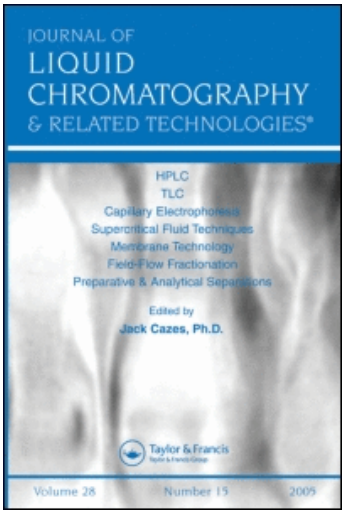

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MICELLAR ELECTROKINETIC CHROMATOGRAPHY OF BILIRUBIN AND RELATED COMPOUNDS IN UNCONJUGATED AND GLYCO-CONJUGATED BILE SALT SOLUTIONS

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ABSTRACT

The interactions of bilirubin, biliverdin, xanthobilirubin, biliverdin dimethyl ester, and xanthobilirubin methyl ester with unconjugated and glyco-conjugated bile salt solutions were investigated by micellar electrokinetic chromatography (MEKC). The capacity factors were measured in solutions of the different bile salts over the pH range of 6.5 to 8.5. The bile salts investigated were deoxycholic, chenodeoxycholic, glycocholic, glycodeoxycholic, and glycochenodeoxycholic acid. These results were compared to previous results in cholic, taurocholic, taurodeoxycholic, and taurochenodeoxycholic acids. Typically, the nature of the bile salt, trihydroxy versus dihydroxy, has a greater effect on the resulting capacity factors than does the nature of the conjugation. The influence on capacity factor of such features as molecular size, shape, and charge are revealed with this group of analytes.

INTRODUCTION

Normal human secretions of bile pigment are mainly composed of 65-85% bilirubin diconjugates with the remainder being monoconjugates and unconjugated bilirubin.[1] While conjugates of bilirubin are water soluble, unconjugated bilirubin (UCB) is relatively insoluble in aqueous solutions at pH 7.0.[2] UCB is found in bile at concentrations exceeding its solubility.[3] These high concentrations of UCB are stabilized in bile solutions through interactions with micelles comprised mainly of bile salts. The concentration of both pigments and bile salts can be altered as a result of any number of physiological conditions. Changes in the chemistry of the gall bladder or liver can thus result in jaundice, gallstone formation, pruritis (excessive itching), or kernicterus.

Bile salts have a steroid backbone that is typically substituted with OH groups at positions 3, 7, and 12. Other substitution patterns exist as well. The monomers can also be conjugated with taurine or glycine creating species with charged headgroups of varying structure and chemistry. The different types of monomers form different types of micelles. The predominant human bile salts are cholic (3, 7, 12-trihydroxy), chenodeoxycholic (3, 7-dihydroxy), and to a lesser extent, lithocholic (3-monohydroxy) acids. In healthy adults all are conjugated, with a typical glycine to taurine ratio of 3:1.[4] However, various physiological conditions, disease or age related, can alter the bile chemistry and resulting distribution of bile salts.

It is therefore of interest to study the interactions of bilirubin with a variety of bile components. By comparing the behavior of bilirubin with different bile components, we may be able to predict how alterations in bile chemistry will affect the fate of heme degradation products. This will lead not only to a better understanding of the chemistry of bilirubin but may also lead to new approaches for the manipulation of bile composition to increase the clearance capabilities of the liver.

We have previously reported on the interactions of bilirubin with bile salt micelles using micellar electrokinetic chromatography (MEKC).[5] Our previous results showed that the capacity factor of bilirubin decreased as the pH increased. This was expected as bilirubin should be an anion at higher pH and the bile salt micelles are anionic. Thus charge repulsion should decrease the ability of bilirubin to interact with these micelles at high pH. In addition, little difference in the capacity factor of bilirubin was observed in dihydroxy and trihydroxy bile salt solutions at pH values where bilirubin is believed to exist as a dianion.

More recently, we have included additional compounds in our studies.[6] These compounds were biliverdin IXa, biliverdin IXa dimethyl ester, xanthobilirubic acid, and xanthobilirubin methyl ester, the structures of which are shown in Figure 1.

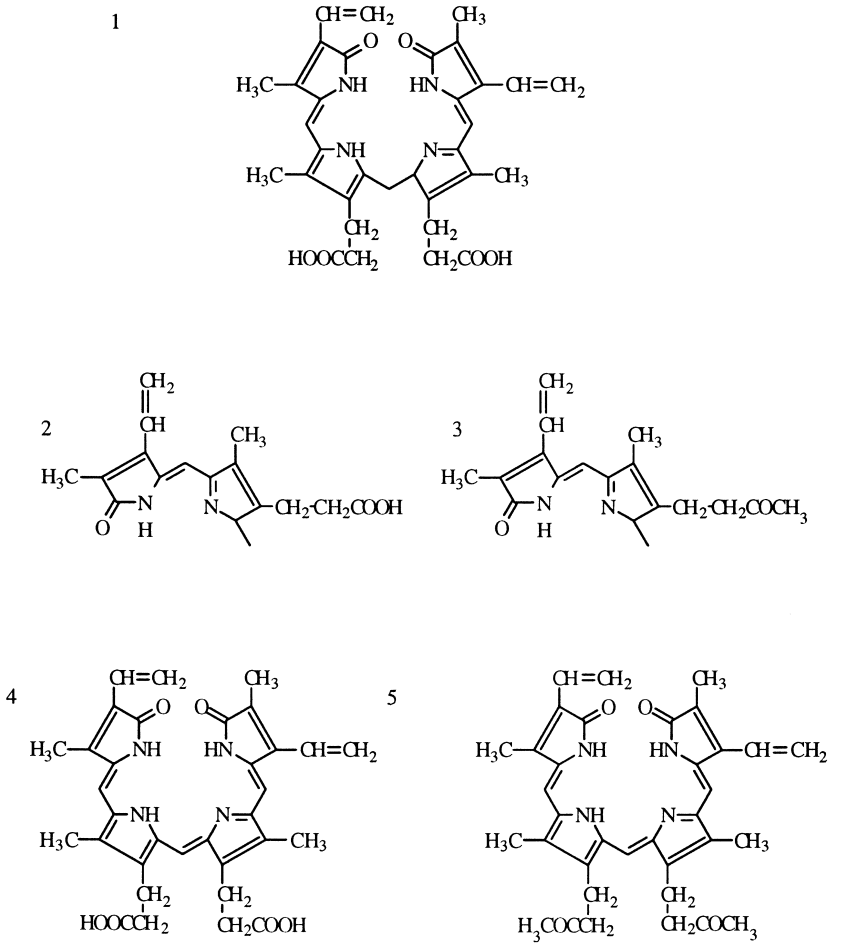

Figure 1. Chemical structures of bilirubin and related analytes. (1) bilirubin, (2) xanthobilirubin, (3) xanthobilirubin methyl ester, (4) biliverdin, (5) biliverdin dimethyl ester.

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 was probed by comparison of the interaction of the parent acids with that of the methyl esters, and the effects of size and shape were studied by comparing bilirubin with biliverdin and xanthobilirubin. Bilirubin is known to adopt a 'ridge-tile' conformation in aqueous solution[7-10] with the planes of the two dipyrrinone units at approximately a 95-100° angle.[8] In this conformation, the acid group of the propionate side chains and the lactam and pyrrinone

groups of the opposite dipyrrole unit engage in intramolecular hydrogen bonding. Biliverdin adopts a helical 'lock-washer' conformation in aqueous solution[11] where the structure is stabilized by hydrogen bonding between the protonated and unprotonated nitrogens in the ring structure.

Our previous MEKC results showed that of the acidic test compounds, only bilirubin exhibits a pH dependence of the capacity factor. Bilirubin and xanthobilirubin showed essentially identical capacity factors in all bile salt solutions. Capacity factors of the methyl esters were elevated above the acids, as would be expected in systems utilizing anionic micelles. More interaction between the analytes and the micelles, as inferred from larger capacity factors, was obtained for the methyl esters in the dihydroxy bile salts, taurodeoxycholic and taurochenodeoxycholic acids, than was obtained in the trihydroxy bile salts of cholic and taurocholic acids. Also, the capacity factor of xanthobilirubin methyl ester and biliverdin dimethyl ester were essentially identical in the trihydroxy bile salts but differed from each other in the dihydroxy bile salts. We postulated that the slight helical nature of biliverdin dimethyl ester allowed it to interact to a greater extent with the more helical dihydroxy bile salts.

In this MEKC study we have investigated the capacity factor of bilirubin and related compounds in glycocholic, glycodeoxycholic, glycochenodeoxycholic, deoxycholic, and chenodeoxycholic acids across the pH range of 6.5 to 8.5. The comparison of the behavior of all these different test analytes yields information on the mode and specificity of interaction of bile pigments with these different bile salts. These results are compared to our previous results obtained with taurine conjugated bile salts.

EXPERIMENTAL

Apparatus and Reagents

Fused-silica capillaries were purchased from Polymicro Technologies (Phoenix, AZ). The capillaries had an I.D. of 75 μm and a length of 69 cm (24 cm from injection tip to window). The power supply was a Spellman CZE 1000R (Plainview NY) with a +/- 30 kV output range. A Series 4500 Microscribe Strip Chart Recorder from The Recorder Company (San Marcos, TX) or a Dell 316SX computer (Austin, TX) fitted with a multifunction data acquisition board, manufactured by National Instruments (Austin, TX) was used to record runs. Software was written in-house using Microsoft Quick Basic (Redmond, WA) and National Instruments' Lab Windows and NI-DAQ software. Detection was performed on either a Varian Instruments Group 2550 UV detector (Walnut Creek, CA) fitted with a deuterium lamp or a Linear Instruments UVIS 200 detector. The detection wavelength in either case was 232 nm.

A Barnstead Nanopure system (Fisher Scientific, Austin, TX) provided deionized water. Biliverdin and biliverdin dimethyl ester were obtained from Porphyrin Products Incorporated (Logan, UT). Xanthobilirubic acid was prepared by hydrolysis[12] of xanthobilirubin methyl ester. The latter was provided courtesy of Dr. David Lightner (University of Nevada, Reno NV). Dimethylsulfoxide was purchased from Fisher; and sodium azide was purchased from Anachemia (Champlain, NY). All other reagents, including bilirubin and the bile salts, were purchased from Sigma (St. Louis, MO). Bilirubin was purified according to the method of McDonagh and Assisi.[13] Other test compounds were used as received. Bile salts were of the highest purity available from the supplier.

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) or a Nalge Company (Rochester, NY) 0.45 μm (25 mm diameter) syringe filter.

Analytes were dissolved individually in DMSO. Typically 1 - 3 mg of each analyte were dissolved in 30 - 300 μL of DMSO. An injection sample was then prepared by adding between 1 - 12 μL of the separately prepared analyte solutions and between 1 - 12 μL of a saturated Sudan III/DMSO solution to 1000 μL of the bile salt solution. The Sudan III functioned as a micelle marker for the separation process. Concentrations of analytes were kept purposely low. Sample solutions were 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.[5]

Capillary Preparation and Run Conditions

A 0.75 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. At the beginning of each day, the capillary was rinsed with 1 M KOH for 10 to 30 minutes, followed by water, and then with the bile salt solution for 10 to 30 minutes. 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 to 10 minutes followed with a deionized water rinse for 5 to 10 minutes. The capillary was stored in deionized water overnight.

The run voltage was 12 kV. Sample injections were performed applying 12 kV for 5-10 seconds. The capillary and the electrode were then wiped clean and placed into the run buffer.

Capacity Factor Calculation

Capacity factors for each compound were calculated as described previously[5] by eq. 1 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

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

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

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

RESULTS

Example electropherograms are shown in Figure 2 for test compounds in deoxycholic acid solutions. Figure 3 shows the results in a graphical format for bilirubin in all bile salts investigated in this study as well as the results from our previous study.[6]

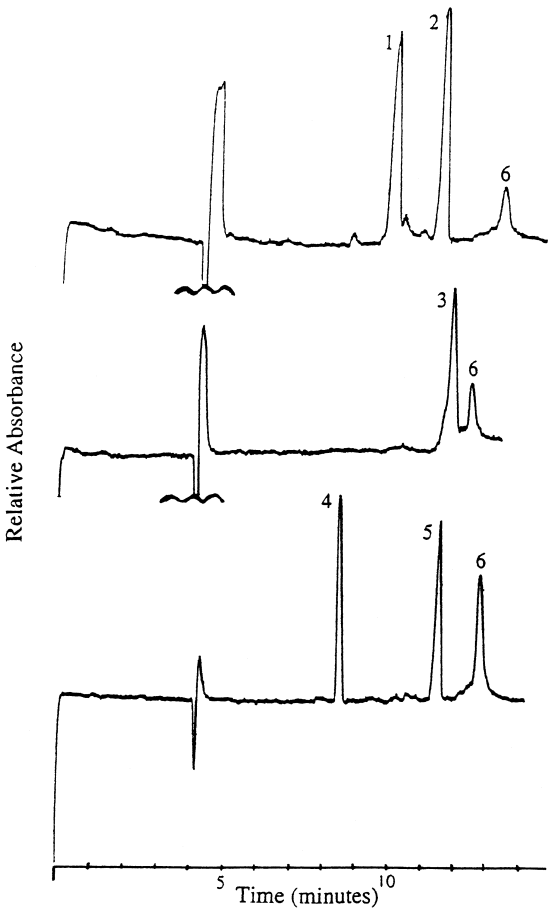

Figure 2. Sample electropherograms in 25 mM deoxycholic acid at pH 7.5. All runs with a 5 second, 12 kV injection. 12 kV run voltage, 232 nm detection wavelength, 31 - 34 μA. All electroosmotic flow peaks are DMSO, peak (6) is Sudan III, and analyte peaks are (1) biliverdin, (2) bilirubin, (3) biliverdin dimethyl ester, (4) xanthobilirubin, (5) xanthobilirubin methyl ester.

Results from nine different bile salt solutions are shown. The bilirubin capacity factor shows a strong pH dependence. In addition, it appears as though there is very little significant change in the capacity factor of bilirubin as the identity of the bile salt is changed.

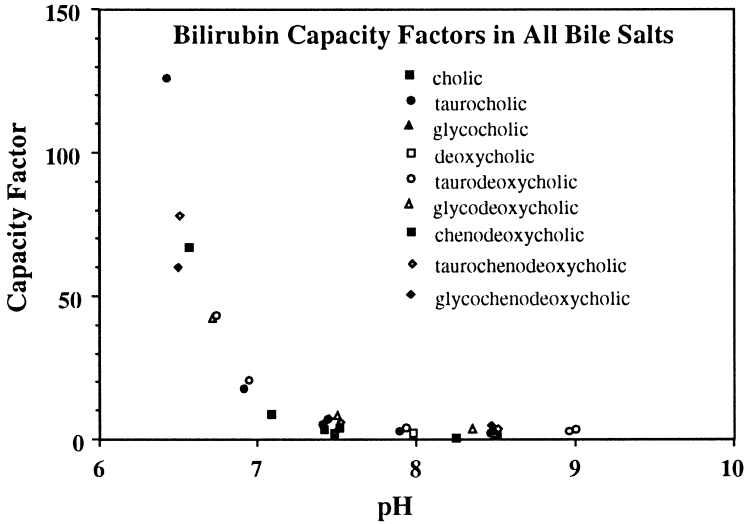

Figure 3. Bilirubin capacity factors in nine bile salt solutions as a function of pH. All bile salts are at 25 mM in phosphate-borate buffer. Capillary dimensions: 75 μm i.d., 69 cm total length, 24 cm separation length. Detection at 232 nm. Separation at 12 kV.

Figure 4 shows the results in a graphical format for the dihydroxy bile salt glycochenodeoxycholic acid. For comparison, the capacity factors for the two methyl esters in taurochenodeoxycholic acid are also indicated.[6] The capacity factors of the two acids in taurochenodeoxycholic acid are not shown because they are essentially identical to the results in glycochenodeoxycholic acid. As can be seen by Figures 3 and 4, there is little effect on capacity factor as the conjugation is changed from taurine to glycine. Only in the case of biliverdin dimethyl ester is there an apparent difference in the capacity factors with the capacity factor being slightly lower in the glycine conjugated version of this bile salt as compared to the taurine version. The P value for a two-tailed t test is 0.0016 at pH 6.5, 0.13 at pH 7.5, and 0.065 at pH 8.5.

Table 1 shows the capacity factors and relative standard deviations for each of the compounds in the glyco-conjugated bile salt solutions: glycocholic, glycodeoxycholic, and glycochenodeoxycholic acids. Two capacity factors are reported in cases where at least three determinations were performed at two slightly different pH values. The results obtained in glycocholic acid are very similar to the results reported previously in cholic and taurocholic acid. The capacity factor values for xanthobilirubin and biliverdin are similar to each other and below a value of 1.6. The capacity factors of the methyl ester species are increased over those of the acid species as would be expected when anionic micelles are used.

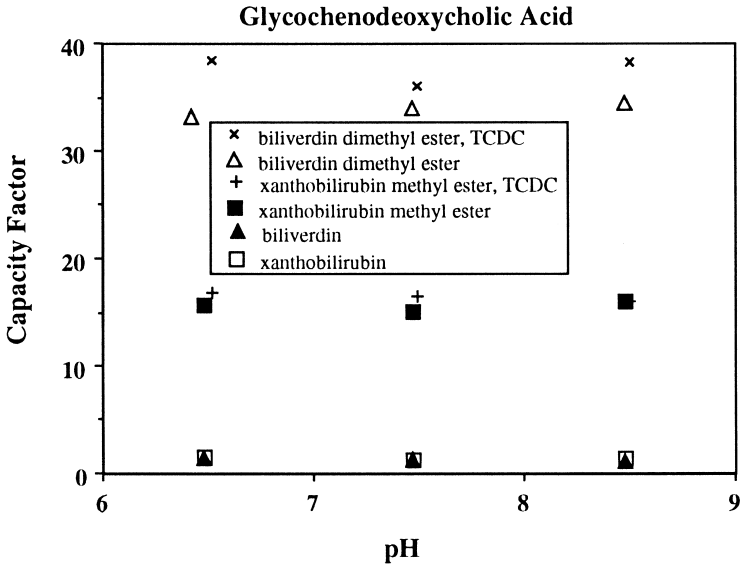

Figure 4. Analyte capacity factors in 25 mM glycochenodeoxycholic acid with taurochenodeoxycholic acid results shown for comparison.

The capacity factor of xanthobilirubin methyl ester is only slightly less than biliverdin dimethyl ester. In the case of this trihydroxy bile salt, conjugation, or the lack of, does not seem to play a role in establishing interaction with these analytes and the trihydroxy bile salts. Only the bilirubin capacity factor shows a pH effect as would be expected since the other test analytes should maintain their neutral or negative charge across this pH range. At pH 6.5 in glycocholic acid, the bilirubin and Sudan III peak co-elute. These compounds were examined in separate electropherograms but a negative capacity factor was nevertheless calculated. A negative capacity factor has no physical meaning. The bilirubin peak does elute just prior to Sudan peak so our conclusion is that the capacity factor under these conditions is very large.

Calculation of a negative capacity factor probably represents a slight uncertainty in the free solution mobility of bilirubin at this pH and/or minor run-to-run variation. This is consistent with our previous data.

Glycodeoxycholic acid results are again very similar to results obtained previously with taurodeoxycholic acid and are similar to the results obtained in glycochenodeoxycholic acid. Bilirubin capacity factors are higher than the methyl esters at low pH and higher than the other acids at high pH. There is little if any difference in the capacity factors of xanthobilirubin and biliverdin and these values are essentially equal to those found in taurodeoxycholic acid.

Table 1

Analyte Capacity Factors in Glyco-Conjugated Bile Salt Solutions

	Capacity Factor (R.S.D./%)		
25 mM Glycocholic Acid	**pH 6.5**	**pH 7.5**	**pH 8.5**
Bilirubin	*	4.76 (4.0)	2.75 (3.0)
Xanthobilirubin	1.21 (3.4)	1.02 (3.3)	0.95 (2.8), 1.02 (6.6)
Biliverdin	1.25 (4.8)	1.15 (1.8)	1.02 (6.6), 1.05 (0.05)
Xanthobilirubin methyl ester	8.38 (3.5)	6.48 (0.57)	6.8 (1.4)
Biliverdin dimethyl ester	9.48 (1.2)	10.2 (3.9)	9.06 (1.9)
25 mM Glycodeoxycholic Acid	**pH 6.4-6.7**	**pH 7.4-7.5**	**pH 8.4-8.6**
Bilirubin	42.5 (7.8)	8.6 (4.4)	3.5 (2.9)
Xanthobilirubin	1.40 (5.1)	1.21 (1.2)	1.06 (1.7)
Biliverdin	1.47 (5.5)	0.81 (10.0)	0.84 (5.0), 0.78 (7.6)
Xanthobilirubin methyl ester	18.2 (6.6)	18.1 (4.9)	17.0 (1.8), 17.0 (1.3)
Biliverdin dimethyl ester	31.7 (5.0)	31.8 (4.3)	29.5 (2.1), 30.1 (1.4)
25 mM Glycochenodeoxycholic Acid	**pH 6.4-6.5**	**pH 7.4-7.5**	**pH 8.5**
Bilirubin	60.1 (21)	7.22 (3.7)	4.95 (1.6)
Xanthobilirubin	1.56 (8.6)	1.28 (0.43)	1.35 (0.69)
Biliverdin	1.42 (11.7)	1.22 (6.7)	16.1 (6.9)
Xanthobilirubin methyl ester	15.7 (1.2)	15.1 (1.0)	1.12 (8.2)
Biliverdin dimethyl ester	33.1 (0.46)	33.9 (4.4)	34.4 (5.2)

* A negative capacity factor is calculated.

Xanthobilirubin methyl ester shows no preference for one of these bile salts over another, with capacity factors of approximately 17 in each system. Biliverdin dimethyl ester shows a slightly decreased capacity factor (approx. 31) in the glyco-conjugated bile salt as opposed to the tauro-conjugated bile salt (approx. 36). P values for a two-tailed t test are 0.05 at pH 6.5, 0.004 at pH 7.5, and 0.12 at pH 8.5.

Table 2 show the capacity factors and relative standard deviations for each of the analytes in the unconjugated bile salts: deoxycholic, and chenodeoxycholic acids. Two values indicate at least three determinations at two slightly different pH values. The two unconjugated bile salts gelled under

Table 2

Analyte Capacity Factors in Unconjugated Bile Salt Solutions

	Capacity Factor (R.S.D./%)		
25 mM Deoxycholic Acid	**pH 7.5***	**pH 8.0**	**pH 8.5**
Bilirubin	4.3 (5.2)	2.1 (3.0)	2.1 (9.0)
Xanthobilirubin	1.1 (1.1)	0.67 (3.7)	0.94 (11.8)
Biliverdin	1.0 (3.0)	0.55 (2.8)	0.92 (10.5)
Xanthobilirubin methyl ester	18.9 (0.52)	15.5 (3.0)	14.0 (8.1), 14.8 (4.3)
Biliverdin dimethyl ester	40.7 (9.6)	33.7 (3.7)	22.3 (7.3), 23.7 (4.8)
25mM Chenodeoxycholic Acid	**pH 7.1***	**pH 7.4-7.6**	**pH 8.2-8.4**
Bilirubin	8.9 (15)	2.65 (5.9)	0.58 (1.8)
Xanthobilirubin	1.2 (3.4)	1.16 (2.9)	1.06 (1.7)
Biliverdin	1.1 (3.7)	1.2 (3.1)	0.81 (2.0)
Xanthobilirubin methyl ester	17.5 (0.89)	17.0 (2.0)	15.1 (1.4)
Biliverdin dimethyl ester	46.8 (2.1)	17.7 (2.0)	16.9 (2.0)

* The bile salt solution gels at pH 6.5.

the conditions used in this work at lower pH values. Data in Table 2 is only presented if the bile salt solution did not show visible signs of gelation after a 24 hour period after the application of applied voltage. The results in these two unconjugated bile salts seem to deviate from the conjugated counterparts. The capacity factors for the acid species are for the most part the same as those obtained in the conjugated systems, but the two methyl ester species seem to show a pH dependence. At higher pH values, biliverdin dimethyl ester clearly has a lower capacity factor than it does in the conjugated versions. And the capacity factor of bilirubin is below that of the methyl ester species at low pH.

DISCUSSION

The pH dependence of the bilirubin capacity factor has been previously discussed at length.[5,6] It is most likely attributed to a change in the ionization state of bilirubin, which would involve a micelle induced pK_a change and/or an aggregation of bilirubin at lower pH values. In any case, as the pH is altered, the ability of bilirubin to effectively interact with bile salt micelles is dramatically altered. It is also interesting to note that, with the exception of the two bile salts which gel at low pH, the capacity factor of bilirubin is always higher than the methyl ester test compounds at the low pH values. Its capacity factor is also higher than the other acids at high pH values. This was found to be true in our previ-

ous investigation but is further proved here in glycocholic, glycodeoxycholic, and glycochenodeoxycholic acids. These results suggest that the charge and conformation of bilirubin allow for the formation of a unique complex with the bile salts which permits increased interaction in comparison to the other test compounds. With regards to the unconjugated dihydroxy bile salts, viscosity measurements were not taken but it is likely that the beginnings of macromolecular assembly are taking place even at pH 7.5 and perhaps at pH 8.0. This could account for the higher capacity factors at low pH for the methyl esters. Since the capacity factor of bilirubin does not also rise abnormally at low pH, and is in fact lower in deoxycholate and chenodeoxycholate than the other bile salts, this indicates that the specifics of the molecular interaction between bilirubin and these unconjugated dihydroxy bile salts and the methyl esters and these unconjugated dihydroxy bile salts differ from each other.

CONCLUSION

The behavior of bilirubin and related analytes in glyco-conjugated bile salts is consistent with our earlier findings in the corresponding tauro-conjugated species at 25 mM bile salt concentrations. Trihydroxy bile salts provide essentially the same results whether the bile salt is conjugated or not. In the case of the dihydroxy bile salts deoxycholic acid and chenodeoxycholic acid, the results are similar to each other as long as the bile salt is conjugated with taurine or glycine. The unconjugated versions seem to behave slightly different with respect to solubilization of these analytes. This difference is attributed to the gelation behavior of the unconjugated dihydroxy bile salts. Consistent with our earlier findings in the tauro-conjugated bile salts, significant differences are observed in the interactions of bilirubin and related species with the glyco-conjugated bile salts. Neutral species show increased capacity factors when compared to anionic analytes. This is consistent with the effects of charge-charge repulsion between analytes and micelles. Of all the compounds studied, only bilirubin shows pH dependent results, most likely due to its unusual conformation in solution, and much lower water solubility compared to the other acidic analytes.

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