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STUDIES ON STEROIDS

CCXVI*. SEPARATION OF BILE ACID 3-GLUCURONIDES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The separation of 3-glucuronides of cholate, chenodeoxycholate, deoxycholate, ursodeoxycholate and lithocholate, and their glyco- and tauro-conjugates, has been carried out by high-performance liquid chromatography on a reversed-phase column. The chromatographic behaviour of bile acid 3-glucuronides was dependent on the type of conjugation. An effect of the pH of the mobile phase on the capacity ratio was observed at higher pH for chenodeoxycholate 3-glucuronide, probably owing to steric interaction of the 7α-hydroxy group with the carboxy group in the glucuronyl moiety. Conversion of the α-hydroxy function on the steroid nucleus into an oxo group resulted in a 50% decrease in the capacity ratio. Bile acid 3-glucuronides were efficiently separated on Shodex ODS Pak F-411 using three kinds of ammonium phosphate buffer–acetonitrile systems.

INTRODUCTION

In recent years, considerable attention has been focused on the metabolism of bile acids in patients with hepatobiliary diseases. Bile acid 3-glucuronides have previously been shown to be present in human plasma and urine[1–4]. The methods commonly used for the quantitation of 3-glucuronides of unconjugated and conjugated bile acids involve prior hydrolysis with β-glucuronidase and then with alkali, followed by the chromatographic separation of deconjugated bile acids[5,6]. These procedures, however, have inevitable disadvantages, such as a lack of reliability and the loss of information about the conjugated form. As bile acid 3-glucuronides are very polar and lacking in volatility and thermal stability, high-performance liquid chromatography (HPLC) on a reversed-phase column appears to be more suitable for the separation and determination of the glucuronides. In previous studies in this series we synthesized 3-glucuronides of unconjugated and conjugated bile acids as authentic

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specimens by unequivocal routes[7,8]. This paper deals with the separation of 3-glucuronides of unconjugated and glyco- and tauro-conjugated bile acids by HPLC. In addition, the chromatographic behaviour of these glucuronides on reversed-phase columns is also described.

EXPERIMENTAL

Materials

The 3-glucuronides of unconjugated and glyco- and tauro-conjugated bile acids were synthesized in these laboratories by the methods reported previously[7,8]. All the chemicals employed were of analytical-reagent grade. Solvents were purified by distillation and deaerated prior to use.

Instruments

The apparatus used was a Hitachi 635A high-performance liquid chromatograph (Hitachi, Tokyo, Japan) equipped with a Hitachi wavelength-tunable effluent monitor (205 nm). μBondapak CN (25 cm × 4.6 mm I.D.) and Shodex ODS Pak F-411 (15 cm × 4.6 mm I.D.) columns were used under ambient conditions.

RESULTS AND DISCUSSION

Initially, the effect of the pH of the mobile phase on the capacity ratio (k') was investigated with a 0.5% phosphate buffer–acetonitrile system on the μBondapak CN column. The k' values of the 3-glucuronides of cholate, chenodeoxycholate, deoxycholate, ursodeoxycholate and lithocholate relative to taurochenodeoxycholate 3-glucuronide were plotted against pH (Fig. 1).

The relative k' values were markedly influenced by the pH of the mobile phase.

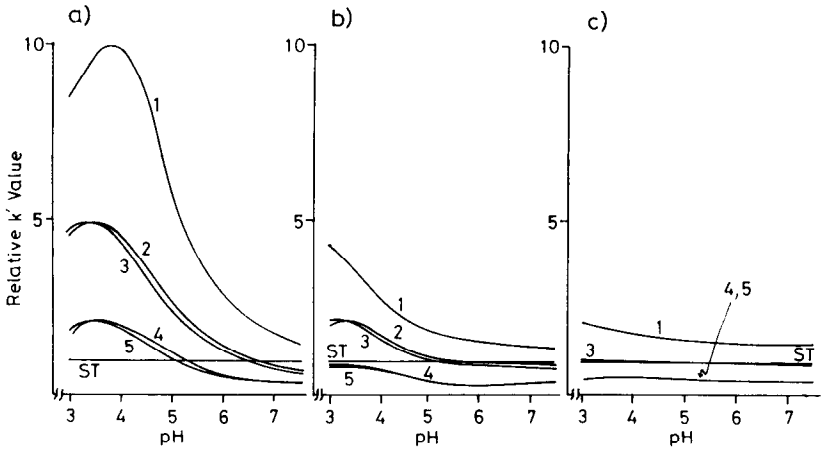

Fig. 1. Effect of pH of mobile phase on k' values of bile acid 3-glucuronides relative to taurochenodeoxycholate 3-glucuronide. 1 = Lithocholate; 2 = chenodeoxycholate, 3 = deoxycholate; 4 = ursodeoxycholate; 5 = cholate; ST (standard) = taurochenodeoxycholate. (a) Unconjugated; (b) glyco-conjugated; (c) tauro-conjugated bile acids. Conditions: column, μBondapak CN; mobile phase, 0.5% phosphate buffer–acetonitrile, 2.0 ml/min; detection, 205 nm.

In the pH range 6.5–7.5, unconjugated and glyco- and tauro-conjugated bile acid 3-glucuronides exhibited similar k' values. On the other hand, the k' values of unconjugated and glyco-conjugated 3-glucuronides increased with decreasing pH from 6.0 and 4.5 respectively. A similar phenomenon has previously been observed with bile acids and their sulphates[9–11]. This chromatographic behaviour can be explained in terms of the dissociation of unconjugated (pK 6.0), glyco-conjugated (pK 4.5) and tauro-conjugated (pK 1.5) bile acids. Irrespective of the type of conjugation, bile acid 3-glucuronides were eluted earlier as the number of hydroxy groups on the steroid nucleus increased. It is of interest that ursodeoxycholate 3-glucuronides having an equatorial β-hydroxy group at C-7 exhibited smaller k' values than other dihydroxylated bile acid 3-glucuronides and nearly identical k' values with cholate 3-glucuronides. In reversed-phase chromatography, an increased polarity of a solute molecule enhances the solubility in the mobile phase, whereas increased hydrophobicity favours affinity with the stationary phase. As for the structure of bile acids, the β-face of steroid nucleus serves for the formation of hydrophobic binding with the stationary phase[12]. Therefore, the presence of a hydroxy group on the β-face, such as in ursodeoxycholate, decreases the hydrophobicity, resulting in an increase in polarity.

The k' values of unconjugated and conjugated deoxycholate 3-glucuronides were smaller than those of corresponding chenodeoxycholate 3-glucuronides in the pH range 4.0–7.5 and, moreover, the elution order of these two was reversed at pH 3.5. It has previously been shown that deoxycholate and its 3-sulphate have larger k' values than the corresponding chenodeoxycholates[9,11]. Inspection of a Dreiding model indicates that the hydroxy function at C-7α is sterically close to the glucuronyl moiety having a pK value of *ca.* 3.5. Therefore, at higher pH, steric interaction may occur between the 7α-hydroxy group and the carboxy group in the glucuronic acid, resulting in an increase in the k' value of chenodeoxycholate 3-glucuronide. Such a

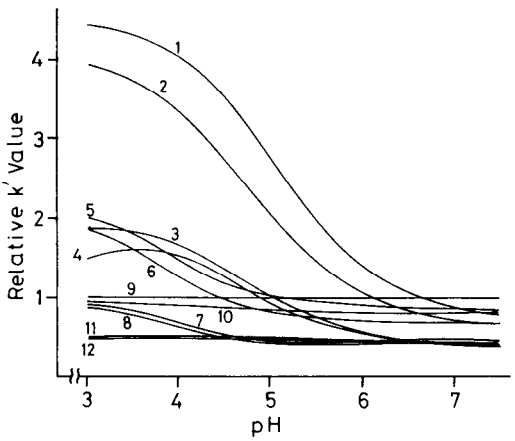

Fig. 2. Effect of pH of mobile phase on k' values of oxo bile acid 3-glucuronides relative to tauro-7-oxolithocholate 3-glucuronide. 1 = 7-Oxolithocholate; 2 = 12-oxolithocholate; 3 = 7-oxodeoxycholate; 4 = 12-oxochenodeoxycholate; 5 = glyco-7-oxolithocholate; 6 = glyco-12-oxolithocholate; 7 = glyco-7-oxodeoxycholate; 8 = glyco-12-oxochenodeoxycholate; 9 = tauro-7-oxolithocholate; 10 = tauro-12-oxolithocholate; 11 = tauro-7-oxodeoxycholate; 12 = tauro-12-oxochenodeoxycholate. Conditions as in Fig. 1.

TABLE I

CAPACITY RATIOS OF OXO BILE ACID 3-GLUCURONIDES RELATIVE TO THE CORRESPONDING BILE ACID 3-GLUCURONIDES

Conditions as in Fig. 1.

Oxo bile acid 3-glucuronide	*Bile acid 3-glucuronide*	*Rk'**		
		pH 3.5	*pH 5.0*	*pH 7.0*
7-Oxolithocholate	Chenodeoxycholate	0.67	0.57	0.53
	Ursodeoxycholate	1.82	1.38	1.10
12-Oxolithocholate	Deoxycholate	0.61	0.51	0.50
7-Oxodeoxycholate	Cholate	0.83	0.63	0.50
12-Oxochenodeoxycholate	Cholate	0.83	0.55	0.48

* $Rk' = k'_{oxo}/k'_{OH}$.

steric interaction has been similarly observed on bile acids and their sulphates between the 12α-hydroxy or 12α-sulphate group and the carboxylic acid or sulphonic acid residue of the side-chain[11,13].

The chromatographic behaviour of 7- and 12-oxo bile acid 3-glucuronides was then investigated on the μBondapak CN column with the 0.5% phosphate buffer–acetonitrile system. The k' values relative to tauro-7-oxolithocholate 3-glucuronide were plotted against pH (Fig. 2). The k' values of these glucuronides were also dependent on the type of conjugation and were influenced by the pH of the mobile phase. The 7-oxo bile acid 3-glucuronides exhibited smaller k' values than the corresponding 12-oxo bile acid 3-glucuronides, with no exceptions. The effect of conversion of the hydroxy function into an oxo group on the chromatographic behaviour is illustrated in Table I. A marked change in the Rk' value was observed for bile acid 3-glucuronides having a 7α- or 12α-hydroxy group but not for ursodeoxycholate 3-glucuronide at higher pH. It is evident from the data that oxidation of the α-hydroxy group decreases the hydrophobic binding with the stationary phase, resulting in a decrease in the k' value by nearly 50%. The Rk' values at pH 3.5 were different from those at pH 5.0 and 7.0, indicating the occurrence of steric interaction of the 12α-hydroxy function with the carboxy group of the side-chain or of the 7α-hydroxy function with the carboxy group in the glucuronyl moiety.

Finally, suitable conditions for the separation of common bile acid 3-glucuronides were investigated. As the 3-glucuronides exhibited nearly identical k' values at higher pH, various combinations of buffer solution (pH 2.5–4.0) with organic solvent were examined on the μBondapak CN column. The use of the 0.5% ammonium phosphate buffer–acetonitrile system appeared to be promising without any significant leading and tailing. Resolution of 3-glucuronides of chenodeoxycholate and deoxycholate was not satisfactorily attained on this column, however. The use of the Shodex ODS Pak F-411 rather than the μBondapak CN column provided more efficient separation of these two. Therefore, the chromatographic behaviour was investigated on this column using 0.5% ammonium phosphate buffer–acetonitrile as the mobile phase. As shown in Fig. 3a, bile acid 3-glucuronides were effectively separated at pH 3.5, except for ursodeoxycholate and glycodeoxycholate 3-glucuronides, whose resolution was achieved at pH 3.0. The k' value of chenodeoxycholate

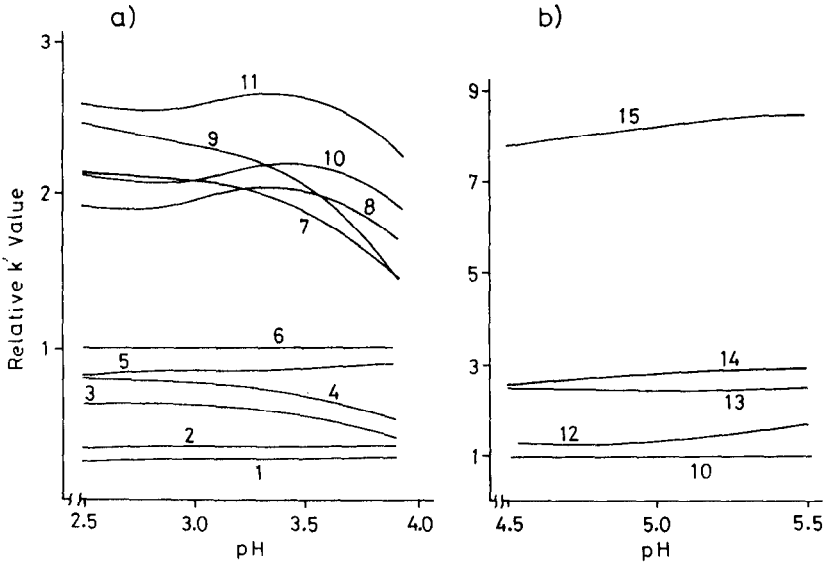

Fig. 3. Effect of pH of mobile phase on k' values of bile acid 3-glucuronides. 1 = Tauroursodeoxycholate; 2 = taurocholate; 3 = glycoursodeoxycholate; 4 = glycocholate; 5 = taurochenodeoxycholate; 6 = taurodeoxycholate; 7 = glycochenodeoxycholate; 8 = ursodeoxycholate; 9 = glycodeoxycholate; 10 = cholate; 11 = taurolithocholate; 12 = glycolithocholate; 13 = deoxycholate; 14 = chenodeoxycholate; 15 = lithocholate. (a) Relative to taurodeoxycholate 3-glucuronide; (b) relative to cholate 3-glucuronide. Conditions: column, Shodex ODS Pak F-411; mobile phase, 0.5% ammonium phosphate buffer–acetonitrile, 1.0 ml/min; detection, 205 nm.

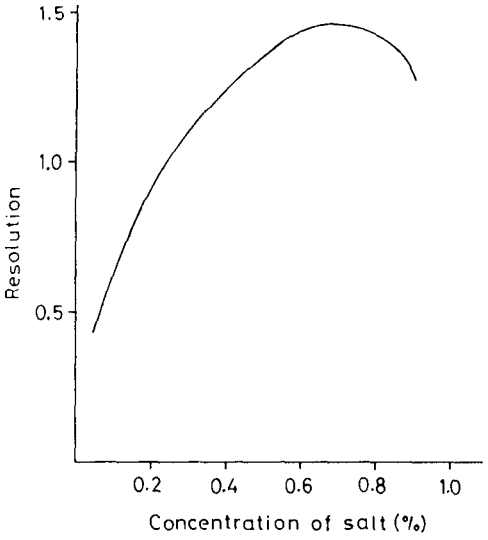

Fig. 4. Effect of salt concentration on resolution of chenodeoxycholate and deoxycholate 3-glucuronides. Conditions: mobile phase, ammonium phosphate buffer (pH 5.0)–acetonitrile. Other conditions as in Fig. 3.

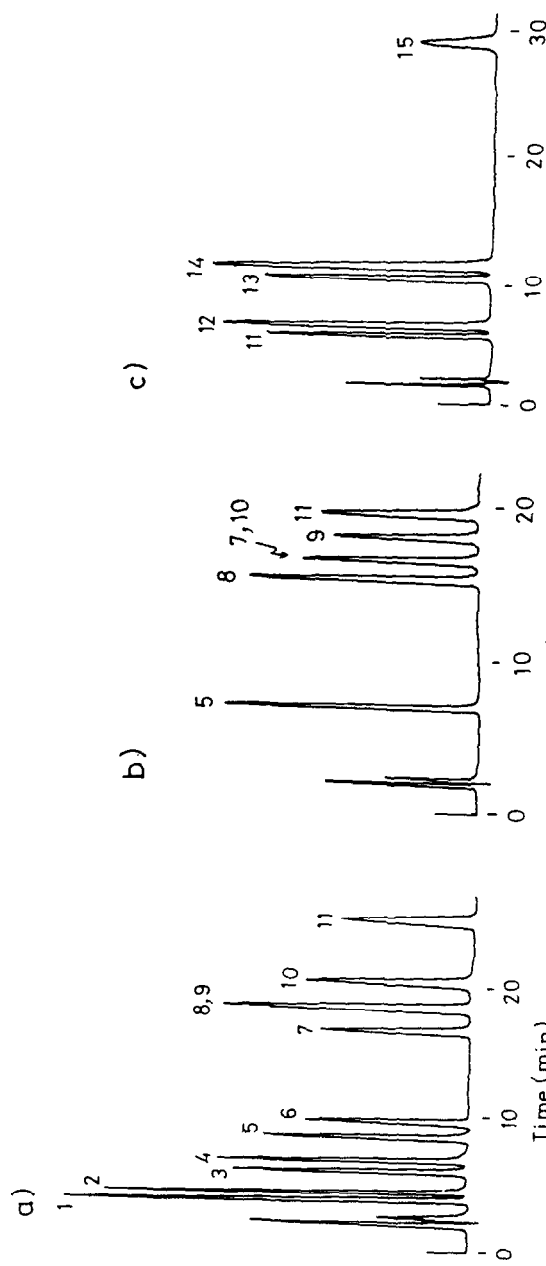

Fig. 5. Separation of a mixture of bile acid 3-glucuronides. Mobile phase: (a) 0.7% ammonium phosphate buffer (pH 3.5)–acetonitrile (150:60); (b) 0.7% ammonium phosphate buffer (pH 3.0)–acetonitrile (135:60); (c) 0.7% ammonium phosphate buffer (pH 5.0)–acetonitrile (125:60). Other conditions and peak identification as in Fig. 3.

3-glucuronides relative to cholate 3-glucuronide increasded with increasing pH, whereas that of deoxycholate 3-glucuronide was almost constant in the pH range 4.5–5.5 (Fig. 3b).

The effect of salt concentration on the resolution of deoxycholate and chenodeoxycholate 3-glucuronides was then examined using ammonium phosphate buffer (pH 5.0)–acetonitrile as the mobile phase (Fig. 4). The resolution (R) increased with increasing salt concentration in the mobile phase, and the maximum R value was found to be *ca.* 1.45 at a 0.7% salt concentration, where the two peaks should be completely resolved. The content of water in the mobile phase influenced to a certain extent the k' value but not the resolution.

Based on these data, 0.7% ammonium phosphate buffer (pH 3.0)–acetonitrile (135:60, v/v), 0.7% ammonium phosphate buffer (pH 3.5)–acetonitrile (150:60, v/v) and 0.7% ammonium phosphate buffer (pH 5.0)–acetonitrile (125:60, v/v) were chosen as suitable mobile phases. A synthetic mixture of 3-glucuronides of unconjugated and glyco- and tauro-conjugated cholate, chenodeoxycholate, deoxycholate, ursodeoxycholate and lithocholate was completely separated, as illustrated in Fig. 5.

It is hoped that this method for the determination of bile acid 3-glucuronides without prior deconjugation may provide much more precise knowledge on the metabolic profile of bile acids. The method is being applied in these laboratories to clinical specimens from patients with hepatobiliary diseases and the results will be reported elsewhere.

ACKNOWLEDGEMENT

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