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ORIGINAL ARTICLE

Characterization of the complexation of tauro- and glyco-conjugated bile salts with γ-cyclodextrin and 2-hydroxypropyl-γ-cyclodextrin using affinity capillary electrophoresis

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Abstract The complexation of seven bile salts, present in the small intestine of rat, dog and man, (taurocholate, tauro-β-muricholate, taurodeoxycholate, taurochenodeoxycholate, glycocholate, glycodeoxycholate and glycochenodeoxycholate) with γ-cyclodextrin and the chemically modified 2-hydroxypropyl-γ-cyclodextrin, was studied using affinity capillary electrophoresis (ACE). The cyclodextrins (CDs) were investigated due to their use in drug formulation as excipients for solubilisation of poorly soluble drugs and drug candidates. Using mobility shift ACE, the bile salt cyclodextrin interactions were characterized demonstrating 1:1 binding stoichiometry with stability constants ranging from 2×10^3 to 8×10^4 M^{-1}. The binding constants showed a systematic dependence on the number and position of hydroxyl groups on the steroid skeleton and the stability constants were in general higher for complexation with the native cyclodextrin than with the modified cyclodextrin. Based upon the size of the complexation constants, it was suggested that the interaction between the CDs and the bile salts takes place at the C and D ring of the steroid skeleton. The complexation of bile salts with the γ-cyclodextrins may compete with drug-γ-cyclodextrin complex formation and, thus, potentially affect drug absorption and efficacy.

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Keywords Affinity capillary electrophoresis · Bile salts · Complexation · Cyclodextrins · γ-Cyclodextrins

Abbreviations

2OHpγCD	2-Hydroxypropyl-γ-CD
ACE	Affinity capillary electrophoresis
CD	Cyclodextrin
GC	Glycocholate
GCDC	Glycochenodeoxycholate
GDC	Glycodeoxycholate
TβMC	Tauro-β-muricholate
TC	Taurocholate
TCDC	Taurochenodeoxycholate
TDC	Taurodeoxycholate

Introduction

In recent years, drug discovery has focused drug optimization on in vitro potency by high-throughput screening, with less attention given to the physico-chemical properties of the compounds. Drug design approaches based on combinational chemistry and quantitative structure–activity relationship, have generated vast numbers of new potent compounds, however, unfortunately often endowed with high molecular weights, high octanol/water partition coefficients (log *P*) and low water solubilities [1]. While these compounds possess high affinity towards the defined drug target, they often have suboptimal biopharmaceutical properties and may fail to progress into in vivo studies, due to solubility problems leading to undesirable pharmacokinetic profiles [2]. Appropriate formulation vehicles for early animal experiments may be challenging when the compound is poorly soluble and more structured formulation efforts are generally needed. Hydrophilic cyclodextrins (CDs) are

widely used in this context to improve the oral bioavailability of poorly soluble compounds in the non-clinical and early phases of drug development [3–7].

CDs, sometimes called cycloamyloses, make up a family of cyclic oligosaccharides, composed of five or more α-D-glucopyranoside units linked 1→4, as in amylose. Typical CDs contain six, seven or eight glucose units and are denoted α-, β- and γCDs, respectively. The CDs take the form of a truncated cone, with an outer hydrophilic surface and an inner hydrophobic cavity in which molecules of comparable size can form inclusion complexes [8]. A number of chemically modified CDs, such as 2-hydroxypropyl-γ-CD (2OHpγCD), have been prepared to improve the inclusion capacity and the physico-chemical properties as compared to the native CDs. A number of drugs have been found to form complexes with the γCDs, e.g. adiphenine, bromodiphenhydramine, chlorocyclizine, cinnarizine, cyclizine, diphenidol, diphenylpyraline, hydroxyzine, meclizine, orphenadrine [9], estradiol, estriol, estrone, ethinyloestradiol [10], flurbiprofen [11], niflumic acid, piroxicam [12], phenolphthaline [13], and resorcinol [14]. However, there is a limited number of studies in the pharmaceutical literature on the use of γ-cyclodextrins as pertains to oral drug administration [9, 15–21]. This is seemingly in contrast with the tendency towards poorly soluble drug candidates of increasing size and the fact that γCDs are capable of forming inclusion complexes with larger molecules than the βCDs.

Westerberg and Wiklund [22] reported a decreased bioavailability of benzo[a]pyrene when dosed orally with increasing amounts of βCD to rats. The authors suggested that this phenomenon was caused by an extensive complex formation between benzo[a]pyrene and the CD. Only the free form of the drug, which is in equilibrium with the complex species, is available for absorption and hence for providing the pharmacological effect of the drug. Thus, while promoting solubility, CDs may essentially prevent or retard drug absorption from the gastrointestinal tract. However, a variety of lipophilic compounds, originating from ingested meals and gastrointestinal secretions, may have the propensity to displace the drug molecule from the CD cavity upon oral administration. Of particular interest are the bile salts (Fig. 1), in connection to the displacement of drugs from the CD complexes as these have hydrophobic moieties and are excreted in large amounts in the duodenum. Thus, bile salt displacement of drug molecules may take place in the duodenum provided that complexation equilibrium favors this [23]. Hence it is important to get a better understanding of complex formation between the CDs and bile salts in order to use these excipients optimally in preformulation and drug formulation. According to Alvaro et al. [24] seven different bile salts are present in the intestines of man, rat and canine. These are: taurocholate (TC), tauro-β-muricholate (TβMC), taurodeoxycholate (TDC), taurochenodeoxycholate (TCDC), glycocholate (GC), glycodeoxycholate (GDC) and glycochenodeoxycholate (GCDC). Further these bile salts are of structural interest for the interaction with the γCDs as they contain variations in the number, position and and orientation of their hydroxyl groups. Studies, investigating the interaction between CDs and bile salts, have primarily been performed with natural CDs using

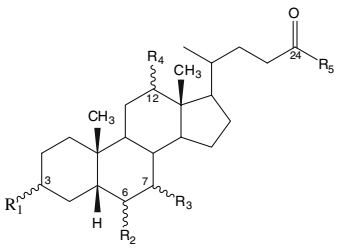

Fig. 1 Schematic structure of glyco- and tauro-conjugated bile salts investigated in the present study. α indicates a sterical orientation below the plan and β above

Bile acid	Abbreviation	R_1 (C-3)	R_2 (C-6)	R_3 (C-7)	R_4 (C-12)	R_5 (C-24)
Glycocholic acid	(GC)	OH (α)	H	OH (α)	OH (α)	$NHCH_2COO^-$
Glycochenodeoxycholic acid	(GCDC)	OH (α)	H	OH (α)	H	$NHCH_2COO^-$
Glycodeoxycholic acid	(GDC)	OH (α)	H	H	OH (α)	$NHCH_2COO^-$
Taurocholic acid	(TC)	OH (α)	H	OH (α)	OH (α)	$NHCH_2CH_2SO^-_3$
Taurochenodeoxycholic acid	(TCDC)	OH (α)	H	OH (α)	H	$NHCH_2CH_2SO^-_3$
Taurodeoxycholic acid	(TDC)	OH (α)	H	H	OH (α)	$NHCH_2CH_2SO^-_3$
Tauro-β-hyocholic acid	(T-β-HC)	OH (α)	OH (α)	OH (β)	H	$NHCH_2CH_2SO^-_3$

calorimetry and NMR [25–32]. To the best of our knowledge, the interaction of TCDC, GCDC and TβMC with natural γCD has not been examined and to our knowledge no reports have been published on the complexation of modified γCDs to any bile salt.

Mobility shift affinity capillary electrophoresis (ACE) has recently been used to determine the stability constants for the interaction of bile salts with neutral βCDs [33]. However, the technique has not previously been used for the study of interactions involving γCDs and bile salts. The purpose of the present study therefore was to (i) investigate the ability of major bile salts present in man, rat and canine, to form complexes with γCD and 2OHpγCD by ACE (ii), evaluate the influence of structural differences among the bile salts and CDs on the determined stability constants, and (iii) to assess the potential of drug-bile salt displacement reactions to occur when applying γCDs as solubilisation agents in early drug development.

Experimental

Materials

The sodium salts of the bile acids were purchased from various sources. Taurocholate (2-([3α, 7α, 12α-trihydroxy-24-oxo-5β-cholan-24-yl) amino]ethanesulfonic acid) was purchased from Fluka (Switzerland), tauro-β-muricholate (2-([4-(3, 6,7-trihydroxy-10, 13-dimethyl-hexadecahydrocyclopenta[a]penathren-17-yl)-pentanoylamino]ethansulfonic acid) was purchased from Steraloids (Newport, RI, USA), taurochenodeoxycholate (2-([3α,7α-dihydroxy-24-oxo-5β-cholan-24-yl]amino)ethanesulfonic acid), taurodeoxycholate (2-([3α,12α-dihydroxy-24-oxo-5β-cholan-24-yl]amino) ethanesulfonic acid), glycocholate (3α,7α,12α-trihydroxy-5β-cholan-24-oic acid N-(carboxymethyl)amide), glycodeoxycholate (3α,12α-dihydroxy-5β-cholan-24-oic acid N-(carboxymethyl)amide) and glycochenodeoxycholate (3α,7α-dihydroxy-24-oxo-5β-cholan-24-oic acid N-(carboxymethyl)amide) from SigmaAldrich (St.Louis, MO, USA) and all were used as received. 2-Hydroxypropyl-γ-cyclodextrin (2OHpγCD) and γ-cyclodextrin (γCD) were purchased from SigmaAldrich (USA). Sodium phosphate buffer, 0.50 M pH 7.00, used as sample and CE buffer were obtained from Agilent (Palo Alto, CA, USA). Ultra pure water used in the CE experiments was obtained from Agilent.

Capillary electrophoresis procedures

CE experiments were performed on an Agilent 3D CE equipped with a diode-array detector. Uncoated fused silica bubble capillaries (150 µm light path; Agilent, USA) 33 cm × 50 µm ID, with a length of 25 cm to the detection window were used for the electrophoresis experiments. Conditioning of new capillaries was conducted by flushing with 1 M NaOH for 60 min followed by 0.1 M NaOH and water for 20 min each. The capillary cassette temperature was set to 22 °C and the voltage was set to +8 kV, which provided a mean capillary temperature of 25 °C (due to Joule heating) as described by Kok [34]. UV detection was performed at 192, 195 and 200 nm and samples were run in triplicate. Along with the UV traces the current was monitored to allow for viscosity corrections. A 0.050 M sodium phosphate, pH 7.00 was used for the sample solutions and the CE experiments. The CDs were added to the phosphate buffer in various concentrations to make up the electrophoresis buffer solutions. The concentrations of the CD solutions were corrected for the amount of adsorbed water in the γCD preparations as determined by Karl Fisher titration using a Metrohm 756 KF coulometer (Herisau, Switzerland). The samples in the phosphate buffer contained the bile salts at a concentration of 5×10^{-6} M and methanol, which served as a marker of the electroosmotic flow (EOF), at a concentration of 0.05% v/v. The samples were introduced into the capillary by applying a pressure of 50 mbar for 2 s. Between the measurements capillaries were flushed with 0.1 M NaOH, 0.050 M phosphate buffer and the electrophoresis buffer solution with or without CD for 1 min each.

Migration theory and binding analysis

Of a charged spherical molecule the effective electrophoretic mobility, μ, is determined by the charge-to-size ratio and the viscosity of the electrophoresis media according to:

$$\mu = \frac{q_{\text{eff}}}{6\pi\eta r} \tag{1}$$

where q_{eff} and r are the effective charge and radius of the analyte, respectively, and η is the viscosity of the electrophoresis media. In capillary electrophoresis the effective electrophoretic mobility, μ, may be obtained from:

$$\mu = \frac{l_c l_d}{V}\left(\frac{1}{t} - \frac{1}{t_0}\right) \tag{2}$$

where l_c is the length of the capillary, l_d is the length of the capillary to the detector, V is the applied voltage and t and t_0 are the peak appearance times of the analyte and the EOF, respectively [35].

The presence of additives such as CDs in the electrophoresis buffer changes the viscosity of the media, which, in turn, will affect the effective electrophoretic mobilities (Eq. 2). Consequently, the effective electrophoretic mobilities should be corrected for viscosity changes due to

the γCDs in the electrophoresis buffer solutions prior to evaluation of the binding isotherms. Viscosity corrections can be based upon the currents measured during electrophoresis or, alternatively, separate viscosity measurements [36]:

$$\mu' = \mu \frac{I_0}{I} = \mu \frac{\eta}{\eta_0} \tag{3}$$

where I_0 and I are the measured currents at zero CD and at a given CD concentration, respectively, and η_0 and η are the electrophoresis buffer viscosities in the absence and presence of the CD, respectively.

The 1:1 complex formation between a bile salt, BS, and a cyclodextrin, CD, in solution can be described by the equilibrium:

$$\mathrm{BS} + \mathrm{CD} \rightleftharpoons \mathrm{BS} - \mathrm{CD} \tag{4}$$

where BS − CD constitutes the formed 1:1 complex. The apparent stability constant $K_{1:1}$ describing the equilibrium (4) may be defined by:

$$K_{1:1} = \frac{[\mathrm{BS} - \mathrm{CD}]}{[\mathrm{BS}] \cdot [\mathrm{CD}]} \tag{5}$$

where [BS], [CD], and [BS − CD] are the equilibrium concentrations of BS, CD, and BS − CD, respectively. The effective electrophoretic mobility, corrected for viscosity changes of the BS, μ', in a CD, containing electrophoresis buffer, is a function of the time spent on free form relative to the time spent in the complexed state [37]:

$$\mu' = \frac{[\mathrm{BS} - \mathrm{CD}]}{[\mathrm{BS}] + [\mathrm{BS} - \mathrm{CD}]} \cdot \mu_{\mathrm{BS-CD}} + \frac{[\mathrm{BS}]}{[\mathrm{BS}] + [\mathrm{BS} - \mathrm{CD}]} \cdot \mu_{\mathrm{BS}} \tag{6}$$

where $\mu_{\mathrm{BS-CD}}$ and μ_{BS} are the electrophoretic mobility of the complex and the free bile salt, respectively. Upon introduction of Eq. 5 into 6 and rearrangement, the binding isotherm can be derived [37, 38]:

$$\mu' = \frac{\mu_{\mathrm{BS}} + \mu_{\mathrm{BS-CD}} K_{1:1} [\mathrm{CD}]}{1 + K_{1:1} [\mathrm{CD}]} \tag{7}$$

Stability constants, $K_{1:1}$, and the electrophoretic mobilities of the BS-CD complexes, $\mu_{\mathrm{BS-CD}}$, were determined by non-linear regression analysis using SigmaPlot (version 9.0, Systat Software, Point Richmond, CA, USA).

Results and discussion

ACE is highly versatile with respect to compounds amendable for study. The most prominent requirement, being that at least one of the interacting species has to carry a charge as is the case for bile salts at near neutral pH. Hence in the present study CE has been used to investigate the interaction between γCD and 2OHpγCD and a number of bile salts present in the intestine of man, canine or rat [24].

Affinity capillary electrophoresis and estimation of the stability contants

ACE may be an attractive approach for assessing molecular interactions. Prominent features of ACE include low sample and reagent requirements, short analysis times and ease of automation. Furthermore, interactions are probed in solution and ACE can be performed without the need for immobilization on chromatographic supports or labelling/derivatsation procedures. Several different ACE modes have been described in the literature, for reviews see [39–42]. The mobility shift ACE format was investigated here because it is most often applicable when the binding stoichiometry is known (or can be limited to a few stoichiometries) and the interaction characterized by rapid on-and-off kinetics. Furthermore, mobility shift ACE has earlier been used to characterize analyte-CD interactions [43–45]. The ACE experiments in the present study were conducted essentially as previously described [33] in 0.050 M sodium phosphate buffer at pH 7.00 and 25 °C. The operating conditions developed for assessing the interactions between bile salts and various β-cyclodextrins were found to be suitable for γCDs as well. Briefly, the mobility of the bile salts was investigated as a function of the γCD concentration in the run buffer. The lowest and highest CD concentrations investigated were 5×10^{-6} M and 2×10^{-2} M, respectively. The γCD concentrations investigated for each system depended on the affinity of the bile salts for the γCDs. The lower end of the CD concentration range (5×10^{-6} to 5×10^{-5} M) was only investigated for bile salts with a stability constant higher than $\sim 10^4$ M^{-1}. To avoid difficulties in the interpretation of the binding data due to competing equilibriums, the samples contained the bile salts at a concentration of 5×10^{-6} M, which is known to be well below the critical micellar concentration of the bile salts [46, 47]. Typical electropherograms are shown in Fig. 2. As can been seen from the depicted electropherograms the bile salt, GC, is just detectable. It is recommended that the analyte (bile salt) concentration should be much smaller than the applied ligand (CD) concentrations [48–52]; otherwise the observed mobility shifts have been found to be concentration dependent and not reflecting binding equilibrium. Interaction between GC and γCD is readily apparent from the electropherograms as a shift in GC peak appearance times relative to the EOF (Fig. 2). The effective electrophoretic mobilities were calculated according to Eq. 2

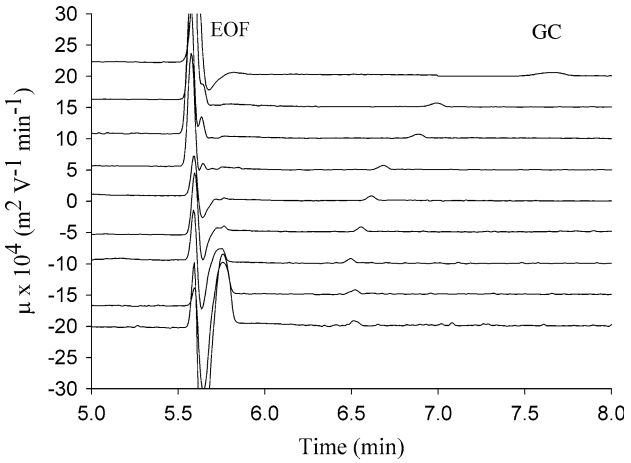

Fig. 2 Stacked electropherograms showing the change in glycocholate (GC) electrophoretic mobility as a function of the γCD concentration in 0.050 M sodium phosphate buffer pH 7.00 at 25 °C. Concentrations of γCD 0, 0.0001, 0.0002, 0.0005, 0.001, 0.002, 0.005, 0.01, 0.02 M from top to bottom. Confer experimental section for details of the CE conditions. The minimum of the EOF signal was taken as the peak appearance time of methanol (EOF marker)

from the peak appearance times of the bile salt and the non-interacting EOF marker, methanol [53]. Further, these mobilities were corrected for the changes in viscosity of the electrophoresis buffer induced by the presence of different CD concentrations by the use of Eq. 3. The corrected electrophoretic mobilities, μ', were depicted as a function of the CD concentration in the electrophoresis buffer. These data were evaluated by non-linear regression analysis using Eq. 7. Correlation coefficients, R^2, were above 0.99 except for GCDC-γCD (0.988) and TCDC-γCD (0.986) indicating that the 1:1 complexation model adequately described the binding data. The estimated stability constants ($K_{1:1}$) are compiled in Table 1 and representative binding isotherms shown in Fig. 3. However, as reported by Bowser et al. [54] high correlation coefficients alone are not sufficient to demonstrate that the 1:1 model is the best to describe the data. However, *x*-reciprocal plots [54–56] were linear and the model assuming 1:2 complexation stoichiometry [54] gave unrealistic binding parameters (negative $K_{1:2}$ and positive 1:2 complex mobilities were obtained) wherefore a 1:1 binding model was accepted.

Inclusion complex formation with γ-cyclodextrin

The present study were conducted in a phosphate buffer at pH 7.00. The pH of the small intestine where the bile salt–γCD interactions are likely to take place in vivo has been reported to 6.2 ± 1.2 [39]. In these pH ranges the conjugated bile salts are all fully ionized. As described above, the seven investigated bile salts all displayed 1:1 complex

Table 1 Complexation constants, $K_{1:1}$ (M^{-1}, mean ± S.E.M.) for 1:1 inclusion complex formation of glyco- and tauro-conjugated bile salts with γCD and 2OHpγCD determined in 0.050 M sodium phosphate buffer pH 7.00 at 25 °C

Bile salt	Cyclodextrin	
	γCD	2OHpγCD
GC	$5.8 \times 10^3 \pm 3.2 \times 10^2$	$1.8 \times 10^3 \pm 1.8 \times 10^2$
	2.1×10^{2a}	
	4.5×10^{3b}	
GCDC	$5.9 \times 10^4 \pm 7.1 \times 10^3$	$7.0 \times 10^4 \pm 6.6 \times 10^3$
GDC	$2.9 \times 10^4 \pm 2.2 \times 10^3$	$1.0 \times 10^4 \pm 7.9 \times 10^2$
	$4.1 \times 10^{3\ b}$	
TC	$4.8 \times 10^3 \pm 1.7 \times 10^2$	$2.1 \times 10^3 \pm 6.8 \times 10^1$
	3.5×10^{3c}	
	4.7×10^{3b}	
TCDC	$8.4 \times 10^4 \pm 1.1 \times 10^4$	$6.0 \times 10^4 \pm 2.4 \times 10^3$
TDC	$2.8 \times 10^4 \pm 2.7 \times 10^3$	$1.4 \times 10^4 \pm 3.6 \times 10^2$
	1.6×10^{4c}	
	1.2×10^{4d}	
	2.6×10^{3b}	
Tβ*M*C	$1.2 \times 10^5 \pm 3.0 \times 10^3$	$7.4 \times 10^4 \pm 1.9 \times 10^3$

[a] Stability constants determined by NMR [31]

[b] Stability constants determined by NMR [25]

[c] Stability constants determined by a phase solubility technique (displacement assay) [27]

[d] Stability constants determined by isothermal titration calorimetry [32]

formation with γCD. This is in accordance with the binding stoichiometry reported for bile salt–γCD complexes in the literature [25, 27, 31, 32]. The stability constants for the complexes formed between γCD and the seven bile salts are presented in Table 1 together with binding parameters found in the literature.

It is apparent from Table 1 that the interaction of the bile salts was essentially unaffected of whether it was glyco- or tauro-conjugated. The stability constant for the two trihydroxy bile salts TC and GC to γCD was $4.8 \pm 0.2 \times 10^3$ and $5.8 \pm 0.3 \times 10^3$ M^{-1} respectively, which is the same range as values reported by others for the same systems [25, 27]. The general trend appears to be that there is a reasonable agreement between the available data obtained by mobility shift ACE and isothermal titration calorimetry and the phase solubility technique whereas stability constants obtained by NMR appears to be significantly lower for some of the bile salt–γCD pairs (Table 1). The stability constants for the two dihydroxy bile salts, TDC and GDC ($2.8 \pm 0.3 \times 10^4$ and $2.9 \pm 0.2 \times 10^4$ M^{-1}) were significantly higher than the stability constants observed for TC and GC and it is clearly possible to discriminate between the two classes of bile salts. The stability constants obtained in the present study are higher

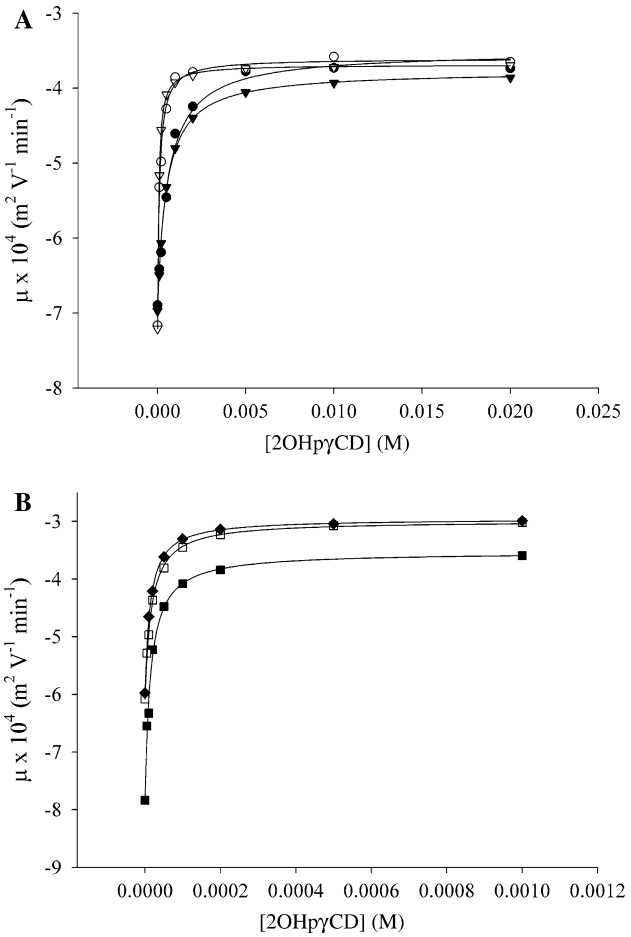

Fig. 3 Binding isotherms. (**a**) Effective electrophoretic mobility of GC (•), GDC (○), TC (▼) and TDC (▽) as a function of the 2OHpγCD concentration. (**b**) Effective electrophoretic mobility of GCDC (■), TCDC (□) and TβMC (♦) as a function of the 2OHpγCD concentration. Solid lines were obtained by non-linear regression analysis (Eq. 7)

than those published by other authors [25, 27, 32], who used NMR, phase solubility studies and calorimetry, respectively. TDC and GDC lack a hydroxyl group on C-7 in the B-ring of the bile salt (Fig. 1), a difference that may contribute to the stronger interaction observed for these bile salts when compared to the binding of TC and GC. The stability constants of GCDC ($5.9 \pm 0.7 \times 10^{-4}$ M^{-1}) and TCDC ($8.4 \pm 1.1 \times 10^{-4}$ M^{-1}), which have not previously been reported in the literature, are higher than the other γCD-bile salts $K_{1:1}$ values reported in the literature [25, 27, 31, 32]. GCDC and TCDC are dihydroxy bile salts, with the hydroxyl groups positioned on the C-3 and C-7, whereas TDC and GDC has the same groups on C-3 and C-12. This structural difference may account for the observed difference in the stability constant, if the interaction occurs at the C and D ring of the steroid backbone of the bile salts. Then the hydroxyl group on the C-7 would be placed at the interface of the cyclodextrin cavity, whereas the hydroxyl group on the C-12 would be inside the cavity, leading to higher steric interference and consequently a weaker stability constant. This interpretation is in accordance with the conclusions drawn by Cabrer et al. [25], based upon investigation of the molecular interaction between γCD and the bile salts TC, GC, TDC and GDC by ROSEY ^{1}HNMR. Cabrer and co-workers reported that the hydroxyl group at C-7 were situated at the opening of the γCD cavity, and that no interactions was seen between the protons of the A-ring in the bile salt and γCD [25]. The trihydroxy bile salt, TβMC, which has its hydroxyl groups on C-3, C-6 and C-7, showed the strongest binding to γCD (Table 1). Again, we observe a correlation between the absence of a hydroxyl group on C-12 and high affinity for γCD.

In a previous study Holm et al. [33] investigated the binding of the same bile salts to βCD and similarly reported that the conjugation of the bile salts had no impact on the strengths of the stability constants. For GC and TC the stability constants are in the same range as those previously reported for complexation with natural βCD measured by ACE [33]. The stability constants determined in this study for the interaction between GCDC and TCDC and γCD are relatively high, which is in accordance with the results previously published for the binding of the same bile salts to βCD [28, 33]. In contrast to this, the stability constants for TDC and GDC were found to be rather different for γ- and βCD. As mentioned above the binding to γCD reported here was $2.8 \pm 0.3 \times 10^4$ M^{-1} and $2.9 \pm 0.2 \times 10^4$ M^{-1}, whereas Holm et al. [33] only reported the same interactions to produce a stability constant of $4.7 \pm 0.6 \times 10^3$ M^{-1} and $4.9 \pm 0.9 \times 10^3$ M^{-1} for the binding to βCD. While a molecular interpretation of this must await structural investigations, it can be noted that the bile salts penetrate deeper into the more accommodating cavity of γCD [25]. Thus, a likely explanation of the difference between the βCD and γCD complexation abilities is the difference in the internal cavity size of the two CDs, which allows more space for the hydroxyl group on C-7 in γCD. This suggestion is in accord with the ROSEY H^{1}NMR data by Cabrer et al. [25].

For βCD the stability constant for TβMC was found to be stronger than the stability constants for GCDC and TCDC [33], but in this study the stability constant for γCD was of similar magnitude for the three bile salts. It is likely that these differences relate to the different orientations of the hydroxyl group on the C-7 in the two classes of bile salts (see Fig. 1). On TβMC the hydroxyl group on C-7 is orientated more parallel with the sterol skeleton than the hydroxyl group on C-7 in GCDC and TCDC, which to a larger degree points out into the room. In βCD these structural differences may impact the binding site on the bile salts, whereas the more spacious cavity of the γCD allows space for both orientations, which may make the

difference less significant, leading to similar stability constants for the three bile salts.

Inclusion complex formation with 2-hydroxypropyl-γ-cyclodextrin

The stability constants for the complexes formed between 2OHpγCD and the conjugated bile salts are presented in Table 1. In spite of 2OHpγCDs potential applicability in oral drug delivery, bile salt interactions with this CD have not previously been explored. The stability constants relating to the modified 2OHpγCD are generally lower than the stability constants found for γCD. The differences in binding constants are moderate, and they are typically lower by a factor of two for 2OHpγCD. More importantly, the relative differences between the seven bile salts, found for γCD, are mirrored in the data for 2OHpγCD, thus suggesting that the interpretations for γCD that are presented above are equally valid for 2OHpγCD. The decrease in the binding constant for TβMC, when interacting with 2OHpγCD instead of γCD is larger than the same decrease for GCDC and TCDC. GCDC and TCDC has only one hydroxyl group at the interphase between the CD cavity and the aqueous phase [25], whereas TβMC's has two hydroxyl groups in this regions, at C-6 and C-7. This may lead to a higher sterical interaction with the 2-hydroxypropyl moieties on the modified γCD, than for GCDC and TCDC and consequently a larger decrease in the stability constant. The general decrease in the stability constants to the modified γCD is analogous to results for βCD and modified βCDs [33] and sterical hindrance due to the 2-hydroxypropyl moieties is generally a likely explanation for the decrease in affinity.

Only the free form of the drug, which is in equilibrium with the complexed species, is available for absorption. In vivo studies have previously demonstrated the importance of bile salts in relation to the increase in drug bioavailability observed upon co-administration of βCDs [23, 57]. It was stated that bile salts are important for the displacement of drugs from the βCD complexes. All the bile salts investigated in the present study interact with the γCDs studied; hence it is likely that they may contribute to the release of complexed drug upon oral administration and thereby ensure that the compound is released at the site of absorption, potentially increasing the bioavailability of the (complexed) drug. Comparison of the stability constants previously published for the interaction between the bile salts and βCDs [25, 27–29, 31–33, 47, 58] with the stability constants presented in this study can indicate whether γCDs may be more or less prone to decrease the bioavailability if given in high doses orally. The largest difference between the two CDs is seen in the strength of the stability constant to TDC and GDC. As these bile salts interact stronger with γCD than βCD, it is less likely that the γCD should decrease the bioavailability of a compound, if dosed orally in excess when compared to the βCDs.

In conclusion this study has presented 1:1 stability constants of the binding of seven biological relevant glyco- and tauro-conjugated bile salts to γCD and the modified 2OHpγCD. Mobility shift ACE was successfully applied for the investigation of bile salt–γCD interactions. For the first time, the stability constants for GC, GCDC, GDC, TC, TCDC, TDC, and TβMC complexation with γCD and 2OHpγCD were determined by the same method enabling direct comparisons and reliable assessments of the relative complex stability. All of the investigated bile salts had a significant affinity for γCD and 2OHpγCD with complexation constants varying from 2×10^3 to 8×10^4 M^{-1}. The study further demonstrated that it was possible for γCDs to recognize conjugated bile salts and distinguish between the structures, and that bile salt complexation with the natural cyclodextrin generally led to higher stability constants than the 2OHpγCD. The presence (or absence) of hydroxyl groups at positions C-7 and C-12 strongly affected the affinity of the bile salts to the CDs, as previously reported in the literature for the βCDs, and this most likely reflects effects of steric hindrance. Hence, the bioavailability of poorly soluble drug substances strongly complexed with γCD or 2OHpγCD may be affected by co-administration of these CDs due to displacement from the inclusion complexes by glyco- and tauro-conjugated bile salts to varying degrees.

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