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Non-surfactant nanospheres of progesterone inclusion complexes with amphiphilic β-cyclodextrins

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Abstract

Amphiphilic β-cyclodextrins were formulated as nanospheres and characterised by particle size, zeta potential and TEM following freeze-fracture. The nanospheres were loaded with progesterone with different loading techniques involving the spontaneous formation of nanospheres from pre-formed inclusion complexes of amphiphilic β-cyclodextrins modified on the primary or secondary face with progesterone. Inclusion complexes were characterised with various techniques including Differential Scanning Calorimetry (DSC), Fast Atom Bombardment Mass Spectrometry (FAB MS) and ^{1}H NMR spectroscopy; and progesterone was believed to be partially included in the CD cavity. Loading properties of conventionally-loaded nanospheres were compared with those prepared directly from pre-formed inclusion complexes and loading technique was found to enhance associated drug percentage significantly ($P < 0.05$). Although both amphiphilic β-cyclodextrins (6-*N*-CAPRO-β-CD and β-CDC6) were capable of high progesterone loading, β-CDC6 displayed slightly higher entrapment efficiency due to the possible higher affinity of progesterone to the 14 alkyl chains surrounding this molecule resulting in higher drug adsorption to particle surface. Progesterone was released within a period of 1 h from all formulations. Progesterone-loaded amphiphilic β-CD nanospheres were proved to be a promising non-surfactant injectable delivery system providing high-quantity of water-insoluble progesterone rapidly within 1 h.

Keywords: Amphiphilic β-cyclodextrin; Nanosphere; Inclusion complex; Progesterone; Loading capacity; Drug release

1. Introduction

Amphiphilic β-cyclodextrins are acylated derivatives of β-cyclodextrin on primary or secondary face in order to obtain self-organising molecules to

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form nanoparticulate systems (Duchene et al., 1999). These derivatives render the hydrophilic surface of the natural cyclodextrins an amphiphilic property, which may increase their contact with biological membranes. Amphiphilic cyclodextrins are obtained by the grafting of acyl chains of various length (C2–C18), structure (linear or branched) with various bond types (ester, ether, amide). Recently, amphiphilic β- and γ-cyclodextrins were reported to give nanocapsules and nanospheres with or without surfactants encapsulating a series of drug molecules (Skiba et al., 1996, 1995; Lemos-Senna et al., 1998).

Progesterone was used as the model drug due to its various characteristics among which its poor aqueous solubility (3.79×10^{-3} M) is the most important. It is a drug that has been complexed to various CD derivatives forming 1:1 or 1:2 drug:CD complexes to consequently achieve an improvement in its solubility (Uekama et al., 1998; Liu et al., 1990). Progesterone:CD complexes were also administered by incorporation into drug delivery systems such as microspheres and solid lipid nanospheres (Cavalli et al., 1999; Monza de Silveira et al., 1998).

Nanoparticles are stable systems that are suitable for drug targeting and bioavailability enhancement of poorly soluble drugs. However, when prepared by conventional methods, drug amount associated to a unit mass of polymer (entrapment efficiency) is often limited, particularly with weakly soluble drugs. This leads to excessive administration of polymeric material limiting their safety and efficacy. Amphiphilic β-CD nanospheres and nanocapsules provide an alternative since they are biodegradable, non-surfactant and display very low haemolytic activity (Wouessidjewe et al., 1996; Memişoğlu et al., 2000, 2002).

The purpose of this work was to develop and characterise a non-surfactant, high-loaded nanoparticulate system for the parenteral administration of progesterone. To improve loading properties of nanospheres, different loading techniques were compared with conventional techniques. Influence of preparation technique on progesterone loading and release was also aimed to evaluate.

2. Materials and methods

2.1. Materials

Progesterone depicted in Fig. 1 was purchased from Fluka Chemie (Germany). Amphiphilic β-cyclodextrins, 6-*N*-CAPRO-β-CD and β-CDC6, represented in Fig. 1 were synthesised and characterised as described previously (Lesieur et al., 2000; Memişoğlu et al., 2000; Ringard-Lefebvre et al., 2002; Memişoğlu et al., 2002). Acetonitrile was of HPLC grade (Carlo Erba, Italy) and all other reagents were of analytical grade and were used without further purification.

2.2. Methods

2.2.1. Preparation of progesterone:amphiphilic β-CD inclusion complexes

Progesterone:amphiphilic β-CD inclusion complexes of 1:2 molar ratio were prepared in a water/ethanol system by co-lyophilisation technique as described previously (Hedges, 1998). Progesterone

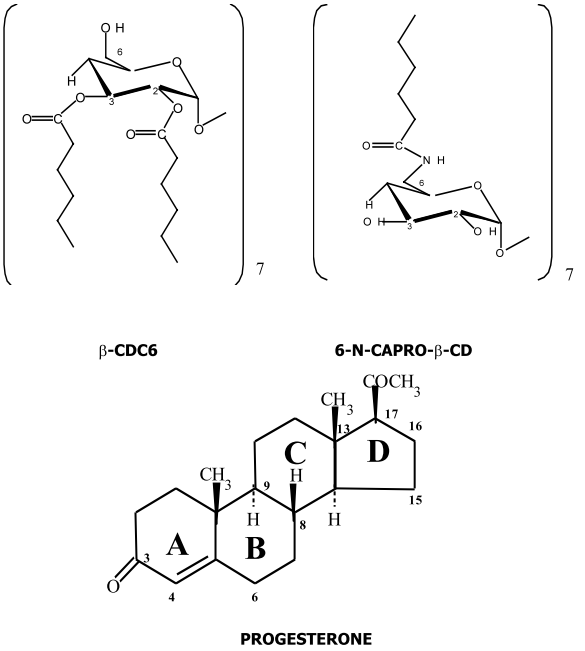


Fig. 1. Model drug; progesterone and amphiphilic β-cyclodextrins modified on the primary or secondary face; β-CDC6 and 6-*N*-CAPRO-β-CD.

and corresponding amphiphilic β-CD (6-*N*-CAPRO-β-CD or β-CDC6) were dissolved in appropriate molar ratio in ethanol and added to equal volume of water. The system was left to equilibrate under constant stirring for 7 days at room temperature. Organic solvent was evaporated under vacuum and aqueous suspension was lyophilised and stored as powder.

2.2.2. Characterisation of inclusion complexes

2.2.2.1. Thermal analysis. Differential Scanning Calorimetry (DSC) was used in order to evaluate physicochemical state of progesterone within 1:2 molar ratio (drug:CD) complexes. DSC thermograms were taken with a DuPont DSC 910 Instrument (DuPont, USA) in a temperature range of 25–200 °C under nitrogen atmosphere. Samples were heated in hermetically sealed aluminium pans at a rate of 10 °C/min.

2.2.2.2. Mass spectrometry. Fast Atom Bombardment Mass Spectrometry (FAB MS) was performed on progesterone and 1:2 drug:CD complexes. FAB MS spectra were taken in positive ion detection mode using Micromass ZabSpec using the FAB/LSIMS technique with Cs ion gun at an anode voltage of 25 kV and *m*-nitrobenzylalcohol as matrix.

2.2.2.3. ^1H NMR spectroscopy. ^{1}H NMR spectra of progesterone and its inclusion complexes with 6-*N*-CAPRO-β-CD and β-CDC6 were analysed with a Brüker DPX 400 Digital FT-NMR spectrophotometer. Chemical shifts are given to external tetramethylsilane at 0 ppm with calibration using solvent signals (DMSO 2.5 ppm, HDO 4.75 ppm, $CDCl_3$ 7.25 ppm).

2.2.3. Formulation of nanospheres

2.2.3.1. Particle size distribution. Effect of molar amphiphilic β-cyclodextrin concentration on nanosphere particle size distribution was evaluated. A concentration range of 0.05–1 mM were used in order to determine the effect of amphiphilic β-CD concentration which is in fact the only component of the nanoparticulate delivery system. Mean diameter and polydispersity index were determined with Coulter N4 Plus (Coultronics, France) at 25 °C with an angle of 90°. Measurements were carried out on samples with intensity between 10^4 and 10^6 cps and were presented as the average of three measures.

2.2.3.2. Zeta potential. Zeta potential (mV) of unloaded nanospheres were measured by Malvern Mastersizer (Malvern Inst., UK) in triplicate in order to elucidate the surface charge and the potential stability of the system.

2.2.3.3. Transmission electron microscopy after freeze-fracture. Freeze-fracture electron microscopy was used to examine the suspension of amphiphilic β-cyclodextrin nanosphere samples: a drop of suspension containing 30% glycerol as a cryoprotectant was frozen in liquid propane, which was cooled with liquid nitrogen at −189 °C. Fracture process was performed with a Balzers BAF 400 T (Balzers, Liechtenstein) at −150 °C and 1.8 mbar of vacuum. The samples were then covered by platinum and carbon (1.9 nm each) unidirectionally. The washing of the samples was carried out in a system of water, ethanol, chloroform and methanol. The replicas were examined and photographed using a LEO 912 transmission electron microscope.

2.2.4. Preparation and loading of amphiphilic β-CD nanospheres

Nanospheres were prepared using the method of nanoprecipitation (Fessi et al., 1988) with modifications on loading technique. Organic phase (1 ml) consisting of 1 mg of amphiphilic β-cyclodextrin (β-CDC6 or 6-*N*-CAPRO-β-CD) or amphiphilic β-CD:progesterone inclusion complex (1:1 or 1:2 molar ratio) dissolved in acetone or ethanol for β-CDC6 and 6-*N*-CAPRO-β-CD, respectively, was added under constant stirring to 2 ml of aqueous phase consisting only of demonised water. Ratio of organic phase to aqueous phase was kept to 1:2 in all formulations.

After stirring for 1 h at room temperature, organic solvent was evaporated under vacuum and the nanosphere dispersion concentrated to the desired volume; which was 1 ml for this study.

Nanospheres of 6-*N*-CAPRO-β-CD (MW: 1812 g/mol) or β-CDC6 (MW: 2596 g/mol) were loaded with model drug Progesterone (MW: 314.4 g/mol)) according to the following methods;

- Pre-loaded nanospheres: nanospheres were prepared directly from drug:CD (1:2 molar ratio) complexes. No further loading was performed.
- Conventionally-loaded nanospheres: nanospheres were prepared from amphiphilic β-CDs only, according to nanoprecipitation technique as described before and were loaded by the addition of 200 μg of progesterone to the organic phase during preparation.
- Highly-loaded nanospheres: nanospheres, which were prepared from pre-formed complexes as described above in the first technique, were overloaded during preparation by dissolving an additional amount of drug (200 μg) in the organic phase.

Free drug in the nanosphere dispersions were separated by centrifugation at 5000 rpm for 10 min. Supernatant was then ultracentrifuged at $120\,000 \times g$ at 25 °C for 1 h by a Sorvall RC28S with fix rotor type S20/20 (DuPont). The precipitate was then lyophilised. Resulting powder was dissolved in ethanol and analysed with an HPLC method using Hewlett–Packard Agilent 1100 apparatus with HPCHEMSTATION software, Bondapak C18 column 300 × 4.6 mm, 5 μM (Interchim, France), mobile phase of acetonitrile/water (70/30 v/v) and a UV detector set at 240 nm with a flow rate of 1 ml/min.

Loading capacity was expressed in terms of entrapped drug quantity, entrapment efficiency and associated drug percentage to give a better outline of the loading capacities of amphiphilic β-CD nanospheres. Entrapped drug quantity is the determined drug quantity in nanosphere dispersion after the elimination of unbound drug by centrifugation.

Entrapment efficiency was calculated according to the following equation;

$$\text{Entrapment efficiency} = \frac{\text{Determined drug quantity (μg)}}{\text{Initial CD quantity (mg)}}$$

Associated drug percentage was also calculated according to the following equation;

$$\text{Associated drug \%} = 100 \times \frac{\text{Determined drug quantity (μg)}}{\text{Initial drug quantity (μg)}}$$

2.2.5. *In vitro release kinetics of progesterone from amphiphilic β-CD nanospheres*

Release kinetics of progesterone from amphiphilic β-CDs were determined after incubation (37 °C) in two different release media; Phosphate Buffered Saline (PBS) pH 7,4 and water:PEG 400 (60:40) providing sink conditions in a thermostated shaker bath system (Memmert, Germany). At given time intervals, 2 ml samples were withdrawn from the system and replaced with equal volume of fresh release medium. Samples were ultracentrifuged at $120\,000 \times g$ for 1 h (SORVALL RC 28S, DuPont) and the supernatant was analysed for free drug with an analytically validated HPLC technique using the above described conditions ($r = 0.99965$).

2.3. *Statistical analysis*

Entrapment data were analysed for significant differences by one-way ANOVA and differences between groups were further analysed by Tukey test.

3. Results and discussion

3.1. *Characterisation of progesterone:amphiphilic β-cyclodextrin inclusion complexes*

Existence and nature of drug:CD complexes have been demonstrated by DSC, FAB MS and ^{1}H NMR spectroscopy. DSC is a technique grequently used to characterise drug:CD inclusion complexes. In Fig. 2, the disappearance of melting endotherm observed at 125 °C for progesterone in complexed form indicates the existence of a complex and the absence of crystalline progetserone in free form. In order to avoid the fact that

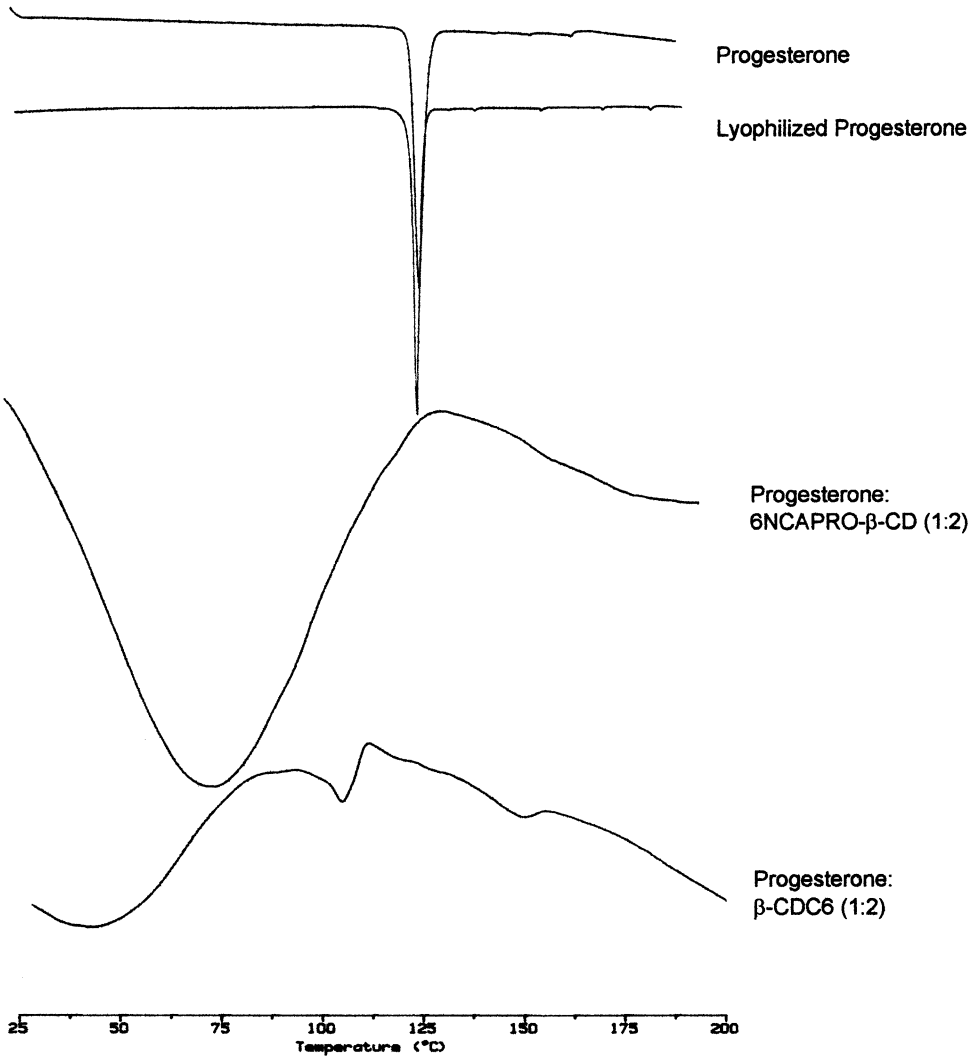


Fig. 2. DSC thermograms of inclusion complexes of amphiphilic β-cyclodextrins with progesterone.

some drugs display dramatic changes in their DSC thermograms after lyophilisation since they pass from crystalline to amorphous state, progesterone alone was subject to all the stages of co-lyophilisation process. It was observed that progesterone melting endotherm is not deplaced, reduced or masked by lyophilisation.

FAB MS was further used for the confirmation of existence of drug:CD complexes. Table 1 displays the principal peaks for progesterone, amphiphilic β-CDs and complexes. As seen in table, progesterone displays its molecular ion peaks at 315 and at 629 m/z. FAB Mass Spectrum of progesterone:β-CDC6 complex, however, displays an uncomplexed CD peak at 2530 m/z and an additional peak at 2640 m/z. Same phenomenon occurred also for progesterone:6-*N*-CAPRO-β-CD complex (Table 2). It can be proposed that three phenyl units comprising the A, B and C rings seen in Fig. 1 are left outside the CD cavity since the cavity is large enough to include only one aromatic ring. In this case, the D-ring and the ethanone moiety is included in the CD molecule. The mass spectra of both complexes confirm this

Table 1
Principal peaks of progesterone, amphiphilic β-CDs and their complexes (DS: substitution degree for over- or underacylated CD derivatives)

Progesterone		6-*N*-CAPRO-β-CD		β-CDC6		Progesterone: 6-*N*-CAPRO-β-CD complex		Progesterone: β-CDC6 complex	
Peak (*m*/*z*)	Interpretation	Peak (*m*/*z*)	Interpretation	Peak (*m*/*z*)	Interpretation	Peak (*m*/*z*)	Interpretation	Peak (*m*/*z*)	Interpretation
315	$[M+H]^+$	1838	$[M+Na+H]^{++}$	2628	[M+Na+H] DS: 15	1836	$[6\text{-}N\text{-CAPRO-β-CD}+Na]^+$	2530	$[β\text{-CDC6}+Na]^+$
629	$[2M+H]^+$	1815	$[M+2H]^{++}$	2531	[M+Na+H]	1946	$[6\text{-}N\text{-CAPRO-β-CD-}C_7H_{10}O+Na]^+$	2640	$[β\text{-CDC6-}C_7H_{10}O+Na]^+$
				2433	[M+Na+H] DS:13				
				2335	[M+Na+H] DS:12				
				2237	[M+Na+H] DS:11				

Table 2
Particle size distribution and zeta potential of amphiphilic β-cyclodextrin nanospheres ($n=3$)

Nanosphere characteristics	Pre-loaded 6-*N*-CAPRO-β-CD nanospheres	Pre-loaded β-CDC6 nanospheres
Particle size (nm) ± S.D.	115 ± 16	130 ± 35
Polydispersity index	0.08	0.07
Zeta potential (mV)	−27.6	−31.6

fact by displaying ion peaks at $[CD\text{-}C_7H_{10}O+Na]^+$ corresponding to D-ring molecular weight added to amphiphilic β-CD. A, B, C rings may be fragmented due to electron bombardment but this needs to be further investigated. FAB MS seems not to be sufficient to totally elucidate the structure of the inclusion complex so H NMR spectroscopy at 400 MHz was also realised.

It is noteworthy that H NMR is a promising tool for the characterisation of complexes and demonstration of total or partial inclusion in CD cavity, which occurs, in a liquid medium. In the case of amphiphilic β-CDs, it is important to demonstrate if the drug is included in the cavity or entrapped within the long aliphatic chains of the molecule. During complexation, the chemical environment of some protons changes and this results in changes in chemical shifts of H NMR lines of the protons that are due to shielding or deshielding effects. Specifically, the cyclodextrin internal protons H-3 and H-5 and the protons of the guest are the most affected (Djedaini and Perly, 1991; Loukas, 1997; Roselli et al., 1999).

For the progestserone:β-CDC6 complex, internal protons H-3 and H-5 were observed for changes in NMR spectra. H-5 shifted from 5.0 to 4.85 ppm but H-3 did not display any shifts and gave proton signals at 4.70 ppm both in free and complex form. Progestserone:6-*N*-CAPRO-β-CD complexd also displayed shifts in CD proton signals. Internal CD proton H-5 signal shifted from 5.90 to 5.15 ppm in complexed from.

Progesterone protons were also evaluated for signal shifts. D-ring protons H-13, H-15, H-16 and 3H for $-COCH_3$ group showed significant upfield

shifts probably due to anisotropic effects of the double bonds and carbonyl groups of the host molecules. It should also be noted that NH proton signal of 6-*N*-CAPRO-β-CD shifted from 8 to 6.6 ppm which may suggest that drug molecule that is included in the CD molecule is mostly close to the primary face and the aliphatic chains grafted to this side of the molecule.

3.2. Characterisation of amphiphilic β-CD nanospheres

Fig. 3a represents the mean diameter versus molar concentration profiles of amphiphilic β-cyclodextrins (Memişoğlu et al., 2002; Ringard-Lefebvre et al., 2002). β-CDC6 is modified on the secondary face with 6C aliphatic esters while 6-*N*-CAPRO-β-CD is modified on the primary face with 6C aliphatic chain linked with an amide bond, both depicted in Fig. 1. It can be observed that 6-*N*-CAPRO-β-CD gives nanospheres of mean diameter around 300–400 nm within a wide concentration range. Self-alignment of this molecule is possible at high concentrations. However, for β-CDC6, formation of large aggregates is inevitable at concentrations higher than 0.6 mM. This confirms previous findings on the molecular areas of β-CDC6 and 6-*N*-CAPRO-β-CD (Ringard-Lefebvre et al., 2002). It was proved that β-CDC6 has a molecular area of 370 $Å^2$ while 6-*N*-CAPRO-β-CD occupies a much less area of 218 $Å^2$ which explains the fact that 6-*N*-CAPRO-β-CD is capable of molecular packing at interfaces at higher concentrations than β-CDC6.

Fig. 3b on the other hand represents polydispersity index versus molar concentration profile demonstrating that very low concentrations of amphiphilic β-CD is not sufficient to form homogeneous nanosphere dispersions since polydispersity index values are quite high. With increase in concentration, polydispersity index is reduced and reaches a minimum at around 0.6 mM for 6-*N*-CAPRO-β-CD and 0.4 mM for β-CDC6 which were chosen as optimum concentrations for the future nanosphere formulations.

Freeze-fracture photomicrogaphs show spherical, smooth-surfaced, regular nanospheric structures seen in Fig. 4 confirming also the particle size of amphiphilic β-CD nanospheres.

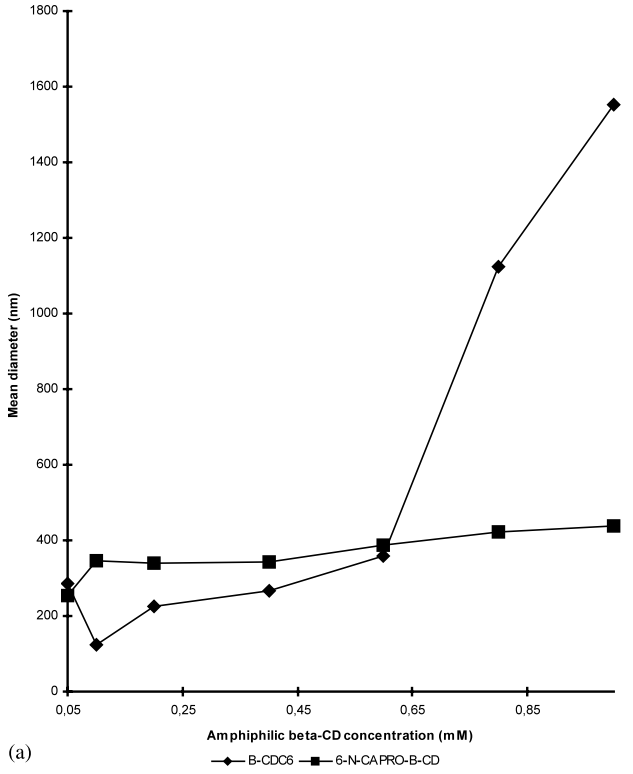
Amphiphilic β-cyclodextrins prepared from progesterone:amphiphilic β-CD inclusion complexes gave nanospheres of appropriate particle size distribution and negative surface charge presented in Table 2. Negative charge at the surface indicates that the molecular aligning of the amphiphilic β-CDs are such that the unsubstitued –OH groups are pointing towards the aqueous surrounding rendering a potential surface hydrophilicity.

Loading properties of amphiphilic β-CD nanospheres were believed to be affected by the following parameters; loading technique and amphiphilic β-CD type. Table 3 displays the entrapment efficiency and drug association properties of the nanospheres prepared with different loading techniques and with different amphiphilic β-CDs.

Amphiphilic β-CD type played a considerable role on loading characteristics. For pre-loaded nanospheres, 6-*N*-CAPRO-β-CD had higher entrapment values. Since drug loading depends mostly on drug included in the cavity for the pre-loading technique, these data suggest a higher inclusion capacity for 6-*N*-CAPRO-β-CD. This confirms the suggestion (Thompson, 1997) stating that modifications on position 6- (primary face) will widen the CD cavity while modifications at 2- and 3 will cause the contrary.

However, when highly-loaded and conventionally-loaded nanospheres are concerned, β-CDC6 is the CD that entraps significantly higher ($P < 0.05$) drug quantities. In these two techniques, nanospheres are loaded with excess amount of drug during preparation. It is highly probable that although a small part of the drug enters the CD cavity, a larger quantity interacts with long aliphatic chain and are entrapped within them. Since aliphatic chain number is 2-fold for β-CDC6, it is possible to entrap more drug molecules into β-CDC6 nanospheres using conventional or high-loading techniques.

A nanoparticle system with maximal drug loading and high entrapment efficiency will reduce the quantity of carrier required for the administration of sufficient amount of active ingredient to the target site as well as drug wastage during manu



(a)

Fig. 3. (a) Mean diameter (nm) vs. amphiphilic β-cyclodextrin concentration in nanospheres ($n = 3$). (b) Polydispersity index vs. amphiphilic β-cyclodextrin concentration in nanospheres ($n = 3$).

facturing. Mainly water-insoluble drugs have been incorporated into nanoparticles using nanoprecipitation technique with typical drug contents being under 5% (Govender et al., 1999). It is also evident that drug: carrier ratio is not more than 10% in conventional nanospheres (Magenheim and Benita, 1991; Couvreur et al., 1996).

Progesterone release behaviour dependent on formulation type is represented in Fig. 5. Conventional and high-loaded nanospheres are loaded with the addition of excess amount of drug solution containing 200 μg of progesterone. It is believed that progesterone introduced at this stage of preparation is largely adsorbed on the particle surface and the alkyl chains of the amphiphilic β-CD molecule. It is evident that drug entrapped in this way will be rapidly released as a function of diffusion to the surrounding medium. This immediate release results from a number of factors including the large surface area of nanospheres (Magenheim and Benita, 1991; Couvreur et al., 1996).

It has been previously reported that conventionally-loaded amphiphilic γ-CD nanospheres studied by Lemos-Senna et al. (1998)entrap drug molecules by adsorption onto particle surface. However, for nanospheres consisting of pre-formed inclusion complexes, drug entrapped in the CD cavity is also released by time dependent mechanisms such as dissociation upon dilution and competitive displacement of drug by buffer constituents and by the effect of solubiliser PEG 400 present in one of the release media. High-loaded nanospheres release the drug adsorbed on the surface immediately but continue the release profile for an additional 1 h by liberating drug entrapped in CD cavity. Differences in release values are significant ($P < 0.05$).

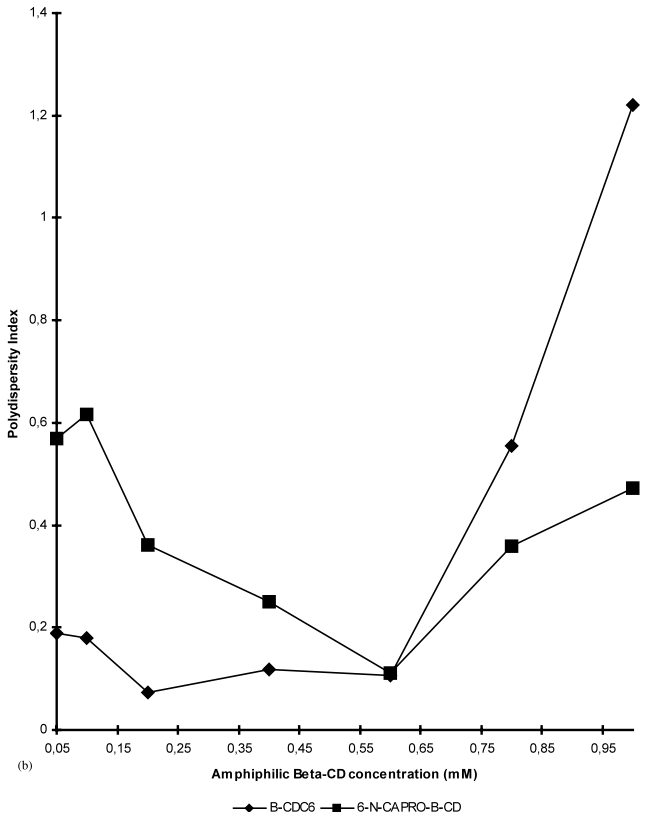


Fig. 3 (*Continued*)

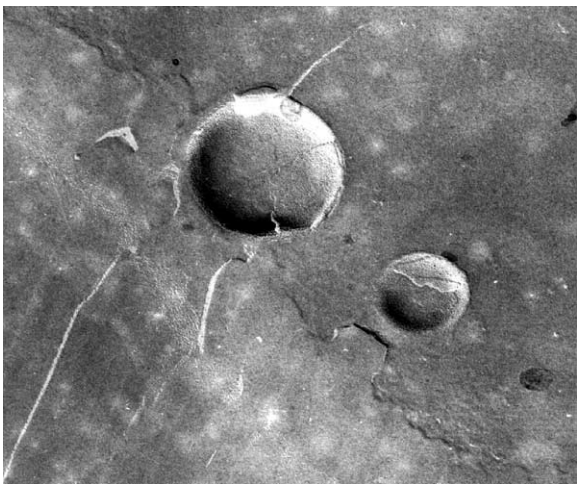


Fig. 4. Freeze-fracture micrograph of amphiphilic β-cyclodextrin nanospheres prior to loading.

Progesterone is rapidly and completely released into water:PEG400 (60:40) system as seen in Fig. 6. The presence of PEG400 as solubiliser influences the rapid release of water-insoluble progesterone from nanospheres. However, in PBS pH 7.4; progesterone showed significantly slower release behaviour for pre-loaded nanospheres in particular.

Although high-loaded nanospheres are also prepared from pre-formed inclusion complexes, the presence of excess amount of surface-adsorbed drug readily released to the medium causes a strong burst effect followed by drug dissociation from complex. Drug release from complex was realised by dissociation of progesterone from complex due to dilution and competitive displacement of drug from cavity by buffer components.

Table 3
Entrapment characteristics of progesterone into amphiphilic β-cyclodextrin nanoparticles prepared by different loading techniques ($n = 8$)

Nanosphere prepared by different techniques	Entrapped drug quantity (μg) ± S.D.	Entrapment efficiency (μg drug/mg CD)	Associated drug (%)
PL 6NC[a]	55 ± 4	65	37
PL β-CDC6[b]	44 ± 2	54	34
HL 6NC[c]	100 ± 9	118	28
HL β-CDC6[d]	119 ± 12	135	33
L 6NC[e]	77 ± 8	77	38
L β-CDC6[f]	85 ± 8	85	42

[a] PL 6NC; pre-loaded 6-*N*-CAPRO-β-CD nanospheres.
[b] PL βCDC6; pre-loaded β-CDC6 nanospheres.
[c] HL 6NC; highly-loaded 6-*N*-CAPRO-β-CD nanospheres.
[d] HL β-CDC6; highly-loaded β-CDC6 nanospheres.
[e] L 6NC; conventionally-loaded 6-*N*-CAPRO-β-CD nanospheres.
[f] L β-CDC6; conventionally-loaded β-CDC6 nanospheres.

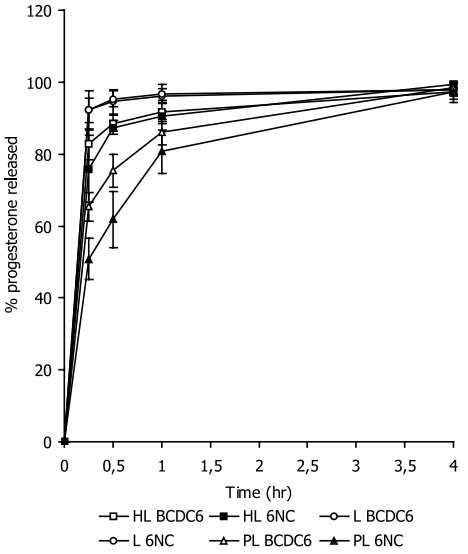


Fig. 5. Progesterone release profile into isotonic phosphate buffer pH 7.4 ($n = 6$).

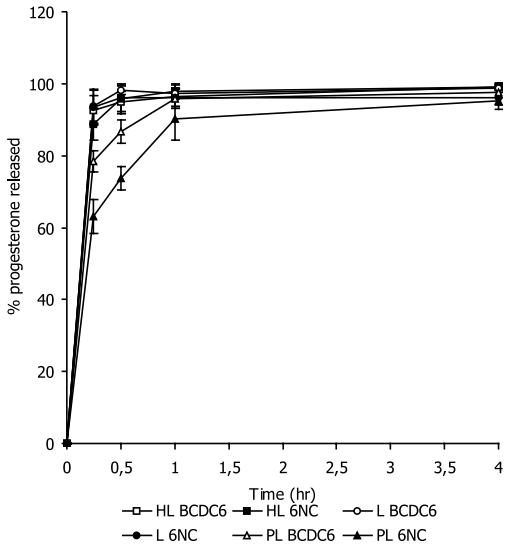


Fig. 6. Progesterone release profile into water:PEG400 (60/40 v/v) ($n = 6$).

4. Conclusion

Progesterone was incorporated into a non-surfactant parenteral nanoparticulate system. Inclusion complexation of progesterone prior to entrapment in nanospheres increased loading properties of this poorly soluble drug. The use of different loading techniques amphiphilic β-cyclodextrin types exerted considerable influence on both loading and release properties. This drug delivery system may be suitable for poorly soluble drugs with parenteral efficacy problems by obtaining rapid and complete release to provide high plasma levels.

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