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ORIGINAL ARTICLE

Thermodynamics of complexation of tauro- and glyco-conjugated bile salts with two modified β-cyclodextrins

René Holm · Jens Christian Madsen · Wei Shi · Kim Lambertsen Larsen · Lars Wagner Städe · Peter Westh

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Abstract The interaction between two modified β-cyclodextrins and bile salts, common for rat, dog and man, was studied using isothermal titration calorimetry. The structural differences in the interaction were investigated by ^{13}C NMR. The two modified β-cyclodextrins were chosen because of their frequent use as oral excipients in drug formulation and in marketed products. All the investigated bile salts had an affinity for the β-cyclodextrins, although there were large variations in the stability constants. The variations in the enthalpic and entropic contributions to the overall Gibbs free energy revealed differences in the binding mode to the investigated bile salts, i.e. the bile salts with a hydroxyl group at C12 interacted differently than bile salts without this hydroxyl group. These observations were supported by ^{13}C NMR, which suggested binding to the D-ring of the steroid structure for bile salts with a hydroxyl group at C12 and to the C-ring for the bile salts without this hydroxyl group. The type of substitution of β-cyclodextrin had significant effects on the thermodynamics of the interaction where especially the entropic changed were affected.

Keywords Isothermal titration calorimetry · Bile salts · Complexation · Cyclodextrin · Modified cyclodextrins · Thermodynamics

Abbreviations

βCD	β-Cyclodextrin
CMC	Critical micelle concentration
GC	Glycocholate
GDC	Glycodeoxycholate
GCDC	Glycochenodeoxycholate
DS	Degree of substitution
HPβCD	2-Hydroxypropyl β-cyclodextrin
ITC	Isothermal titration calorimetry
mβCD	2-*O*-methyl β-cyclodextrin
SBEβCD	Sulfobutylether β-cyclodextrin
TC	Taurocholate
TβMC	Tauro-β-muricholate
TDC	Taurodeoxycholate
TCDC	Taurochenodeoxycholate

Introduction

Enzymes belonging to the group cyclodextrin cycloglycosyltransferases (GCRase, EC 2.4.1.19) convert starch into cyclic oligosaccharides, known as cyclodextrins (CDs).

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R. Holm (✉)
Preformulation, H.Lundbeck A/S, Ottiliavej 9,
2500 Valby, Denmark
e-mail: rhol@lundbeck.com

J. C. Madsen
Compound ID & Purification, H.Lundbeck A/S,
Ottiliavej 9, 2500 Valby, Denmark

W. Shi · P. Westh
NSM, Research Unit for Functional Biomaterials, Roskilde University, Universitetsvej 1, 4000 Roskilde, Denmark

K. L. Larsen · L. W. Städe
Section of Chemistry, Department of Biotechnology, Chemistry and Environmental Engineering, Aalborg University, Sohngaardsholmsvej 57, 9000 Aalborg, Denmark

These structures are composed of α-(1,4) linked glucopyranose subunits. α, β, or γ-CDs, consisting of six, seven, or eight glucose units respectively and are the most investigated CDs [1, 2]. The CD molecule has the shape of a torus and a spatial distribution of the polar hydroxyl groups on the outer rime and apolar (relative to water) glucoside oxygens and hydrogens in the cavity, hence the CD molecule have a hydrophilic outside and a hydrophobic cavity. As a consequence of this structure, CDs have the ability to form inclusion complexes through molecular encapsulation with a wide range of organic compounds [1, 2]. This special characteristic makes CDs valuable in a number of scientific disciplines, including pharmaceutics, where the increased solubility of complexes with poorly soluble drugs is exploited [1, 3–13], and is therefore a solution to a frequently encountered problem in modern drug discovery and development [14].

Natural βCD has an aqueous solubility of only 18.5 g/L [15], which is believed to be a reflection of the very rigid structure formed through H-bonding of the C2-hydroxyl of one glucopyranose unit with the C-3 hydroxyl of an adjacent unit [16]. In the βCD molecule a complete set of seven intra-molecular H-bounds can be formed, effectively lowering the thermodynamic drive for interaction with the solvent [17–21]. Natural βCD can be chemically modified by e.g. hydroxylation, alkylation or sulfoalkylation, thereby breaking this belt of H-bonds around the βCD increasing the solubility. Different chemically modified CDs with improved physical chemical properties have been prepared and commercialised. Two uncharged βCD derivatives, 2-hydroxypropyl β-cyclodextrin (HPβCD) and 2-*O*-methyl βCD (mβCD), have been widely investigated in the pharmaceutical literature on account of their fast dissolution rate, high solubility in water and low toxicity [22–24], and have been used in a number of marketed pharmaceutical products [4, 5]. See Fig. 1 for the natural βCD and potential position of substitutions.

Upon oral administration, xenobiotics complexed with CD are not absorbed in the gastrointestinal tract. The drug molecule must be displaced from the complex to be available for absorption, as CDs are only absorbed to a very small extent from the gastrointestinal tract [2]. In the small intestine this displacement of the xenobiotics is thought to depend upon bile salts [25–27], which may bind competitively to the CD. Insight into the interaction between bile salts and CDs is consequently a general prerequisite for rational formulation design of oral drug products containing CDs.

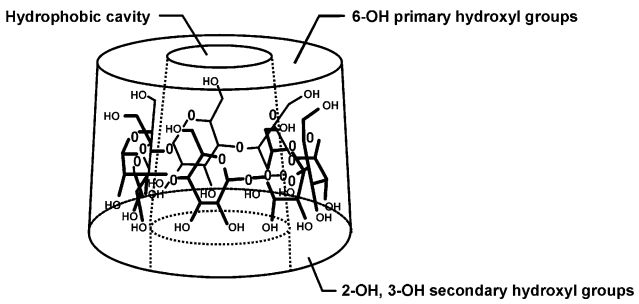

Fig. 1 Chemical structure of βCD, where 2, 3, and 6-hydroxyls of a glucopyranose unit may have a substituent attached

Most studies on the interaction of CDs and bile salts have used natural CDs [26–33]. Very limited information is available on the thermodynamics of the complexation between bile salts and modified βCDs [33–35], despite the extensive use of modified CDs. A few complexes between modified βCDs and bile salts have been studied earlier [33, 34], but variations due to differences in materials (e.g. the polydispersity of modified CDs) and experimental approaches are too great to single out effects of the CD modifications on the binding constant and other thermodynamic parameters. The experimental setup and the bile salts used here are the same as those in our earlier work on natural CDs [36], consequently it follows that the results may form the basis for a detailed comparative analysis, which can elucidate interrelationships of chemical modification of βCDs and the binding of bile salt. The purpose of the current study was therefore to investigate differences in binding thermodynamics and mode or place of binding of bile salts present in man, rat and dog [37] to the two pharmaceutically important modified βCDs; mβCD and HPβCD, using isothermal calorimetry (ITC) and ^{13}C NMR.

Experimental

Chemicals

Seven different bile salts of interest for man, rat and dog [37] were investigated. These are, taurocholate (TC), tauro-β-muricholate (TβMC), taurodeoxycholate (TDC), taurochenodeoxycholate (TCDC), glycocholate (GC), glycodeoxycholate (GDC) and glycochenodeoxycholate (GCDC), see Fig. 2. 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-(3α,6β,7β-trihydroxy-10,13-dimethylhexadecahydro-cyclopenta[a]phenanthren-17-yl]-pentanoylamino)ethansulfonic acid) from Makaira Ltd (London, UK), taurochenodeoxycholate (2-([3α,7α-dihydroxy-24-oxo-5β-cholan24-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)

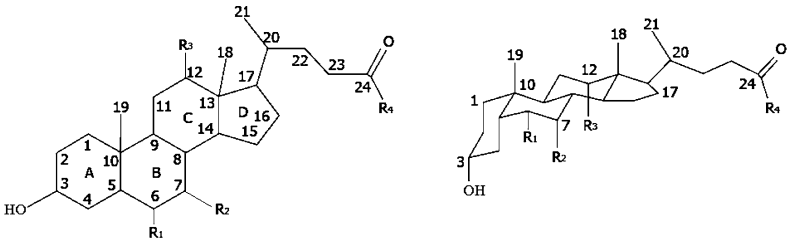

Fig. 2 Schematic structure and structure showing orientation of the glyco- and tauro-conjugated bile salts investigated in the present study. α Indicates a sterical orientation below the plane and β above

Bile acid	Abbreviation	R_1 (C-6)	R_2 (C-7)	R_3 (C-12)	R_4 (C24)
Taurocholic acid	TC	H	OH (α)	OH	$NHCH_2CH_2SO^-_3$
Glycocholic acid	GC	H	OH (α)	OH	$NHCH_2COO^-$
Taurodeoxycholic acid	TDC	H	H	OH	$NHCH_2CH_2SO^-_3$
Glycodeoxycholic acid	GDC	H	H	OH	$NHCH_2COO^-$
Taurochendeoxycholic acid	TCDC	H	OH (α)	H	$NHCH_2CH_2SO^-_3$
Glycochendeoxycholic acid	GCDC	H	OH (α)	H	$NHCH_2COO^-$
Tauroβhyocholic acid	TβMC	OH	OH (β)	H	$NHCH_2CH_2SO^-_3$

amide), glycodeoxycholate (3α,12α-dihydroxy-5β-cholan-24-oic acid *N*(carboxymethyl)amide) and glycochenodeoxycholate (3α,7α-dihydroxy-24-oxo-5β-cholan-24oic acid *N*-(carboxymethyl)amide) from SigmaAldrich (St. Louis, MO, USA). All bile salts had stated purities greater than 97% and were used as provided. 2-*O*-methyl β-cyclodextrin (mβCD; degree of substitution according to supplier information of 3.99, i.e. average number of glucopyranose units with a substitution group) was the generous gift from Roquette (Le Strem, France) and 2-hydroxypropyl β-cyclodextrin (HPβCD; degree of substitution according to supplier information 4.55) was purchased from the same company. Sodium phosphate and D_2O were obtained from SigmaAldrich (St. Louis, MO, USA). The water used in the experiments was obtained from a Millipore purification system.

MALDI-TOF MS

MALDI-TOF MS was performed using a fast evaporating nitro-cellulose (FENC) matrix in a Reflex III (Bruker Daltonics, Bremen, Germany) to determine the average degree of CD substitution. A thin layer of freshly prepared saturated α-cyano-4-hydroxycinnamic acid (CCA) in acetone was deposited on a MALDI target plate and allowed to dry. The modified βCD was then deposited directly onto the MALDI target plate by adding a 1 μL droplet of a 1:1 solution of the modified βCD (1 mM) and a 1:4 (v/v) mixture of nitro-cellulose and saturated CCA in an aqueous solution of 0.1% triflouroacetic acid and 80% acetonitrile.

The average degree of substitution (DS) of the modified CD's is calculated from the MALDI-TOF MS spectrum using the following equation:

$$\mathrm{DS} = \frac{\sum_i I_i \cdot \mathrm{DS}_i}{\sum_i I_i} \quad (1)$$

where "I_i" denotes the intensity of the i'th peak and "DS_i" denotes the degree of substitution corresponding to the i'th peak.

Isothermal titration calorimetry

Microcalorimetric titrations were performed at atmospheric pressure and 25 °C in an aqueous 50 mM phosphate buffer solution, pH 7.0, using a MicroCal VP-ITC titration microcalorimeter (MicroCal, Northhampton, MA, USA). The isothermal titration calorimeter was calibrated electronically. The bile salts were loaded into the calorimetric cell and titrated with twenty 10 μL aliquots of CD solution with 240 s of separation. All the solutions were degassed using a ThermoVac (MicroCal, Northhampton, MA, USA) before the titration experiment. The heat of dilution was estimated from the heat change at high CD concentrations, where practically all bile salt had been bound, and subtracted from the raw data prior to non-linear regression analysis. The latter was performed with the ORIGIN software package (version 7.0). The fitted parameters are the binding constant (K_s), the change in binding enthalpy (ΔH) and the binding stoichiometry (n). Knowledge of the binding constant and molar reaction enthalpy enabled

calculation of the standard Gibbs free energy of binding (ΔG°) and the change in entropy (ΔS°) according to:

$$\Delta G^\circ = -RT \ln K_s = \Delta H^\circ - T\Delta S^\circ$$

where R is the gas constant and T is the absolute temperature.

All ITC experiments were conducted at (or below) 1 mM as bile salts form micelles with a critical micelle concentration (cmc) in the range of 2–10 mM [32, 33, 38–41]. This ensured that the measured enthalpy change represented the complex formation without contributions from a concomitant dissolution of micellar aggregates. The experimental setup for the ITC procedures was similar to those previously used for similar systems [33, 34, 36]. In order to obtain useful data for TβMC, which showed a very strong binding to both mβCD and HPβCD, a TβMC concentration of 0.25 mM was used.

NMR spectroscopy

All the NMR experiments were carried out at 25 °C on a Bruker Avance-600 NMR spectrometer operating at 14.1 T and equipped with a cryogenically cooled probe. Characterisation of the CDs, 6144 scans were acquired for the APT experiments while for the two-dimensional HSQC, H2BC, COSY and HMBC experiments, 48, 96, 96, and 64 scans, respectively, were acquired for each of the 128 t_1 increments (at a concentration of 10 mM per component in D_2O). The assignment of the CDs was achieved by a combination of APT, HSQC, H2BC, and HMBC experiments, which were in agreement with results previously published by Mucci et al. [42] for HPβCD.

The assignment of the ^{13}C chemical shifts for the free and the complexed bile salts was achieved by a combination of APT, HSQC, H2BC, and HMBC experiments, which were in agreement with results previously published by Ijare et al. [43] for all bile salts except TβMC, where no previous assignment could be found. For the uncomplexed and complexed bile salts (at 10 mM in D_2O), 1024 scans were acquired for the APT experiments while for the two-dimensional HSQC, H2BC, and HMBC experiments, 8, 32, and 32 scans, respectively, were acquired for each of the 128 t_1 increments. The experiments with TβMC were carried out at 5 mM for both the bile salt and the CD due to lack of compound. The lower concentration of the complexes with TβMC necessitated twice the number of scans for all experiments.

Results and discussion

Modified CDs are extensively used in both drug discovery, drug development and in marketed pharmaceutical products to improve the bioavailability or chemical stability of the pharmacological active compounds [1, 3–13]. Upon oral administration, bile salts are thought to play an important role in the release of these drugs from the CD complexes [25–27]. The thermodynamic properties of the bile-CD complexes are thus important both for the basic understanding of the binding process and rational drug delivery.

Cyclodextrin characterisation

The two modified β-CDs were characterised by MALDI-TOF MS, ^1H NMR, APT, COSY and ^1H–^{13}C HSQC NMR in order to determine the molecular weight and degree and position of the substituents. The MALDI-TOF MS and the NMR spectra used for the characterisation of the investigated CD can be found in the Supplementary data.

For both modified βCDs the MALDI-TOF MS revealed a mixture of up to 8 substitutions, see Table 1. For HPβCD the highest signal intensity was found for the tetra substituted CD. The average molecular weight (m_w) was 1417.44 and the DS was 4.46. For mβCD the penta substituted CD gave the highest signal, while the average m_w was 1227.1 and the DS was 4.82.

In the NMR experiments, the methylation is clearly seen in the APT and HSQC spectra of the mβCD sample, where a new ^{13}C signal appears at $\delta(^{13}C) = 60$ ppm, when compared to the natural βCD, corresponding to the methyl group of mβCD. The methylation induces changes in the chemical shifts in a number of neighbouring ^1H and ^{13}C nuclei in mβCD. Since the NMR sample contains both methylated and non-methylated βCD molecules, we observe a doubling of several of the $\delta(^1H)$ and $\delta(^{13}C)$

Table 1 Calculated and measured masses of the two modified βCDs including average degree of substation (DS) calculated according to Eq. 1

Number of substitution	HPβCD (m/z)		mβCD (m/z)	
	Theoretical	Measured	Theoretical	Measured
1	1215.04	1214.30		
2	1273.12	1272.32	1185.03	1185.10
3	1331.20	1330.39	1199.04	1199.01
4	1389.28	1388.46	1213.07	1213.07
5	1447.36	1446.46	1227.10	1227.12
6	1505.44	1504.55	1241.12	1241.13
7	1563.52	1562.53	1255.15	1255.16
8	1621.59	1620.50	1269.18	1269.13
Average mw	1418.32	1417.44	1227.09	1227.10
$DS = \frac{\sum I(d \cdot S)}{\sum I}$	4.46		4.82	

values, and this is particularly apparent in the two-dimensional HSQC and COSY spectra where some of the correlation peaks are doubled.

The HSQC spectrum provides a convenient way of measuring the substitution-induced chemical-shift differences ($\Delta\delta$) for ^{1}H and ^{13}C simultaneously. If $\Delta\delta$ for a given nucleus is defined as its chemical shift in the substituted form minus its chemical shift in the unsubstituted form, a large $\Delta\delta$ for the C-1, H-1, C-2, and H-2 nuclei (-2.5, $+0.19$, $+9.3$ and -0.27 ppm, respectively) is observed, which can be attributed to the substitution of the 2-OH. For the C-3 and H-3 nuclei, only small $\Delta\delta$ values of -1.0 and $+0.6$ ppm, respectively, was observed. Based on these observations, the conclusion is that the 2-OH is the primary position for substitution. However, the presence of several small signals in the NMR spectra reveals that a minor degree of substitution also occurs at the 3-OH and 6-OH positions. From the APT measurement a DS of 4.24 was estimated by calculating the ratio between the integrated signals from H-1 and the methyl group. This differs significantly from the DS obtained from the MALDI-TOF MS measurements. As there are several parameters that may affect the ionisation of the modified CDs, the value obtained by NMR was considered the most reliable and used for the calculations in the ITC experiments.

For the HPβCD a splitting pattern similar to mβCD was observed in the ^{1}H and HSQC spectra. The $\Delta\delta$ for the C-1, H-1, C-2, and H-2 nuclei was -2.2, $+0.15$, $+8.3$ and -0.15 ppm, respectively, while the C-3 and H-3 nuclei had a $\Delta\delta$ of -0.75 and $+0.10$ ppm, respectively. In addition there was a splitting of the H-6 signal (0.2 ppm). As the CH and CH_2 proton signals from the hydroxylpropyl groups are found in the same region as the signals from H-2 to H-6 from the CD, the COSY spectrum could not be used for assignment of the neighbouring protons and the position of substitution was therefore exclusively based on the splitting patterns in the ^{1}H and ^{1}H–^{13}C HSQC spectra. The preferential position for substitution was the 2-OH. The DS obtained from the CH_3 signal in the APT spectrum was 4.48 in accordance with the value calculated from the MALDI-TOF measurements as well as the value provided by the supplier.

Calorimetry

Figure 3 shows a typical enthalpogram for a complex with high stability constant. Panel A shows the raw data while panel B illustrates the ability of the non-linear regression to account for the measurements. Regression parameters for triplicate measurements are listed in Table 2 (mβCD) and 3 (HPβCD), respectively. The following sections present the main results from these tables.

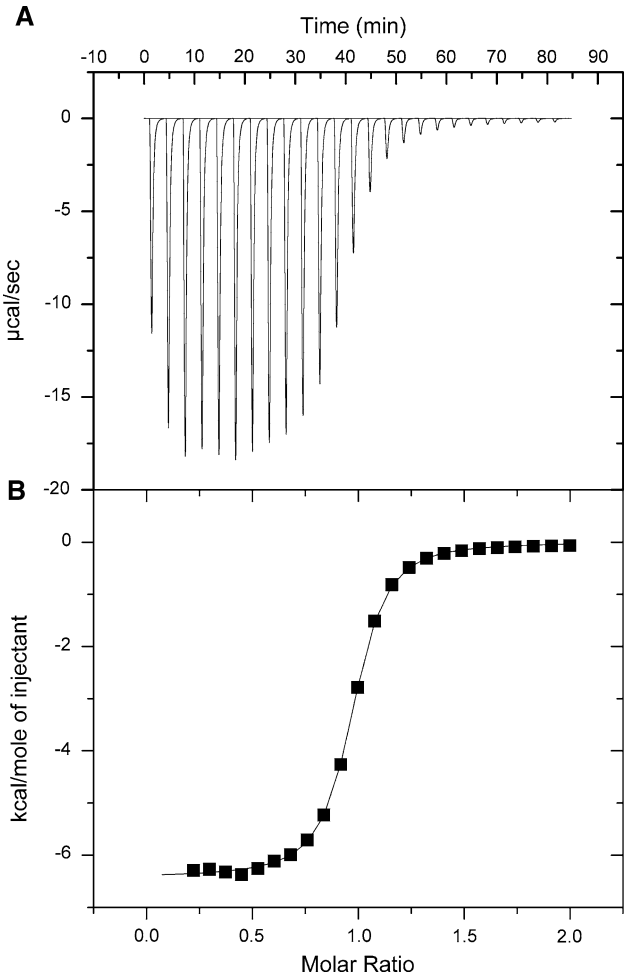

Fig. 3 Calorimetric titration of TCDC with mβCD in 50 mM phosphate buffer (pH 7.0) at 298.15°K. **A** Raw data for sequential 10 µL injections of mβCD solution (10 mM) into the bile salt solution (1 mM). **B** Heats of reaction as obtained from the integration of the calorimetric traces

Binding stoichiometry

The stoichiometric parameter, n, is derived directly from the non-linear regression from the ITC experiment and fell in the range 0.9–1.1 for all the investigated systems. This suggests that the interaction was strongly dominated by a 1:1 complex in the concentration range studied here, in accordance with earlier reports on modified βCDs [33–35, 44, 45].

Stability constants

The measured stability constants are presented in Tables 2 and 3, including literature values were available. Generally, the stability constant for the bile salts and the two modified CDs are lower than the reported values for natural

Table 2 Complex stability constant (K), standard free energy ($\Delta G°$), enthalpy ($\Delta H°$) and entropy changes ($\Delta S°$) of 1:1 inclusion complexes of bile salts with mβCD (degree of substitution 0.61). Values are given as mean ± range from three different experiments in 50 mM phosphate buffer (pH 7.0) at 298.15°K (mean ± SD, $n = 3$). Data given in italic are literature data for comparison

Guest	K (M^{-1})	$\Delta G°$ (kJ mol^{-1})	$\Delta H°$ (kJ mol^{-1})	$\Delta S°$ (J K^{-1} mol^{-1})
TC	2847 ± 21	−19.70 ± 0.02	−19.88 ± 0.13	−0.63 ± 0.05
	2148[a]	*−19.0*[a]	*−7.2*[a]	*39.6*[a]
GC	3033 ± 81	−19.83 ± 0.07	−20.80 ± 0.38	−3.17 ± 1.45
	1958[a]	*−18.8*[a]	*−7.2*[a]	*46.6*[a]
TDC	6383 ± 387	−21.69 ± 0.15	−21.49 ± 0.43	0.68 ± 1.89
	2944[b]	*−19.8*[b]	*−12.9*[b]	*23.4*[b]
GDC	7487 ± 659	−22.09 ± 0.22	−21.39 ± 0.54	2.47 ± 1.92
TCDC	155,333 ± 7572	−29.60 ± 0.12	−27.47 ± 0.58	7.16 ± 2.05
GCDC	170,667 ± 13,577	−29.83 ± 0.20	−28.25 ± 0.19	5.31 ± 0.87
TβMC	572,333 ± 29,263	−32.83 ± 0.12	−32.96 ± 0.38	−0.44 ± 1.67

[a] Literature data determined by isothermal titration calorimetry from [34]

[b] Literature data determined by isothermal titration calorimetry from [33]

Table 3 Complex stability constant (K), standard free energy ($\Delta G°$), enthalpy ($\Delta H°$) and entropy changes ($\Delta S°$) of 1:1 inclusion complexes of bile salts with HPβCD (degree of substitution 0.64). Values are given as mean ± range from three different experiments in 50 mM phosphate buffer (pH 7.0) at 298.15°K (mean ± SD, $n = 3$). Data given in italic are literature data for comparison

Guest	K (M^{-1})	$\Delta G°$ (kJ mol^{-1})	$\Delta H°$ (kJ mol^{-1})	$\Delta S°$ (J K^{-1} mol^{-1})
TC	1533 ± 64	−18.20 ± 0.10	−13.23 ± 0.34	16.66 ± 1.17
	1399[a]	*−18.0*[a]	*−8.75*[a]	*31.0*[a]
GC	1637 ± 45	−18.33 ± 0.68	−14.52 ± 0.52	12.78 ± 1.94
	1764[a]	*−18.5*[a]	*−8.2*[a]	*34.5*[a]
TDC	3547 ± 236	−20.24 ± 0.16	−15.01 ± 0.49	17.55 ± 2.08
	1967[b]	*−18.8*[b]	*−11.7*[b]	*23.8*[b]
GDC	3803 ± 206	−20.41 ± 0.13	−15.39 ± 0.35	16.86 ± 1.22
TCDC	46,433 ± 2757	−26.61 ± 0.15	−21.60 ± 0.28	16.81 ± 1.39
GCDC	52,733 ± 2056	−26.92 ± 0.10	−18.93 ± 0.98	26.53 ± 3.05
TβMC	104,567 ± 29,263	−28.62 ± 0.10	−27.57 ± 0.19	3.53 ± 0.97

[a] Literature data determined by isothermal titration calorimetry from [34]

[b] Literature data determined by isothermal titration calorimetry from [33]

βCD [27, 28, 30, 32, 46]. The stability constant was higher for mβCD than HPβCD, which is in accordance with previously published data [33–35]. The size of the stability constant reported in the present study was in the same range as literature data investigating the interaction between bile salts and modified βCDs [33, 34], though higher in some cases. The CDs used by Cooper et al. [33] had a higher degree of substitution than the CDs used in the present study, whereas similar information is not available for the work by Ollila et al. [34]. A possible explanation for these differences may therefore be due to variations in the degree or position of substitution. Supporting this hypothesis, Zia et al. [47] reported a decrease in the stability constants for four different steroids (hydrocortisone, prednisolone, methylprednisolone and testosterone) interacting with sulfobutylether-β-cyclodextrin (SBEβCD) as the degree of substitution increased [47]. Further, Müller and Brauns [48] reported that hydroxylpropyl βCDs with lower degrees of substitution were more prone to form stronger complexes with hydrocortisone. This is also in accordance with other previous investigations [49–51], collectively suggesting that the stability of steroids and other polyaromatics are generally hampered by the substitution of the CD.

For both investigated CDs, and all pairs of bile salts, the affinity of the tauro-conjugated bile salts was slightly (but statistically significant) lower than its glycol-conjugated counterparts. Similar findings have previously been

reported for the interaction between βCD and TC, GC, TDC and GDC, whereas the difference between TCDC and GCDC was not observed when the interaction occurred with βCD [36]. Holm et al. [36] demonstrated that the interaction of TCDC and GCDC with βCD occurred at the C and D ring of the sterol. Hence, the bile salt was placed so deep in the CD cavity that the bile salt conjugation was in the bulk. However, for the modified βCDs the cavity is extended by the addition of the methyl or hydroxyl-propyl groups [52]. The differences between βCD and the modified βCD may therefore have emerged as the conjugation of the bile salts is situated in or at the proximity of the extended CD cavity. Hereby the chemical differences in bile salt conjugations are reflected in the magnitude of the stability constant as an interaction now occurs, assuming that the interaction still occurs at the C and D ring of the bile salt.

The stability constants for TC and GC were lower than the stability constants for TDC and GDC for both investigated CDs. These findings are in contradiction to results published for the interaction of these bile salts and natural βCD, where the stability constants were similar for the four bile salts [36]. The structural difference between the two classes of bile salts is the hydroxyl group on C7, which is present on TC and GC but not in TDC and GDC. The extension of the CD cavity in the modified CDs may again be a part of the explanation for these observed differences, as it may give rise to additional contacts in the complexes affecting the stability constant.

Although variations were found between the stability constants for TC/GC and TDC/GDC the most noticeable differences in Table 2 and 3 correlate with the presence of a hydroxyl group at C12 on the bile salt (Fig. 2), where the interaction has been suggested to occur with βCDs [28, 29, 35, 36]. The stability constants for TCDC, GCDC and TβMC are significantly higher than for TC, GC, TDC and GDC, which is consistent with earlier reports with mβCD and HPβCD [35]. The hydroxyl group at C12 is highly important for the structural positioning of the bile salt within the modified CDs due to electrostatic or steric hindrances, hence explaining the distinct negative impact on the stability constant of this group. Modification of the CDs has no significant implication on this as it happens within the CD cavity.

Enthalpy and entropy changes

A compensatory relationship between enthalpy and entropy changes is frequently seen in the complex formation between series of related guest molecules and CDs [53–55]. The molecular ("extra-thermodynamic") mechanism underpinning this linear relationship between ΔH and ΔS has been extensively discussed [56–70] as no explicit

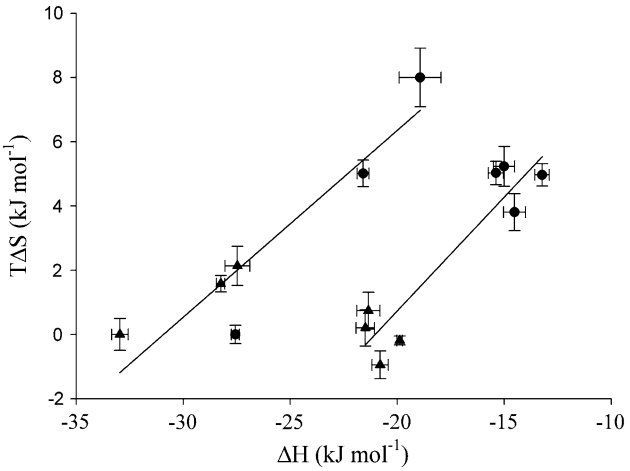

Fig. 4 Compensation plot showing ΔH as a function of $T\Delta S$ for the complex formation between the seven investigated bile salts with either mβCD, *filled triangle*; and HPβCD, *filled circle*

relationship between these parameters can be derived from fundamental thermodynamics. Empirical analysis of enthalpy–entropy compensation plots for bile salts complexes with natural βCD suggested that the weakly bound bile salts (the cholates and deoxycholates) fall into one group with a mode of interaction qualitatively different from the strongly bound bile salts [35, 36]. For the two current modified CDs a similar pattern was observed, as demonstrated in the compensation plot in Fig. 4, hence TC, GC, TDC and GDC seem to interact differently with the modified CDs than TCDC, GCDC and TβMC. It has been suggested that the slope (α) and the intercept ($T\Delta S°$) of the enthalpy–entropy compensation plot can be related to the degree of conformational change and the extent of desolvation induced upon complexation, respectively [53–55, 71]. As the data in the present study are limited, a thorough enthalpy–entropy analysis would be an over interpretation. Nevertheless, Fig. 4 does suggest a relative large intercept ($T\Delta S°$) for both classes of bile salts, which is in accordance with reported values for simple modified CDs possessing a flexible hydrophilic sidearm [55]. This large intercept indicates an extensive desolvation effect in the interaction between the investigated species.

The enthalpy change is negative for all the host–guest pairs investigated, with values in the −15 to −30 kJ/mol range, and ΔH is the dominant contribution to $\Delta G°$. Thus, it was found that the net affinity is proportional to the size of the (negative) enthalpy change. Previously published ΔH values for TC, GC and TDC are lower than the current results particularly for mβCD. This may again be the result of different batches with a different extent and/or placement of CD substitution. Comparison of the data in Tables 2 and 3 with analogous results for natural βCD [36] shows no major differences in ΔH for mβCD, whereas

ΔH was generally smaller for 2OHpβCD. The introduction of a hydrophilic side chain on the CD expands the hydrophobic cavity of the CD to some extent [52] and thus, judged from the thermodynamic parameters found in the present study, co-operative van der Waals and hydrophobic interactions must occur in inclusion complexation with 2OHpβCD. This phenomenon is also previously reported on the interaction of long-chain carboxylic acids interaction with 2OHpβCD [72] and mαCD [73]. The value of ΔH is determined by several effects [71, 74–76], which may differ for 2OHpβCD when compared to mβCD and natural βCD. More water interactions needs to be disrupted due to the extension of the cavity, but also the adaptation of the bile salt into the CD cavity may be less optimal.

Interestingly, the lower enthalpic contribution towards association with HPβCD is almost fully compensated by a more positive entropy change. This observation is interesting in the light of the expanding effect of the hydroxylpropyl substitution on the dimensions of the cavity [52], which is discussed above. Generally, ΔS for the interaction between 2OHpβCD and the bile salts contributed to the Gibbs free energy with a positive value larger than the change in entropy observed for mβCD. Holm et al. [36] recently reported large negative entropy for the interaction between βCD and TC, GC, TDC and GDC, and entropy around zero for TCDC, GCDC and TβMC. This indicates that the longer and more branched the side on the CD, the smaller the entropic penalty or the larger the contribution to the total free energy. According to the classical hydrophobic interactions [77], the difference between ΔS for the three CDs may, be explained by the disruption between the CD side chain and associated water generating a lower degree of order, thereby leading to higher degrees of freedom and consequently a favourable entropy. Another plausible common interpretation would be that bile salt complexes with HPβCD compared to the natural βCD are characterised by moderately expanded hydrophobic contact between the two species leading to an increased ΔS and a less optimal spacial fit, e.g. placement of the hydroxyl groups in the bile salts close to the hydroxyl group in the CD or sterical hindrance to the CD cavity from the CD side chains.

^{13}C-NMR

^{13}C-NMR spectra were obtained to identify the location of the sterol structure in the CD cavity. A typical spectrum is presented in Fig. 5. The change in the chemical shift (ppm, $\Delta\delta$) when including bile salts into either mβCD or HPβCD in D_2O can be found in the supportive data. Earlier NMR studies on the interaction between bile salts and modified CDs have been conducted in methanol–d_4 solutions [42, 44, 45, 78, 79], as the low solubility of the studied unconjugated bile salts prevented the investigations in D_2O [80]. These studies showed distinctly lower $\Delta\delta$ values than the present work. Solvent is known to influence the CD complexes [81–84] and the difference in the size of $\Delta\delta$ may, therefore, be a reflection of the differences in polarity of the continuous phase, affecting the stability of the complexes.

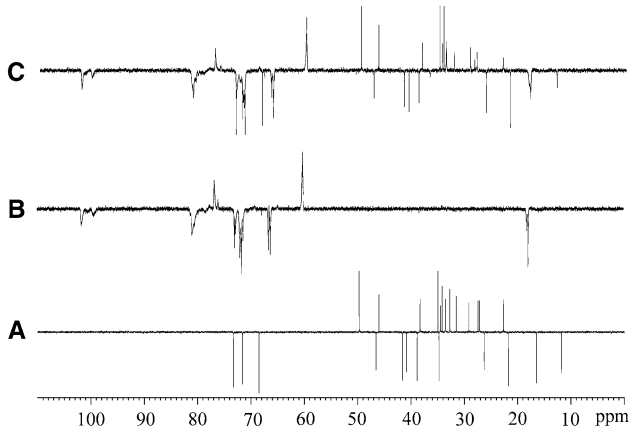

Fig. 5 Partial ^{13}C NMR spectra of **A** TC, **B** HPβCD, **C** the complex of TC in HPβCD; all measured in D_2O

The carbon signals that underwent the largest shifts for TC, GC, TDC and GDC are all from on the conjugation chain situated at C17. High $\Delta\delta$ at C13, C16, C17 and C18 points out that the interaction extends to the cyclopentane ring (the D-ring). For C11 a relatively high shift is observed as well, but since no other interactions were seen from the C-ring, this may indicate a strong interaction of C11 with the hydroxylpropyl or methyl chains in the modified CDs. The small shifts of the remaining carbons indicate a weak interaction of the other parts of the bile salts with the two modified βCD. For TDC and GDC some weak interactions with C1 and C2 in the A-ring seem present; an observation also previously reported for natural βCD [27, 28, 36]. These observations have led to the suggestion of 2:1 complexes with βCD [27, 28], however, the second interaction site was not confirmed by the ITC results. This suggests that the stability of the second complex is so low that it does not become significantly populated in the concentration regime ($\sim$1 mM) investigated in the ITC trials.

For TCDC, GCDC and TβMC high $\Delta\delta$ shifts were seen at the same carbon atoms as in other bile salts, but interactions were also observed at C14 and C12, suggesting that the bile salts get deeper into the CD cavity. Very small shifts were seen for the carbon atoms in the A- and B-rings of the bile salt, i.e., only weak interactions occurred between the CDs and the atoms in these rings. The overall structure of the bile salt complexes formed by the two modified CDs therefore seems to be similar to the complexes of natural βCD [36]. More exhaustive analysis,

however, identified interactions of the tauro- and glycol-conjugated part of the bile salts and the modified CD, an interaction not observed for the natural βCD. This may contribute to the slight differences in the thermodynamics between TCDC and GCDC, as discussed above.

Though interactions of the bile salts with the methyl or hydroxylpropyl groups added to the CDs were observed, it cannot be concluded that the relative large differences in the thermodynamics between βCD, mβCD and HPβCD rely solely on these small structural differences. The changes in both the stability constant and the enthalpic and entropic contribution to the Gibbs free energy may therefore be a complex mixture of minor differences in physical orientation within the CD cavity, CD flexibility and interactions with different substituents on the rims of the CDs, but apparently also through differences in the hydration effects. However, what can be firmly stated is that the thermodynamics is affected by CD substitution in a rather systematic way.

Conclusion

In conclusion, this study has presented 1:1 stability constants and thermodynamic parameters for the binding of seven biologically relevant glyco- and tauro-conjugated bile salts to the two modified βCDs, mβCD and HPβCD, studied by ITC. All of the investigated bile salts had a significant affinity for the two CDs, with stability constants varying from 1.5×10^3 to 5×10^5 M^{-1}; hence the interaction must be considered highly relevant for the release of CD-formulated drugs upon oral administration. Complex formation was found to be enthalpy driven for all the bile salts, with typical ΔH values typical in the range of -15 to 30 kJ/mol. The entropy changes were highly sensitive to the chemical structure of the host molecule. This may suggest an important role of the extension and altered flexibility of the CD cavity, as possibly hydrophobic contacts between the bile salt and the conjugated moieties and to some extent also through additional dehydrating effects. The presence or absence of hydroxyl groups at C-12 strongly affected the affinity of the bile salts for the CDs, but also increased the entropy penalty. ^{13}C-NMR experiments suggested a plausible structure for the inclusion complex formed between the investigated bile salts and the two modified βCDs investigated, which were similar to the structures reported for natural βCD. Differences in the thermodynamic parameters of natural and modified βCDs could not be fully rationalised through the observed structural variations.

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