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The effect of non-ionic surfactant vesicle (niosome) entrapment on the absorption and distribution of methotrexate in mice

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Non-ionic surfactant vesicles (niosomes) prepared from a non-ionic surfactant, cholesterol and dicetyl phosphate and containing methotrexate (MTX) have been administered to mice. Given intravenously the niosomes prolong the levels of MTX in the blood, large amounts of the drug being taken up by the liver. There was also an increased uptake of MTX into the brain, perhaps due to an effect of the niosome components on the permeability of the blood brain barrier. Absorption of the drug from the gastrointestinal tract following oral ingestion, appeared to be increased at some doses; most of the entrapped MTX was taken up by the liver, but uptake of MTX into the brain was also increased. The metabolic profile of the drug is altered by the niosomes which appear to prevent the rapid formation of 7-hydroxy methotrexate.

Encapsulation of a drug in vesicular structures can be predicted to prolong the existence of the drug in the systemic circulation and thus enhance penetration into target tissue, and perhaps reduce toxicity if selective uptake can be achieved (Todd et al 1982).

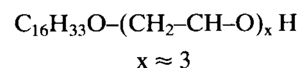
Earlier we reported (Vanlerberghe et al 1978; Handjani-Vila et al 1979) a series of novel non-ionic surfactants capable of forming lamellar vesicles (niosomes) on admixture with cholesterol and dicetyl phosphate and subsequent hydration in aqueous media. We now describe a method of small scale preparation of niosomes and the results of the first in-vivo investigation of their properties as alternative drug carriers to liposomes. We discuss the absorption, distribution and elimination of methotrexate when administered in two niosome preparations varying slightly in composition and compare the influence of the niosomes with the effects of the non-ionic surfactant, polysorbate 80, which is incapable of forming vesicular structures in solution.

Various synthetic lipids such as the didodecyl-, dioctadecyl- and didocosyldimethylammonium bromides form bilayer structures in aqueous dispersions (Kunitake et al 1977; Kunitake & Okahata 1977, 1978) but so far it appears that none have been used as drug delivery vehicles. The toxicity of ionic surfactants may preclude such use. Methotrexate was selected as the model drug because of previous work with it in this laboratory and also to allow direct comparison with published work on liposomal MTX.

MATERIALS AND METHODS

Materials

Chemicals and reagents. Methotrexate injection (MTX) was obtained from the Lederle Corporation. Polyoxyethylene sorbitan mono-oleate (Polysorbate 80) manufactured by Honeywill Atlas and dicetylphosphate were obtained commercially from Sigma Chemical Company. The non-ionic surfactant, I, and cholesterol used to prepare the niosomes were supplied by L'Oreal and Prolabo, France respectively. All other chemicals and solvents were of analytical or HPLC grade. Compound I has the structure,



$$C_{16}H_{33}O\text{–}(CH_2\text{–}CH\text{–}O)_x\,H \quad x \approx 3$$

$$CH_2OH$$

(I) Mol. wt ≈ 473

Animals. Porton (BKW) mice, 8–10 weeks old, 25–35 g, were purchased from Bantin and Kingman Ltd, UK.

Methods

Preparation of niosomes

In preparing the niosomes, Compound I (71·25 mg), cholesterol (71·25 mg) and dicetylphosphate (7 mg)

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to give a ratio of 47·5 : 47·5 : 5 were used as the lipid ingredients for niosomes of type A, and Compound I (97·5 mg), cholesterol (45 mg) and dicetylphosphate (7·5 mg) to give a ratio of 60 : 30 : 5 were used for niosomes of type B. These ingredients were dissolved in chloroform–methanol (1 : 1, 2 ml v/v). The solvents were evaporated at room temperature (20 °C) using a rotary evaporator, leaving a thin layer solid mixture deposited on the wall of a round bottom flask. MTX (2·5 ml of a 10 mg ml^{-1} solution prepared by diluting MTX injection with distilled water) was added to the flask in a 7 °C water bath and shaken about 15 min, until a good dispersion of the mixture was obtained. The suspension was then sonicated for 3 × 30 s to form unilamellar niosomes.

Hardy et al (1980) used a dialysis method to separate liposomes from unwanted electrolytes. The niosome-entrapped MTX could also be separated from the free MTX by this method which takes about 5 h. The prepared niosomes were filled into Visking 8/32 dialysis bags (Scientific Instrument Centre Ltd, UK) and the free MTX dialysed for 30 min each time into 500 ml 0·9% NaCl (saline). The dialysis of free MTX was always complete after about 10 changes of saline, when no MTX was detectable in the recipient solution. By this method the concentration of encapsulated MTX was well above that required for administration, so saline was used to dilute to suitable concentrations.

Measurement of niosome size by photon correlation spectroscopy

A Malvern photon correlation spectrometer (Malvern Instrument 48 channel, 7023 single clipped photon correlation spectrometer) was used for determining the average diameter of sonicated niosomes. 10 μl of the niosome suspension was diluted with 2·5 ml distilled water into cleaned absorbance cells. The decay curve of the diluted niosome suspension was measured using a He/Cd 10 mW laser λ = 441·6 nm at 25 °C and scattering angle θ = 90° following the method of Baillie et al (1979).

The average measured size of niosomes A and niosomes B was 115·2 ± 7·0 nm and 123·6 ± 6·6 nm respectively; the difference was not significant.

Analysis of methotrexate by reverse-phase high performance liquid chromatography (HPLC)

A stainless steel analytical column (25 × 0·45 cm) was packed with Hypersil (silica 5 μm particles coated with octadecylsilane). This reverse-phase column was used in all subsequent assays of MTX.

To every millilitre of serum or gram of tissue, acetonitrile–water (9 : 1, 1·5 ml) was added. The tissue samples were homogenized in the presence of acetonitrile–water by a Citenco variable speed homogenizer. The serum–acetonitrile–water mixture and homogenized tissue samples were shaken in a table-top Buchler vortex (Buchler Instruments, USA) for 20 min. An equal volume of chloroform was added to the shaken samples which were further shaken for 30 min, centrifuged for 30 min at 2000 rev min^{-1}, and 100 μl of the resulting supernatant, containing MTX, applied to the column. The samples were eluted with 15% methanol in 0·5 M Tris-phosphate buffer at a constant flow rate of 1 ml min^{-1} and detected by uv spectrometry at 303 nm, for MTX.

The effect of niosomes on the kinetics of methotrexate after oral and intravenous administration

After preparation and purification, the niosomes A were ultrafiltered and reconstituted in saline. The niosome suspension was administered orally to one group of mice and intravenously to another group. To two other groups of mice, free MTX solution was administered orally or intravenously. The volume of niosome suspension or MTX solution administered was 5 ml kg^{-1}, equivalent to 6·2 mg MTX kg^{-1}. Six mice from each group were killed at intervals and the blood samples were collected. The serum was separated from the other blood components by centrifugation and the amount of MTX in serum was determined by the EMIT method.

Effects of polysorbate 80 and niosomes on the absorption and distribution of methotrexate after oral and intravenous administration

Purified niosomes A and B were diluted with saline to produced systems equivalent to 0·545 mg MTX ml^{-1}. Solutions containing 0·545 mg MTX ml^{-1} water or 6% polysorbate 80 were also prepared. Different groups of mice received different types of preparation either intravenously through the tail vein or by the oral route. The dose given was 5 ml kg^{-1} (equivalent to 2·72 mg MTX kg^{-1}. At intervals, six animals from each group of mice were killed: blood, liver, and brain samples were collected and the amounts of MTX in these tissues were determined by HPLC.

RESULTS

Fig. 1 shows typical multilamellar niosomes formed after the hydration process of the lipid thin layer before sonication. Upon sonication these multi-

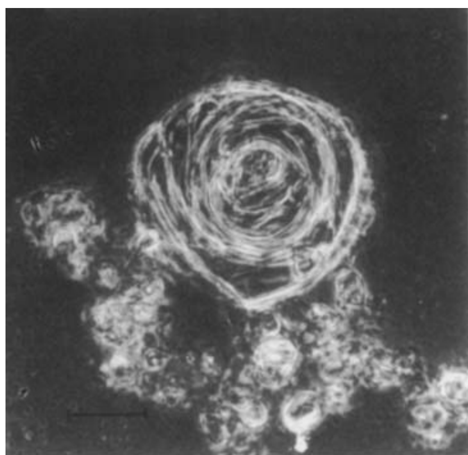

FIG. 1. Photomicrograph of niosomes following hydration but before sonication. Marker is 10 μm.

lamellar niosomes will break down to form the unilamellar or small multilamellar niosomes with an average diameter ranging from 110–130 nm. The presence of dicetylphosphate in the formulation seems to be needed to consistently produce niosomes with diameter greater than 100 nm. The niosomes prepared by the procedure described are able to entrap between 20–30% of the MTX used in the hydration process.

The effect of polysorbate 80 and niosomes on the oral absorption and distribution of methotrexate

Fig. 2 shows the concentrations of MTX in the serum of mice after oral administration of different preparations: serum MTX in mice given the drug in 6% polysorbate 80 was significantly higher than in mice given the drug without surfactant in serum collected at 0·5, 1 and 3 h post administration ($P < 0{\cdot}01$, 0·01 and 0·05 respectively), confirming the anticipated effect of the surfactant of oral absorption (Azmin et al 1982). Significantly higher serum MTX levels were found in mice given niosomes of type A and type B than in mice given drug-polysorbate, or drug solution, in serum collected at 3, 4, 6, 8 and 24 h post administration (all $P < 0{\cdot}01$). The levels between the niosomes were not significantly different.

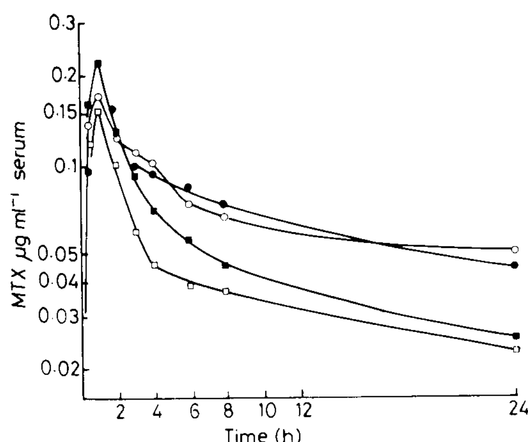

FIG. 2. Levels of methotrexate in the serum of mice after oral administration of 2·72 mg MTX kg⁻¹ as free MTX (□—□) MTX in 6% polysorbate 80, (■—■); MTX entrapped in niosomes, type A (○—○) and MTX entrapped in niosomes type B (●—●).

The results suggest that niosomes can improve the absorption of MTX from the gastrointestinal tract of the mouse, but this may be dependent on MTX concentration.

Methotrexate in liver

The administration of MTX in niosomes (A and B) results in levels of MTX in the liver of mice significantly higher at all times sampled ($P < 0{\cdot}01$) than in mice given MTX in solution or polysorbate 80 (Fig. 3). Significantly higher liver MTX levels occurred after administration of the type A niosomes than type B.

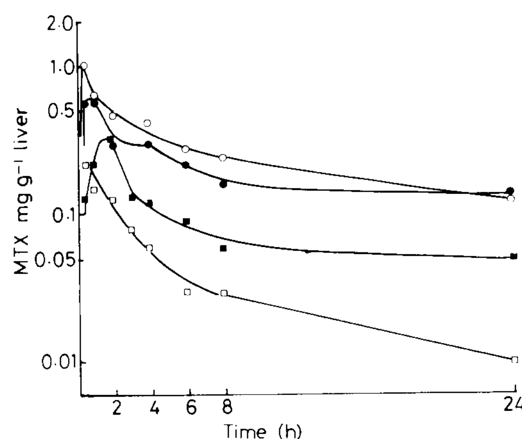

FIG. 3. Methotrexate levels (μg g⁻¹) in the liver of mice following oral administration of 2·72 mg MTX kg⁻¹. Symbols and systems as for Fig. 2.

Methotrexate in brain

Although less clear cut, significant differences were obtained in the levels of MTX in the brain between the four formulations, the surfactant-containing systems producing higher levels, while the difference between the two niosomes preparations was not significant, except at 1 h (Fig. 4). The difference

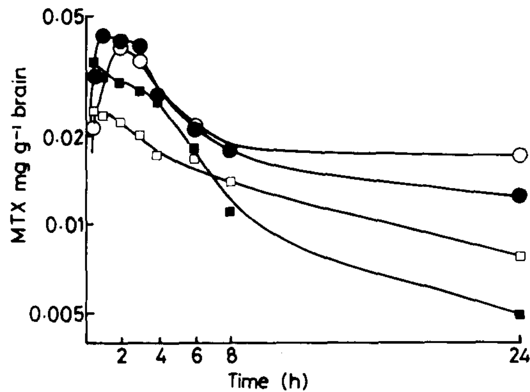

FIG. 4. Levels of methotrexate in the brain of mice after oral administration of 2·72 mg MTX kg^{-1} in four different systems. Symbols as for Fig. 2.

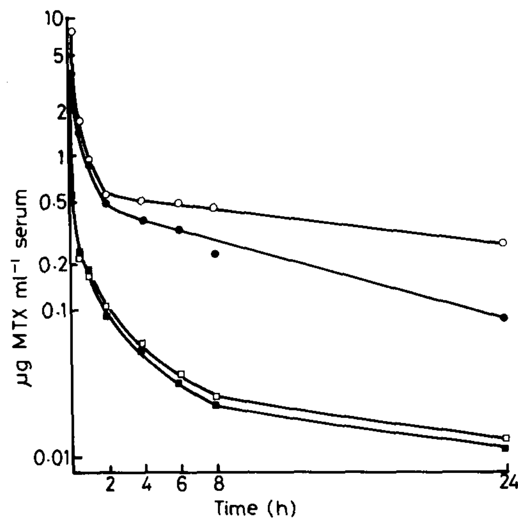

FIG. 5. Serum levels of methotrexate in mice after intravenous administration of 2·72 mg MTX kg^{-1} in the four preparations as before.

between the niosomes and the polysorbate 80 was as follows:

Levels of MTX were significantly higher in the brain of mice given MTX in 6% polysorbate 80 than in mice given niosomes A, in sera taken 0·5 and 1 h after administration ($P < 0.05$ and 0·02), but the reverse was true in 2, 3, 8 and 24 h sera ($P < 0.05$, 0·05 and 0·02). Levels of MTX were significantly higher in mice given niosomes B in 1, 2, 3 and 24 h sera ($P < 0.01$, 0·01, 0·02 and 0·05) than in mice given 6% polysorbate 80.

The effects of polysorbate 80 and niosomes on the distribution of methotrexate in mice after intravenous injection

Because of the complication of interpreting the effect of niosomes and polysorbate 80 on absorption and distribution when the preparations were administered orally, the formulations were given intravenously to eliminate the first absorptive stage. The results first show that polysorbate 80 does not significantly affect the levels of the drug in comparison with the free MTX solution, whereas the nonionic vesicles markedly alter the pharmacokinetic profile of the drug (Fig. 5). Moreover the levels of MTX observed when the drug was given in the type A niosomes were significantly higher than when given in the type B niosomes in serum collected at 10 min, 45, 6, 8 and 24 h post injection ($P < 0.01$, 0·05, 0·01, 0·02 and 0·01 respectively).

The α and β half-lives for MTX given as solution with and without polysorbate 80 were about 5 and 70 min respectively, while the $\gamma t_{\frac{1}{2}}$ after 8 h was about 17 h. The elimination of MTX from the serum of mice given niosomes type A and B was slower during the first 2 h with α and $\beta t_{\frac{1}{2}}$ of about 24 and 75 min. The $\gamma t_{\frac{1}{2}}$ values after 2 h were about 20 and 11 h respectively.

Methotrexate in the liver

Niosomes induced higher levels of MTX in the liver than did the aqueous or polysorbate solutions (Fig. 6).

These results largely mirror those obtained with liposomes, in that the levels of MTX are maintained in the blood by the niosomes, and that most of the niosomes containing MTX appear to be taken up by

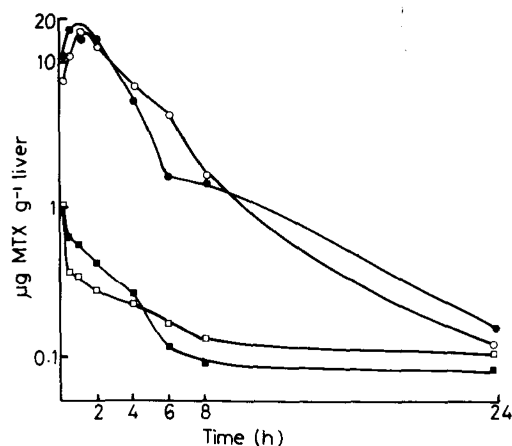

FIG. 6. Levels of methotrexate in mouse liver after intravenous injection of 2·72 mg MTX kg^{-1} in the four systems as before.

the liver. The profile of MTX levels suggests that the liver might act as a depot, releasing free MTX into the circulation after the niosomes are broken down, hence the observed maintenance of serum MTX levels from 2 h post injection, as shown in Fig. 6, following the initial rapid drop in levels. For the first few hours post-administration, polysorbate 80 induced higher liver concentrations than the aqueous solution. The drop after 4 h to significantly lower levels might indicate facilitated elimination through biliary excretion.

Methotrexate in the brain

After intravenous injection of various MTX preparations, niosomes, in particular type A, there was an increased penetration of MTX into the brain tissue (Fig. 7). Levels achieved by the type A systems were significantly different from those achieved by the type B preparations up to 60 min post-injection, for reasons as yet unknown. These results give a clear indication that niosomes enhance the uptake of MTX into the brain and confirm earlier findings that polysorbate 80 also has a direct effect in enhancing the uptake of MTX into the brain.

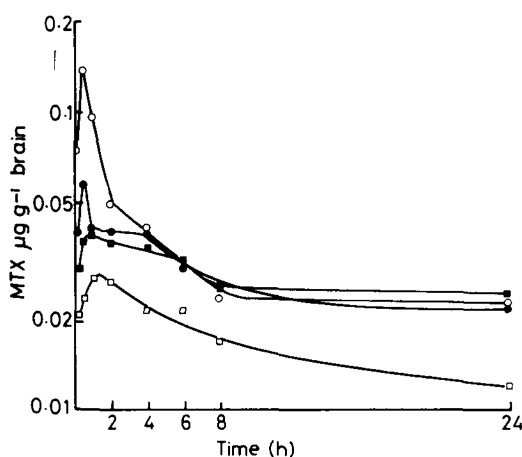

FIG. 7. Levels of methotrexate in the mouse brain (μg g^{-1} brain tissue) after intravenous administration of free MTX solution (□—□) MTX in 6% polysorbate 80 solution (■—■) MTX in niosomes type A (○—○) and type B (●—●) as before.

Formation of 7-hydroxymethotrexate

Niosome entrapment reduces the excretion of MTX into the urine and bile (unpublished results). Monitoring of the levels of MTX and the 7-hydroxy metabolite indicates that entrapped MTX is protected from rapid metabolism in-vivo particularly in the niosomes but to a small degree when delivered in micellar systems of polysorbate 80 as seen in Fig. 8.

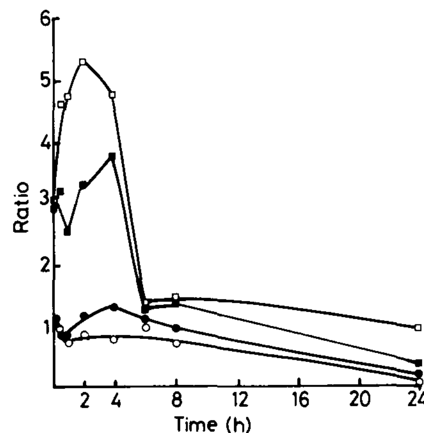

FIG. 8. The ratio of 7-hydroxy MTX to unchanged MTX, measured in the liver of mice after intravenous injection of 2·72 mg MTX kg^{-1} as free MTX solution (□); MTX in 6% polysorbate 80 solution (■) MTX entrapped in niosomes, type A (○) and MTX entrapped in niosomes type B (●).

DISCUSSION

The experiments clearly showed that polysorbate 80 had an effect in increasing the absorption of MTX from the gastrointestinal tract of mice and confirmed the ability of this surfactant to enhance the uptake of the drug into the brain. The increased uptake into the brain after oral administration could be due to the increased blood levels, but the increased uptake into the brain after intravenous injection could result from the direct effect of polysorbate 80 in altering the blood-brain barrier permeability. Or, as Levy & Anello (1969) have shown that oleic acid is able to alter membrane permeability in the goldfish, the role of this metabolite of polysorbate 80 in enhancing absorption by altering the bio-membrane permeability, cannot be ruled out.

The results showed that niosomes could be useful in maintaining the levels of MTX in the blood after intravenous injection. Several workers report (Kimelberg 1976; Kimelberg et al 1976; Kimelberg & Atchison 1978; Freise et al 1981; Puisieux & Benita 1982) similar phenomena with MTX encapsulated in liposomes.

We found an initial rapid elimination of MTX from the blood during the first 15 min followed by an intermediate rate for about 2 h and a slow elimination from 2 h onward. Similar patterns were observed for colchicine entrapped in liposomes (Juliano & Stamp 1975), liposome-entrapped protein (Gre-

goriadis & Neerunjun 1974) and liposome-encapsulated MTX (Kimelberg & Atchison 1978).

It appears that a large amount of MTX entrapped in niosomes is taken up by the liver. Others (Gregoriadis & Neerunjun 1974; Rahman & Wright 1975; Kimelberg 1976; Kimelberg & Atchison 1978; Freise et al 1981) have shown the ability of liver to take up most of the administered drug entrapped in liposomes. Gradual decline of MTX levels in the liver (Fig. 6) and the maintenance of the levels in the blood (Fig. 5) seem to suggest that the liver acts as a depot for the drug. Gregoriadis et al (1974) and Rahman & Wright (1975) have shown that liposomes are taken up intact into the liver cells, probably by endocytosis. It is likely that the niosomes are also taken up intact by the liver where they are broken down gradually by lysosomal lipase to release the free MTX which then re-enters the circulation.

The results showed that there is an increased uptake of MTX into the brain when it is administered intravenously as niosomes. Similar enhancement of MTX uptake into the brain was observed when MTX entrapped in liposomes was injected intravenously into rats (Freise et al 1981). Rahman & Wright (1975) have observed the increased uptake of EDTA into rat brain when it is administered as EDTA entrapped in liposomes. The ability of niosomes to increase the uptake of MTX into the brain could be due to the effect on the membrane barrier as observed with the liposome effect on Ehrlich ascites cell membranes (Fry et al 1979).

The experiments on oral uptake from niosomes probably need confirmation because of the nature of MTX absorption from the gastrointestinal tract. However, the pattern of results, especially that seen with liver and brain levels, is confirmed by the intravenous data.

In the experiments carried out, no adverse effects were observed. These first in-vivo experiments on drug delivery by means of synthetic non-ionic surfactant vesicles are pointers to their potential in this field. The possibility that metabolic patterns of the drug may be altered by its administration in niosomes provides additional hope that these systems will find a use in therapy, but knowledge of the toxicity of niosome-encapsulated drug and the effect on tumour kill in whole animals is needed before further conclusions can be drawn.

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