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ORIGINAL ARTICLE

Effect of self-aggregation of γ-cyclodextrin on drug solubilization

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Abstract The purpose of this study was to investigate the physicochemical properties of drug-saturated aqueous cyclodextrin (CD) solutions. Phase solubility profiles of different drugs were determined in aqueous solutions containing γ-cyclodextrin (γCD) and/or hydroxypropyl-γ-cyclodextrin (HPγCD) in absence or presence of water-soluble polymers. ^{1}H-NMR and turbidity analysis were performed as well as permeation studies. Phase solubility diagrams showed that the observed γCD content (1–20% w/v) was only slightly different from the theoretical values for aqueous solutions that had been saturated with indomethacin, diclofenac sodium or amphotericin B, all displayed A-type profiles, while it was less than the theoretical value in solutions that had been saturated with corticosteroids (hydrocortisone and dexamethasone) that displayed B_S-type profiles. In the latter case self-assemble of drug/CD complexes decreased the overall CD solubility. Water-soluble polymers enhanced aqueous solubility of the drugs tested by stabilizing the drug/CD complexes, i.e. enhancing their stability constants, without affecting the observed aqueous γCD solubility. When the drug solubility leveled off (the B_S-type profiles) the amount of dissolved γCD increased and approached the theoretical values. Hydrocortisone formed partial inclusion complex with γCD and HPγCD and no non-inclusion or aggregates could be detected in diluted solutions by ^{1}H-NMR. Both permeation and turbidity studies showed that formation of dexamethasone/γCD complex promoted CD aggregation. All these observations indicate that CD aggregate formations play a role in CD solubilization of lipophilic and poorly water-soluble drugs and that the water-soluble polymers enhance the complexation efficiency of γCD and HPγCD by stabilizing the self-assembled drug/CD nanoparticles and promote non-inclusion complex formation.

Keywords Self-aggregation · γ-Cyclodextrin · Solubilization · Polymer

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Introduction

Cyclodextrin (CDs) are cyclic oligosaccharides obtained by the enzymatic degradation of starch. The most common naturally occurring CDs are composed of six (αCD), seven (βCD) and eight (γCD) glucose residues, respectively. CDs form a shallow truncated cone with a hydrophobic center cavity and a hydrophilic outer surface. Due to the reversible dynamic process of drug/CD complex formation the complexation improves in general biological availability of drugs without hampering their biological activities [1, 2]. CDs are known to self-aggregate to form nanostructures. Recent studies have shown that the parent CDs self-aggregate in water even in relatively dilute solutions [3–5]. Both the parent CDs and their derivatives form CD aggregates that can be detected by microscopic techniques [6]. The diameters of naturally CD aggregates (αCD, βCD and γCD) have been reported to be in the range of 200–300 nm by dynamic light scattering (DLS) [7]. Studies have shown that interactions between CD molecules play an important role in formation of CD complexes and that self-aggregation of CDs can reduce their ability to interact with drug molecules [8]. Fluorescence anisotropy, NMR spectroscopy, photon correlation spectroscopy (PCS), circular dichoism (CD), transmission electron microscopy (TEM), dynamic light scattering (DLS), and X-ray crystallographic analysis are

analytical tools that can be applied to characterize nanoparticles [7, 9–18]. The group of Bonini and Rossi used Cryo-TEM, static and dynamic light scattering (DLS) and electron spin resonance (ESR) spectroscopy to confirm self-aggregation of βCD [3, 19]. Previously we have used three different techniques to detect and characterize drug/CD complex aggregates, i.e. drug permeation through semi-permeable membrane, determination of changes in the value of activity coefficients of drug/CD complex solutions and TEM analysis [20].

Although previous studies have reported self-aggregation of CDs in aqueous media, their focus has been on pure aqueous CD solutions and non-pharmaceutical systems. Here we describe the self-assemble of γCD and drug/γCD complexes in pure solutions as well as in aqueous eye drop formulation.

Materials and methods

Materials

Dexamethasone (Dx) was purchased from Fagron group (Amsterdam, Netherlands), hydrocortisone (HC) from ICN Biomedicals (Aurora, OH, USA), amphotericin B (AmB), diclofenac sodium (DC-Na) and indomethacin (IDM) from Sigma (St. Louis, MO, USA), γ-cyclodextrin (γCD) and 2-hydroxypropyl-γ-cyclodextrin (HPγCD) with molar substitution 0.6 (Mean MW 1576 Da) from Wacker Chemie (Munich, Germany), disodium edetate dehydrate (EDTA) and sodium chloride from Merck (Darmstadt, Germany), benzalkonium chloride, sodium lauryl sulfate (SLS), hydroxypropylmethylcellulose (HPMC), sodium carboxymethylcellulose (Na CMC) and hexadimethrine bromide (HDMBr) from Sigma (St. Louis, MO, USA). Semi-permeable cellophane membranes (SpectaPor®, molecular weight cut off (MWCO) 3500, 6000–8000 and 12000–14000) were purchased from Spectrum Europe (Breda, Netherlands). All other chemicals used were of analytical reagent grade purity. Milli-Q (Millipore, Billerica, MA, USA) water was used for the preparation of all solutions.

Phase-solubility profiles

The solubility of the drugs tested was determined by the heating method [21]. The degradation of dexamethasone, hydrocortisone, indomethacin and diclofenac sodium in γCD and HPγCD solutions were determined to be less than 1% by HPLC after heating in autoclave (121 °C for 20 min). Amphotericin B was chemically unstable during heating in an autoclave and, thus, its solutions were saturated through heating in a sonicator (60 °C for 30 min). Excess amount of the drug to be tested was added to an aqueous eye drop solution containing 0–20% (w/v) CD (i.e. pure γCD, pure HPγCD, or a mixture of γCD and HPγCD 80/20 and 20/80 (weight ratios)), benzalkonium chloride (0.02% w/v), EDTA (0.1% w/v) and sufficient sodium chloride to obtain isotonicity. The drug suspensions were heated in an autoclave (121 °C, 20 min), or in the case of amphotericin B in a sonicator (60 °C for 30 min), in order to promote drug and in some cases CD saturation and then allowed to cool to room temperature. Then small amount of the solid drug was added to the suspensions and the pH adjusted to 7.4 with concentrated sodium hydroxide solution. The suspensions were allowed to equilibrate at room temperature (22–23 °C) for 7 days under constant agitation. After equilibrium is attained, the suspensions were filter through 0.45 μm cellulose membrane filter, the filtrates diluted with the mobile phase and analyzed by HPLC.

To investigate the effect of polymers on dexamethasone solubility and the amount of dissolved γCD, the phase-solubility of dexamethasone was determined in pure aqueous γCD solutions containing various water-soluble polymers. In this study, HPMC, Na CMC and HDMBr were chosen as neutral, anionic and cationic polymer, respectively. The polymer (HPMC, Na CMC or HDMBr, all 0.25% w/v) was dissolved in water and 0–20% w/v γCD dissolved in the polymer solution. Then excess dexamethasone was added to form drug suspension. To examine the effect of second drug on the amount of dissolved γCD in the dexamethasone saturated solutions, an excess dexamethasone and amphotericin B were added to aqueous solutions containing 0–20% w/v γCD or γCD/HPγCD 80/20 (weight ratio) and the suspension formed heated in a sonicator (60 °C for 30 min). The phase-solubility was determined as described above. The stability constant of the drug/CD complexes and complexation efficiency (CE) were calculated from the phase-solubility profiles [21].

Quantitative determinations

The quantitative determinations of the individual drugs were performed on a reversed-phase HPLC component system from Hewlett Packard Series 1100, consisting of a G132A binary pump with a G1379A solvent degasser, a G13658 multiple wavelength detector, a G1313A auto sampler, and Phenomenex Luna 5μ C18 reverse-phase column (150 x 4.6 mm). The HPLC chromatographic conditions were shown in Table 1.

Quantitative analysis of γCD and HPγCD content was also determined by HPLC. The liquid chromatographic system (Dionex UltiMate 3000, USA) was equipped with a differential refractive index detector (Shodex RI-101, Japan) with sensitivity of 600 μRIU. CHROMELEON® software version 6.80 for LC integration was used for data

Table 1 HPLC conditions

Drugs	Mobile phase[a]	Flow rate (ml/min)	Wavelength (nm)	Retention time (min)
Dexamethasone	ACN:THF:Water (33:1:66)	1.5	241	5.1
Hydrocortisone	ACN:THF:Water (33:1:66)	1.5	254	3.0
Diclofenac sodium	ACN:1.0% Acetic acid (60:40)	1.5	282	4.0
Indomethacin	ACN:0.5% Acetic acid (50:50)	1.5	240	6.9
Amphotericin B	ACN:0.25 mM EDTA (37:63)	1.0	403	3.2

ACN acetonitrile, *THF* tetrahydrofuran, *Acetic acid* aqueous acetic acid solution, *EDTA* aqueous disodium edetate dehydrate solution

[a] Volume ratios

integration. The column used was Luna NH_2 100A (10 μm, 250 × 4.6 mm) (Phenomenex, USA). The HPLC conditions were as follows. Mobile phase: 67% (v/v) acetonitrile in pure water; flow rate: 1 ml/min; injection volume: 20 μl; and column oven temperature: 25 °C. γCD content in solution was calculated by using peak area while estimated HPγCD content was determined by peak height. HPγCD consist of mixture of isomers with a mean molar substitution of 0.6. Consequently, the HPγCD chromatogram showed somewhat broad HPγCD peak.

^{1}H-NMR spectroscopic studies

The ^{1}H-NMR spectra were recorded on a Brucker AVANCE 400 (Brucker Biospin GmbH, Karlsruhe, Germany) operated at 500 MHz. The D_2O solutions of hydrocortisone, γCD, HPγCD, γCD/HPγCD mixtures (80/20 and 20/80 weight mixtures) (total CD concentration 1.7×10^{-3} M) and hydrocortisone/CD complexes at the molar ratio 1:1 (concentration 1.7×10^{-3} M) were subjected to analysis. Chemical shifts are expressed as ppm (δ).

Turbidity studies

Dexamethasone saturated aqueous γCD or HPγCD solutions (1–20% w/v) were prepared by the heating method in an autoclave (121 °C, 20 min). Pure aqueous γCD and HPγCD solutions in the same concentration range, as well as pure water, were used as reference solutions. Sample preparations were allowed to equilibrate under constant agitation for 7 days and then the suspensions were filtered through 0.45 μm cellulose membrane filter. All samples were left on upright position for 24 h and finally carried out by measuring the absorbance at 420 nm on a UV–VIS spectrophotometer (Model Lambda 35, PerkinElmer, USA). The turbidity determinations were done in triplicate.

Permeation studies

The permeability of dexamethasone or indomethacin from drug saturated aqueous γCD solutions (1–20% w/v) were carried out using Franz diffusion cell apparatus consisting of a donor and a receptor compartments (FDC 400 15FF, Vangard International, Neptune, USA) separated by a semi-permeable cellophane membrane. The membrane was soaked overnight in the receptor phase that consisted of pH 7.4 phosphate buffer saline containing 1% sodium lauryl sulfate. Sodium lauryl sulfate (a drug solubilizer) was added to maintain sink condition. The receptor phase was sonicated under vacuum to remove dissolved air before it was placed in the receptor chamber. The study was conducted at room temperature (22–23 °C) and under continuous stirring for 6 h by a magnetic stirring bar rotating at 300 rpm. An aliquot of receptor medium (150 μl) was withdrawn at 30, 60, 120, 180, 240 and 360 min and replaced immediately with an equal volume of fresh receptor phase. The drug concentration in the receptor phase was determined by HPLC. The steady state flux was calculated as the slope (dq/dt) of linear section of the amount of drug in the receptor chamber (q) versus time (t) profiles according to Eq. 1:

$$J = \frac{dq}{A \cdot dt} \quad (1)$$

where A is the surface area of the mounted membrane (1.77 cm^2). However, in case of γCD content, the amount of γCD in receptor phase was below the detection limit of the HPLC method, and thus the donor phase was collected at the end of the experiment and the amount of γCD left in the donor phase determined by HPLC. The amount of γCD transported through membrane was calculated by the difference between the initial and final γCD concentration values. The results of the permeation studies are presented as in vitro permeation flux of drug and amount of γCD transported through different MWCO of membranes *vs* time. Concentration changes due to volume changes (donor phase) or dilution (receptor phase) were monitored and corrected for.

Results and discussion

The phase-solubility diagrams of the drugs were determined in the aqueous eye drop medium, i.e. an aqueous solution containing 0–20% (w/v) CD, 0.02% (w/v)

benzalkonium chloride, 0.10% (w/v) EDTA and sufficient sodium chloride to make the solution isotonic. Figure 1 displays the phase-solubility profiles of drugs tested including the theoretical and observed solubility of CD in aqueous eye drop solution saturated with drug. The theoretical CD concentrations were derived from their aqueous solubilities at 25 °C while the observed CD concentrations were obtained from the quantification analysis by HPLC. All the phase-solubility diagrams were of type A except the diagrams of dexamethasone and hydrocortisone in γCD and 80/20 γCD/HPγCD solutions that were of B_S-type according to the Higuchi-Connor classification system indicating the precipitation of an insoluble complex at high CD concentration (Fig. 1). The CE of γCD, HPγCD and their mixtures are high (0.26–1.26 and 0.31–1.90 for dexamethasone and hydrocortisone, respectively), indicating a strong interaction between drug and CD. Interestingly the observed γCD solubility in drug saturated aqueous γCD and γCD/HPγCD (80/20) solutions did not follow the drug solubility, i.e. did not decrease with increasing amount of CD added to the complexation media (Fig. 1A and B). In all other cases the observed CD concentration agreed well with the theoretical value. It appears that the limited aqueous solubility of γCD and the hydrophobic nature of the steroids promote precipitation of the steroid/CD complex while the enhanced aqueous solubility of the γCD derivative and hydrophilic nature of the other drugs tested decrease the tendency of precipitation.

Polymers can enhance the CD complexation of drugs and they can enhance the drug permeation through biological membranes, possibly through formation of ternary complexes or co-complexes and enhanced mucoadhesion of the enlarged co-complexes [22–26]. However, limited information exists on the effects of polymers on the CD solubility. Therefore, the effects of three different types of polymers, i.e. HPMC, Na CMC and HDMBr (non-ionic, anionic and cationic polymer, respectively) on the γCD solubility were investigated. Table 2 shows the stability

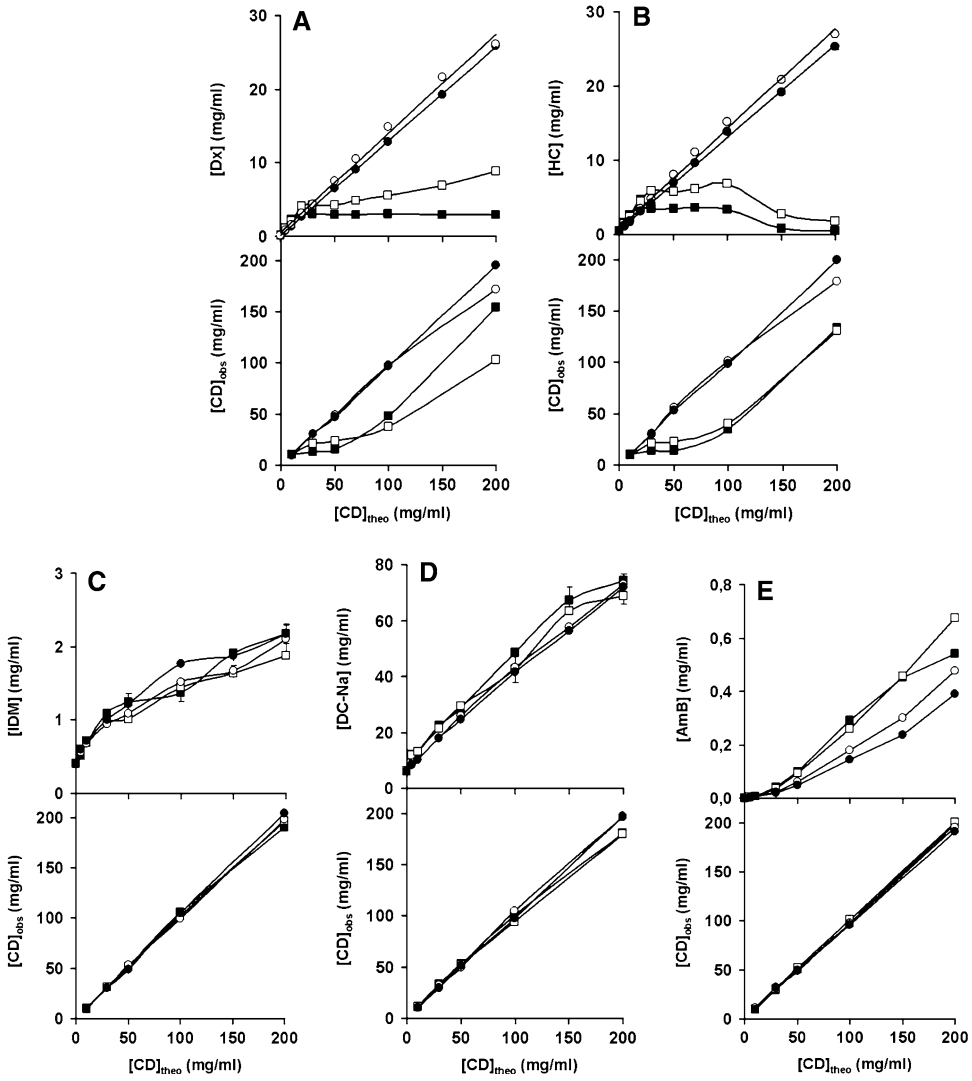


Fig. 1 Phase-solubility profiles of drugs and cyclodextrins in eye drop complexation medium; dexamethasone (**A**); hydrocortisone (**B**); indomethacin (**C**); diclofenac sodium (**D**); amphotericin B (**E**); γCD (*filled square*); HPγCD (*filled circle*); γCD/HPγCD 80/20 (*open square*); γCD/HPγCD 20/80 (*open circle*); observed cyclodextrin concentration ($[CD]_{obs}$); theoretical cyclodextrin concentration ($[CD]_{theo}$)

Table 2 Apparent stability constant ($K_{1:1}$) and the complexation efficiency (CE) of dexamethasone/γCD complexes in pure aqueous solution without or in the presence of a polymer (0.25% w/v) at room temperature (22–23 °C)

Polymer	Slope[a]	Correlation coefficient	$K_{1:1}$ (M^{-1})	CE	CE ratio
–	0.204	0.904	1210	0.26	1.00
HPMC	0.461	0.910	2620	0.86	3.42
Na CMC	0.431	0.938	3330	0.76	2.92
HDMBr	0.493	0.894	3830	0.97	3.73

HPMC hydroxypropyl methylcellulose, *Na CMC* carboxymethylcellulose sodium salt, *HDMBr* hexadimethrine bromide

[a] Slope of the linear phase-solubility diagram (0–23 mM)

constant and CE of dexamethasone/γCD complexes in aqueous γCD solutions without or in the presence of the polymers. As expected, both parameters increased upon addition of polymer to the complexation medium. The γCD content determined by HPLC in complexation medium saturated with dexamethasone is displayed in Table 3. The tested polymers did not solubilize insoluble γCD or dexamethasone/γCD complexes. This observation indicated that the polymers enhance the solubilizing effect of γCD through formation ternary complexes or co-complexes rather than by increasing the solubility of γCD or disturbing self-aggregated γCD or γCD complexes. This observation is in agreement with a study of Valero et al. where the authors concluded that the polymer molecules act as bridges between CD complexes [27].

In our previous studies the CE of dexamethasone was increased by as much as 76% when amphotericin B was introduced to the complexation media [28]. Figure 2 shows the observed amount of dissolved γCD versus the theoretical γCD value (i.e. the amount of CD added to the complexation media) in aqueous γCD solutions saturated with dexamethasone or combination dexamethasone-amphotericin B. At low γCD concentrations the solubility of γCD is increased in the presence of amphotericin B but it decreases at higher (>10% w/v or >7.7 × 10^{-2} M) γCD concentrations. Similar observation was made when a mixture of γCD and HPγCD was studied. This indicates that the enhanced CE in the presence of amphotericin B is mainly due to aggregate formation although enhanced CE due to γCD solubilization at low γCD concentrations cannot be ruled out.

Hydrocortisone was selected to investigate further the inclusion complexes in aqueous solution. The interaction between hydrocortisone and either γCD and/or HPγCD was investigated in D_2O by magnetic resonance spectroscopy. The concentration of both the drug and the CDs was 1.7 × 10^{-3} M that corresponds to 0.2 and 0.3% (w/v) of γCD and HPγCD, respectively. The H-3 and H-5 protons of the glucose units are facing to the interior of the hydrophobic CD cavity, whereas H-6 protons are located at the rim adjacent to the primary alcohols (the narrow cavity opening), and H-2 and H-4 are at the opposite end (Table 4). The ^{1}H chemical shift displacements of the CDs were determined in the presence of hydrocortisone. The ^{1}H signals of the inner protons, H-3 of both γCD and HPγCD shifted significantly upfield whereas ^{1}H signals of H-5 did not show any shifts. The outer H-1, H-2, H-4 and H-6 protons changed slightly. In case of HPγCD, the H-3 and H-5 protons could not be detected due to overlapping with other signals in the multicomponent mixture. These results indicated that hydrocortisone is shallowly included in CD cavity, depositing it around the secondary hydroxyl groups, which is in agreement with previous studies of hydrocortisone complexes with β-CD and its derivatives [29]. At this very low concentration (1.7 × 10^{-3} M corresponding to 0.06% w/v) the drug forms inclusion complexes with both γCD and HPγCD. No non-inclusion complexes or complex aggregates were observed at this low CD concentration.

UV spectrometry is unsurpassed for precision and sensitivity for quantifying aggregates. Figure 3 shows the turbidity of aqueous CD solutions, either pure or after they had been saturated with dexamethasone. After preparation the solutions were filtrated through a 0.45 μm membrane filter and, thus, the aggregates detected were formed after the filtration. The turbidity of the aqueous γCD solutions increased with increasing γCD concentration while the HPγCD solutions remained essentially non-turbid. These results indicate

Table 3 Effect of a polymer (0.25% w/v) on the solubility of γCD in aqueous γCD solution saturated with dexamethasone

Theoretical total γCD content (mg/ml)	Observed concentration of dissolved γCD ± standard deviation (mg/ml)			
	No polymer	HPMC	Na CMC	HDMBr
10	10.2 ± 0.5	9.8 ± 0.4	9.6 ± 0.7	10. 8 ± 0.8
30	12.7 ± 0.3	10.4 ± 1.0	13.6 ± 0.7	15.1 ± 1.8
50	15.8 ± 0.0	12.2 ± 2.1	11.4 ± 0.6	15.8 ± 2.2
100	48.3 ± 1.6	45.6 ± 1.6	49.8 ± 1.8	46.0 ± 2.0
150	n.d.	105.8 ± 2.1	112.4 ± 1.5	118.9 ± 2.8
200	154.5 ± 0.5	141.3 ± 3.7	143.2 ± 1.7	149.4 ± 1.6

n.d. not determined, *HPMC* hydroxypropyl methylcellulose, *Na CMC* carboxymethylcellulose sodium salt, *HDMBr* hexadimethrine bromide

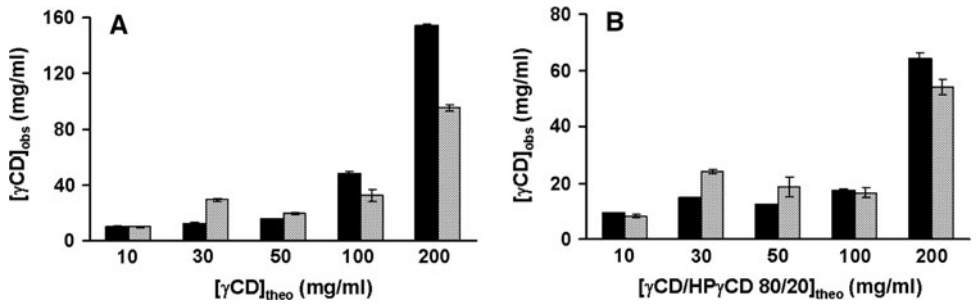
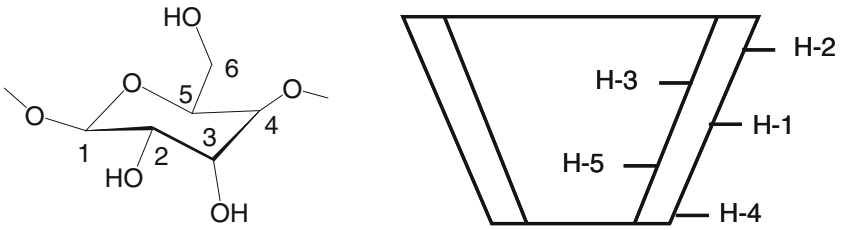


Fig. 2 Observed amount of dissolved γCD ($[\gamma CD]_{obs}$) versus theoretical γCD value ($[\gamma CD]_{theo}$ or $[\gamma CD/HP\gamma CD\ 80/20]_{theo}$) in aqueous solutions saturated with dexamethasone (*black column*) or dexamethasone-amphotericin B (*grey column*); aqueous γCD solutions (**A**) and aqueous γCD/HPγCD mixture (80/20) solutions (**B**)

Table 4 ^{1}H-NMR chemical shift (Δδ, ppm)[a] displacements of γCD, HPγCD or mixtures of γCD and HPγCD (total cyclodextrin conc. 1.7×10^{-3} M) by addition of hydrocortisone (1.7×10^{-3} M) in D_2O at 25 °C



^{1}H assignment[b]	γCD	HPγCD	γCD/HPγCD Mixture	
			80/20	20/80
H-1	−0.02	−0.03	−0.02	−0.01
H-2	+0.01	0.00	+0.01	+0.02
H-3	−0.18	–[c]	−0.20	−0.19
H-4	+0.01	−0.01	+0.01	+0.01
H-5	0.00	–[c]	0.00	0.00
H-6	−0.01	−0.01	0.00	0.00
H-7		0.00		0.00
H-8		−0.05		−0.02

[a] Chemical shift displacements were expressed as $\Delta\delta = \delta_{CD\ complex} - \delta_{CD\ free}$

[b] H-7 and H-8 are located on the hydroxypropyl moiety of HPγCD

[c] Could not be determined due to overlapping with other signals

that formation of dexamethasone/γCD complexes enhances the aggregate formation in aqueous γCD solutions.

Aggregate formation can be detected by permeation measurements through semipermeable membranes [20, 30, 31]. The molecular weights of 1:1 dexamethasone/γCD or indomethacin/γCD complexes are approximately 1700 Da and, thus, they should be able to permeate semipermeable membrane with a molecular weight cut off of 3500, even as dimers. Higher order aggregates should be able to permeate membranes with MWCO of 6000–8000 and 12000–14000. Figure 4 displays the in vitro permeation flux of dexamethasone and indomethacin, and amount of γCD transported at 6 h, through semipermeable membrane with MWCO of 3500, 6000–8000 and 12000–14000 Da. The dexamethasone fluxes did increase with increasing γCD concentrations up to 5% (w/v) (3.9×10^{-2} M) and then the flux levels off while the amount of transported γCD still increases up to the highest γCD concentration tested (20% (w/v) γCD) (Fig. 4A). The total solubility (i.e. both free and in a γCD complex) increases with increasing γCD concentration and formation complex aggregates is negligible at these relatively low γCD concentrations (≤5%). At higher γCD concentrations (5–20% w/v corresponding to $3.9–15.4 \times 10^{-2}$ M) γCD form water-insoluble complexes with dexamethasone leading to a B_S-type phase-solubility diagram (Fig. 1A). In other words, the dexamethasone/γCD

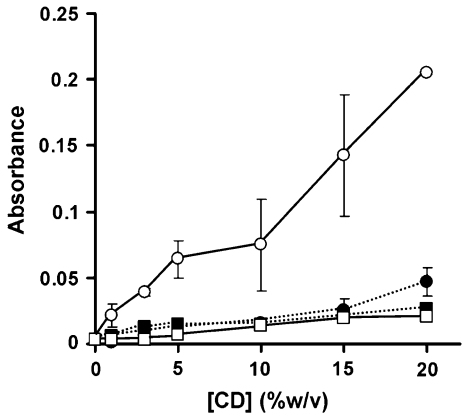


Fig. 3 Turbidity analysis of aqueous cyclodextrin solutions with and without saturated dexamethasone after 0.45 μm membrane filtration and storage for 24 h at 420 nm; γCD (*filled circle*); HPγCD (*filled square*); dexamethasone/γCD (*open circle*); dexamethasone/HPγCD (*open square*); each point represent mean ± std

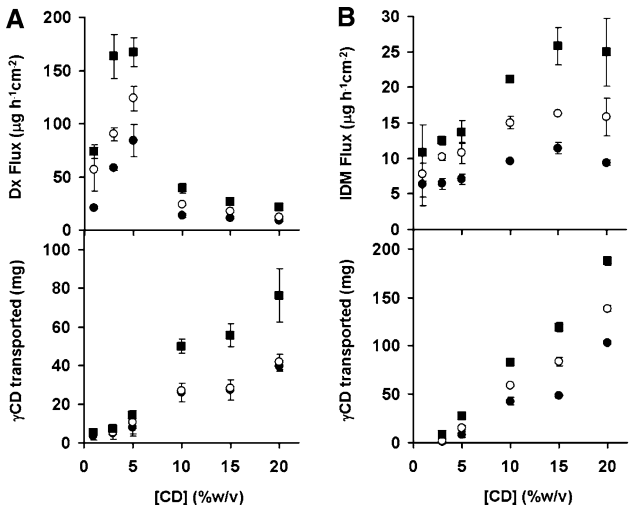


Fig. 4 In vitro drug permeation flux profiles, and the amount of γCD at 6 h, transported through semi-permeable membranes MWCO 3500 Da (*filled circle*); 6000–8000 Da (*open circle*); 12000–14000 Da (*filled square*); dexamethasone (**A**); indomethacin (**B**)

complexes form large aggregates that precipitate out from the solutions. Indomethacin forms an A_N-type phase-solubility diagram with γCD (Fig. 1C). The flux profiles (Fig. 4B) indicate that this could be due to formation of indomethacin/γCD complex aggregates that are both small and water-soluble at low γCD concentrations but become both larger and less water-soluble at higher γCD concentrations. These observations are in agreement with our previous studies as well as with ^{1}H-NMR spectroscopic studies that indicate that CD starts to form aggregates at concentration at about 5.4% w/v [16].

Conclusions

The results indicate that CD aggregate formations play a role in CD solubilization of lipophilic and poorly water-soluble drugs and that the water-soluble polymers enhance the complexation efficiency of γCD and HPγCD by stabilizing the self-assembled drug/CD nanoparticles and promote non-inclusion complex formation.

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