International Journal of Pharmaceutics, 17 (1983) 91–98
Elsevier

The stability of a prodrug, liposome and a prodrug–liposome complex in simulated biological conditions

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(Received February 10th, 1983)
(Modified version received May 4th, 1983)
(Accepted May 10th, 1983)

Summary

The release rates of cortisone hexadecanoate from liposomes were determined in the presence of a rat skeletal muscle homogenate. The presence of such biological material had very little effect on the stability of the cortisone hexadecanoate–DPPC liposome complex, which indicates that an intramuscular injection of liposomes is unlikely to be adversely influenced by enzymes encountered at the injection site.

Stability to biodegradation of DPPC liposomes in rabbit blood was enhanced by the incorporation of cholesterol and to a lesser extent cortisone hexadecanoate. All stability assessments in blood were in respect of the leakage rates of an aqueous entrapped marker, namely [^{14}C]polyethyleneglycol 4000.

Prodrug stability was assessed by determining the hydrolysis rate of the cortisone-21-aliphatic esters in 50% rabbit plasma. The rate of hydrolysis was inversely related to acyl chain length.

Introduction

It is now well established that the release of hydrophilic liposomally entrapped species is enhanced in the presence of blood, plasma and serum and constituent proteinaceous fractions (Scherphof et al., 1980). However, the effect of a biological environment upon the release rates of bilayer incorporated, lipophilic materials, or the attendant liposome stability conferred by such encapsulation is less well understood.

The effects of cholesterol in stabilizing liposomes to the presence of biological material is now well defined but the possible effects of following the incorporation of other lipid-soluble moieties has not been considered. Kirby et al. (1980) have reported that the stability of small unilamellar vesicles in whole blood is greater than that in either plasma or serum. The release of lipophilic materials from disrupted liposomes is delayed but the release of hydrophilic materials is immediate (Julianc and Stamp, 1979). It has been suggested that few molecules of molecular weight in excess of 1000 daltons leak from intact liposomes (Ryman and Tyrell, 1979). Hence the appearance in the external phase of such an entrapped hydrophilic marker serves as an ideal indication of liposome disruption.

The prodrugs employed in this study are esters of cortisone. The rate of hydrolysis is an important criterium determining the distribution and biological activity following either dissolution or release from the depot site such as a liposomal formulation of the steroid ester. The hydrolysis of some aliphatic hydrocortisone-21-esters has been investigated in the presence of plasma or carboxylesterase when it was found that those esters of an intermediate chain length were hydrolyzed most rapidly (C_3–C_9) (O'Neill and Carless, 1980).

This paper will therefore examine the influence of a skeletal muscle homogenate on the release rates of cortisone hexadecanoate from liposomes. This should indicate whether an intramuscular injection of liposomes will be adversely influenced by enzymes, and proteinaceous fractions encountered at the proposed injection site.

In addition, the stability of liposomes will be assessed in rabbit blood to assess lipid phase composition upon stability to biological disruption.

Materials and Methods

Lipid and cortisone ester (tritiated and non-tritiated) sources and purity were as previously reported (Arrowsmith et al., 1983). [^{14}C]Polyethylene glycol 4000 ([^{14}C]PEG 4000), spec. act. 40 mCi/mmol was obtained from Amersham, U.K. Soluvone was obtained from Dista, U.K. and Heparin 1 in 1000 (Blue), 5000 units/ml from Duncan Flockhart, U.K. Toluene and ethylacetate were Analar Grade (BDH, U.K.).

Determination of cortisone ester release from liposomes in muscle homogenate

An equilibrated suspension of cortisone hexadecanoate incorporated into dipalmitoylphosphatidylcholine (DPPC) liposomes was prepared as previously described (Arrowsmith et al., 1983). A 5% w/v rat muscle homogenate was prepared as follows. 20 g of skeletal muscle was dissected from the hind legs of female Wistar rats and chopped roughly with a scalpel. 1 g portions were homogenized (Tissue Homogenizer M.S.E., U.K.) with 5 ml of sterile saline in a 22 ml universal vial until smooth. The homogenate produced from 5 g of muscle was diluted to 90 ml with sterile normal saline and 150 μl of a 250,000 units $\cdot$ ml^{-1} solution of Soluvone added. After temperature equilibration at 37°C, 10 ml of the liposome suspension was mixed with the muscle homogenate and solute efflux followed by sample

centrifugation (Arrowsmith et al., 1983). 0.5 ml samples of the supernatant were assayed by scintillation counting. The time zero state of partitioning was measured by centrifugation of samples of the liposome suspension prior to dilution with tissue homogenate.

It was found that a constant amount of steroid was lost from solution due to binding to muscle protein and this was compensated for in calculation of the efflux rate.

The stability of DPPC liposomes in rabbit blood

To 50 μCi [^{14}C]PEG 4000, supplied dissolved in 0.25 ml 3% sterile ethanol in water, was added 1 ml of 1.125% (w/v) saline, sterilized by passage through a 1 μm pore size millipore filter. 1 ml of the resulting normal saline, containing 40 μCi [^{14}C]PEG, was added to films of DPPC (Table 1). Liposomes were produced by gentle shaking at 60°C for 5 min, followed by brief mechanical shaking to complete suspension. The suspension was centrifuged at 50,000 $\times g$ for 10 min and the supernatant collected and used to prepare the next suspension. The pellet, containing encapsulated [^{14}C]PEG was resuspended in sterile normal saline (2 ml) and recentrifuged (as above). This process was repeated and the washed liposomes finally distributed in 5 ml of normal saline and 50 μl taken for scintillation counting. The remainder was added to 20 ml of rabbit blood (Lop strain) which was obtained by marginal ear vein cannulation and homogeneity maintained with 2 ml of heparin in a saline solution (500 units $\cdot$ ml^{-1}) per 100 ml of blood. The blood–liposome mixtures were shaken at 37°C and the appearance of [^{14}C]PEG was followed by periodic centrifugation (50,000 $\times g$ for 15 min of 2.5 ml samples. Duplicate 0.5 ml of the supernatant was assayed by scintillation counting.

10 μl vols. of [^{14}C]PEG 4000 (40 μCi $\cdot$ ml^{-1}) were mixed with 3 ml aliquots of blood and the distribution of free PEG 4000 between supernatant and plasma established by centrifugation at 50,000 $\times g$ for 15 min. No sedimentation of [^{14}C]PEG 4000 occurred following similar centrifugation in saline.

The hydrolysis rates of cortisone aliphatic esters in rabbit plasma

10 μCi of cortisone derivatives were rotary-evaporated (40°C) to a film from chloroform solution in 10 ml Erlenmeyer flasks. 2.5 ml of sterile normal saline was

TABLE 1

Liposome composition	Weight of DPPC (mg)	Weight of additive (mg)
DPPC	20	–
DPPC + 9 mole % cortisone hexadecanoate	18.2	1.47
DPPC + 9 mole % cortisone octanoate	18.2	1.19
DPPC + 9 mole % methylhexadecanoate	18.2	0.66
DPPC + 30 mole % cholesterol	14	3.16

added (pH 7) and the flask shaken at 37°C overnight. The amount of material dissolved was assayed by scintillation counting of a 10 μl sample. 0.25 ml of cortisone derivative solution was diluted with water to 1.25 ml and extracted with 2 × 10 ml of chloroform. The aqueous phase was discarded, the chloroform pooled and 1 ml of a mixed methanol–chloroform solution of 0.1 mg · ml^{-1} cold cortisone and cold cortisone derivative added prior to rotary-evaporation. The steroid was redissolved in the minimum of chloroform and spotted onto TLC. plates (silica gel GF 254, Merck) which were developed in toluene–ethyl acetate (13 : 7). Cortisone ($R_f = 0.15$) and cortisone ester spots (C_2, $R_f = 0.30$; C_4, $R_f = 0.33$; C_6, $R_f = 0.35$; C_8 $R_f = 0.37$; C_{16}, $R_f = 0.41$; C_{22}, $R_f = 0.44$) were identified under UV light and removed for scintillation counting and an equivalent area for background counting. The assay procedure is as previously described (Arrowsmith et al., 1983). The initial state of hydrolysis was calculated from these data.

Blood was removed by cannulation (Butterfly cannula, Abbot Ireland, Ireland) of the marginal ear vein of New Zealand White rabbits. Further samples were obtained by cardiac puncture using a 25-gauge 25 mm hypodermic needle and a 5 ml disposable syringe. Clotting was prevented by the addition of 2 ml of heparin per 50 ml of blood and the plasma collected following centrifugation at 1000 × g for 30 min.

1 ml of the rabbit plasma and 1 ml of the cortisone ester saline solution, prepared above, was mixed in clean glass scintillation vials and incubated at 37°C for 45 min. The composition of 1 ml of this mixture was assessed as described above.

Calculations

During the 20 h dissolution period of the cortisone esters some small amount of hydrolysis occurred. Hence this must be compensated for when estimating the percentage hydrolyzed in 45 min.

$$100 \times \frac{(C_{45} - C_0)}{(100 - C_0)} = H_{45}$$

where C_0 = % hydrolyzed at t = 0, C_{45} = % hydrolyzed at t = 45 min, and H_{45} = % hydrolyzed during 45 min in plasma.

C_0 and C_{45} are calculated from the ratio of cortisone spot to total radioactivity, i.e. cortisone plus ester spot disintegrations · min^{-1}.

Results and Discussion

The release profiles of cortisone-21-hexadecanoate from DPPC liposomes into normal saline and into saline containing a 5% w/v suspension of rat skeletal muscle is shown in Fig. 1. Due to microbial growth it was possible to follow the release from the latter preparation for a period of 2 days only. However, it is evident that the presence of such biological material has very little effect on the stability of the

DPPC liposomes–cortisone hexadecanoate complex. The two profiles are virtually identical, the release in the presence of muscle having a slightly higher first-order rate constant ($k = 11.7 \times 10^{-3}\ h^{-1}$) than the release into saline ($10.8 \times 10^{-3}\ h^{-1}$). Knight and Shaw (1979) report the stability of the hydrocortisone hexadecanoate–DPPC liposome complex in aspirated, cell-free, human rheumatoid synovial fluid. However, it was found that steroid loss from fluid liposomes bearing hydrocortisone octanoate was very rapid in the same medium.

It is possible that liposome integrity is lost in the muscle homogenate but that the cortisone hexadecanoate remains adhered to liposomal fragments.

Arakawa et al. (1975) report that the efflux of the antibiotic cefazolin sodium from cholesterol-rich liposomes was not increased by the presence of a rat muscle homogenate. This information and the data presented in Fig. 1 leads to the conclusion that the muscle environment causes little increase in the fragility of liposomes administered to this site.

DPPC liposomes proved to be fairly stable in the presence of rabbit blood, reflecting the similarity of release profiles seen in Fig. 1. The liposomes containing 30% cholesterol can be used as a reference to the stability of the other liposomes as the stability of such a formulation is known to be good. This is again demonstrated in Fig. 2 where following a small initial drop in [^{14}C]PEG 4000 entrapment, very little material is subsequently lost. The release of marker from DPPC liposomes is faster than from those containing cholesterol but the DPPC liposome stability in rabbit blood at 37°C appears satisfactory. It was possible, due to the proximity of physiological temperatures to the phase transition temperature of DPPC liposomes

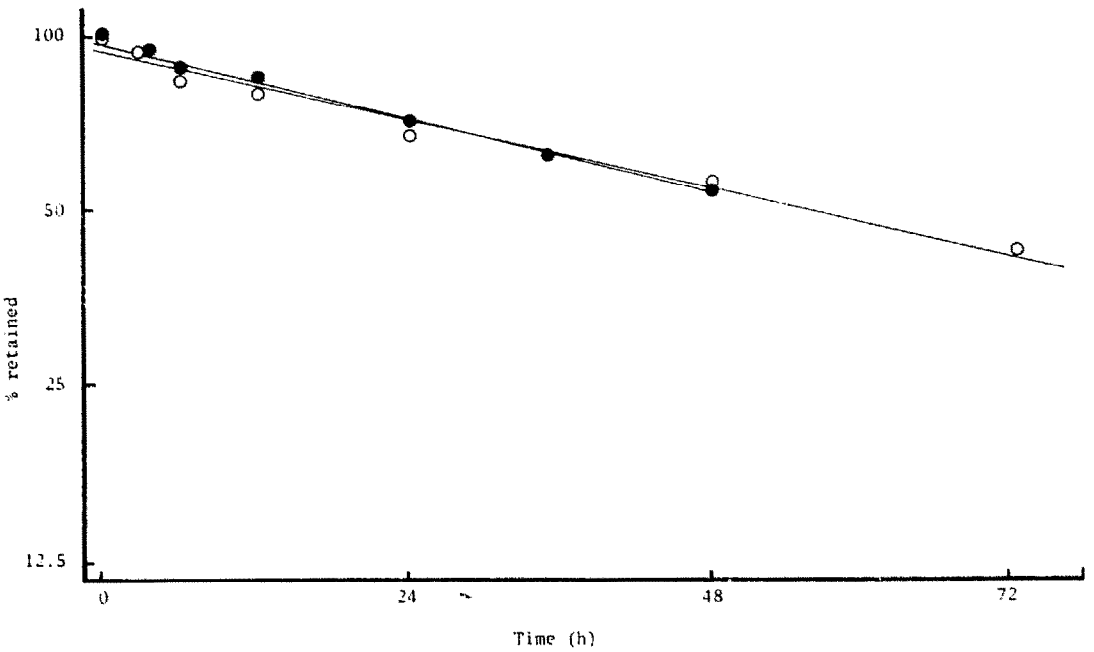

Fig. 1. The release of cortisone hexadecanoate from DPPC liposomes at 37°C. ○, Buffer; ●, 5% muscle homogenate.

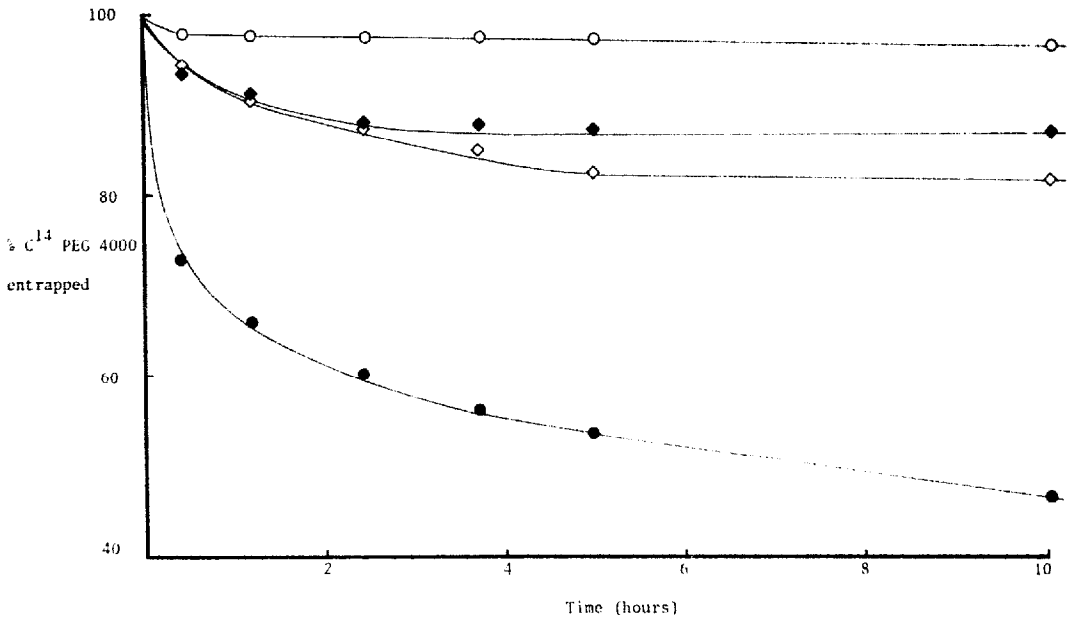

Fig. 2. The release of a non-diffusible marker ([^{14}C]PEG 4000) from DPPC liposomes associated with different lipophilic solutes. ○, 30 mole % cholesterol; ◆, 9 mole % cortisone hexadecanoate; ◇, 100% DPPC; and ●, 9 mole % methyl hexadecanoate.

in the presence of additives, that in biological media, such liposomes may exhibit a great fragility.

The presence of 9 mole % of cortisone octanoate did not alter the rate of appearance of [^{14}C]PEG 4000 in the external phase. Indeed the profile produced was virtually identical to that resulting from DPPC alone and has not been included in Fig. 2 for the sake of clarity. It is possible that cortisone octanoate does not interact sufficiently strongly with the bilayer to affect a general structure, a statement not supported by differential scanning calorimetry (D.S.C.) evidence (Arrowsmith et al., 1983). However, some of the initial inclusion of 9 mole % of this material may have been washed out of the liposomes during the procedures to remove unentrapped polymer. It is likely that liposomal cortisone palmitate is also lost during these procedures, but since this ester has a greater lipid affinity, such losses are probably relatively small.

The remaining cortisone palmitate renders DPPC liposomes slightly more stable than those of DPPC alone but they are more fragile than those containing cholesterol. This biological stability, and therefore predictability of release from DPPC liposomes, is a useful property in a prospective vehicle for sustained release.

The aqueous marker was rapidly lost from liposomes containing 9 mole % of methyl hexadecanoate. It is surprising that this additive should induce such a marked destabilization of the bilayer in the rabbit blood as the D.S.C. thermograms showed little disturbance compared with DPPC alone (Arrowsmith et al., 1983). Also it has been reported that additives of equivalent length to the bilayer optimize packing into the alkyl chain array (Mabrey and Sturtevant, 1976).

However, these comparative fragility effects supply further evidence that corti-

sone hexadecanoate packs into the lecithin bilayer in a different manner to methyl hexadecanoate.

It has been suggested that cholesterol confers its effects through a reordering of liposomal bilayers to a state intermediate between fluid and gel phases (Ladbrooke and Chapman, 1969). The evidence that rates of permeation across fluid bilayers are reduced, while rates across gel phase bilayers are increased, by the presence of cholesterol (Papahadjopoulos et al., 1973) supports this theory (Papahadjopoulos et al., 1972). Kirby et al. (1980) have suggested that cholesterol-ordering effects are responsible for the stability conferred upon its inclusion into egg lecithin liposomes and subsequently exposed to blood fractions. However, were this the sole mode of cholesterol stabilization of lecithin bilayers to biodegradation, then it would be expected that its incorporation into gel liposomes such as DPPC would lead to reduced stability, in conjunction with the reduction in bilayer order.

Similarly, if order were the sole criterion of rate of biodegradation then the inclusion of bilayer cortisone palmitate would be likely to induce fragility; the presence of a foreign molecule altering the packing of the bilayer. It is possible that the presence of cholesterol, and perhaps also cortisone hexadecanoate, renders phospholipid molecules less susceptible to removal from the bilayer by high density lipoproteins due to some affinity between sterol/steroid and the phospholipid.

The results shown in Table 2 for the hydrolysis of cortisone esters in Lop rabbit plasma, bear comparison with those of O'Neill and Carless (1980) for hydrocortisone esters in human plasma. The pattern of hydrolysis rate is generally the same, except the Lop plasma has a much greater ability to hydrolyze the acetate ester. From compilation of these results it is evident that corticosteroid esters of chain length 14–22 carbon atoms are relatively poor substrates for circulating esterases or lipases. Their ultimate hydrolysis in vivo may occur essentially in the liver where fatty acid esters are routinely dismantled.

Hydrocortisone hexadecanoate had previously been shown by Shaw (1978) to be much more stable in rheumatoid synovial fluid than hydrocortisone octanoate (c.f. Table 2 for comparable cortisone esters in plasma). He further reports that encapsulation of the shorter ester in egg lecithin liposomes did not prevent hydrolysis, this is probably due to its tenuous affinity for this liposome formulation.

TABLE 2

THE HYDROLYSIS OF SOME CORTISONE ESTERS IN 50% RABBIT PLASMA AFTER INCUBATION AT 37°C FOR 45 MIN

Ester	% Hydrolyzed
Cortisone acetate	94
Cortisone butyrate	75
Cortisone hexanoate	70
Cortisone octanoate	67
Cortisone hexadecanoate	6
Cortisone docosanoate	2

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