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Drugs-in-cyclodextrins-in-liposomes: an approach to controlling the fate of water insoluble drugs in vivo

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Abstract

Distearoyl phosphatidycholine liposomes containing entrapped complexes of ^{14}C-labelled hydroxypropyl-β-cyclodextrin (HPβ-CD) with ^{3}H-labelled dehydroepiandrosterone (DHEA), retinol (RET) and dexamethasone (DEX) were prepared and incubated with rat blood plasma at 37°C. The rate of drug dissocation and release in the media was minimal for DEX/HPβ-CD (11%), modest for RET/HPβ-CD (23%) and considerable for DHEA/HPβ-CD (56%, 60 min). However, the HPβ-CD moiety of each of the complexes was retained by liposomes quantitatively. Intravenous injection of free complexes into rats led to their rapid clearance from the circulation with up to 94% of HPβ-CD recovered in 24 h urine together with lesser and variable amounts of drug (up to 46% of the dose, DHEA < RET < DEX). A proportion of the drugs (up to 25% of the dose), but very little HPβ-CD, was removed by the liver where drugs were catabolised rapidly, presumably following complex dissociation in the blood and drug transport to the tissue via plasma proteins. After injection of complexes entrapped in liposomes, these were found to alter the pharmacokinetics of the complexes with only 6–13% of HPβ-CD and a moderate proportion of drugs (up to 26% of the dose) recovered in 24 h urine. Much of the HPβ-CD moiety was recovered in the liver (up to 83%) and spleen (up to 13% of the dose) 30 min after injection, together with a variable proportion of drugs (DHEA < RET < DEX). In longer term (up to 24 days) experiments with liposome-entrapped complexes, there was removal of HPβ-CD from the tissues albeit at a very slow rate. Moreover, the metabolism of individual drugs, both the liver and spleen following vesicle disintegration appeared to depend directly on the rate of complex dissociation. Administration of drug/cyclodextrin inclusion complexes via liposomes could serve as a means to control the action of a wide range of therapeutic agents.

Keywords: Liposome; Cyclodextrin; Dehydroepiandrosterone; Retinol; Dexamethasone; Drug delivery

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1. Introduction

Adequate aqueous solubility, a property not readily encountered in the diverse and complex organic structures typically found in pharmacologically active agents, is required for successful delivery of drugs in therapy. Consequently, traditional parenteral formulations for poorly water-soluble compounds have necessitated the use of either prodrugs or organic co-solvents such as ethanol and propylene glycol, non-ionic surfactants such as Tween 80 or Cremophor®, or extreme pH, conditions that may not solubilise enough drug for therapeutic efficacy and can cause local irritation or even systemic toxicity (Ennis et al., 1986; Frosch and Czametzki, 1987).

An alternative approach to improve the water-solubility of drugs involves the use of cyclodextrins (CD), which can form water soluble inclusion complexes with hydrophobic molecules. Although used by the pharmaceutical industry almost since their discovery early this century, mainly to protect labile agents from isomerisation or oxidative and photolytic degradation (Schlenk et al., 1958), it is only during the last 30 years that their potential as a drug carrier and delivery system has been recognised (Uekama and Otagiri, 1987; Duchene, 1991). Cyclodextrins are cyclic oligosaccharides possessing six, seven or eight $\alpha(1-4)$ linked glucopyranose units designated α, β and γ, respectively. The exterior face is hydrophilic while the interior cavity is hydrophobic and can entrap apolar drugs. However, β-cyclodextrin (β-CD), the most useful and widely researched cyclodextrin, has a very low aqueous solubility (1.85 g/100 ml at 25°C) (Muller and Brauns, 1985) and furthermore, is known to cause renal necrosis when administered intravenously to rats (Frank et al., 1976; Hiasa et al., 1981; Perrin et al., 1978), thus limiting its use to oral applications. Chemical modification, most commonly with hydroxypropyl and sulphoalkyl ether groups, have produced derivatives with improved safety features (Thompson, 1997), increased aqueous solubility ($>50\%$) and sometimes more powerful solubilising capacity than the parent β-CD (Muller and Brauns, 1985; Pitha and Pitha, 1985; Pitha et al., 1986). A number of toxicity studies with rats and dogs on the parenteral use of hydroxypropyl β-cyclodextrin (HPβ-CD) in particular have shown excellent safety profiles (Coussement et al., 1990; Frijlink et al., 1990; Monbaliu et al., 1990) as have tests on human volunteers (Szathmary et al., 1990).

When drug/CD inclusion complexes are injected intravenously, rapid dissociation takes place either because of dilution or because other blood components displace the included drug. The released drug is then metabolised as free drug while the cyclodextrin moiety is excreted through the kidneys (Szejtli, 1987). Therefore, while cyclodextrins are useful in improving drug formulations (Szejtli et al., 1983; Gal-Furzy et al., 1984) and increasing bioavailability (Hassan et al., 1990; Puglisi et al., 1991), the pharmacokinetics of complexed drugs are not altered.

The in vivo behaviour of liposomes and entrapped solutes has been well documented (Gregoriadis, 1988, 1995a,b; Gregoriadis and Florence, 1993). Liposomes can entrap hydrophilic drugs in the aqueous phase or hydrophobic drugs in the lipid bilayers and retain them en route to their destination. Moreover, their composition can be adapted to achieve predetermined clearance rates and tissue distribution (Gregoriadis, 1988) and attachment of appropriate ligands renders targeting to accessible specific cell receptors possible. However, accommodation of lipophilic compounds in the lipid phase can be problematic as some drugs interfere with bilayer formation and stability, thus limiting the range and amount of valuable drugs that can be associated with liposomes. Entrapping water-soluble drug/CD inclusion complexes into the aqueous phase of liposomes would avoid such drawbacks while preventing complex dissociation or renal excretion which are often inevitable with the free complexes. Thus, combining liposomes and cyclodextrins in a complementary carrier system could broaden the scope and accessibility of present and future therapeutic agents not only for topical use as recently applied in this laboratory in order to protect labile drugs against U.V. radiation (Loukas et al., 1995) and light-induced oxidation (Loukas et al., 1996), but also in vivo for the control of drug fate and metabolism (McCormack and Gregoriadis, 1994a,b, 1996).

To that end, inclusion complexes were formed between HPβ-CD and three different water-insoluble drugs of appropriate size: dehydroepiandrosterone (DHEA), retinol (RET) and dexamethasone (DEX), and the complexes were entrapped in the aqueous phase of liposomes. Following intravenous injection of free and entrapped complexes into rats, comparative studies were carried out on their fate and biodistribution by monitoring the concentrations of both HPβ-CD and drug moieties in the urine, blood, liver, kidneys and spleen over time. A parallel study was also carried out with DEX and DHEA entrapped in the bilayers as such. Results show that administration of drug/HPβ-CD complexes via liposomes prevents complex dissociation and loss of drug into the urine and contributes to significant drug uptake by tissues. These metabolise drugs at rates which are dependent on the dissociation constant of the complex in situ.

2. Materials and methods

2.1. Materials

Distearoyl phosphatidylcholine (DSPC) was obtained from Sygena (Liestal, Switzerland). Dehydroepiandrosterone (DHEA), retinol (RET), dexamethasone (DEX) and 2-hydroxypropyl-β-cyclodextrin (HPβ-CD) were purchased from Sigma-Aldrich (Dorset, UK). [1,2-^{3}H(N)] dehydroepiandrosterone ([^{3}H]DHEA, 37 MBq) and [11,12-^{3}H(N)] retinol ([^{3}H]RET, 9.25 MBq) were from DuPont (UK) New England Nuclear Products (Herts, UK). [1,2,4-^{3}H], tritiated dexamethasone ([^{3}H]DEX, 9.25 MBq) was from Amersham (Amersham, UK) and 2-hydroxypropyl-β-cyclodextrin labelled with ^{14}C in the hydroxypropyl group ([^{14}C]HPβ-CD), 3.7 MBq) from Cyclodextrin Technologies Development (Gainsville, FL).

2.2. Formation of inclusion complexes

DHEA (25 mg), RET (10 mg) and DEX (18 mg) were each dissolved in 2 ml chloroform/ethanol and known amounts of their respective tracers mixed. Following evaporation of the solvent under a stream of oxygen-free nitrogen 2 ml deionised water containing HPβ-CD (100 mg in the case of DHEA and RET and 50 mg for DEX) plus a known amount of ^{14}C-HPβ-CD were added to each of the dried films and allowed to stir for up to 4 days at 20°C. The drug/HPβ-CD suspensions were then centrifuged at $70\,000 \times g$ for 60 min in a refrigerated Sorvall Plus Combi ultracentrifuge to yield pellets and clear supernatants. Radioactivity measurements showed that in each case the supernatants contained the inclusion complexes and the pellets consisted of free, uncomplexed drugs.

2.3. Entrapment of inclusion complexes into liposomes

The dehydration–rehydration procedure (Kirby and Gregoriadis, 1984) with modifications (McCormack and Gregoriadis, 1994a,b, 1996) was used to entrap each of the three complexes into the aqueous phase of liposomes. In brief, small unilamellar vesicles (SUV) made from 32 μmol DSPC at 60°C were mixed with each of the doubly radiolabelled complexes, diluted to 10 ml with deionised water and freeze–dried overnight. Following controlled rehydration (Kirby and Gregoriadis, 1984) of the dried materials, multilamellar dehydration–rehydration vesicles (DRV) were formed. Non-entrapped material was separated from entrapped by diluting the suspensions in 0.15 M sodium phosphate buffered saline (PBS, pH 7.4) and centrifuging at $36\,000 \times g$ for 25 min, removing the supernatants, washing the pellets and centrifuging again. The final DRV pellets were suspended in 1 ml PBS.

2.4. Incorporation of DEX into liposomal bilayers

DEX (2 mg), with a known amount of [^{3}H]DEX, was dissolved with 32 μmol DSPC in chloroform/ethanol. Following evaporation of the dried film and its dispersion in 2 ml deionised water, SUV were prepared and these were used to generate DRV as described in Section 2.3. DEX incorporation into the bilayers was 46.9% of the amount used.

2.5. Assay of radioactivity

All measurements in the present study were based on radioactivity estimations. Drug entrapment values were calculated by mixing samples of pellets and supernatants with 4 ml Hisafe solution and assaying for ^{3}H (drugs) and/or ^{14}C (HPβ-CD) in a Wallac liquid scintillation counter programmed for the simultaneous analysis of both isotopes. Concentrations of drugs and HPβ-CD in urine, blood plasma and homogenised tissues were determined likewise.

2.6. In vitro stability of entrapped complexes in plasma

Fresh rat plasma (2 ml) obtained from male Wistar rats and warmed to 37°C was mixed with 0.4 ml of each of the three liposome-entrapped drug/HPβ-CD complexes and incubated at this temperature. Samples were taken after 2 min and 60 min, diluted to 5 ml with PBS and centrifuged at $36\,000 \times g$ for 25 min at 4°C. Samples from the supernatants and pellets were monitored for release of drugs and HPβ-CD into the media by ^{3}H and ^{14}C measurements, respectively.

2.7. Experiments in vivo

Randomised groups of male Wistar rats (8 per group, 125–150 g body weight) were injected into the tail-vein with: (a) 0.5 ml free drug/HPβ-CD complexes; (b) free HPβ-CD; (c) liposome-entrapped complexes and (d) liposome-entrapped HPβ-CD. One half of the rats per group were killed 30 min later while the remaining animals were kept in individual metabolic cages and urine collected over 24 h after which they were killed. Additional groups of rats were injected as above with liposome-entrapped DEX/HPβ-CD complexes and killed at 3, 7, 14 and 24 days, or with DEX incorporated into DSPC bilayers and killed 30 min and 24 h later. Blood was collected at death and tissues (liver, kidneys and spleen) were removed and homogenised individually in 0.1% Triton X-100 using a Polytron PT 3000 (Kinematica AG) homogeniser at speeds of up to 15 000 rpm. They were then made up to a known volume with deionised water. Urine, blood plasma and tissue samples were all assayed for ^{14}C and/or ^{3}H levels as above.

3. Results and discussion

3.1. Formation of inclusion complexes

DHEA ($M_w = 288.4$), RET ($M_w = 286.5$) and DEX ($M_w = 392.4$) were chosen as model drugs on the basis of their poor water solubility and because their size and structure predisposes them to fit into the cyclodextrin cavity. DHEA/HPβ-CD and DEX/HPβ-CD inclusion complexes were formed by mixing both components in a 1:1 molar ratio allowing in theory for one drug molecule to interact with one HPβ-CD molecule. Steroids in general, among the many lipophilic molecules known to form inclusion complexes, have a particular affinity for complexation (Pitha et al., 1983; Uekama and Otagiri, 1987) so rapid equimolar complexes would be expected. RET and HPβ-CD were mixed in a 1:2.5 molar ratio, preliminary experiments having indicated a lesser tendency towards 1:1 complexation. In practice, inclusion complexes in solution form equilibria in which free drug and empty cyclodextrin molecules may be present and where two or more cyclodextrin molecules may attach to other groups on the drug molecule. The final molar ratios in the complexes were 1:1.2 (DHEA), 1:4.5 (RET) and 1:1.8 (DEX), values which were consistent over many experiments.

3.2. Entrapment of inclusion complexes into liposomes

The phospholipid used was distearoyl phosphatidylcholine (DSPC), a choice based on previous experiments with a variety of different phospholipids (McCormack and Gregoriadis, 1994a). Due to its high gel-liquid-crystalline transition temperature (T_c) (56°C) DSPC produces rigid vesicles which appear to be more resistant to phospholipid sequestration by entrapped CDS than more fluid vesicles. Entrapped complexes were presumed to be intact when the ratio of

Table 1
Release of drugs (^{3}H) and HPβ-CD (^{14}C) from liposomes in the presence of plasma

Liposome-entrapped complex	Released radioactivity (%)			
	2 min		60 min	
	^{3}H	^{14}C	^{3}H	^{14}C
DHEA/HPβ-CD	30.7	1.2	56.2	0.6
Retinol/HPβ-CD	10.5	0.8	22.8	1.5
Dexamethasone/HPβ-CD[a]	2.8 ± 0.8	0.8 ± 0.1	11.0 ± 3.2	1.4 ± 0.2

Liposomes composed of DSPC and containing ^{3}H-drug/^{14}C-HPβ-CD inclusion complexes were incubated with rat blood plasma at 37°C. Values of released ^{3}H (drug) and ^{14}C (HPβ-CD) radioactivity are percentage of total in the incubated DRV preparations.
[a] Values given are from three different experiments (McCormack and Gregoriadis, 1996).

radioactivity measurements (in terms of percentage entrapment) for both isotopes approached unity.

Liposomes passively entrap solutes in their aqueous phase and in general increasing the concentration of solutes results in greater amounts being entrapped (Kirby and Gregoriadis, 1984). However, an essential step in the DRV method necessitates freeze–drying a mixture of SUV and solutes. This leads to vesicle fusion and the formation of larger liposomes. Thus, any compounds, such as sugars, present at appropriate concentrations, which interfere with the process of fusion through cryo-protection, could greatly reduce entrapment efficiency. This was minimised by limiting the cyclodextrin to lipid ratio and by increasing the volume of the suspension prior to freeze–drying (Kirby and Gregoriadis, 1984). Bearing in mind that excessive dilution of inclusion complexes leads to dissociation (Mesens and Putteman, 1993), results from experiments with varying concentrations of inclusion complexes and varying pre-dehydration volumes were optimal when 5–22 μmol HPβ-CD per 32 μmol DSPC with 5–15 ml range of dilution were used (results not shown). In the current work, 15–20 μmol HPβ-CD and 10 ml pre-dehydration volumes were adopted. Entrapment values based on radioactivity measurements (three preparations for each complex) were $24.2 \pm 5.2\%$ (DHEA/HPβ-CD), $19.0 \pm 1.3\%$ (RET/HPβ-CD) and $26.4 \pm 0.8\%$ (DEX/HPβ-CD) of the complex used.

3.3. Stability of liposome-entrapped complexes in the presence of plasma

When entrapped complexes were exposed to plasma, a degree of complex dissociation occurred with variable leakage of each of the three drugs into the media. Phospholipids can displace drugs from the CD cavity (Szejtli et al., 1986) to an extent that seems to depend upon the drug/CD stability constant. Displaced drugs can be accommodated in the lipid bilayers rendering them more fluid (Castelli et al., 1984) and permeable (Gregoriadis, 1995b) and it has been suggested (McCormack and Gregoriadis, 1994a) that displaced drugs localise at or near the vesicle surface to associate preferentially with plasma proteins. Of the entrapped complexed DHEA, 30% was found in the media after 2 min (Table 1) increasing to 56% by 60 min. Less RET was released after 2 min (10%) although this more than doubled after 60 min, while only 2.8% of DEX was released initially with a further small increase (to 11%) by 60 min. In each case, insignificant amounts of HPβ-CD were found (up to 1.5%) in the media even after 60 min indicating no vesicle destabilisation. This is in agreement with data from earlier work (McCormack and Gregoriadis, 1994a) with co-entrapped carboxyfluorescein, a marker for liposomal stability (Gregoriadis and Davis, 1979), which showed minimal release. The degree of drug dissociation from DSPC liposomes seems to indicate stability constants in the order DHEA < RET < DEX.

Table 2
Plasma levels of drugs and HPβ-CD after intravenous injection of free and liposome-entrapped inclusion complex solutions

Injected material	Free material (%)		Liposomal material (%)	
	^{3}H	^{14}C	^{3}H	^{14}C
DHEA/HPβ-CD complex	6.2 ± 0.5 (4)	8.3 ± 0.7 (4)	4.9 ± 1.3 (3)	0.4 ± 0.2 (3)
RET/HPβ-CD complex	16.3 ± 1.7 (4)	14.9 ± 1.3 (4)	1.3 ± 0.2 (4)	1.0 ± 1.0 (4)
DEX/HPβ-CD complex	9.9 ± 0.4 (4)	7.2 ± 0.8 (4)	1.0 ± 0.1 (4)	0.2 ± 0.0 (4)
Hpβ-CD	—	11.1 ± 1.2 (4)	—	0.6 ± 0.1 (4)

Rats were injected intravenously with free inclusion complexes of drugs with HPβ-CD, free HPβ-CD, liposome-entrapped complexes or entrapped HPβ-CD and killed 30 min later. Drugs and HPβ-CD were radiolabelled with ^{3}H and ^{14}C, respectively. Values are % $\pm$ S.D. of the injected radioactivity in total blood plasma. Blood volume was estimated as 7.4% of body weight with a heamatocrit value of 50%.
Numbers in parentheses denote number of animals used. Radioactivity values 24 h after injection were nil (McCormack and Gregoriadis, 1996).

3.4. In vivo fate of free and liposome-entrapped drug/HPβ-CD complexes

On intravenous injection of drug/HPβ-CD complexes, rapid dissociation occurs through: (a) dilution by the plasma proteins and extracellular fluids (Mesens and Putteman, 1993); (b) displacement of the drug by endogenous lipids, particularly cholesterol which has a high affinity for the CD cavity (Frijlink et al., 1991) and (c) competition between HPβ-CD and plasma proteins which will favour a drug/protein association (Pitha, 1993).

Thus, while CDS increase the bioavailability and enhance the pharmacological effects of complexed drugs, the pharmacokinetics of the drugs themselves are not changed by their combination with CDS as, once dissociated, they are cleared from the circulation and metabolised as free. Liposomes are taken up preferentially by the reticuloendothelial system (RES), particularly liver and spleen cells and, therefore, drugs in entrapped complexes would be expected to exhibit altered biodistribution and pharmacokinetics as they adopt the fate of their carrier.

Radioactivity values 30 min after injection of free complex suggested small amounts of drug (6–16%) and HPβ-CD (7–15% of the dose) present in the plasma indicating rapid clearance (Table 2) and values for both were nil after 24 h. In all cases, most (73–94%) of the HPβ-CD was, as expected (Frijlink et al., 1990), recovered in the urine after 24 h together with variable amounts of the three drugs (Fig. 1). In agreement with data of stability studies (Table 1), the extent of recovery of the drugs in urine was DHEA < RET < DEX reflecting the degree of complex dissociation for each of the drugs. Liposome-entrapped complexes were also cleared rapidly from the circulation with only 4.9% of (leaked) DHEA and trace amounts of RET and DEX found in plasma after 30 min (Table 2). However, although up to 25% of dissociated drugs were recovered in the urine over 24 h, only small quantities (6–13%) of HPβ-CD were present (Fig. 1). HPβ-CD, when given as such or entrapped, followed a fate that was similar to that of free and entrapped complexed cyclodextrin (Fig. 1).

Fig. 2 shows values for the free complexes in the liver 30 min after injection. Very low values for HPβ-CD confirm its quantitative clearance by the kidneys (Fig. 1) while the hepatic levels of each of the drugs (19–26% of the dose) were probably the result of complex dissociation in the blood and subsequent transport to the tissue via plasma proteins. Nil values for all three drugs in the liver after 24 h suggest complete catabolism and excretion of metabolites. A very different result is seen in Fig. 3. Within 30 min post injection, a large proportion of the entrapped complexes are found in the liver, presumably transported to the tissue via liposomes. The high values for HPβ-CD (61–82% of the dose) indicate that liposomes can preempt its total excretion into

the urine and curtail complexed drug dissociation. The extent of this dissociation appears to be directly related to the stability constant of the complex, as the pattern observed in the in vitro stability studies (Table 1) is also reflected here, i.e. DHEA < RET < DEX. It is surmised that the entrapped complexes were taken up by the liver within minutes of injection allowing little time for dissociation to occur in the circulation. Once in the liver, liposomes are known (Gregoriadis, 1995a) to disintegrate thus releasing HPβ-CD with drug still complexed. The amount of the complexed entity appears to be dependent on its stability constant, for instance, only 33% DHEA is found in the liver after 30 min compared to

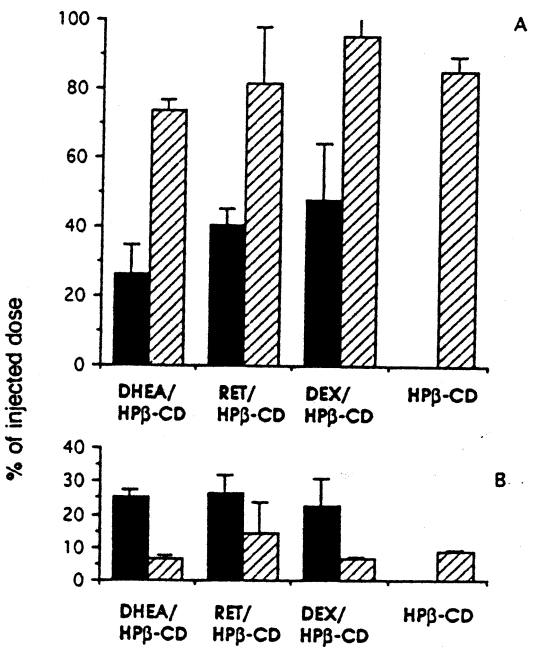

Fig. 1. Drug (^{3}H) and HPβ-CD (^{14}C) radioactivity in 24 h urine collections from rats injected with free (A) or liposome-entrapped (B) complex solutions or HPβ-CD. Rats in groups of four were injected with free complexes of [^{14}C]HPβ-CD (0.6–1.96 mg) with DHEA (0.52 mg), retinol (0.12 mg) or dexamethasone (0.12 mg), liposome (3.5 mg DSPC)-entrapped complexes of [^{14}C]HPβ-CD (0.20–1.00 mg) with DHEA (0.13 mg), retinol (0.20 mg) or dexamethasone (0.10 mg), free HPβ-CD (0.60 mg) or liposome-entrapped HPβ-CD (0.24 mg). Range of ^{3}H and ^{14}C dpm injected per animal was 0.20–1.75×10^6 and $0.10–1.0 \times 10^6$, respectively. Values are expressed as % ± S.D. of injected radioactivity. For other details see Section 2.

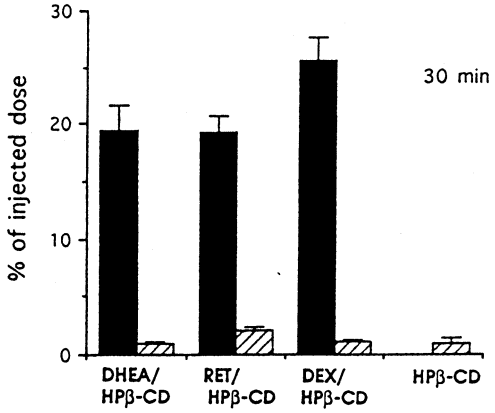

Fig. 2. Hepatic levels of drug (^{3}H) (filled bars) and HPβ-CD (^{14}C) (shaded bars) radioactivity 30 min after intravenous injection of doubly radiolabelled drug/HPβ-CD inclusion complexes or free [^{14}C]HPβ-CD. Values are % ± S.D. of injected dose in total liver. Drug radioactivity values in the liver of similarly treated rats at 24 h were nil. For other details see the legend to Fig. 1 and Section 2.

53% of DEX. In view of the small amount (4.9%) of DHEA recovered in plasma after 30 min (Table 2), it is possible that a portion of the hepatic DHEA (33%) was previously protein-bound (following its dissociation from liposomes) or bilayer-associated as the drug value in the liver was nil after 24 h (Fig. 3). It is likely that some DHEA reached the liver still complexed but was gradually dissociated intracellularly. This could also apply to RET albeit to a lesser extent as its value in the liver after 24 h is half its 30 min value (cf. stability studies, Table 1). Hepatic DEX found after 30 min (53% of the dose) was probably all complexed as levels decreased to only 51% after 24 h.

On examination of the fate of liposome-entrapped DEX/HPβ-CD and HPβ-CD over days (Table 3) it seems that DEX is steadily dissociating from HPβ-CD in situ (liver) and only then being broken down, so that after 14 days 4.6–6.4% of the injected drug remains complexed. HPβ-CD is also being degraded, albeit more slowly. From a value of 74.3 at 24 h, 36–38% remains after 14 days, declining to about 21% after 24 days. As Coussement et al. (1990) have reported no toxicity associated with HPβ-CD, this gradual biodegradation over weeks would

appear to be acceptable. Table 4 gives the values of both DEX and HPβ-CD in the spleen. These are in agreement with the proportionately lesser amounts expected from splenic uptake of liposomes, and DEX is shown to dissociate from HPβ-CD at a rate that parallels that of hepatic DEX, although HPβ-CD disappears more rapidly.

Although all three drugs can be processed rapidly by the liver when present in the free form (see Fig. 2 and legend), their rate of metabolism by the tissues as entrapped inclusion complexes appears to also depend on the rate with which drugs dissociate from the complexes. It is conceivable on the other hand, that (a) the slow removal

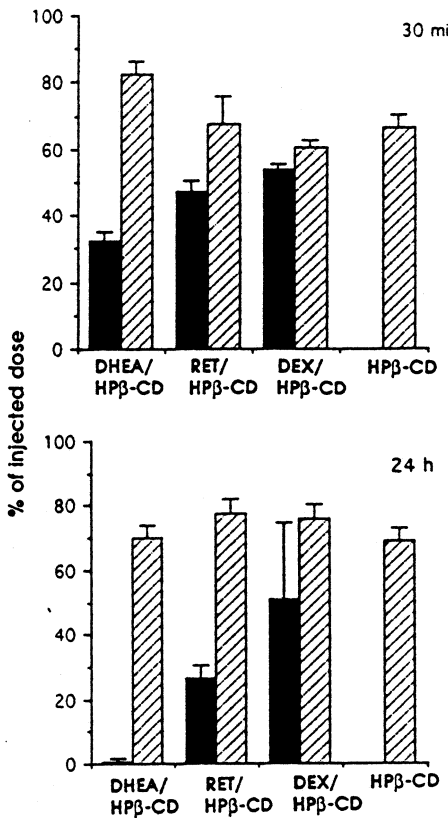

Fig. 3. Hepatic levels of drug (^{3}H) (filled bars) and HPβ-CD (^{14}C) (shaded bars) radioactivity 30 min and 24 h after intravenous injection of liposome-entrapped doubly radiolabelled drug/HPβ-CD inclusion complexes or liposome-entrapped [^{14}C]HPβ-CD. Values are % ± S.D. of injected dose in total liver. For other details see the legend to Fig. 1 and Section 2.

Table 3
Hepatic and splenic values of DEX (^{3}H) and HPβ-CD (^{14}C) at time intervals after intravenous injection of liposome-entrapped doubly radiolabelled DEX/HPβ-CD inclusion complex

Time (days)	Liver		Spleen	
	^{3}H	^{14}C	^{3}H	^{14}C
1	51.1 ± 4.3	74.3 ± 3.1	4.9 ± 0.90	8.5 ± 1.1
3	20.0 ± 2.1	64.4 ± 3.2	1.6 ± 0.20	4.9 ± 0.9
7	8.6 ± 1.2	44.5 ± 2.3	0.7 ± 0.10	2.1 ± 0.3
14	4.6, 6.4	36.4, 38.2	0.4, 0.5	1.2, 1.9

Rats in groups of four (two animals for day 14) were injected intravenously with liposome (4 mg DSPC)-entrapped [^{3}H]DEX/[^{14}C]HPβ-CD complex (0.11 mg DEX, 0.6 mg HPβ-CD; $0.9–1.2 \times 10^6$ ^{3}H or ^{14}C dpm) and killed at time intervals. Values in total liver and spleen are % ± S.D. of injected radioactivity. For other details see legend to Fig. 1. HPβ-CD (^{14}C radioactivity) values in two rats at 24 days were 21.6 and 20.3% (liver) and 1.2% (spleen) (McCormack and Gregoriadis, 1996).

of DEX from tissues does not entirely reflect its rate of dissociation from HPβ-CD but also a slow rate of liposome disintegration in situ. Such disintegration, whilst apparently not crucial for DHEA (and to some extent RET) which appears to escape from intact liposomes (Table 1), may be a prerequisite for DEX dissociation and processing of the drug's metabolism to commence; (b) DEX is dissociated more rapidly than it is assumed here but its rate of metabolism and/or excretion from the tissues is slower than for DHEA and RET. The latter notion does not necessarily contradict findings of complete drug (including DEX) removal from the liver by 24 h of injection of the free complexes (Fig. 2 and legend) as the mode of drug uptake by, and cellular and intracellular location in, the tissues for the free and entrapped complexes are likely to differ. As alluded to earlier, drugs dissociate from free complexes and reach the tissues as such, probably via proteins whereas liposome-entrapped complexes are known (McCormack and Gregoriadis, unpublished observations) to end up in the lysosomal apparatus following endocytosis of the carrier (Gregoriadis, 1995a). Both possibilities were examined using similar liposomes incorporating

Table 4
Drug (^{3}H) and HPβ-CD (^{14}C) radioactivity in the spleen of rats injected with free or liposome-entrapped drug/HPβ-CD complexes or HPβ-CD alone

Injected material	Time (h)	DHEA/HPβ-CD		RET/HPβ-CD	DEX/HPβ-CD	Hpβ-CD		
		^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{14}C
Free	0.5	0.10 ± 0.00	0.1 ± 0.00	0.95 ± 0.18	0.35 ± 0.10	0.5 ± 0.21	0.18 ± 0.05	0.18 ± 0.05
	24	0.08 ± 0.03	0.08 ± 0.0	0.18 ± 0.10	0.07 ± 0.10	0.15 ± 0.06	0.13 ± 0.05	0.14 ± 0.05
Entrapped	0.5	2.00 ± 0.22	9.53 ± 1.92	8.23 ± 1.68	12.93 ± 2.04	5.48 ± 0.80	9.88 ± 0.86	6.25 ± 0.70
	24	0.25 ± 0.19	6.85 ± 1.16	4.48 ± 1.12	10.80 ± 0.59	5.15 ± 2.13	9.03 ± 2.80	4.63 ± 1.20

Rats in groups of four were injected intravenously with free or liposome-entrapped ^{3}H drug/^{14}C HPβ-CD complexes or [^{14}C]HPβ-CD and killed 0.5 or 24 h later. Values are % ± S.D. of injected radioactivity per whole spleen. For other details see legend to Fig. 1 (McCormack and Gregoriadis, 1996).

DEX in their bilayers and expected (Gregoriadis, 1995a) to transport the drug to the lysosomes of the liver and spleen. Results (Table 5) indicate that, in contrast to the liposome-entrapped DEX/HPβ-CD complex where hepatic (Fig. 3) and splenic (Table 4) drug values remained virtually unchanged 24 h post injection, corresponding values for DEX incorporated as such in the bilayers of liposomes were reduced by about 70 (liver) and 80% (spleen). It thus appears that slow complex dissociation rather than rate of vesicle disintegration or rate of DEX removal from the liver and spleen accounts for much of the high DEX content of these tissues following the initial 24 h period in rats injected with the liposome-entrapped complex.

Table 5
Hepatic and splenic values of DEX (^{3}H) radioactivity in rats injected with liposomes incorporating DEX in their bilayers

Time (h)	Liver	Spleen
0.5	48.1 ± 5.2	6.8 ± 1.3
24	15.5 ± 1.5	1.4 ± 0.4

Rats in groups of three were injected intravenously with liposomes (4 mg DSPC) incorporating [^{3}H]DEX (0.15 mg; 0.5×10^6 dpm) in their bilayers and killed at time intervals. Values are % ± S.D. of the dose in total liver and spleen (McCormack and Gregoriadis, 1996).

4. Conclusions

While recognising the importance of HPβ-CD as an effective solubiliser of many poorly water-soluble drugs and its superiority as a safe and efficient carrier in parenteral applications, entrapping drug/HPβ-CD complexes into liposomes (thus providing an additional versatile carrier) must be considered a further progressive development. As drug/HPβ-CD complexes dissociate rapidly in the circulation, the pharmacokinetics of drugs is expected to be the same as that of free drugs. On the other hand, entrapping these drug complexes into liposomes curtails their dissociation and contributes to altered pharmacokinetics. The degree of this change of pharmacokinetics seems to depend on the stability constant of the complex. For instance, DHEA which appears to dissociate easily from HPβ-CD, is completely catabolised after 24 h whether given as a free complex, incorporated into liposomal bilayers (McCormack and Gregoriadis, unpublished observations) or as a liposome-entrapped complex. DEX, on the other hand, which has a high stability constant, concentrates in liver and spleen when given as an entrapped complex and is metabolised at a rate dependent upon its dissociation from HPβ-CD: only that portion of the drug which is not included in the cyclodextrin cavity is available for processing (Frömming and Szejtli, 1994). In other words, a slow release of drugs from HPβ-CD (as a result of a high stability constant)

prolongs their presence and consequently their pharmacological action in situ. This appears to be a significant advantage that entrapped complexed drugs have over drugs incorporated into liposomes as such: the biodistribution is similar but the rate of metabolism for drugs in entrapped complexes is directly related to their rate of dissociation from cyclodextrins following vesicle disintegration. In contrast, the metabolism of bilayer-included drugs is dependent on liposome disintegration, the rate of which is expected to be common to all drugs.

Although liposomes are a useful vehicle for the parenteral delivery of hydrophobic drugs, some of these may be incompatible with vesicle formation or can be accommodated in the bilayers only in limited amounts. Increasing the lipid load in order to incorporate sufficient drug for adequate therapeutic efficacy may not be acceptable, particularly with chronic use. Entrapping such drugs complexed to cyclodextrins in the aqueous phase of liposomes could overcome these problems. Moreover, the present approach could be a means to control the duration of drug action in situ in cases where the dissociation constants of cyclodextrin-drug complexes can be tailored appropriately.

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