aqueous solution or free KG liposomes.

These data agree with those on the relative distribution of MTX (Vaidyanathan et al., 1985) 14 days after application of MTX in 50% propylene glycol, but are much higher than delivery of MTX from microemulsions or after iontophoretic experiments (Alvarez-Figueroa and Blanco-Mèndez, 2001).

The use of deformable liposomes for non-invasive drug delivery is widely reported. These vesicles encapsulating calcitonin (Schätzlein et al., 1997), insulin (Cevc et al., 1995), diclofenac (Cevc and Blume, 2001), oestradiol (El Maghraby, 2000) improved skin delivery and up to 100% of the applied insulin was transported through the intact skin without significant composition or size modification of the liposomes (Cevc et al., 1998).

In accordance with the results of a previous study, where KG liposomes, applied non-occlusively, significantly improved in vitro skin delivery of KG compared with aqueous solution, the relative amount of MTX transported into the skin after 24 h by means of deformable liposomes was about 40–50% of the applied dose.

It is supposed that KG-liposomes can penetrate the skin whole, carrying the MTX entrapped in the hydrophilic core. Subsequently, a partition of intact vesicles into the deeper layers of the stratum corneum (Van den Berg et al., 1999; Honeywell-Nguyen et al., 2002) leads to high drug accumulation and only little vesicle materials probably reaches the deepest layers of the corneum and then the viable epidermis. However, other determinations, such as composition and vesicle size in donor and receiving medium, freeze-fracture electron microscopy or confocal laser scanning microscopy of the skin, will be necessary to confirm this hypothesis.

In conclusion, deformable liposomes obtained using KG, a safe surfactant widely used in cosmetics, applied non-occlusively, improve in vitro skin delivery of MTX compared to either aqueous solution or normal liposomes. The enhanced accumulation of MTX within the skin might help to optimize targeting of this drug, creating new opportunities for well-controlled and modern topical application of MTX in the treatment of psoriasis.

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