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Retention of bile salts in micellar electrokinetic chromatography: relation of capacity factor to octanol–water partition coefficient and critical micellar concentration

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Abstract

The capacity factors of 16 anionic cholates (from six bile salts, including their glyco- and tauro-conjugates) were determined in a micellar electrokinetic chromatography (MEKC) system consisting of buffer, pH 7.5 (phosphate–boric acid; 20 mmol/l) with 50 mmol/l sodium dodecyl sulfate (SDS) as micelle former and 10% acetonitrile as organic modifier. The capacity factors of the fully dissociated, negatively charged analytes (ranging between 0.2 and 60) were calculated from their mobilities, with a reference background electrolyte (BGE) without SDS representing ''free'' solution. For comparison, the capacity factors were derived for a second reference BGE where the SDS concentration (5 mmol/l) is close to the critical micellar concentration (CMC). The capacity factors are compared with the logarithm of the octanol–water partition coefficient, log P_{OW}, as measure for lipophilicity. Clear disagreement between these two parameters is found especially for epimeric cholates with the hydroxy group in position 7. In contrast, fair relation between the capacity factor of the analytes and their CMC is observed both depending strongly on the orientation of the OH groups, and tauro-conjugation as well. In this respect the retention behaviour of the bile salts in MEKC seems to reflect their role as detergents in living systems, and might serve as model parameter beyond lipophilicity.

Keywords: Bile salts; Cholates

1. Introduction

Bile acids are steroid compounds, hydroxy-derivatives of 5-β-cholan-24-oic acid. They have different physico–chemical properties according to the number, position and orientation of their hydroxyl groups, and the type of conjugation with glycine and taurine, which form the glyco- and tauro-derivatives. These factors influence their solubility, detergent properties and lipophilicity.

Ursodeoxycholic acid, cholic acid, chenodeoxycholic acid, deoxycholic acid, lithocholic acid, and their glyco- and tauro-conjugate forms (Fig. 1) play an important role in biological systems under both, physiological and pathophysiological conditions [1–3]. Their biological functions are principally associated with lipid digestion and absorption, solubilisation of cholesterol and bile formation influencing its

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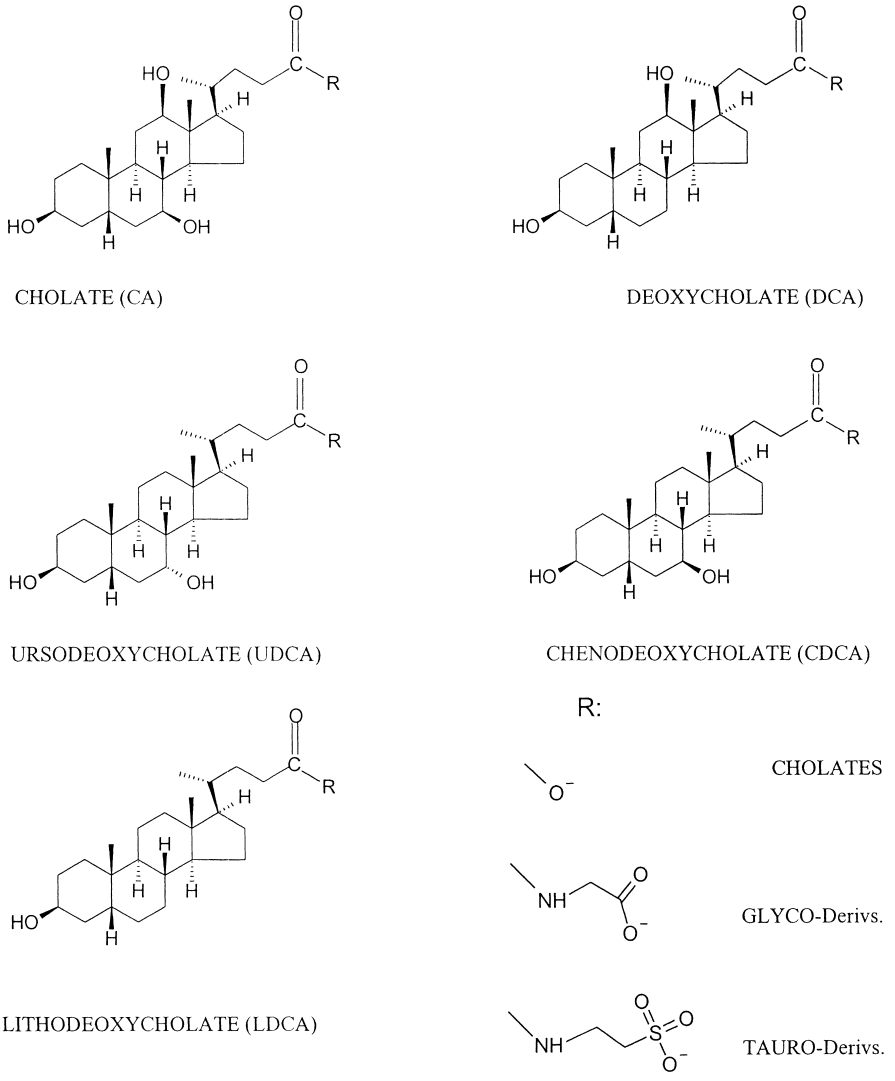

Fig. 1. Chemical structures of the bile salts and their glyco- and tauro-derivatives.

volume and composition. Some of the compounds are used as therapeutic agents [4], ursodeoxycholic acid and chenodeoxycholic acid, e.g., are administered in the treatment of cholesterol gallstones. Dehydrocholic acid is offered in pharmaceutical formulations associated with other components as a choleretic agent in the treatment of liver dysfunctions [5,6].

The knowledge of the biotransformation of bile acids and their role in healthy metabolism [7], on the one hand, and in dysfunctions especially in hepatobiliary disease [6,8], on the other hand, is highly important. There are several attempts to relate the chemical structure of a compound to its biological behaviour related to absorption, interaction in biomembranes and transportation [5,7,9]. For such purposes parameters like the partition coefficient, P_{OW}, between octanol and water, or the chromatographic retention in reversed-phase systems have been used. In addition to classical column liquid chromatography, micellar electrokinetic chromatography (MEKC) was introduced as a simple and

inexpensive analytical tool with the aim of describing the hydrophobicity of chemical compounds. Several papers apply this technique to drug research, using the capacity factor as hydrophobic parameter, or to predict indirectly P_{OW} values, or to compare both [10–13]. In most works the analytes are neutral. However, bile acids exist as anions under physiological conditions. Therefore, in order to mirror the in vivo situation, the ionic forms rather than that of the uncharged species are taken as analytes. Thus, in the present work the retention of free and derivatised bile salts was determined in a MEKC mode with sodium dodecyl sulfate (SDS) as micelle former; under the chosen conditions these analytes are present as anionic species throughout. It was the topic of this work to prove whether or not this retention, expressed by the capacity factor, shows correlation with log P_{OW}. Additionally, the agreement with the critical micellar concentration (CMC) of the bile salts as pure compounds was evaluated.

2. Experimental

2.1. Reagents

All bile acids (except glyco-ursodeoxycholic acid) and SDS were purchased from Sigma (St. Louis, MO, USA). Glyco-ursodeoxycholic acid was obtained from Tokyo Tanabe (Tokyo, Japan). Methanol, acetonitrile (both HPLC grade), anthracene, sodium tetraborate, sodium monohydrogenphosphate and 85% phosphoric acid, all analytical grade, were supplied by E. Merck (Darmstadt, Germany). Ultrapure water was obtained from an EASYpure RF system (Barnstead, Dubuque, IA, USA). Solutions were filtered through a 0.45 μm nylon membrane (MSI, Westboro, MA, USA) and degassed before use.

2.2. Instrumentation

A Capillary Ion Analyser (Waters, Milford, MA, USA) was used for the electrophoretic measurements; data were processed by Millennium software (Waters). An uncoated fused-silica capillary of 60 cm (53 cm to detector)×75 μm I.D. (Waters) was used. Injection was done hydrostatically (10 cm height) for 18 s; the operating voltage was +25 kV, temperature 25°C. UV absorbance was detected at 185 nm (mercury lamp).

Determination of the mobilities was carried out from the migration times as usual. The mobility of the electroosmotic flow (EOF) was determined from the water dip, that of the micelle by the aid of anthracene.

At the beginning of each day the capillary was rinsed with 0.1 mol/l potassium hydroxide for 3 min, then washed with water for 10 min and with background electrolyte (BGE) for 10 min. Between runs, the capillary was conditioned with BGE for 3 min. At the end of each day the capillary was flushed with 0.1 mol/l potassium hydroxide for 3 min and finally with water for 10 min.

2.3. Buffering electrolytes

The electrophoretic mobilities were determined in the following BGEs: (1) SDS-free, sodium tetraborate–sodium monohydrogenphosphate buffer, 10 mmol/l each, pH 7.5, 10% acetonitrile; (2) 5 mmol/l SDS, sodium tetraborate–sodium monohydrogenphosphate buffer, 10 mmol/l each, pH 7.5, 10% acetonitrile; and (3) 50 mmol/l SDS, sodium tetraborate–sodium monohydrogenphosphate buffer, 10 mmol/l each, pH 7.5, 10% acetonitrile.

2.4. Stock and standard solutions

Stock solutions of each bile acid containing 2.0 mg/ml were prepared in methanol and a standard solution of 400 μg/ml of each bile acid was obtained by appropriate dilution with 1 mmol/l tetraborate–1 mmol/l phosphate buffer.

3. Results and discussion

The extent of interaction of an analyte, *i*, with the stationary phase in a chromatographic system is determined by the mass partition coefficient, the capacity or retention factor, k_i. For systems with a pseudo-stationary phase like micelles or soluble polymeric additives, an analogous description is reasonable. Here the mobile phase is the ''free'' solvent, and the stationary phase is the micelle in

case of MEKC. In the equation to derive the capacity factor the measured mobility, μ_i^{meas}, of each analyte consists of two contributions: (i) the mobility in ''free'' solution, μ_i^{free}; this is governed by the (vector) sum of the electrophoretic (effective) mobility of the ionic species, μ_i^{eff}, and that of the EOF, μ_{eof}. Note that in the present case dealing with fully deprotonated, anionic analytes the effective mobility, μ_i^{eff}, is equal to the electrophoretic mobility, μ_i^{eph}. (ii) The (measured) mobility of species, *i*, in the micelle, μ_i^{mic}, which can be considered as being equal to the (measured) mobility of the micelle, μ_{mic}^{meas}. The latter is composed from its own electrophoretic mobility and that of the EOF. The capacity factor, k_i, is then:

$$k_i = \frac{\mu_i^{meas} - \mu_i^{free}}{\mu_{mic}^{meas} - \mu_i^{meas}} = \frac{\mu_i^{meas} - \mu_i^{eph} - \mu_{eof}}{\mu_{mic}^{eph} + \mu_{eof} - \mu_i^{meas}} \quad (1)$$

In this equation the mobilities must be treated as signed quantities. For convention, mobilities of positively charged particles have positive, negatively charged ones have negative sign. The electrophoretic mobilities, although related to the conductivity, are signed, too, as soon as the particle transport is considered. The EOF mobility has positive sign when electroosmosis is flowing towards the cathode. When the net migration of the analyte is directed towards the cathode (as in the present case) its mobility has positive sign as well. Eq. (1) allows the calculation of the capacity factors for ionic analytes from the measured mobility, the electrophoretic mobility in free solution, and the (electrophoretic) mobility of the micelle.

3.1. Mobilities

The measured, total mobilities of the bile salts in the micellar system containing 50 mmol/l SDS in the BGE are presented in Table 1. The data for all hydroxy cholates range between 16 and 28 units (i.e., 10^{-9} m^2 V^{-1} s^{-1}). The electrophoretic mobility, μ_i^{eph}, of the analytes in ''free'' solution is needed for the calculation of the capacity factor according to Eq. (1). In a strict sense this is the mobility in a BGE with the same composition as the MEKC system, but without containing the micelles. In the simplest approach the mobilities in an SDS-free system are used. However, probably more appropriate is a buffering BGE with dispersed molecular SDS at a

Table 1
Measured (total) and electrophoretic mobilities, μ_i, of the cholates in the BGEs with different SDS concentrations, and capacity factor, k_i, in 50 mmol/l SDS

Abbreviation	Analyte	μ_i^{meas} 50 mmol/l SDS	μ_i^{eph} 0 mmol/l SDS	μ_i^{eph} 5 mmol/l SDS	k_i
DHCA	Dehydrocholate	47.46	−17.86	−18.81	0.200
UDCA	Ursodeoxycholate	27.79	−17.23	−19.52	2.17
G-UDCA	Glyco-ursodeoxycholate	24.88	−15.71	−18.31	3.38
T-UDCA	Tauro-ursodeoxycholate	23.53	−15.91	−21.78	4.25
CA	Cholate	23.68	−16.66	−20.79	3.83
G-CA	Glyco-cholate	20.59	−15.52	−22.27	6.75
T-CA	Tauro-cholate	18.46	−15.55	−23.38	13.9
CDCA	Chenodeoxycholate	18.64	−16.94	−22.16	10.8
G-CDCA	Glyco-chenodeoxycholate	18.41	−15.95	−23.68	13.4
T-CDCA	Tauro-chenodeoxycholate	16.12	−15.42	−24.34	59.9
DCA	Deoxycholate	18.48	−17.23	−23.84	10.9
G-DCA	Glyco-deoxycholate	18.62	−16.12	−23.87	11.1
T-DCA	Tauro-deoxycholate	16.16	−16.75	−26.54	17.7
LCA	Lithocholate	16.99	−17.82	−26.47	14.9
G-LCA	Glyco-lithocholate	17.65	−16.76	−27.70	16.2
T-LCA	Tauro-lithocholate	15.51	−17.47	−27.41	39.7

Mobilities are in 10^{-9} m^2 V^{-1} s^{-1}. Temperature = 25°C.
Measured mobilities are the mean of three replicate injections of each day, and determined at 3 different days. Relative standard deviation is lower than 1%.

concentration close to the CMC. This difference might be of relevance, because SDS even in sub-micellar concentrations could change the retention of the analytes. Such effect has been observed, e.g., for neutral analytes and cationic micelle-forming additives [14,15].

The electrophoretic mobilities of the cholates determined in a BGE with 20 mmol/l phosphate-borate, pH 7.5, with 10% acetonitrile, but without SDS, are given in Table 1. For comparison the respective mobilities with 5 mmol/l SDS are shown, too. This SDS concentration is close to the CMC in the present BGE. In water it is 8 mmol/l, in an electrolyte system of similar composition like the present buffer (without acetonitrile) it is 4 mmol/l [16]. Presence of acetonitrile might influence the CMC further [17]. However, even when a small part of SDS is present as micelle at 5 mmol/l, the main part is dispersed in a non-micellar form in this BGE. Assuming 1 mmol/l of SDS being present as micelle, its contribution compared to the 50 mmol/l BGE used for MEKC seems insignificant.

It is clear that in the considered system the associate, the analyte and the dispersed non-micellar SDS are in equilibrium. The observed mobility in this system is thus the weighted average of the mobility of the analyte and its associate with SDS [15]. However, these details are out of the scope of the present paper.

It is worth mentioning that the EOF mobility was essentially constant during the measurements: its average value was 69.64 units, with a standard deviation of 0.73 units, which is around 1% only. For improved accuracy of the capacity factors the mobilities of both, the analytes and the EOF, were determined in the same run.

3.2. Capacity factors

The calculated capacity factors of the analytes in the MEKC system consisting of 50 mmol/l SDS are presented in Table 1. They are related to the SDS-free system, and to a mobility of 54.0 units for the SDS micelle (the average from 15 measurements with a standard deviation of about 1%). They cover the wide range between 0.2 and 60. In order to depict a possible difference of the capacity factors depending on the choice of the reference electrolyte system,

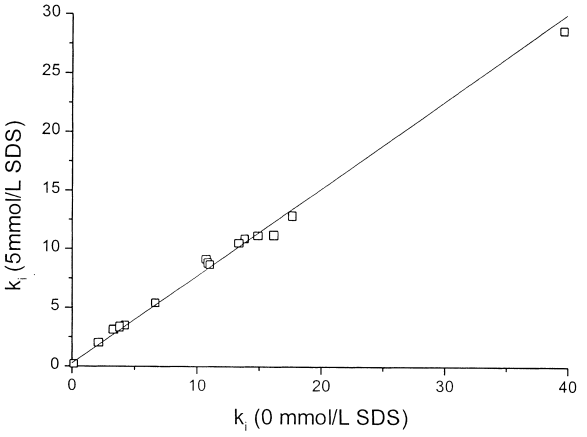

Fig. 2. Relation of the capacity factors (Eq. (1)) calculated for mobilities with the reference BGE without SDS as ''free'' solution, and for those with 5 mmol/l SDS (close to the CMC), respectively.

the correlation of the k_i values derived with the SDS-free, and with the 5 mmol/l SDS containing BGE is shown in Fig. 2. It can be seen that the capacity factors based on both reference BGEs are highly correlated (linear correlation coefficient is 0.999). The reason for this result seemingly lies in the relatively small variation of the mobilities in these two systems compared to those in the micellar system. The retention is dominated by the mobilities in the systems containing a high concentration of micelles.

The effect of the micelle on the resolution of analytes is seen in Fig. 3 for one example. Under the conditions shown in Fig. 3A the analytes are moving solely by capillary zone electrophoresis according to their own electrophoretic mobilities. Association with SDS present in concentration close to the CMC changes the selectivity of the system (Fig. 3B), and separation is strongly increased in the common MEKC system (Fig. 3C).

3.3. Relation between capacity factor, lipophilicity and detergency

In Table 2 the capacity factors of the analytes are sorted in increasing order. In the same table the position and direction of the OH groups in the bile salts are indicated. That compound with double bonded oxygen (DHCA, see Fig. 1) has the lowest k_i

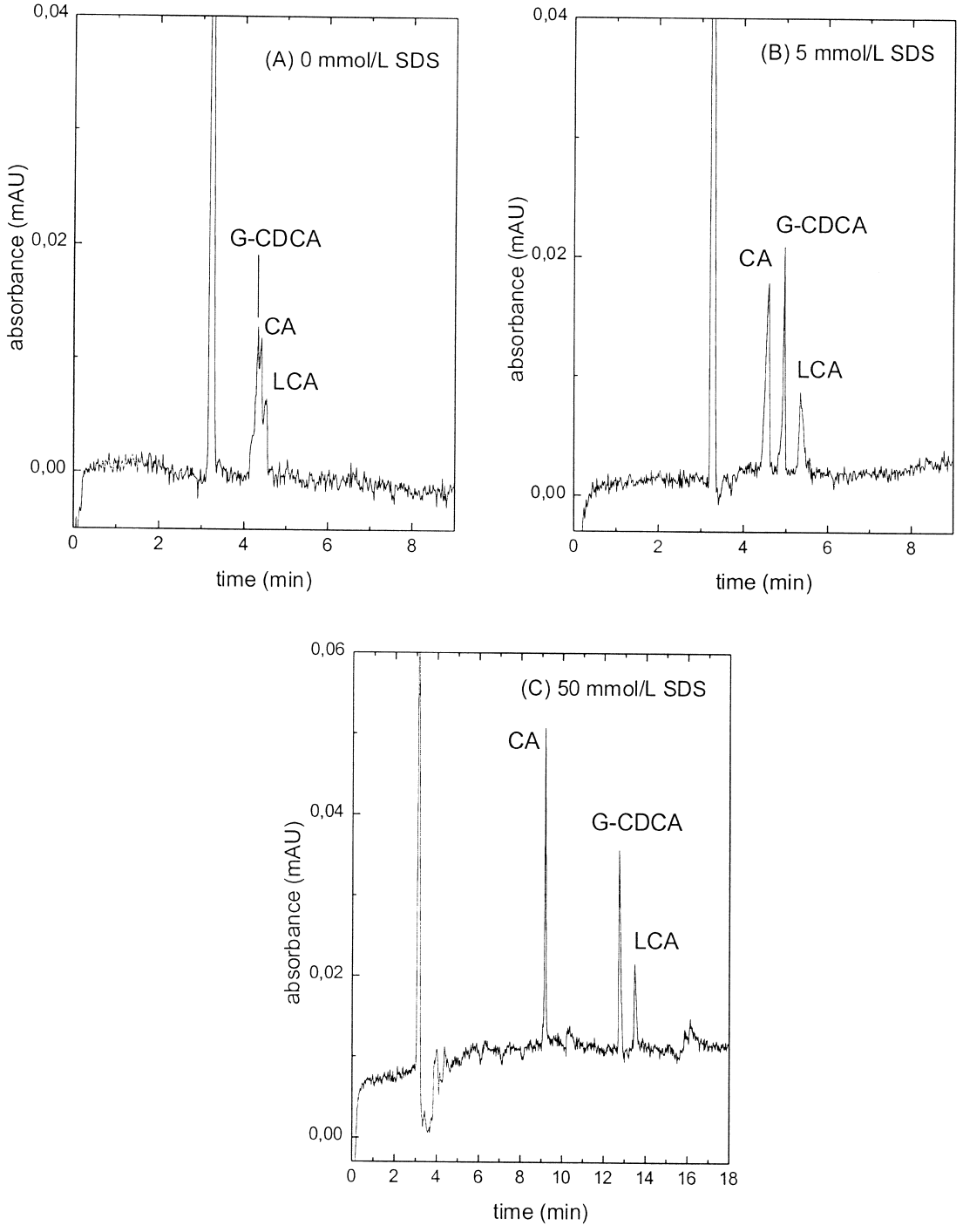

Fig. 3. MEKC of cholate (CA), glyco-chenodeoxycholate (G-CDCA) and lithocholate (LCA) in three different BGE systems. Conditions: uncoated fused-silica capillary, 60 cm (53 cm to detector)×75 μm I.D. Temperature 25°C, voltage +25 kV, UV detection at 185 nm. BGE sodium tetraborate–sodium monohydrogenphosphate buffer, 10 mmol/l each, pH 7.5, 10% acetonitrile. (A) SDS-free BGE; (B) 5 mmol/l SDS; (C) 50 mmol/l SDS.

Table 2
List of cholates in increasing order of capacity factors

Analyte	Number of OH	Position/orientation of OH group	k_i	log P_{OW}	CMC (mmol/l)
DHCA		3.7.12-trioxo	0.200	–	–
UDCA	2	3α7β	2.17	2.20	7.0
G-UDCA	2	3α7β	3.38	0.20	4.0
CA	3	3α7α12α	3.83	1.10	11.0 (9)
T-UDCA	2	3α7β	4.25	–	2.2
G-CA	3	3α7α12α	6.75	−0.40	10.0
CDCA	2	3α7α	10.8	2.25	4.0
DCA	2	3α12α	10.9	2.65	3.0
G-DCA	2	3α12α	11.1	0.80	2.0
G-CDCA	2	3α7α	13.4	0.45	2.0
T-CA	3	3α7α12α	13.9	−0.50	6.0 (7)
LCA	1	3α	14.9	–	0.6[a]
G-LCA	1	3α	16.2	–	–
T-DCA	2	3α12α	17.7	–	2.4 (0)
T-LCA	1	3α	39.7	–	–
T-CDCA	2	3α7α	59.9	–	3.0 (2)

Orientation of the OH groups in the molecules are give together with log P_{OW} and CMC. Symbols as in Table 1.
Values for CMC (in 0.15 mol/l NaCl solutions) and log P_{OW} taken from Refs. [18–20]. Values in parentheses from Ref. [27]. – No data available.
[a] Value for 7β OH, as all 3α OH and 7α OH not sufficiently soluble [18].

value. Roughly, all other compounds show increasing capacity factors with decreasing number of OH groups. Exceptions are UDCA and its glyco-derivative; note that these two cholates have OH in β direction. Although they possess the same number of two OH groups like the epimeric CDCA and G-CDCA (with α OH in position 7), they have even smaller k_i values than the trihydroxy cholates. It can be further derived from Table 2 that the tauro-derivatives in general interact relatively strongly with the micelles, as most of them are found in the high k_i range of the list.

3.3.1. Relation of k_i to log P_{OW}

Interaction between SDS micelles and analytes is normally related to their lipophilicity, a property expressed by log P_{OW}. For nine analytes in their anionic form values for this coefficient were available from the literature [18–20]. They are given in Table 2. As expected, log P_{OW} is lower for the unsubstituted cholates (UDCA, CA, DCA) as more OH groups they possess. Note that the epimeric CDCA and UDCA have about the same log P_{OW} (2.25 and 2.20), which means that this parameter is seemingly not sensitive to the orientation of the OH group. In pronounced contrast are the capacity factors (10.8 and 2.17) that differ by nearly a factor of 5.

Transformation of the unsubstituted cholates into their glycine and taurine conjugates causes a significant reduction of log P_{OW}, by about 1.5 to 2 units, allowing the conclusion that the G- and T-groups reduce lipophilicity. This effect on log P_{OW} is in marked contrast to the k_i values, which increased upon conjugation. Thus k_i values do not mirror the lipophilicity of at least a major part of the present analytes in the MEKC system.

3.3.2. Relation of k_i to CMC

A pronounced property of bile salts is their ability for self-association and micelle formation. For this reason they have been used as micelle- or co-micelle formers in MEKC [21–28]. Thus it could be assumed that cholates are distributed between an aqueous phase and the SDS-micelle in a somewhat different manner than other lipophilic compounds. Based on nuclear magnetic resonance (NMR) data of Wiedmer and co-workers [28,29] it has been deduced that at low concentration cholate is incorporated into the SDS micelle not randomly, but under

orientation of the ionic group towards the outside of the micelle [24]. It seems thus reasonable to relate the extent of chromatographic partitioning of these analytes (their k_i values) to their ability for micelle formation (the CMC of the pure cholates). We take the CMC values from the literature in electrolyte solutions (0.15 mol/l NaCl) rather than those from pure water, as the former reflect the situation in MEKC better.

From Table 2 it can be seen that (not unexpectedly) the lower the number of OH groups in the molecule, the lower is the CMC (the higher is the detergency). There is agreement between the CMC and k_i for the taurine derivatives of a particular bile salt: compared to the unconjugated form taurine conjugation decreases the CMC, and indeed the T-forms have also the highest k_i values.

Most strikingly, the CMC reflects the strong influence of the orientation of the OH group of the epimeric UDCA and CDCA [20], in contrast to log P_{OW} as pointed out above. UDCA with orientation 7β has nearly a two times larger CMC than CDCA (as also observed with the G-conjugates). Note that the same tendency is found for the k_i values, where β orientation lowered the capacity factors compared to α orientation (see above). It can be concluded that the capacity factors of the bile salts are related much more pronouncedly to the their CMC values than to their log P_{OW}. Due to the complexity of the partioning in MEKC, the relation between k_i and CMC is not linear; however, at least a general tendency can be observed. In this respect the retention behaviour of the bile salts in MEKC seems to reflect their role in living systems as detergents, and might serve as model parameter far beyond lipophilicity as commonly understood.

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