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Incorporation of alphaxalone into different types of liposomes

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Abstract—The poor solubility of steroid anaesthetics in water has been a serious drawback in the development of clinically acceptable intravenous formulations. The use of Cremophor EL to solubilize steroids such as alphaxalone led to unacceptable hypersensitivity reactions and consequent withdrawal of this anaesthetic. In principle, liposomes can act as a safe solvent for the intravenous administration of alphaxalone. We report the incorporation of [^{14}C]acetylated alphaxalone in both multilamellar vesicles and stable plurilamellar vesicles prepared from a range of amphiphiles including synthetic polyhydroxyl lipids. For both types of preparations, addition of cholesterol to phosphatidylcholine-based lipids caused an increase in encapsulation efficiency. Maximum encapsulation was achieved with the stable plurilamellar vesicle preparation of 1-stearyl-2-myristylglycerate-3, *N*-methylglucamine:cholesterol:egg phosphatidylcholine (78%). The rate of efflux of this anaesthetic from a range of liposomes was measured in serum. The highest rate (85% after 30 min) was observed with an equimolar egg phosphatidylcholine:cholesterol stable plurilamellar vesicle preparation. From these studies it can be concluded that liposomes offer a suitable alternative for intravenous delivery of steroidal anaesthetics.

The anaesthetic properties of steroids have been known for a number of years (Selye 1942). They have two major advantages over barbiturates; a greater therapeutic ratio and the property of progressive elimination from the blood by the liver, thereby ensuring that recovery from anaesthesia does not rely on redistribution (Dundee & Clarke 1980). The major practical drawback of these molecules is their insolubility in water. Atkinson et al (1965) demonstrated that rapid induction of anaesthesia and high potency of steroid anaesthetics were both associated with the presence of a free 3α-hydroxy group in the steroid molecule. All attempts to solubilize such steroids by esterification produced compounds with reduced potency and increased induction time. One of the most potent compounds tested is 3α-hydroxy-5α-pregnane-11,20-dione (alphaxalone). Since alphaxalone is insoluble in water, attempts were made to dissolve the steroid in biologically acceptable media. One formulation is based on the use of Cremephor EL (polyoxyethylated castor oil), a non-ionic surfactant which forms the basis of the intravenous anaesthetic propanidid. However a number of adverse reactions which resemble immediate hypersensitivity and anaphylaxis have been reported for both alphaxalone and propanidid (Du Cailar 1972; Avery & Evans 1973; Mehta 1973; Notcutt 1973; Horton 1973; Sutton et al 1974; Clarke et al 1975; Watkins et al 1976). Subsequently Cremephor EL was shown to be responsible for these reactions (Fisher 1976).

Recently a soya bean and egg phosphatide emulsion has been used as the basis of a safe solvent for the intravenous administration of diazepam and propofol. This has led to preliminary studies on the use of this colloid system for formulating the steroid anaesthetic, pregnanolone (Hogskilde et al 1987a, b). We feel that liposomes offer a suitable alternative to Cremephor EL. In this paper we report conditions leading to the incorporation of alphaxalone into a range of liposome systems and compare the rate of efflux of this steroid from these lipid preparations.

Materials and methods

Compounds. Dimyristylphosphatidylcholine (DMPC), distearylphosphatidylcholine (DSPC), egg phosphatidylcholine (egg PC), sphingomyelin (SM) and cholesterol were obtained from Sigma Chemical Co. Ltd (Poole, UK). 1-Stearyl-2-myristylglycerate-3,*N*-methylglucamine ($C_{18:0}$,$C_{14:0}$-GMG), 1,2-distearylglycerate-3,*N*-methylglucamine ($C_{18:0}$,$C_{18:0}$-GMG) and 1-stearyl-2-behenylglycerate-3,*N*-methylglucamine ($C_{18:0}$,$C_{22:0}$-GMG) were prepared as previously described (Assadullahi et al 1991). Alphaxalone was kindly donated by Glaxo Research Group Ltd (Greenford, UK). Sodium chloride, tris(hydroxymethyl)-aminoethane (Tris) and all solvents were purchased from BDH (Poole, UK).

Experimental procedure. Alphaxalone efflux from liposomes was first measured by UV absorbency; however, the results were unreliable and lacked reproducibility due to the low extinction constant of the steroid. To enable the accurate measurement of steroid efflux, an isotopically labelled derivative, [^{14}C]acetylated alphaxalone, was prepared. Although the acetylated derivative will have slightly different partitioning properties to that of native alphaxalone, the difference is likely to be minimal.

[^{14}C]Acetic anhydride (50 μCi, 0·01 mmol) was added to alphaxalone (40 mg) in dichloromethane (10 mL). After standing 18 h at room temperature (21 C), the resulting solution was diluted to 50 mL with dichloromethane and washed with sodium bicarbonate (0·1 M) and water. After treatment with sodium sulphate the organic layer was evaporated to dryness. The dry product was dissolved in 5 mL dichloromethane. The specific activity of the acetylated alphaxalone was determined to be 1·9 μCi mg^{-1}. The label was located on the hydroxy function of alphaxalone.

Liposome preparation. Stable plurilamellar vesicles. The method used was based on the work of Gruner et al (1985). The lipids (total weight 50 mg) were dissolved in a mixture of chloroform and diethylether (5 mL, 75:25, v/v). Alphaxalone (5 mg) was added to this organic phase. The aqueous hydrating solution (0·3 mL, sodium chloride: 130 mM, Tris-HCl: 20 mM, pH 7·4) was added and the mixture emulsified at room temperature using a bath-type sonicator, during which time a gentle stream of nitrogen was passed over the mixture. This procedure was continued until the organic solvent was completely evaporated. The resulting emulsion was then resuspended in more hydrating solution (5 mL) and the suspension was vortexed using a Spiromix (Denley Instruments, Billingshurst, UK). The suspension was pelleted by centrifugation (15 min, 3000 rev min^{-1}) and washed three times with the hydrating solution.

Multilamellar vesicles. We used a modification of the technique developed by Bangham & Horne (1964). Lipids (total weight 50 mg) were dissolved in chloroform and alphaxalone (5 mg) in dichloromethane was added. The organic solvents were removed at 30 °C by rotary evaporation, leaving a thin film deposited on the wall of the flask. The aqueous hydrating solution (as described for stable plurilamellar vesicles) was then added. The suspension was vortexed using a Spiromix until a fine milky suspension was obtained. The suspension was then centrifuged (15 min, 3000 rev min^{-1}) and washed as described for the stable plurilamellar vesicle preparation.

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Table 1. Percentage encapsulation efficiency of acetylated alphaxalone (5 mg) into multilamellar vesicles (MLVs) and stable plurilamellar vesicles (SPLVs) with different lipid composition (total weight 50 mg).

		Percentage encapsulation efficiency	
Lipid composition	Molar ratio	MLVs	SPLVs
Egg PC	1	25	33
Egg PC:cholesterol	1:1	34	47
DSPC:DMPC:cholesterol	1:1:1	42	48
($C_{18:0}$,$C_{14:0}$-GMG):cholesterol	1:1	45	56
($C_{18:0}$,$C_{14:0}$-GMG):cholesterol:egg PC	1:1:1	53	78
($C_{18:0}$,$C_{18:0}$-GMG):cholesterol	1:1	28	34
($C_{18:0}$,$C_{18:0}$-GMG):cholesterol:egg PC	1:1:1	46	53
($C_{18:0}$,$C_{22:0}$-GMG):cholesterol	1:1	40	49
($C_{18:0}$,$C_{22:0}$-GMG):cholesterol:egg PC	1:1:1	46	62

Each experiment was carried out four times; the values for percentage efficiency ranged between $\pm 0{\cdot}2$ and $\pm 0{\cdot}4$ for both types of preparation.

Determination of encapsulation efficiencies. [^{14}C]Acetylated alphaxalone (1 μCi) was added to a 10 mM solution of the non-radioactive acetylated alphaxalone (this was prepared using the same procedure as that of the radioactive steroid) in the organic phase of liposome preparation. The liposomes were pelleted by centrifugation (15 min, 3000 rev min^{-1}) and resuspended in 2 mL of the hydrating solution. The percentage encapsulation efficiency was calculated as:

$$\frac{\text{Counts min}^{-1}\text{ in the washed pellet}}{\text{total counts min}^{-1}} \times 100$$

Electron microscopy. Negative stain electron micrographs were prepared by mixing an equal volume of liposome suspension and ammonium molybdate (2% w/v). The resulting suspension (100 μL) was applied to a mesh copper grid with 15 nm carbon film. The copper grid was then placed on a filter paper and allowed to dry. Samples were photographed at 100 kV using a Phillips EM 301C transmission electron microscope.

Freeze-drying procedure. Samples of liposome suspension whilst being vortexed, were frozen in a mixture of dry ice and acetone and placed on a Birchover Instrument freeze-drier, condenser capacity 3·5 L and operated at -50 C. The machine was evacuated to approximately 50 mTor and the sample was left overnight until reduced to a white translucent film. Liposomes were subsequently rehydrated using the aqueous hydrating solution.

Efflux studies. For efflux studies each liposome suspension (5 mL) containing the steroid was incubated at 0, 0·5, 1 and 2 h at 37°C. At each time point the liposome suspension was pelleted by centrifugation (15 min, 3000 rev min^{-1}) and the radioactivity in the supernatant was monitored.

Results and discussion

[^{14}C]Acetylated alphaxalone (1 μCi, total: 5 mg in each case) was incorporated into stable plurilamellar vesicles and multilamellar vesicles prepared from a range of lipids (Table 1). It is evident that in all cases encapsulation efficiencies were markedly higher for the stable plurilamellar vesicle preparations. For both preparations the trends in encapsulation efficiency are influenced by altering the lipid composition in a similar manner since the differences noted are primarily due to changes in chain lengths and saturation of lipid structures. The addition of cholesterol to phosphatidyl choline (PC)-based lipids causes an increase in encapsulation. In principle this could be explained by the ability of cholesterol to stabilize bilayers, thereby reducing the efflux of entrapped molecules and hence increasing the encapsulation efficiency. In addition, cholesterol can cause some ultrastructural effects on the packing of the bilayers with respect to each other.

Although encapsulation by novel lipid-containing liposomes was relatively low in the presence of cholesterol alone, encapsulation was significantly enhanced by the presence of both egg PC and cholesterol. Because of the ease of preparation and the higher efficiency of encapsulation, stable plurilamellar vesicles were considered to be superior to multilamellar vesicles and they were used in the other experiments reported in this study.

The rate of efflux of acetylated alphaxalone from stable plurilamellar vesicles with a range of lipids was measured in

Table 2. Percentage efflux of the incorporated acetylated alphaxalone in stable plurilamellar vesicle preparations from different lipids.

	Percentage efflux		
	0·5 h	1 h	2 h
Egg PC:cholesterol	85 ± 5	92 ± 3	97 ± 1
DSPC:DMPC:cholesterol	22 ± 8*	38 ± 13*	59 ± 10*
($C_{18:0}$,$C_{14:0}$-GMG):cholesterol	23 ± 6*	35 ± 16*	32 ± 5*
($C_{18:0}$,$C_{14:0}$-GMG):cholesterol:egg PC	$17{\cdot}5 \pm 10$*	21 ± 7*	33 ± 10*

In each experiment equimolar ratios of lipids were used. Each experiment was carried out five times in duplicate. *$P = 0{\cdot}02$ for egg PC: cholesterol liposomes compared with all others at different times. The difference between other types of liposomes was not statistically significant.

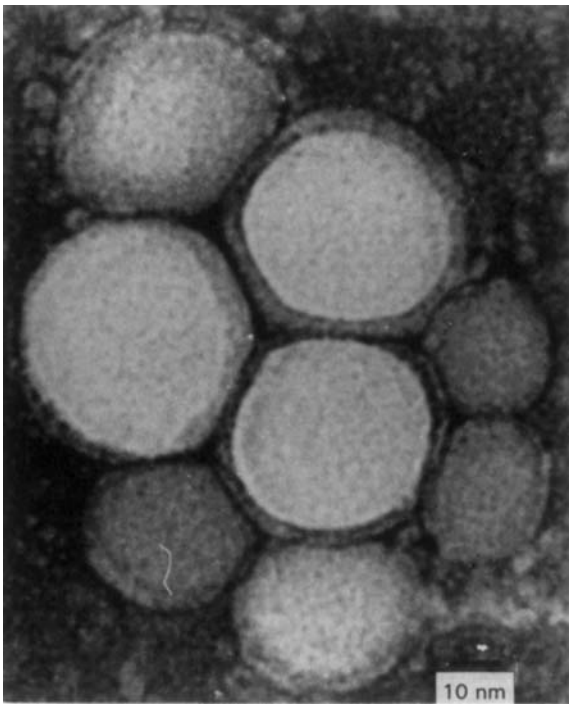

FIG. 1. Electron micrograph of acetylated alphaxalone incorporated stable plurilamellar vesicles after freeze-drying, and negatively stained with ammonium molybdate (× 26000) 3·1–3·3 μm.

human serum. The results (Table 2) indicate that amongst the lipids investigated, the rate of efflux was highest with egg PC:cholesterol, where up to 85% of the incorporated alphaxalone was detected in serum within the first 30 min. This was increased to 93 and 98% at 1 and 2 h, respectively. In contrast with DSPC:DMPC:cholesterol-containing stable plurilamellar vesicles, a gradual and an almost linear increase was noted. The highest release rate with this system at 2 h was 60%. On comparing this value with egg PC:cholesterol-containing stable plurilamellar vesicles, it is evident that egg PC:cholesterol-containing liposomes release more steroid after 30 min, than the synthetic PC liposomes at 2 h. This effect is primarily due to tighter packing of the saturated PCs, DMPC and DSPC. With novel lipids, the increase in the rate of efflux was also gradual. However, the overall release of alphaxalone from these lipid preparations was much slower (approx. 35% in 2 h). These observations are in good agreement with those obtained for serum stability of liposomes prepared from these novel lipids (Assadullahi et al 1991). Thus egg PC:cholesterol containing stable plurilamellar vesicles, which possess a short half-life in serum (releasing over 90% of their incorporated alphaxalone within 1 h), would appear to offer a suitable system for intravenous delivery of alphaxalone. The results presented (Table 2) will be an underestimate of alphaxalone efflux rate, due to the chemical modification of the parent compound.

A prerequisite for the successful introduction of alphaxalone liposome preparations is a stable and long shelf-life. In principle a freeze-dried preparation of this lipophilic steroid can provide this feature. The encapsulation efficiencies of alphaxalone-incorporated stable plurilamellar vesicles was similar following rehydration of the freeze-dried material. Direct visualization by electron microscopy showed that the integrity of such liposomes did not change following the freeze-drying procedure (Fig. 1.). Moreover, the rate of efflux remained the same when compared with that of liposomes which had not been freeze-dried. This is an important advantage with lipophilic drugs, since once incorporated into liposomes they can be freeze-dried and have a long shelf-life.

From these studies it can be concluded that egg PC:cholesterol-containing stable plurilamellar vesicles are suitable for intravenous administration of alphaxalone. However, liposomes prepared from egg PC are susceptible to oxidation and this will shorten their shelf-life. Therefore, it is unlikely that such liposomes will be selected for the formulation of alphaxalone, lipids which are not susceptible to oxidation being preferred. Certain groups of novel lipids such as 1-stearyl-2-laurylglycerate-3,*N*-methylglucamine offer such characteristics.

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