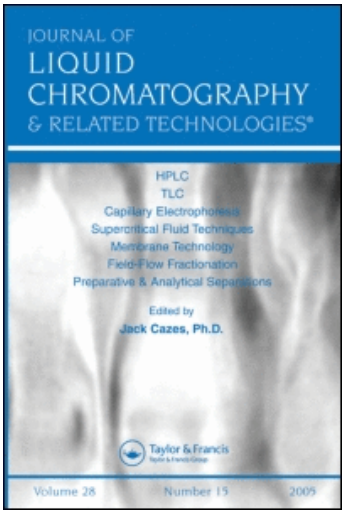

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SEPARATION OF MONOSULFATED BILE ACIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Separation of 3-, 7- and 12-monosulfates of cholate, chenodeoxycholate, deoxycholate, lithocholate and ursodeoxycholate and their glyco- and tauro-conjugates by high-performance liquid chromatography on a reversed phase column has been carried out. Effects of pH and salt concentration of a mobile phase on the k' value of sulfated bile acids were investigated with the μBondapak C_{18} and ODS SC-02 columns. The 3-sulfated bile acids were efficiently separated on ODS SC-02 using three aqueous ammonium carbonate/acetonitrile systems. The chromatographic behaviors of 7- and 12-sulfated bile acids are also briefly discussed.

INTRODUCTION

Since the first report on the occurrence of sulfated bile acids in man (1), considerable attention has been drawn to the metabolic significance of sulfation of bile acids in liver diseases. The separation of the 3-sulfates of major bile acids by thin-layer chromatography has been attempted, but the satisfactory results have not yet been obtained (2,3). Gas-liquid chromatography of the sulfates involving prior hydrolysis and/or solvolysis followed by derivatization of deconjugated bile acids has also been carried out (4,5). This method, however, has the inevitable disadvantages, such as the lack of reliability (6,7) and the loss of information

about the conjugated form. In the previous papers of this series we reported a method for the simultaneous determination of unsulfated bile acids in bile without hydrolysis and derivatization using high-performance liquid chromatography (HPLC) on a reversed phase column (8,9). This paper deals with the separation of the 3-monosulfates of unconjugated, glyco- and tauro-conjugated bile acids by HPLC. In addition the chromatographic behaviors of 7- and 12-monosulfated bile acids are also described.

EXPERIMENTAL

Materials

The 3-, 7- and 12-monosulfates of unconjugated, glyco- and tauro-conjugated bile acids were synthesized in these laboratories by the methods previously reported (10). All of the reagent used were of analytical-reagent grade. Solvents were purified by distillation prior to use.

Instruments

The apparatus used for this work was a JASCO Model Tri Rotar high-performance liquid chromatograph (Japan Spectroscopic Co., Tokyo) equipped with a Model UV-100 II ultraviolet (UV) detector monitoring the absorbance at 205 nm. The ODS SC-02 (25 cm x 4.6 mm I.D.) (Japan Spectroscopic Co.) and μBondapak C_{18} (1 ft. x 1/4 inch I.D.) (Waters Assoc., Milford, Mass.) columns were employed under ambient conditions.

RESULTS AND DISCUSSION

The separation of unsulfated bile acids by reversed phase chromatography on the octadecylsilyl (ODS) bonded column has been reported by several groups (9, 11-13). Among the typical columns commonly used for the analysis of polar compounds, μBondapak C_{18} and ODS SC-02 were chosen in this work for use in the separation of sulfated bile acids.

Initially, the effect of pH of a mobile phase on the capacity ratio (k') was investigated with the 0.5% phosphate buffer/acetonitrile system on the μBondapak C_{18} column. The k' values of monosulfated chenodeoxycholate, deoxycholate and cholate relative to taurodeoxycholate were plotted against the pH value (Fig. 1).

The close similarity in the chromatographic behavior was observed among the three 3-sulfated bile acids. In the pH range of 7.0 to 7.5 the unconjugated, glyco- and tauro-conjugated sulfates exhibited the similar k' values one another and the definite elution order within each of the three bile acids. In contrast, the elution order of these three was reversed in the lower pH region. The relative k' value of the unconjugated bile acid sulfates raised with decreasing pH to 4.5 and arrived at the plateau. As for the sulfated glyco-conjugates the inflection of the relative k' value was observed at pH 5.0, exhibiting a marked increase of this value toward the lower pH region.

It is evident from the data that the k' value of sulfated bile acids is dependent upon the structure of the side chain at C-17. Difference in the chromatographic behavior between unconjugated and glyco-conjugated bile acids may be explained in terms of the pK value, i. e. 6.4 for the former and 4.4 for the latter. These results are substantially compatible with the previous report by Horváth et al. (14). It was demonstrated that the k' value of organic acids obtained with the ODS column is dependent upon the acidity of an eluent and hence can be calculated from the pK values of both organic acid and eluent. Irrespective of the structure of the side chain sulfated bile acids were eluted in the order with decreasing number of the hydroxyl group on the steroid nucleus. Sulfated ursodeoxycholate having an equatorial hydroxyl group at C-7 showed a somewhat smaller k' value than its C-7 epimer, that is sulfated chenodeoxycholate. It is of particular interest that the elution order of 3-sulfated bile acids is identical with that of unsulfated bile acids (8). On the basis of these results the mobile phase in the pH range of 6.5 to 8.0 was chosen for use in

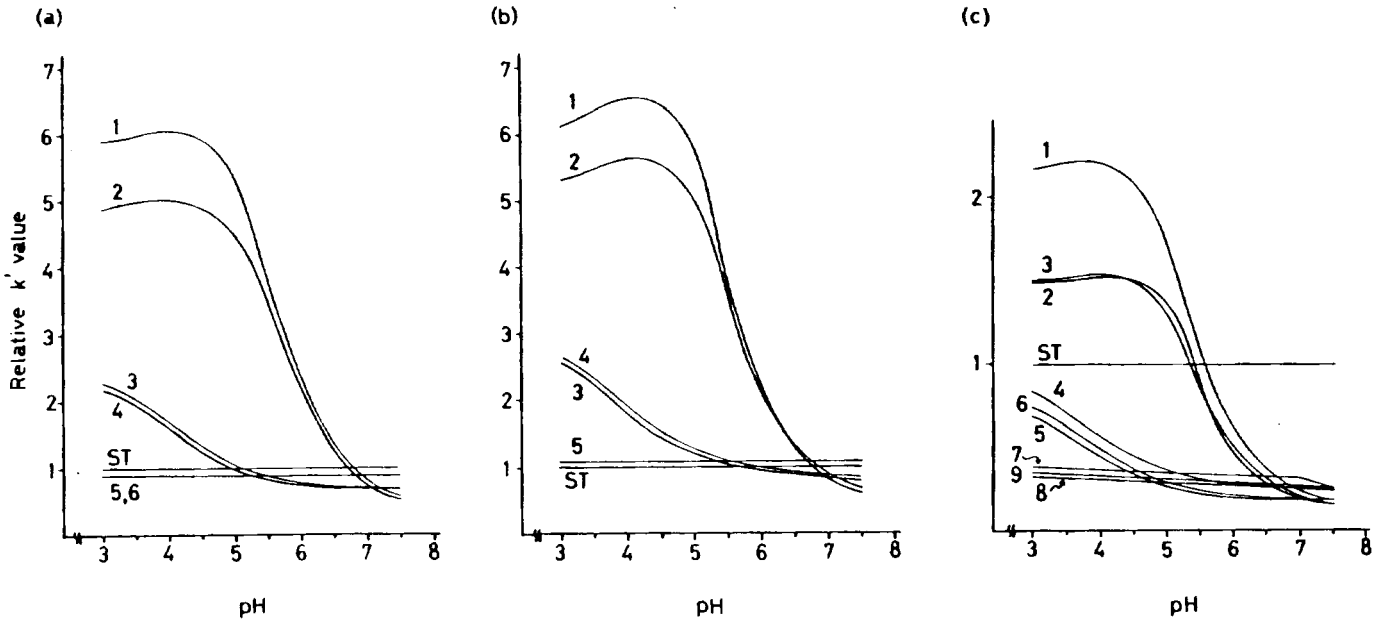

FIGURE 1. Effect of pH on Relative k' Value of Monosulfated Bile Acids to Taurodeoxycholate 3-Sulfate. (a) Sulfated chemodeoxycholates: 1=3-sulfate, 2=7-sulfate, 3=glyco-3-sulfate, 4=glyco-7-sulfate, 5=tauro-3-sulfate, 6=tauro-7-sulfate. (b) Sulfated deoxycholates: 1=3-sulfate, 2=12-sulfate, 3=glyco-3-sulfate, 4=glyco-12-sulfate, 5=tauro-12-sulfate. (c) Sulfated cholates: 1=3-sulfate, 2=7-sulfate, 3=12-sulfate, 4=glyco-3-sulfate, 5=glyco-7-sulfate, 6=glyco-12-sulfate, 7=tauro-3-sulfate, 8=tauro-7-sulfate, 9=tauro-12-sulfate. ST(standard)=taurodeoxycholate 3-sulfate. Conditions: column, μBondapak C_{18} (1 ft. x ¼ in. I.D.); mobile phase, 0.5% phosphate buffer/acetonitrile, 2.0 ml/min; detection, 205 nm.

the simultaneous analysis of 3-sulfated bile acids.

Various combinations of buffer solution (pH 7.0-7.8) and organic solvent were examined to choose the suitable mobile phase on the μBondapak C_{18} column. The use of the 0.5% ammonium carbonate/acetonitrile system appeared to be promising without any significant leading and tailing. The resolution of 3-sulfated chenodeoxycholate and deoxycholate, however, was not satisfactorily attained on this column. The use of the ODS SC-02 column rather than μBondapak C_{18} provided more efficient separation of unsulfated chenodeoxycholate and deoxycholate.* The chromatographic behavior of the 3-sulfates was therefore investigated on this column using 0.5% phosphate buffer/acetonitrile as a mobile phase. As can be seen in Fig. 2, the k' values of 3-sulfated glycochenodeoxycholate and taurochenodeoxycholate relative to chenodeoxycholate 3-sulfate raised with increasing pH whereas that of deoxycholate 3-sulfate was almost constant in the whole pH range of 7.0 to 8.0.

The effect of salt concentration on the retention value and resoltuion of 3-sulfated chenodeoxycholate and deoxycholate was then examined using aqueous ammonium carbonate solution (pH 7.8)/ acetonitrile as a mobile phase. The k' values of 3-sulfated cholate, chenodeoxycholate and deoxycholate relative to the corresponding value obtained with 0.05% ammonium carbonate were determined (Fig. 3). The relative retention value raised with the increasing salt concentration of the eluent remarkably up to 0.3% and then gently. Relatively distinct difference in the relative k' value between 3-sulfated chenodeoxycholate and deoxycholate was observed at the higher salt concentration.

The resolution of two peaks is dependent upon not only k' value but also peak shape. In this study the value of peak width/ (t_R-t_0) was taken as a parameter to represent the nature of a peak.

* Difference in the resolution between these two columns may be ascribable to adsorption on the uncoated silanol group of the supports.

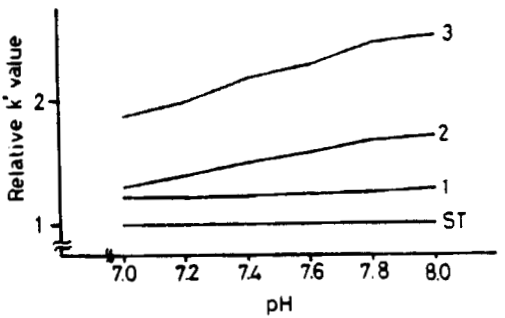

FIGURE 2 Effect of pH on Relative k' Value of 3-Sulfated Bile Acids to Chenodexoycholate 3-Sulfate
1=Deoxycholate, 2=glycochenodeoxycholate, 3=taurochenodeoxycholate. ST(standard)=chenodeoxycholate 3-sulfate. Conditions: column, ODS SC-02 (25 cm x 4.6 mm I.D.). Other conditions as in Fig. 1.

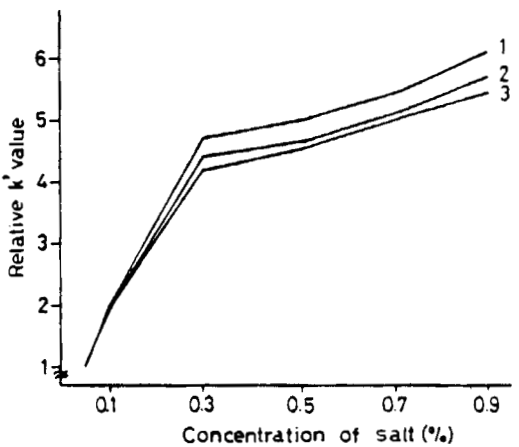

FIGURE 3 Effect of Salt Concentration on Relative k' Value of 3-Sulfated Bile Acids
1=Deoxycholate, 2=chenodeoxycholate, 3=cholate. The k' value obtained with 0.05% ammonium carbonate was taken as a standard. Conditions: mobile phase, ammonium carbonate/acetonitrile (26:8), 2.1 ml/min. Other conditions as in Fig. 2.

In order to establish the suitable condition for the resolution, the influence of the concentration of ammonium carbonate on the peak shape was investigated with closely related 3-sulfated chenodeoxycholate and deoxycholate. As shown in Fig. 4, chenodeoxycholate 3-sulfate exhibited almost the constant value in the salt concentration range of 0.1 to 0.7% whereas deoxycholate 3-sulfate showed a minimum value at 0.5%. The maximum resoltuion factor (R) for the two bile acid 3-sulfates was found to be ca. 1.4 at 0.5% salt concentration where the two peaks should be completely resolved. The content of water in the mobile phase exerted an influence on the capacity ratio to a certain extent but not on the resolution.

Based upon these data 0.5% ammonium carbonate (pH 7.5-7.8)/ acetonitrile (26:8 or 20:8, v/v) was chosen as a suitable mobile phase. A synthetic mixture of 3-sulfates of unconjugated, glyco- and tauro-conjugated cholate, chenodeoxycholate, deoxycholate and lithocholate were thus completely separated as illustrated in Fig. 5. The sulfated ursodeoxycholate and cholate groups exhibited nearly identical k' value with the above mobile phases. These

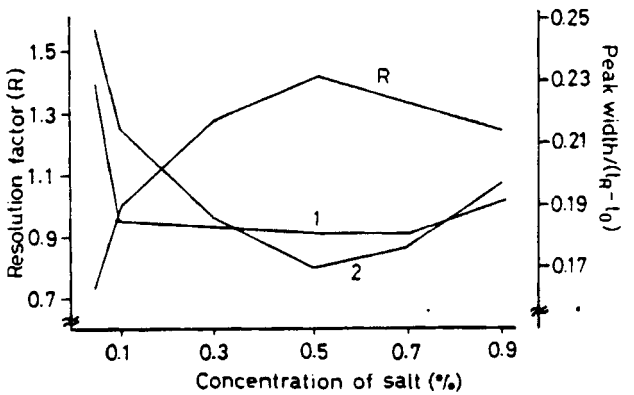

FIGURE 4 Effect of Salt Concentration on Resolution of 3-Sulfated Chenodeoxycholate and Deoxycholate
1=Chenodeoxycholate, 2=deoxycholate. The peak width/ (t_R-t_0) value was taken as a parameter to represent the peak shape. Conditions as in Fig. 3.

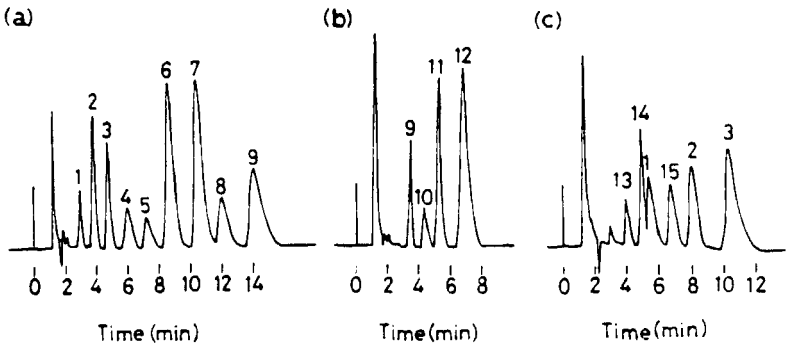

FIGURE 5 Separation of a Mixture of 3-Sulfated Bile Acids 1=Cholate, 2=glycocholate, 3=taurocholate, 4=chenodeoxycholate, 5=deoxycholate, 6=glycochenodeoxycholate, 7=glycodeoxycholate, 8=taurochenodeoxycholate, 9=taurodeoxycholate, 10=lithocholate, 11=glycolithocholate, 12=taurolithocholate, 13=ursodeoxycholate, 14=glycoursodeoxycholate, 15=tauroursodeoxycholate. Conditions: mobile phase (a) 0.5% ammonium carbonate/acetonitrile (26:8), 2.1 ml/min; (b) 0.5% ammonium carbonate/acetonitrile (20:8), 2.0 ml/min; (c) 0.04% ammonium carbonate /acetonitrile (36:8), 1.6 ml/min. Other conditions as in Fig. 2.

two, however, were efficiently separated when 0.04% ammonium carbonate (pH 7.8)/acetonitrile (36:8, v/v) was employed. The capacity ratios of 3-sulfated bile acids observed under the three conditions are listed in Table 1. The detection limits of 3-sulfated glyco- and tauro-conjugates determined by the UV monitoring were 50 and 100 ng, respectively, while that of unconjugated bile acids was 500 ng.

Effect of pH on the k' value of 7- and 12-sulfates relative to taurodeoxycholate 3-sulfate was similarly investigated on the μBondapak C_{18} column using 0.5% phosphate buffer/acetonitrile as a mobile phase. It is evident from the data in Fig. 1 that the chromatographic behaviors of the 7- and 12-sulfates were quite similar to that of the 3-sulfate with an only exception of deoxycholate 12-sulfate. The 12-sulfates of glyco- and taurodeoxycholates exhibited the larger relative k' value than the corresponding 3-sulfates in the whole pH range. As for unconjugated deoxycholate

TABLE 1

Capacity Ratios of 3-Sulfated Bile Acids

Compound	Capacity ratio (k') A	B	C
3-Sulfate			
Cholate	2.4	4.0	
Glycocholate	3.5	6.3	
Taurocholate	4.7	8.4	
Chenodeoxycholate	6.6		
Glycochenodeoxycholate	10.1		
Taurochenodeoxycholate	14.4		
Deoxycholate	7.6		
Glycodeoxycholate	13.3		2.5
Taurodeoxycholate	17.0		2.9
Ursodeoxycholate	1.8	2.7	
Glycoursodeoxycholate	2.4	3.6	
Tauroursodeoxycholate	3.4	5.2	
Lithocholate			3.8
Glycolithocholate			4.8
Taurolithocholate			6.5

Conditions : column, ODS SC-02 (25 cm x 4.6 mm I.D.); mobile phase, (A) 0.5% ammonium carbonate/acetonitrile (26:8), 2.1 ml/min, (B) 0.04% ammonium carbonate/acetonitrile (36:8), 1.6 ml/min, (C) 0.5% ammonium carbonate/acetonitrile (20:8), 2.0 ml/min; detection, 205 nm. t_0: (A)0.9,(B)1.1,(C)0.9 min.

the elution order of the 12- and 3-sulfates was reversed at pH 6.5. Inspection of Dreiding model provides a plausible explanation for this phenomenon in such a way that the sulfate group at C-12 is sterically close to the carboxylic acid or sulfonic acid residue of the side chain and the steric interaction between these two groups may be reflected to the unusual chromatographic behavior. The most suitable separation of 7- and 12-sulfated bile acids was attained on the ODS SC-02 column using 0.5% ammonium carbonate/ acetonitrile (26:8, v/v) as a mobile phase. The capacity ratios observed under the condition are listed in Table 2.

It should be emphasized that the novel method which is capable

TABLE 2

Capacity Ratios of 7- and 12-Monosulfated Bile Acids

Compound	Capacity ratio (k')
7-Sulfate	
Cholate	2.4
Glycocholate	3.3
Taurocholate	4.3
Chenodeoxycholate	7.9
Glycochenodeoxycholate	11.7
Taurochenodeoxycholate	15.9
Ursodeoxycholate	4.0
Glycoursodeoxycholate	5.3
Tauroursodeoxycholate	7.4
12-Sulfate	
Cholate	2.5
Glycocholate	3.7
Taurocholate	4.7
Deoxycholate	10.6
Glycodeoxycholate	16.3
Taurodeoxycholate	21.8

Conditions : mobile phase, 0.5% ammonium carbonate/acetonitrile (26:8), 2.1 ml/min. Other conditions as in Table 1. t_0 : 0.9 min.

of determining the sulfates of unconjugated and conjugated bile acids without prior deconjugation may provide more precise knowledge of the metabolic profile of bile acids. Application of the present method to clinical specimens of the patients with hepatobiliary diseases will be a fertile field to be investigated.

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