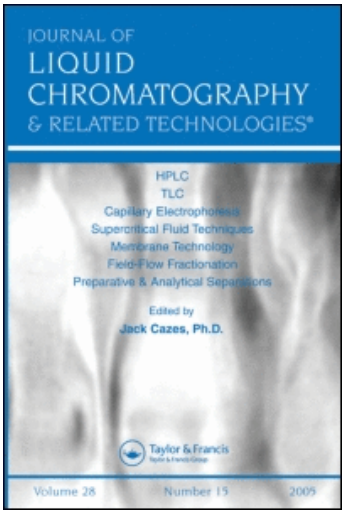

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Niwa, Toshifumi , Koshiyama, Tomoyoshi , Goto, Junichi and Nambara, Toshio(1993) 'High-Performance Liquid Chromatographic Separation of Bile Acid N-Acetylglucosaminides', Journal of Liquid Chromatography & Related Technologies, 16: 2, 331 — 341
To link to this Article: DOI: 10.1080/10826079308020916
URL: http://dx.doi.org/10.1080/10826079308020916

JOURNAL OF LIQUID CHROMATOGRAPHY, 16(2), 331-341 (1993)

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF BILE ACID N-ACETYLGLUCOSAMINIDES

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ABSTRACT

The separation of 3-N-acetylglucosaminides of cholate, chenodeoxycholate, deoxycholate, ursodeoxycholate and lithocholate, and their glycine- and taurine-conjugates has been carried out by high-performance liquid chromatography on a reversed-phase column. The chromatographic behavior of bile acid 3-N-acetylglucosaminides was dependent on the number and positions of hydroxyl groups and the structure of the side-chain. The bile acid 3-N-acetylglucosaminides were efficiently separated according to their conjugated form on a Cosmosil 5C_{18} column using a 0.3% potassium phosphate buffer-acetonitrile system. The chromatographic separation of ursodeoxycholate 3- and 7-N-acetylglucosaminides is also discussed.

INTRODUCTION

In connection with hepatobiliary diseases, considerable attention has been directed to the metabolism of bile acids.

Recently, the presence of ursodeoxycholic acid N-acetylglucosaminide, a novel conjugate, in human urine was disclosed (1, 2) and a particular interest has been focused on the physiological significance of N-acetylglucosaminidation toward the hydroxyl group on the steroid nucleus. Since bile acid N-acetylglucosaminides are comparatively polar and lack volatility and thermostability, high-performance liquid chromatography (HPLC) on a reversed-phase column appears to be most suitable for the separation and determination of these conjugates in biological fluids. In our previous work, N-acetylglucosaminides of unconjugated, glycine- and taurine-conjugated bile acids were synthesized as authentic specimens (3). This paper deals with the high-performance liquid chromatographic behavior of bile acid 3-N-acetylglucosaminides and their efficient separation on a reversed-phase column.

EXPERIMENTAL

Meterials

The N-acetylglucosaminides of unconjugated and glycine- and taurine-conjugated bile acids were synthesized in these laboratories by the methods previously reported (3). All the chemicals used were of analytical-reagent grade. Solvents were purified by distillation prior to use.

Apparatus

The apparatus used was a 510 solvent delivery system (Waters Chromatography Div., Millipore Co., Milford, Mass.,

U.S.A.) equipped with a 875-UV detector (JASCO, Tokyo, Japan) monitoring the absorbance at 205 nm. HPLC was carried out on a Cosmosil $5C_{18}$ column (5 μm, 150 x 4.6 mm i.d.) (Nacalai Tesque, Inc., Kyoto, Japan) at ambient temperature.

RESULTS AND DISCUSSION

Initially, the effect of pH of the mobile phase on the capacity ratio (k') was investigated with a 0.3% phosphate buffer-acetonitrile system. The k' values of 3-N-acetylglucosaminides of cholate, chenodeoxycholate, deoxycholate and ursodeoxycholate relative to taurodeoxycholate 3-N-acetylglucosaminide were plotted against pH (Fig. 1). The relative k' values were markedly influenced by pH of the mobile phase. In the pH range 6.5-7.5, unconjugated and glycine- and taurine-conjugated bile acid 3-N-acetylglucosaminides exhibited similar k' values. On the other hand, the k' values of unconjugated and glycine-conjugated bile acid 3-N-acetylglucosaminides increased with decreasing pH from 6.0 and 4.5, respectively. The similar chromatographic behavior has previously been observed with bile acids, their glucuronides, mono- and disulfates (4-8), and can be explained in terms of dissociation of unconjugated (pKa 6.0), glycine- (pKa 4.5) and taurine-conjugated bile acids (pKa 1.5) (9). Irrespective of the structure of the side-chain, bile acid 3-N-acetylglucosaminides were eluted earlier with increasing number of the hydroxyl

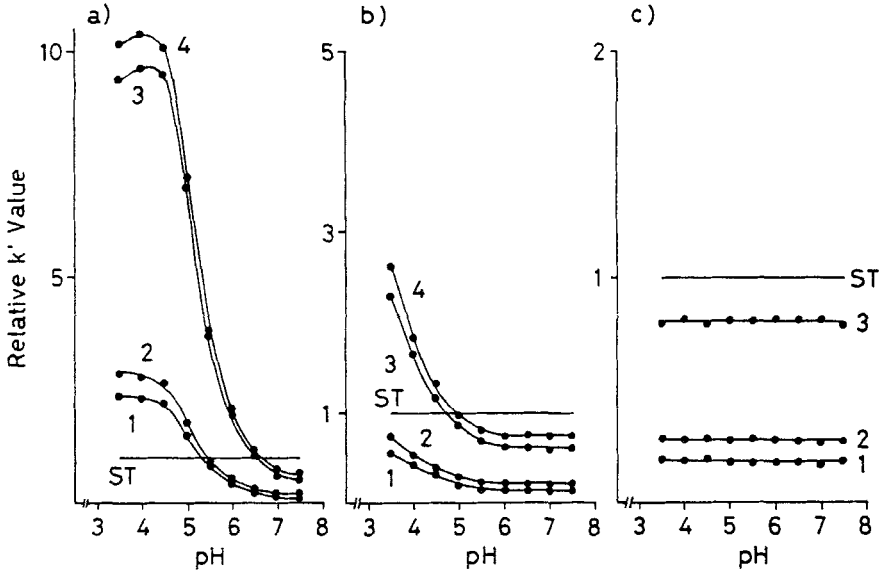

FIGURE 1. Effect of pH of mobile phase on k' values of bile acid 3-N-acetylglucosaminides relative to taurodeoxycholic acid 3-N-acetylglucosaminide. 1= Ursodeoxycholate, 2=cholate, 3=chenodeoxycholate, 4=deoxycholate, ST(standard)=taurodeoxycholic acid. a) Unconjugate, b) glycine-conjugate, c) taurine-conjugate. Conditions: column, Cosmosil $5C_{18}$; mobile phase, 0.3% potassium phosphate buffer-acetonitrile, 2.0 ml/min; detection, 205 nm.

group on the steroid nucleus. Ursodeoxycholates having an equatorial β-hydroxyl group at C-7 showed much smaller k' values than corresponding dihydroxylated bile acids and almost identical k' values with those of corresponding cholates. It is well known that the β-face of the steroid nucleus serves for hydrophobic interaction with the stationary phase in reversed-phase partition chromatography (10, 11). Thus, the presence of a hydroxyl group on the β-side of the molecule, such as in ursodeoxycholate, results in a decrease of the interaction, and hence, of the k' value.

The pH of the mobile phase also influenced the separation of two positional isomers, chenodeoxycholate and deoxycholate 3-N-acetylglucosaminides (Fig. 2). In the pH range 4.5-6.0, unconjugated bile acids showed similar k' values, while these were efficiently resolved under the neutral or weakly alkaline condition. In contrast, glycine- and taurine-conjugated deoxycholates exerted larger k' values than those of corresponding chenodeoxycholates in the whole pH range investigated. Such a characteristic chromatographic behavior has also been observed for bile acids and their sulfates (7, 12). This phenomenon can be explained in termes of steric interaction between the α-hydroxyl group at C-12 and the carboxylic or sulfonic acid residue of the side-chain (12). On the basis of these data, 0.3% potassium phosphate buffer (pH 7.5)-acetonitrile (75:25 or 73:27, $^{v}/_{v}$) was chosen as a suitable mobile phase for the separation of these 3-N-acetylglucosaminides. As

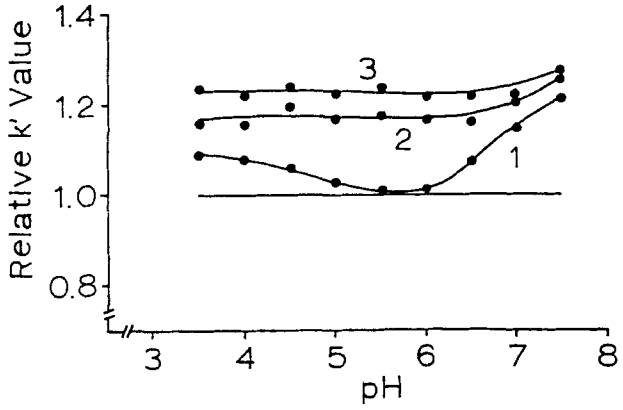

FIGURE 2. Effect of pH of mobile phase on *k'* values of deoxycholate 3-N-acetylglucosaminides relative to corresponding chenodeoxycholate 3-N-acetylglucosaminides. 1=Unconjugate, 2=glycine-conjugate, 3=taurine-conjugate. Conditions as in Fig. 1.

illustrated in Fig. 3, 3-N-acetylglucosaminides of cholate, chenodeoxycholate, deoxycholate and ursodeoxycholate were completely separated according to their conjugated form. On this condition, unconjugated, glycine- and taurine-conjugated lithocholate 3-N-acetylglucosaminides were eluted at 35 to 40 min.

In 1988, ursodeoxycholic acid N-acetylglucosaminide in human urine was first characterized by means of gas chromatography-mass spectrometry (MS) and FAB-MS (1). However, the complete structure involving the position and nature of the conjugate still remains unclear. In the previous

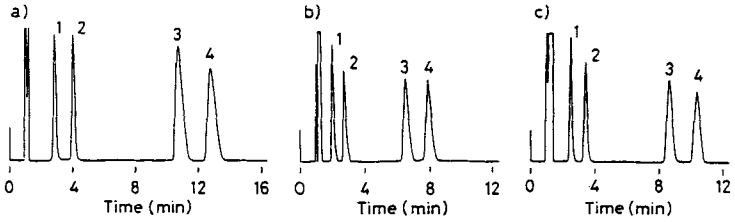

FIGURE 3. Separation of bile acid 3-N-acetylglucosaminides. 1=Ursodeoxycholate, 2=cholate, 3=chenodeoxycholate, 4=deoxycholate. a) Unconjugate, b) glycine-conjugate, c) taurine-conjugate. Conditions: column, Cosmosil $5C_{18}$; mobile phase, 0.3% potassium phosphate buffer (pH 7.5)-acetonitrile ($^{v}/_{v}$) a) 75:25, b) and c) 73:27. Other conditions as in Fig. 1.

work of this series, we prepared unconjugated, glycine- and taurine-conjugated ursodeoxycholate 7-N-acetylglucosaminides as well as their C-3 isomers. Therefore, the resolution of 3- and 7-N-acetylglucosaminides of ursodeoxycholates was then undertaken using a 0.3% phosphate buffer-acetonitrile system. As shown in Fig. 4, the 7-N-acetylglucosaminides exhibited larger k' values than those of corresponding C-3 isomers under the neutral condition. On the other hand, k' values of C-7 isomers of unconjugated and glycine-conjugated bile acids decreased with decreasing pH from 6.0 and 4.5, respectively. Inspection of a Dreiding model indicates that the primary

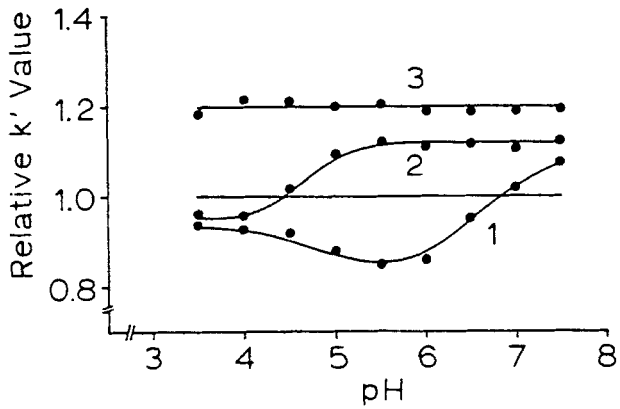

FIGURE 4. Effect of pH of mobile phase on k' values of ursodeoxycholate 7-N-acetylglucosaminides relative to corresponding C-3 isomers. 1=Unconjugate, 2=glycine-conjugate, 3=taurine-conjugate. Conditions as in Fig. 1.

hydroxyl function on the sugar moiety at 7β is sterically close to the acidic residue of the side-chain. Accordingly, the steric interaction may occur between the primary hydroxyl group of the N-acetylglucosamine moiety and the carboxylic or sulfonic acid residue of the side-chain under the dissociated condition, resulting in an increase in k' values of 7-N-acetylglucosaminides. In the lower pH region, the acidic residue is undissociated and the sugar moiety at the β-side of the steroid nucleus decreases the hydrophobic surface area, resulting in earlier elution. As shown in Fig. 5, the resolution of urso-

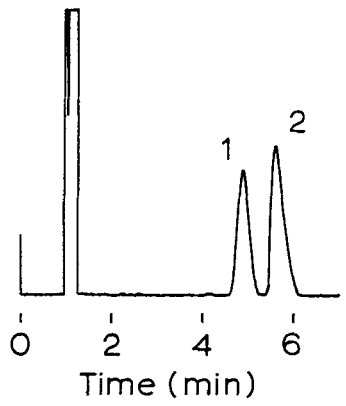

FIGURE 5. Separation of ursodeoxycholate 3- and 7-N-acetylglucosaminides. 1=7-N-Acetylglucosaminide, 2=3-N-acetylglucosaminide. Conditions: mobile phase, 0.3% potassium phosphate buffer (pH 6.0)-acetonitrile 11:4 ($^{v}/_{v}$). Other conditions as in Fig. 1.

deoxycholate 3- and 7-N-acetylglucosaminides is attained by the use of the weakly acidic or neutral mobile phase on a Cosmosil $5C_{18}$ column. These findings on the chromatographic behavior may be useful for the characterization of bile acid N-acetylglucosaminides in biological fluids.

It is hoped that the newly developed method for the separation of bile acid N-acetylglucosaminides without prior deconjugation may provide more precise knowledge on the metabolic profile of bile acids. Applications of the present method to clinical specimens from patients with hepatobiliary

diseases are being conducted in these laboratories, and the details will be reported elsewhere.

ACKNOWLEDGMENTS

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

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Received: April 5, 1992
Accepted: April 13, 1992