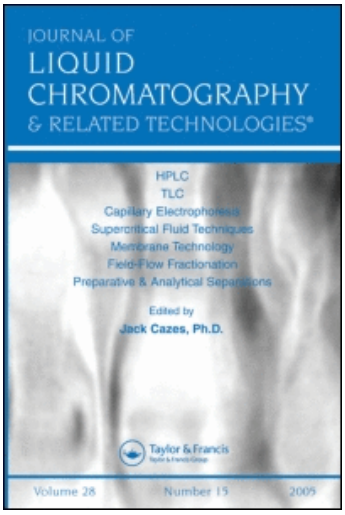

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STUDIES ON STEROIDS CLXIX. HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC BEHAVIOR OF SULFATED BILE ACIDS

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ABSTRACT

The elution behavior of sulfated bile acids in high-performance liquid chromatography on the octadecylsilyl bonded column with acetonitrile/0.5% phosphate buffer has been investigated. A significant influence of pH of the mobile phase on the capacity ratio (k') was observed in the higher pH region for bile acid 12-sulfates. Blockage of the 12α-hydroxyl group in sulfated bile acids by acetylation produced a marked decrease in the k' values relative to their parent compounds in the pH range above 6.0. The k' values of dehydrocholate monosulfates and their acetates were measured and the increments exerted by transformation into the acetates were estimated. Remarkable increments were observed for dehydrocholate monosulfates with the 3α-hydroxyl group but not for those with the 7α- or 12α-hydroxyl group. The effect of pH of the mobile phase on chromatographic behavior has been discussed from the stereochemical point of view.

INTRODUCTION

In a previous paper of this series we reported the elution behavior of 3-, 7- and 12-sulfated bile acids in high-performance liquid chromatography (HPLC) on the octadecylsilyl bonded (ODS) column (1). The 7- and 12-sulfates showed behavior similar to

that of the 3-sulfates with mobile phases of varying pH, exhibiting a smaller k' value than the corresponding 3-sulfates with the exception of deoxycholate* 12-sulfates. The glyco- and tauro-deoxycholate 12-sulfates showed larger k' value than the corresponding 3-sulfates in the whole pH range. As for the unconjugated deoxycholate monosulfates, the elution order of the 12- and 3-sulfates was reversed at pH 6.5. A plausible explanation for these phenomena has not yet been offered.

Recently, the occurrence of the 3-sulfates of keto bile acids in human urine was demonstrated by means of gas chromatography-mass spectrometry (2). These sulfates are not so stable under the hydrolysis or solvolysis condition. It is, therefore, desirable to develop a method for the direct analysis of the intact sulfates in biological materials. Previously, the synthesis of the 3-, 7- and 12-sulfates of bile acids having a keto and/or an acetoxyl group in the steroid nucleus was reported (3).

This paper describes the relationship between elution behavior in HPLC and structure of sulfated bile acids. In addition, chromatographic behavior of 3-, 7- and 12-dehydrocholate monosulfates and their acetates has also been investigated and discussed from the stereochemical point of view.

EXPERIMENTAL

Materials

The 3-, 7- and 12-sulfates of bile acids were synthesized in these laboratories by the methods previously reported (3,4). All

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* The following trivial names are used in this paper: deoxycholate, 3α,12α-dihydroxy-5β-cholan-24-oic acid; chenodeoxycholate, 3α,7α-dihydroxy-5β-cholan-24-oic acid; cholate, 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid; 3-dehydrocholate, 7α,12α-dihydroxy-3-oxo-5β-cholan-24-oic acid; 7-dehydrocholate, 3α,12α-dihydroxy-7-oxo-5β-cholan-24-oic acid; 12-dehydrocholate, 3α,7α-dihydroxy-12-oxo-5β-cholan-24-oic acid.

the reagents used were of analytical-reagent grade. Solvents were purified by distillation prior to use.

Apparatus

The apparatus used for this work was a Waters 6000A solvent delivery system (Waters Assoc., Milford, Mass.) equipped with a Model Uvidec-100 II ultraviolet detector (Japan Spectroscopic Co., Tokyo) monitoring the absorbance at 205 nm. The ODS SC-02 (25 cm x 4.6 mm I.D.) (Japan Spectroscopic Co.) and Radial-Pak A (10 cm x 8 mm I.D.) (Waters Assoc.) columns were employed under ambient conditions. Acetonitrile/0.5% potassium phosphate buffer (pH 3.0-7.5) (8:27-8:17) were used as mobile phases at a flow rate of 2 ml /min.

RESULTS AND DISCUSSION

Initially, the unusual chromatographic behavior of bile acid 12-sulfates was examined in general for dehydrocholate 12-sulfates and their acetates. The effect of pH of the mobile phase on the capacity ratio (Rk') relative to cholate 3-sulfate was investigated on the SC-02 column with the acetonitrile/0.5% phosphate buffer system. The ratio of Rk' value of the 12-sulfate at a certain pH to that of the corresponding 12-sulfate at pH 3.5 was plotted against the pH values (3.5-7.5). As illustrated in Figure 1, the ratio increased with increasing pH, particularly above pH 6.5. The pH effect, however, was not distinctly observed for 3-dehydrocholate 12-acetate 7-sulfate. It is evident from these data that the pH dependence for the capacity ratio is common to bile acid 12-sulfates. The glycine- and taurine-conjugated bile acids have smaller pK values than the unconjugated and are almost completely dissociated in this pH region. Conjugated deoxycholate 12-sulfates exhibited larger k' value than the corresponding 3-sulfates in the whole pH range and somewhat different chromatographic behavior from unconjugated bile acid 12-sulfates.

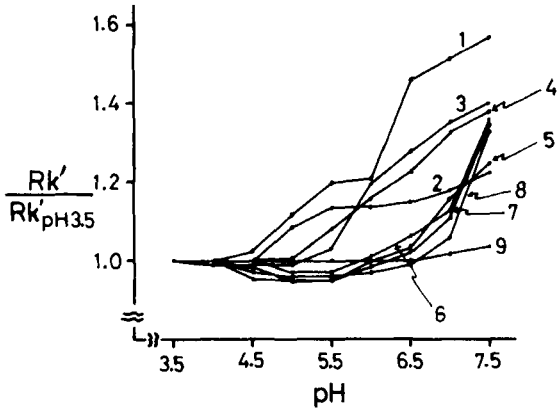

FIGURE 1 Effect of pH of Mobile Phase on Relative k' Values of Sulfated Bile Acids
1, deoxycholate 12-S; 2, cholate 12-S; 3, cholate 3-acetate 12-S; 4, cholate 7-acetate 12-S; 5, 3-dehydrocholate 12-S; 6, 3-dehydrocholate 7-acetate 12-S; 7, 7-dehydrocholate 12-S; 8, 7-dehydrocholate 3-acetate 12-S; 9, 3-dehydrocholate 12-acetate 7-S

Inspection of a Dreiding model indicates that the hydroxyl function at C-12 is sterically close to the carboxylic acid or sulfonic acid residue of the side chain. Therefore, the effect of blockage of the 7α- and 12α-hydroxyl groups by acetylation on chromatographic behavior was investigated with various sulfated cholate derivatives. The ratio of the Rk' values of the acetylated bile acid sulfate to the corresponding non-acetylated bile acid sulfate was plotted against the pH values (see Figure 2). Acetylation of the 12α-hydroxyl group exerted a marked decrease in the Rk' value in the pH region above 6.0. This result strongly implies the presence of steric interaction between the hydroxyl group at C-12 and carboxylic acid moiety at C-24 in the higher pH region.

It has previously been demonstrated that unsulfated deoxycholate and chenodeoxycholate were efficiently resolved under the weakly alkaline condition on the ODS column (5,6). Since it was supposed that a similar steric interaction would exist in deoxy-

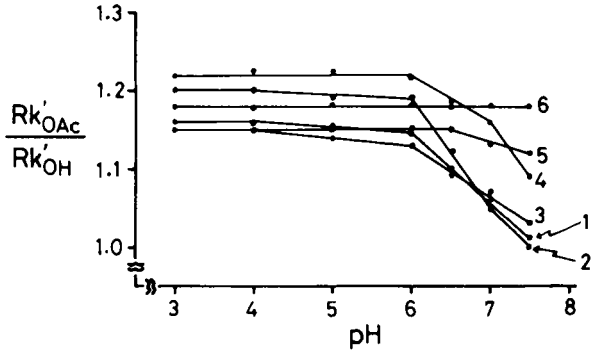

FIGURE 2 Effect of Acetylation on Relative k' Values of Sulfated Bile Acids
1, cholate 12-acetate 3-S; 2, 7-dehydrocholate 12-acetate 3-S; 3, cholate 12-acetate 7-S; 4, 3-dehydrocholate 12-acetate 7-S; 5, cholate 7-acetate 12-S; 6, 3-dehydrocholate 7-acetate 12-S

cholate, the effect of pH of the mobile phase on chromatographic behavior of unsulfated bile acids was also investigated on the Radial-Pak A column. The k' values of unconjugated, glyco-, and taurodeoxycholate relative to the corresponding chenodeoxycholate were plotted against the pH values, respectively (see Figure 3). Unconjugated deoxycholate showed a larger k' value than chenodeoxycholate in the higher pH region, while both gave almost identical values in the lower pH region. On the other hand, glyco- and taurodeoxycholate exhibited larger k' values than conjugated chenodeoxycholate in the whole pH range. These data lend a support on the assumption that the separation of deoxycholate from chenodeoxycholate on the ODS column is ascribable to the steric interaction between the hydroxyl group at C-12 and acidic moiety of the side chain.

Finally, the chromatographic behavior of dehydrocholate sulfates and their acetates were investigated on the SC-02 column with the acetonitrile / 0.5% phosphate buffer system at pH 3.5, 5.0, and 7.0. The k' values of these bile acids relative to cholate

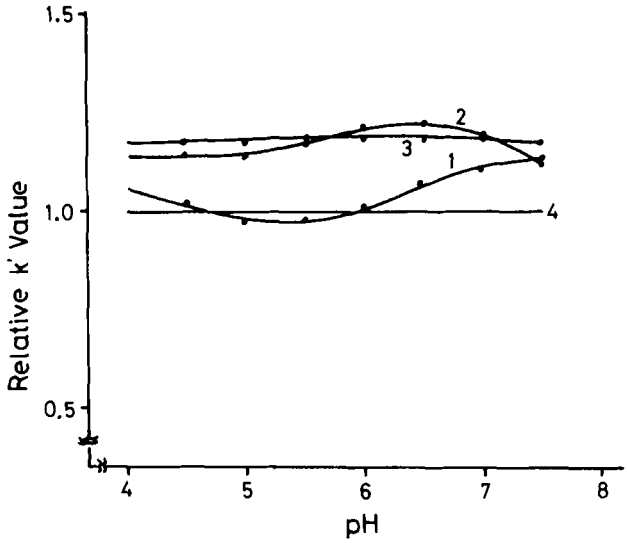

FIGURE 3 Effect of pH of Mobile Phase on k' Values of Bile Acids Relative to Chenodeoxycholate
1, deoxycholate; 2, glycodeoxycholate; 3, taurodeoxycholate; 4, unconjugated, glyco-, and taurochenodeoxycholate

3-sulfate are listed in Table 1. The 3-, 7- and 12-dehydrocholate monosulfates exhibited no significant difference in the Rk' values at each pH, and complete separation was not accomplished. The effect of acetylation on chromatographic behavior was then investigated with six dehydrocholate monosulfates. A marked change in the Rk' values was observed for dehydrocholate monosulfates with the 3α-hydroxyl group but not for those with the 7α- or 12α-hydroxyl group. This result implies that dehydrocholate monosulfates can be efficiently separated by acetylation prior to HPLC. An increment in the Rk' value exerted by acetylation, i.e. ΔRk' value, was estimated (see Table 1). In the lower pH region, the values for the 3α-, 7α-, and 12α-hydroxyl groups were 0.48-0.67, 0.03-0.06, and 0.10-0.14, respectively. At pH 7.5, ΔRk' values were different from those at pH 3.5 and 5.0, indicating the participation of

TABLE 1

Relative Capacity Ratios of Sulfated Dehydrocholates and Their Acetates

Compound	pH 3.5		pH 5.0		pH 7.5	
	Rk'	ΔRk'	Rk'	ΔRk'	Rk'	ΔRk'
7-Dehydrocholate 3-S	0.54		0.50		0.52	
12-Dehydrocholate 3-S	0.47		0.43		0.42	
3-Dehydrocholate 7-S	0.62		0.57		0.69	
12-Dehydrocholate 7-S	0.46		0.39		0.47	
3-Dehydrocholate 12-S	0.64		0.61		0.80	
7-Dehydrocholate 12-S	0.37		0.35		0.50	
7-Dehydrocholate 12-acetate 3-S	0.65	0.11	0.60	0.10	0.52	0.00
12-Dehydrocholate 7-acetate 3-S	0.50	0.03	0.47	0.04	0.44	0.02
3-Dehydrocholate 12-acetate 7-S	0.76	0.14	0.70	0.13	0.76	0.07
12-Dehydrocholate 3-acetate 7-S	1.13	0.67	1.05	0.66	1.26	0.79
3-Dehydrocholate 7-acetate 12-S	0.69	0.05	0.67	0.06	0.94	0.14
7-Dehydrocholate 3-acetate 12-S	0.87	0.50	0.83	0.48	1.17	0.67

steric interaction with the carboxylic acid group of the side chain.

The current methods for the determination of bile acid sulfates involve prior solvolysis and hydrolysis (7,8), but information about the conjugated form and position is thus lost. Having investigated the chromatographic behavior of sulfated bile acids with mobile phases of varying pH, we have now clarified that it depends on the position of the sulfate and hydroxyl groups. The k' values of 12-sulfates increase significantly in the higher pH region, owing to steric interaction with the acidic moiety on the side chain. We have also demonstrated that the effect of acetylation on the k' value depends upon the position of the hydroxyl

function. In particular, the k' values of bile acids having a 3α-hydroxyl group are significantly influenced by acetylation. These findings may be useful in the structural elucidation of sulfated bile acids and in the characterization of sulfotransferases in biological materials. The availability of an excellent method for the analysis may provide more precise knowledge on the metabolic profile of sulfated bile acids in patients with hepatobiliary diseases.

ACKNOWLEDGEMENTS

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