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Bile salt micellar electrokinetic chromatography of bilirubin and related compounds

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Abstract

The interaction of bilirubin, biliverdin, bilirubin dimethyl ester, biliverdin dimethyl ester, xanthobilirubic acid, and xanthobilirubin methyl ester with trihydroxy and dihydroxy bile salt solutions is investigated by micellar electrokinetic chromatography (MEKC). The capacity factor of each compound is measured in solutions of the different bile salts over the pH range of 6.5–9.0. The capacity factor of bilirubin increases with pH below 7 in all bile salt solutions. Biliverdin and xanthobilirubin show essentially identical capacity factors for all bile salts. Biliverdin dimethyl ester and xanthobilirubin methyl ester also have very similar capacity factors, which are greater than those of the carboxy analogs, in trihydroxy bile salts. The capacity factors of these esters are higher in the dihydroxy bile salts, with the capacity factor of biliverdin dimethyl ester being twice that of xanthobilirubin methyl ester. Factors involved in the MEKC analysis of these compounds are discussed. © 1997 Elsevier Science B.V.

Keywords: Bilirubin; Biliverdin; Bile salts; Cholic acid; Taurocholic acid; Taurodeoxycholic acid; Taurochenodeoxycholic acid

1. Introduction

Heme, the prosthetic group of hemoglobin and other mammalian hemeproteins is degraded by oxidation in the liver to produce biliverdin IXα. Biliverdin then undergoes reduction at the methylene bridge to form bilirubin IXα [1]. The structures of bilirubin and biliverdin are shown in Fig. 1.

Although the chemical structures of bilirubin and biliverdin are very similar, their solution conformations differ greatly. These structures are more closely approximated in Fig. 2. Bilirubin undergoes intramolecular hydrogen bonding between the carboxylic acid groups of the propionate side chains and the lactam and pyrrole groups of the opposite dipyrrole units [2]. It is thus considerably more lipid soluble than biliverdin. The extent of hydrogen bonding in aqueous solutions at physiological pH is currently under debate, but it is well established that bilirubin adopts this 'ridge-tile' conformation [2–5] in aqueous solutions. The planes of the two dipyrrole units are at approximately a 95–100° angle [3] and bilirubin rapidly interconverts between two chiral enantiomers [4]. In contrast, biliverdin adopts a helical 'lock-washer' conformation in aqueous solution [6]. This structure is stabilized by hydrogen bonding between the protonated and unprotonated nitrogens in the ring structure. The structure of

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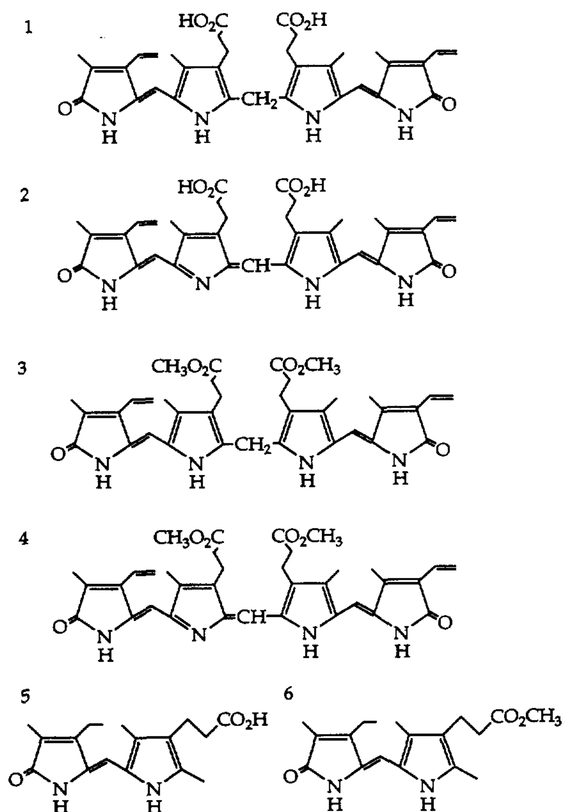

Fig. 1. Chemical structures of bilirubin and related analytes. (1) Bilirubin, (2) biliverdin, (3) bilirubin dimethyl ester, (4) biliverdin dimethyl ester, (5) xanthobilirubic acid, (6) xanthobilirubin methyl ester.

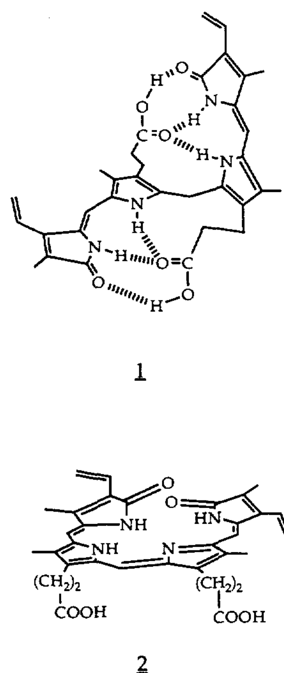

Fig. 2. Preferred conformations of bilirubin (1) and biliverdin (2) showing intramolecular hydrogen bonding.

biliverdin dimethyl ester is a similar type of helix [7]. Bilirubin dimethyl ester is also structurally similar to the parent acid in polar solvents, except that the 'ridge-tile' is flatter in the dimethyl ester [8].

Bilirubin is esterified with D-glucuronic acid in the liver prior to excretion. The conjugated sugar moieties prevent the formation of intramolecular hydrogen bonds and render conjugated bilirubin water soluble. The 1–3% of bilirubin that is not conjugated to sugars is solubilized in bile through interactions with bile salt micelles and monomers [9]. The precipitation of this unconjugated bilirubin in the form of calcium salts is hypothesized to be an important step in pigment gallstone formation [10–13]. It is therefore of interest to better understand the interaction of bilirubin and bile salts.

We have previously reported the interactions of bilirubin with bile salt micelles using micellar electrokinetic chromatography (MEKC) [14]. Our previous results showed that the capacity factor of bilirubin decreased as the pH increased. In addition, little difference in capacity factor for bilirubin was observed in dihydroxy and trihydroxy bile salt solutions at pH values where bilirubin is believed to exist as a dianion.

The additional compounds included in the present study are bilirubin IXα dimethyl ester, biliverdin IXα, biliverdin IXα dimethyl ester, xanthobilirubic acid, and xanthobilirubin methyl ester, the structures of which are shown in Fig. 1. These compounds were chosen to help explore the influence of structure, shape, size, and charge on the extent of interaction of bilirubin with bile salts. The influence of charge can be probed by comparison of the interaction of the parent acids with that of the methyl esters, and the effects of size and shape can be studied by comparing bilirubin with biliverdin and xanthobilirubin.

The four bile salts investigated are cholic, taurocholic, taurodeoxycholic, and taurochenodeoxycholic

acids. Taurodeoxycholic acid was not used in our previous study. While bile salts have been studied for many years, and they are finding increased application in capillary methods, few of their three-dimensional micellar structures have been completely solved. Bile salt monomers have a steroid backbone that can be substituted at positions R1, R2, and R3, as shown in Fig. 3. The monomers can also be conjugated with taurine or glycine at position R4. Two of the bile salts studied, taurochenodeoxycholic acid and taurodeoxycholic acid are dihydroxy bile salts. These two bile salts, and their micelles, should be slightly more hydrophobic than the two trihydroxy bile salts. Deoxycholate micelles are believed to have a rodlike helical structure, with a left-handed twist [15–17]. The charged side chains are in the center of this helix with associated cations and the monomers stack with all the hydroxyl groups facing in one direction. The trihydroxy bile salts appear to form a different type of micellar structure. Cholic acid is believed to be a bilayer with hydrophobic and hydrophilic channels created by the stacking of the cholate monomers [18,19]. The fundamental unit of this micelle is a dimer that can form chains and/or sheets. A model for micellar solubilization of analytes by taurocholate involves participation of dimers and higher order multimers [20].

In this study we investigate the capacity factor of bilirubin and related compounds in solutions of four different bile salts across the pH range of 6.5–9.0. The comparison of the behavior of the different test analytes yields information on the mode and specificity of interaction of bile pigments with these different bile salts.

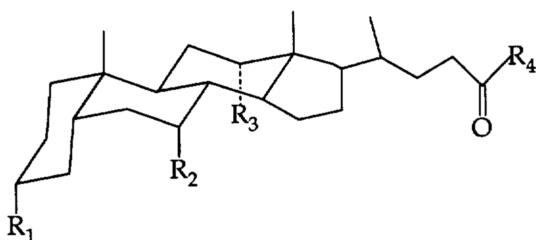

Fig. 3. Bile salt monomer unit: cholic acid, R1, R2, R3, R4=OH; taurocholic acid, R1, R2, R3=OH, R4=$-NHCH_2CH_2SO_3H$; taurodeoxycholic acid, R1, R3=OH, R2=H, R4=$-NHCH_2CH_2SO_3H$; taurochenodeoxycholic acid, R1, R2=OH, R3=H, R4=$-NHCH_2CH_2SO_3H$.

2. Experimental

2.1. Apparatus and reagents

Fused-silica capillaries were purchased from Polymicro Technologies (Phoenix, AZ, USA). The capillaries had an I.D. of 75 μm and a length of 69 cm (24 cm from injection tip to window). A Varian Instruments Group 2550 UV detector (Walnut Creek, CA, USA) was used and fitted with a deuterium lamp. The detection wavelength was 232 nm. The power supply was a Spellman CZE 1000R (Plainview NY, USA) with a ±30-kV output range. A Series 4500 Microscribe Strip Chart Recorder from The Recorder Company (San Marcos, TX, USA) was used to record runs. The apparatus was not thermostatted and so all solutions and analyses were at ambient temperature, typically 22–25°C.

A Barnstead Nanopure system purchased through Fisher Scientific (Austin, TX, USA) provided deionized water. Biliverdin, biliverdin dimethyl ester, and bilirubin dimethyl ester were obtained from Porphyrin Products Incorporated (Logan, UT, USA). Xanthobilirubic acid was prepared by hydrolysis [21] of xanthobilirubin methyl ester. The latter was provided courtesy of Dr. David Lightner (University of Nevada, Reno, NV, USA). Dimethylsulfoxide was purchased from Fisher; and sodium azide was purchased from Anachemia (Champlain, NY, USA). 1-Nitropyrene as purchased from Aldrich (Milwaukee, WI, USA). All other reagents, including bilirubin and the bile salts, were purchased from Sigma (St. Louis, MO, USA). Bilirubin was purified according to the method of McDonagh and Assisi [22]. Other test compounds were used as received.

2.2. Buffer and sample preparation

A 20 mM monobasic sodium phosphate solution and a 20 mM sodium tetraborate solution were made, each containing 0.005% sodium azide. These solutions were mixed with each other until the desired running buffer pH was obtained. The resulting solutions were used to prepare 25 mM solutions of the desired bile salt. The bile salt solutions were then filtered with a Gelman Sciences (Ann Arbor, MI, USA) or a Nalge Company (Rochester, NY, USA) 0.45-μm (25-mm diameter) syringe filter.

Analytes were dissolved individually in DMSO. Typically 1–3 mg of each analyte were dissolved in 300 μl of DMSO (bilirubin and biliverdin), or 50 μl of DMSO (bilirubin dimethyl ester and biliverdin dimethyl ester), or 30 μl of DMSO (xanthobilirubin and xanthobilirubin methyl ester). An injection sample was then prepared by adding between 1 and 12 μl of the separately prepared analyte solutions and between 1 and 12 μl of a saturated Sudan III/DMSO solution to 1000 μl of the bile salt solution. Injected sample solutions were in the concentration range of 10^{-3} to 10^{-5} *M* for the various analytes. The Sudan III functioned as a micelle marker for the separation process. This mixture was then filtered with a 0.45- or 0.2-μm (13-mm diameter) syringe filter (Gelman Sciences). During analysis, Sudan III and a test compound occasionally co-eluted. If co-elution made it difficult to measure peak times, Sudan III and the test compound were analyzed separately. In addition, if test compounds co-eluted with each other, they were analyzed separately.

Analytes were also prepared in buffer solutions without bile salts, after dissolution in DMSO, in order to obtain free solution mobilities of the anions, and to confirm that neutral analytes were behaving as neutral compounds in our system at the experimental pH values. In the case of the methyl esters, insufficient analyte dissolved in aqueous solution to make possible the measurement of the free solution mobility. These values were assumed to be zero. In the case of bilirubin, an extrapolated free solution mobility was used as described previously [14].

2.3. Capillary preparation and run conditions

A 0.5-cm detection window was made on the fused-silica capillary by suspending a drop of fuming sulfuric acid on the polyimide coating. A short injection tip was made in a similar manner. The capillary was then wiped with deionized water, methanol, and again with deionized water.

At the beginning of each day, the capillary was rinsed with 1 *M* KOH for 15–30 min and then with the bile salt solution for 15–30 min. Solutions were aspirated through the capillary with a Nalgene hand pump (Fisher Scientific) attached to a solution trap. At the end of each day the capillary was rinsed with 1 *M* KOH for 5–10 min followed with a deionized water rinse for 5–10 min. The capillary was stored in deionized water overnight.

The run voltage was 12 kV. The sample injection was performed by placing the electrode and capillary tip into the sample vial and applying 12 kV for 10 s. The capillary and the electrode were then wiped clean and placed into the run buffer.

2.4. Capacity factor calculation

Capacity factors for each compound were calculated as described previously [14] by the equation shown below, where μ is the electrophoretic mobility of the analyte in the presence of micelles, μ_o is the electrophoretic mobility of the analyte in the absence of micelles, μ_{mc} is the electrophoretic mobility of the micelles. For each compound with a non-zero free solution electrophoretic mobility, the mobility of the electroosmotic flow was

$$k' = \frac{\mu - \mu_o}{\mu_{mc} - \mu} \quad (1)$$

subtracted from the apparent electrophoretic mobility to obtain the true electrophoretic mobilities used in Eq. (1). For neutral species, the electrophoretic mobility in the absence of micelles is zero and Eq. (1) reduces to the more familiar form shown in below as Eq. (2).

$$k' = \frac{\mu}{\mu_{mc} - \mu} \quad (2)$$

3. Results and discussion

Example electropherograms are shown in Fig. 4 for all compounds in a variety of bile salt solutions. Table 1 shows the capacity factors for each of the analytes in solutions of the two trihydroxy compounds, cholic acid and sodium taurocholate. Additional data for bilirubin and biliverdin which fall outside the pH ranges given in the table are shown in Fig. 5 and Fig. 6, respectively. Table 2 shows the capacity factors for each of the compounds in the two dihydroxy bile salt solutions. Again, additional pH data for bilirubin and biliverdin are shown in Fig. 5 and Fig. 7, respectively. It should be emphasized that all measurements were done at a bile salt concentration of 25 m*M* regardless of the CMC

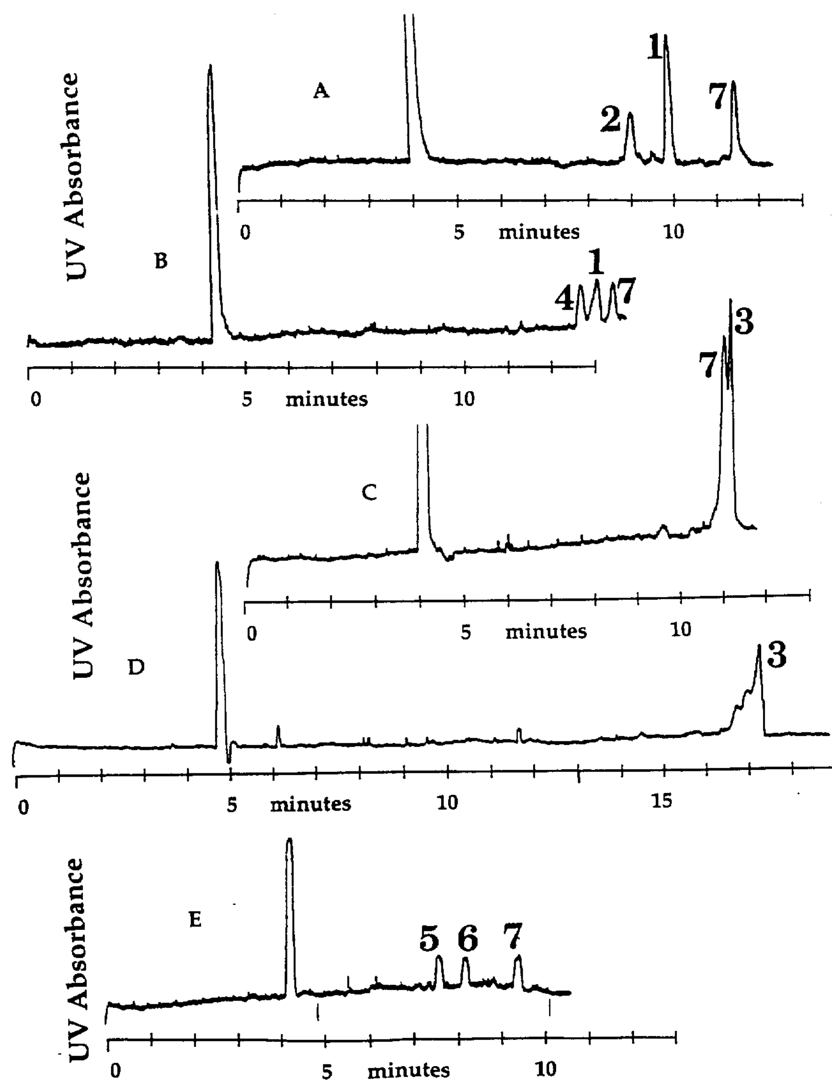

Fig. 4. Sample electropherograms. All runs done with a 10-s, 12-kV injection; 12-kV run voltage; 232-nm detection wavelength. All electroosmotic flow peaks are DMSO; peak (7) is Sudan III; and analyte peaks are labeled as in Fig. 1. (A) 25 m*M* taurodeoxycholate at pH 8.5; (B) 25 m*M* taurodeoxycholate at pH 6.7; (C) 25 m*M* taurodeoxycholate at pH 9.0; (D) 25 m*M* taurochenodeoxycholate at pH 6.5; (E) 25 m*M* taurocholate at pH 8.4.

value or partial molar volume of the different bile salt micelle solutions. Thus, the observed trends in capacity factor do not necessarily reflect the same trends in partition coefficient. We are currently investigating the actual partition coefficient of bilirubin in different bile salts as a function of pH. Those results will be discussed in a later publication [23].

Several trends are apparent in the results. First, only bilirubin shows a significant dependence of capacity factor on pH. In our previous work we ascribed this pH dependence to bilirubin ionization. Assuming bilirubin is a dianion at higher pH, it follows that the dianion should have a lower capacity factor due to charge repulsion with the anionic micelles. If bilirubin is protonated as the pH is lowered, then the capacity factor should increase. Our data shows this trend as the capacity factor of bilirubin is significantly greater at lower pH than it is

Table 1
Analyte capacity factors in trihydroxy bile salt solutions

Analyte	Capacity factor (R.S.D., %)					
	25 m*M* cholic acid			25 m*M* taurocholic acid		
	pH 6.5–6.7	pH 7.3–7.8	pH 8.4–8.8	pH 6.3–6.7	pH 7.1–7.5	pH 8.4–8.5
Bilirubin	67[a]	3.3 (8.8)	1.83 (3.3)	126 (16.4)	5.4 (5.7)	2.4 (2.3)
Bilirubin dimethyl ester	60[a], 116[a]	20.8[a], 38.4[a]	18.5[a], 28.5[a]	29 (11), 88 (22)	27 (12), 70 (19)	28 (4.3)
Biliverdin	1.38 (3.6)	0.73 (9.6)	0.70 (4.5)	0.61 (4.1)	1.59 (3.4)	0.8 (19)
Biliverdin dimethyl ester	9.4 (2.0)	7.8 (4.7)	7.08 (1.3)	8.2 (1.5)	pH 7.1, 9 (1.8); pH 7.4, 7.97 (0.6)	
Xanthobilirubic acid	0.80 (3.8)	0.81 (2.5)	0.76 (11.3)	1.13 (1.9)	1.10 (2.7)	1.09 (2.2)
Xanthobilirubin methyl ester	7.25 (0.27)	7.78 (1.10)	6.6 (3.7)	7.28 (0.64)	7.5 (4.6)	7.5 (1.45)

[a]Capacity factors determined from runs where analytes were run separately from Sudan III.

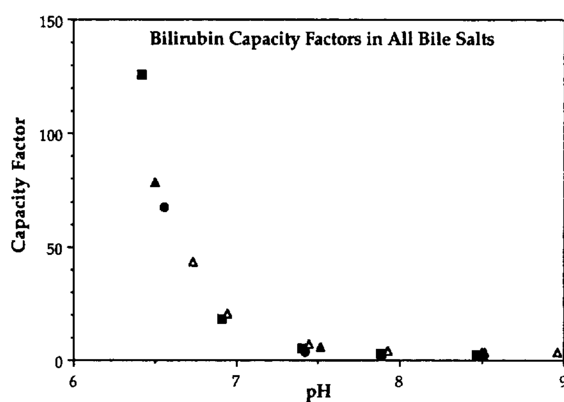

Fig. 5. Bilirubin capacity factors in four bile salt solutions: (●) 25 m*M* cholic acid; (■) 25 m*M* taurocholate; (△) 25 m*M* taurodeoxycholate; (▲) 25 m*M* taurochenodeoxycholate.

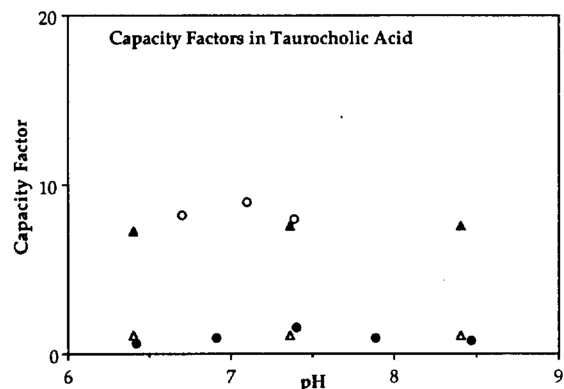

Fig. 6. Analyte capacity factors in 25 m*M* taurocholate: (●) biliverdin; (○) biliverdin dimethyl ester; (△) xanthobilirubin; (▲) xanthobilirubin methyl ester.

at high pH. This interpretation is not consistent, however, with recent determinations of the pK_a values of bilirubin. Results using ^{13}C NMR suggest the bilirubin pK_a values are 4.2 and 4.9 in aqueous solution [6]. If these pK_a values apply to bilirubin in bile salt solution, then the molecule will be ionized over the entire pH range of this study. However, it is possible that the binding of bilirubin to micelles alters its apparent pK_a values. There is evidence for micelle-induced shifts in pK_a values of bilirubin and other species [10,24,25]. An alternative explanation is that bilirubin aggregates at lower pH values. Evidence for the aggregation of bilirubin also exists in the literature [26,27]. Such aggregates may have a higher micelle affinity or higher free solution mobility, which is not accounted for in our calculations, than bilirubin monomers. However, aggregation at lower pH would seem to be best explained by a change in ionization as well. Since bilirubin is insoluble in aqueous solution at lower pH values, this is a difficult hypothesis to examine in a CZE format. Regardless of the explanation, bilirubin exhibits a significant change in capacity factor, for all the bile salts tested, as the pH is lowered. Also, little difference in bilirubin's capacity factor is seen as the bile salt changes from a tri- to dihydroxy or from an unconjugated to a conjugated form.

The capacity factor of biliverdin does not show a dependence on pH. This is consistent with the compound being fully ionized across the pH range of this study. This could be explained if the pK_a values for biliverdin were lower than those of bilirubin, but ^{13}C NMR studies suggest this is not the case [6].

Table 2
Analyte capacity factors in dihydroxy bile salt solutions

Analyte	Capacity factor (R.S.D., %)					
	25 m*M* taurodeoxycholic acid			25 m*M* taurochenodeoxycholic acid		
	pH 6.3–6.7	pH 7.1–7.8	pH 8.4–8.9	pH 6.4–6.6	pH 7.4–7.6	pH 8.4–8.6
Bilirubin	pH 6.3, ∞ pH 6.7, 43.6 (2.6)	7.04 (3.0)	3.5 (1.0)	78 (7)	5.9 (3.8)	3.7 (6.6)
Biliverdin	1.48 (4.1)	1.21 (1.5)	1.10 (2.0)	1.86 (2.3)	1.4 (4.4)	1.27 (4.1)
Bilirubin dimethyl ester	−218 (8.2)	pH 7.4, −172 (5.8) pH 7.9, −167 (11)	pH 8.5, −216[a] pH 9.0, −140 (15)	−150[a]	−95[a]	−81[a]
Biliverdin dimethyl ester	35 (3.1)	36.3 (1.6)	36 (10)	38.4 (2.4)	36 (3.9)	38 (3.8)
Xanthobilirubic acid	1.36 (0.73)	1.18 (5.9)	1.10 (7.9)	1.40 (1.24)	1.35 (1.14)	1.36 (4.1)
Xanthobilirubin methyl ester	17.3 (1.3)	17.8 (2.1)	19.0 (2.1)	16.8 (1.9)	16.5 (2.0)	16.1 (0.87)

[a] Capacity factors determined from runs where analytes were run separately from Sudan III.

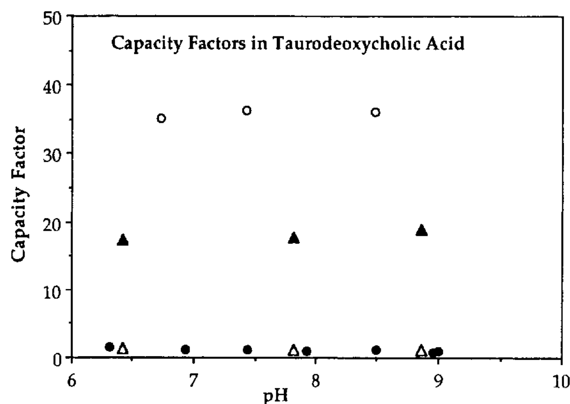

Fig. 7. Analyte capacity factors in 25 m*M* taurodeoxycholate: (●) biliverdin; (○) biliverdin dimethyl ester; (△) xanthobilirubin; (▲) xanthobilirubinmethylester.

Thus, there appears to be a significant difference in the mechanics of the interaction of biliverdin and bilirubin with all of the bile salts studied. Three-dimensional structure likely plays a role in these interactions, since the structure of biliverdin is substantially different from the structure of bilirubin. Alternatively, biliverdin may not show the same aggregation properties as bilirubin at low pH. It also appears as though the interaction between biliverdin and the bile salts is slightly different from fully ionized bilirubin as the capacity factors for bilirubin are all slightly larger than biliverdin in every bile salt. This is likely due also to the structural differences of these two compounds. The identity of the bile salt does not appear to affect the capacity factors of biliverdin. It should also be noted that the capacity factor of biliverdin dimethyl ester was always significantly higher than that of biliverdin at all pH values. These results all suggest that the double negative charges on both deprotonated bilirubin and biliverdin prevent strong interaction between these compounds and the anionic micelles.

Results obtained with bilirubin dimethyl ester were disappointing. In the trihydroxy bile salts, two small, poorly defined peaks are seen. The relative intensity of these peaks changes with time, so it seems likely that this analyte is degrading in these bile salt solutions. In the dihydroxy bile salts a single fronted peak is often seen, but this peak overlaps with the Sudan III peak. The peak maximum for biliverdin dimethyl ester always appears slightly after the Sudan maximum. This results in a negative capacity factor. A negative capacity factor has no physical meaning, and its occurrence may be explained in several ways. First, Sudan III may not be an accurate marker for these micelles. Although nitropyrene [28] and a homologous series approach [29] have both also been used previously as micelle markers in bile salt MEKC, Sudan III is commonly accepted as the micelle marker [30–32]. However, there has been no in-depth study as to whether Sudan III is truly an adequate marker in bile salt MEKC under a variety of conditions. Presumably, Sudan will migrate close to the micelle but there may be more appropriate markers. We examined the suitability of 1-nitropyrene as a micelle marker at a pH of 7.35 in taurocholic acid and a pH of 7.41 in taurodeoxycholic acid. In both cases, the nitropyrene peak eluted prior to the Sudan peak by 15 and 21 s, respectively. The second explanation for a negative capacity factor is that the micelle structure may change depending upon the analyte present, thus changing the mobility of the micelle. Third, interactions with the surfactant monomer are not accounted for in the development of the capacity factor equations. Should these interactions be important in this circumstance then the capacity factor calculation would be incorrect. It can be concluded, however, that bilirubin dimethyl ester exhibits significant interaction with the dihydroxy bile salts, and greater interaction than bilirubin, across this pH range. The increased interaction in comparison to bilirubin is most easily attributed to the lack of charge–charge repulsion with the anionic micelles. The slightly flattened structure of the dimethyl ester suggests that shape may also play a role. There is evidence that the bilirubin 'ridge-tile' is perturbed when bound to taurocholate micelles at pH 8 [33].

The most intriguing results are observed when comparing the behavior of xanthobilirubin with that of biliverdin. In the trihydroxy bile salts, biliverdin and xanthobilirubin exhibit essentially identical capacity factors across the pH range. No pH dependence is seen and the low value is consistent with effects of charge–charge repulsion between the anionic analytes and anionic micelles. The fact that the xanthobilirubin capacity factor values so closely match the biliverdin values may indicate that only a

small portion of the biliverdin molecule, perhaps half of it, interacts with the micelles. Similar results are obtained with dihydroxy bile salts. As expected, the capacity factors for biliverdin dimethyl ester and xanthobilirubin methyl ester both increase relative to the free acids. This is easily explained by the removal of charge–charge repulsion effects. It is curious that these capacity factors are so similar to each other. This implies that no more interaction with the micelle occurs for biliverdin dimethyl ester than for xanthobilirubin dimethyl ester, despite the significantly increased surface area of the former. Results obtained with the dihydroxy bile salts are very different. The capacity factors of xanthobilirubin methyl ester in the dihydroxy systems are roughly double that found in the trihydroxy systems. This may be explained by the increased hydrophobic character of the dihydroxy bile salts due to the smaller number of hydroxy groups. The capacity factors of biliverdin dimethyl ester with dihydroxy bile salts increase roughly four-fold over that found in the trihydroxy systems. A stronger interaction between this compound and the dihydroxy bile salts, compared to xanthobilirubin methyl ester, is also evident. One possible explanation is that the helical nature of the dihydroxy bile salt micelles facilitates more interaction with the 'lock-washer' configuration of the biliverdin species than does the trihydroxy micelle structures.

4. Conclusions

Significant differences are observed in the interactions of bilirubin and related species with four bile salts at micellar concentrations. Nonionic species show increased capacity factors when compared to anionic species. This is consistent with the presumed effects of charge–charge repulsion between analytes and micelles. While charge effects are significant, there is also evidence that the size and three-dimensional structure of the analytes also plays a role in governing interactions with bile salt micelles. The capacity factors of the free carboxylic acids are essentially identical in all of the bile salt systems studied, while there is significantly more interaction of the methyl ester derivatives with the dihydroxy bile salts as compared to the trihydroxy bile salts. This latter effect may reflect the more hydrophobic microenvironment presented by dihydroxy bile salts. Of all the compounds studied, only bilirubin shows pH-dependent results, most likely due to its unusual conformation in solution.

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