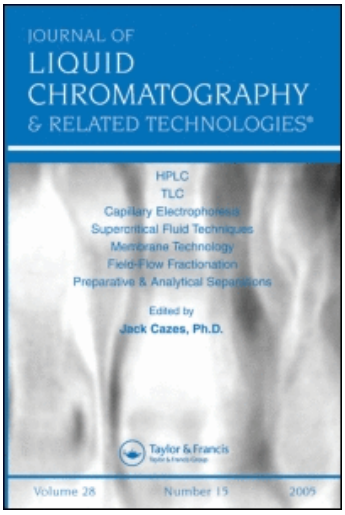

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Separation and Characterization of Ursodeoxycholate 7-N-Acetylglu-Cosaminides in Human Urine by High-Performance Liquid Chromatography with Fluorescence Detection

Toshifumi Niwa[a]; Kazuhiro Fujita[a]; Junichi Goto[a]; Toshlo Nambara[a]
[a] Pharmaceutical Institute Tohoku University Aobayama, Sendai, Japan

To cite this Article Niwa, Toshifumi , Fujita, Kazuhiro , Goto, Junichi and Nambara, Toshlo(1993) 'Separation and Characterization of Ursodeoxycholate 7-N-Acetylglu-Cosaminides in Human Urine by High-Performance Liquid Chromatography with Fluorescence Detection', Journal of Liquid Chromatography & Related Technologies, 16: 12, 2531 — 2544
To link to this Article: DOI: 10.1080/10826079308019590
URL: http://dx.doi.org/10.1080/10826079308019590

JOURNAL OF LIQUID CHROMATOGRAPHY, 16(12), 2531-2544 (1993)

SEPARATION AND CHARACTERIZATION OF URSODEOXYCHOLATE 7-N-ACETYLGLUCOSAMINIDES IN HUMAN URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

TOSHIFUMI NIWA, KAZUHIRO FUJITA, JUNICHI GOTO*, AND TOSHIO NAMBARA
Pharmaceutical Institute
Tohoku University
Aobayama, Sendai 980, Japan

ABSTRACT

The separation and characterization of unconjugated, glycine- and taurine-conjugated ursodeoxycholate 7-N-acetylglucosaminides in human urine without prior deconjugation have been carried out by high-performance liquid chromatography (HPLC) on a reversed phase column. The bile acid fraction was obtained from a urine specimen by the combined use of a Sep-Pak C_{18} cartridge for solid phase extraction and lipophilic gel, piperidinohydroxypropyl Sephadex LH-20 (PHP-LH-20), for ion-exchange chromatography. Bile acid N-acetylglucosaminides were derivatized quantitatively into the corresponding fluorescent esters through the inherent primary hydroxyl group by treatment with 9-anthroyl cyanide. The derivatives were separated into unconjugated, glycine- and taurine-conjugated fractions on PHP-LH-20. Subsequent resolution into individual N-acetylglucosaminides was attained

by HPLC with fluorescence detection on a Cosmosil $5C_{18}$ column using 0.3% potassium phosphate buffer-methanol as a mobile phase. Unconjugated, glycine- and taurine-conjugated ursodeoxycholate 7-N-acetylglucosaminides in human urine from a patient with primary biliary cirrhosis were unambiguously identified on the basis of their chromatographic behavior using mobile phases of different pHs and organic modifiers. It has proved that ursodeoxycholate 7-N-acetylglucosaminides in urine were dominantly conjugated with glycine, while any 3-N-acetylglucosaminides of ursodeoxycholates as well as other common bile acids were not present.

INTRODUCTION

In general, conjugation is one of the major metabolic pathways for endo- and xenobiotics. Bile acids, biosynthesized from cholesterol in liver, are conjugated with glycine or taurine at the C-24 acidic residue and excreted into bile. Further metabolic conjugation with sulfuric acid or glucuronic acid also takes place at the hydroxyl group on the steroid nucleus. In hepatobiliary diseases, the levels of these bile acids in blood and urine are elevated. Since the occurrence of bile acid N-acetylglucosaminides, a novel type of conjugate, in human urine was disclosed (1,2), a particular interest has been focused on the physiological significance of this new conjugation. The N-acetylglucosaminides of steroids in biological fluids have been reported as double conjugates with sulfuric acid or glucuronic acid (3-5). On the other hand, bile acid N-acetylglucosaminides in urine were found in the unconjugated form at C-24.

The method involving prior hydrolysis followed by chromatographic separation of deconjugated bile acids has inevitable disadvantages, such as loss of information about the conjugated form and position. High-performance liquid chromatography (HPLC) is a powerful tool for separation and determination of polar compounds. However, the reliable method for determination of bile acid N-acetylglucosaminides requires elimination of interferences due to common bile acids, their sulfates and glucuronides which exist predominantly in biological fluids. N-Acetylglucosaminides have a characteristic primary hydroxyl group which is more reactive toward acylation than secondary hydroxyl groups on the steroid and sugar moieties. In previous studies of this series, we prepared 3- and 7-N-acetylglucosaminides of bile acids as authentic samples (6) and developed a suitable pre-column derivatization method through the inherent primary hydroxyl group using 9-anthroyl cyanide as a labeling reagent for the trace analysis of these bile acids (7). The fluorescent derivatives were separated into individual N-acetylglucosaminides according to their conjugated form at C-24 on a reversed phase column and monitored by fluorescence detection, the limit of detection being 100 fmol. This paper deals with separation and characterization of ursodeoxycholate 7-N-acetylglucosaminides in human urine by HPLC with pre-column fluorescence labeling.

EXPERIMENTAL

High-performance liquid chromatography

The apparatus used in this study was a 510 solvent delivery system (Waters Chromatography Div., Millipore Co., Milford, Mass., U.S.A.) equipped with a 650-10LC fluorescence spectrophotometer (excitation wavelength 362 nm; emission wavelength 470 nm) (Hitachi, Ltd., Tokyo, Japan) or an 875-UV detector (205 nm) (JASCO, Tokyo, Japan). HPLC was carried out on a Cosmosil $5C_{18}$ column (5 μm, 150 mm x 4.6 mm i.d.) (Nacalai Tesque, Inc., Kyoto, Japan) at ambient temperature.

Materials

The N-acetylglucosaminides of unconjugated, glycine- and taurine-conjugated bile acids were synthesized in these laboratories by the method previously reported (6). All the chemicals used were of analytical-reagent grade. Solvents were purified by distillation prior to use. Piperidinohydroxypropyl-Sephadex LH-20 (PHP-LH-20) (acetate form, 0.5 mequiv./g) (8) and 9-anthroyl cyanide (9) were prepared in the manner previously reported. The Sep-Pak C_{18} cartridge (Waters) was washed successively with ethanol, water, 5% aqueous bovine serum albumin solution and then water prior to use. All glassware used were silanized with trimethylchlorosilane.

Procedure for chromatographic separation and characterization of N-acetylglucosaminides in human urine

A urine specimen (5 ml) from a patient with primary biliary cirrhosis (PBC) administered ursodeoxycholic acid (600 mg/day for 2 weeks) was diluted with 0.5M potassium phosphate buffer (pH 7.0) (5 ml) and passed through a Sep-Pak C_{18} cartridge. After washing with water (10 ml), bile acids were eluted with 90% ethanol (5 ml) and the eluate was applied to a column (18 mm x 6 mm i.d.) of PHP-LH-20 (100 mg). Elution was carried out at a flow rate of 0.2 ml/min. After removal of neutral and basic compounds by washing with 90% ethanol (5 ml), bile acids were eluted with 0.3M acetic acid-potassium acetate (pH 6.7) (8 ml). The bile acid fraction thus obtained was applied to a Sep-Pak C_{18} cartridge in the manner described above to remove inorganic salts. The eluate was subjected to derivatization with 9-anthroyl cyanide (400 μg) in acetonitrile (100 μl) containing 0.1% quinuclidine at 50°C for 30 min (7). The excess reagent was decomposed by addition of methanol (50 μl) and the mixture was concentrated under a nitrogen gas stream, redissolved in 90% ethanol and then applied to a column (18 mm x 6 mm i.d.) of PHP-LH-20 (100 mg). After washing with 90% ethanol (5 ml), derivatized N-acetylglucosaminides were separated into unconjugated, glycine- and taurine-conjugated fractions by stepwise elution with 0.15M acetic acid in 90% ethanol (8 ml), 0.2M formic acid

in 90% ethanol (8 ml) and 0.3M acetic acid-potassium acetate (pH 6.7) (8 ml), respectively. Each dried eluate obtained was redissolved in methanol and an aliquot was subjected to the HPLC analysis on a Cosmosil $5C_{18}$ column using 0.3% potassium phosphate buffer-methanol as a mobile phase. Bile acid N-acetylglucosaminides were monitored by fluorescence detection.

RESULTS AND DISCUSSION

Clean-up of N-acetylglucosaminides in urine

Since bile acid N-acetylglucosaminides are comparatively polar and unstable, separation of these conjugates is markedly influenced by the clean-up procedure. In the previous papers, we reported that the combined use of solid phase extraction with ODS bonded silica and group separation on a lipophilic ion-exchange gel, PHP-LH-20, was effective for the analysis of bile acids and their conjugates in biological fluids (10,11). In the present study, a Sep-Pak C_{18} cartridge was used for extraction of N-acetylglucosaminides in urine. A synthetic mixture of N-acetylglucosaminides of unconjugated, glycine- and taurine-conjugated bile acids dissolved in phosphate buffer (pH 7.0) was applied to the cartridge. The eluate obtained with 90% ethanol was then subjected to the HPLC analysis on a Cosmosil $5C_{18}$ column (12). The N-acetylglucosaminides were recovered quantitatively in an initial 3 ml of the effluent.

When a neutral or weakly alkaline mobile phase, by which N-acetylglucosaminides can be satisfactorily resolved according to their conjugated form at C-24, is employed, little difference in the capacity ratio (k') is observed among unconjugated, glycine- and taurine-conjugated bile acids. Therefore, group separation on PHP-LH-20 prior to HPLC was prerequisite. A synthetic mixture of derivatized bile acid N-acetylglucosaminides was dissolved in 90% ethanol and applied to a column of PHP-LH-20. After removal of neutral compounds with 90% ethanol, derivatized N-acetylglucosaminides were fractionated into the three groups by stepwise elution with 0.15M acetic acid in 90% ethanol, 0.2M formic acid in 90% ethanol and 0.3M acetic acid-potassium acetate (pH 6.7). As illustrated in Fig. 1, unconjugated, glycine- and taurine-conjugated ursodeoxycholate N-acetylglucosaminides were thus completely resolved.

Chromatographic separation and characterization of ursodeoxycholate 7-N-acetylglucosaminides in human urine

The separation and characterization of bile acid N-acetylglucosaminides in urine were carried out according to the procedure shown in Fig. 2. The urine sample from a patient with PBC administered ursodeoxycholic acid was extracted with a Sep-Pak C_{18} cartridge and then chromatographed on PHP-LH-20 to remove coexisting substances which would interfere the derivatization reaction. The eluate was then subjected to the condensation reaction with 9-anthroyl cyanide according to the procedure previously described (7). The fluorescent *deriva-*

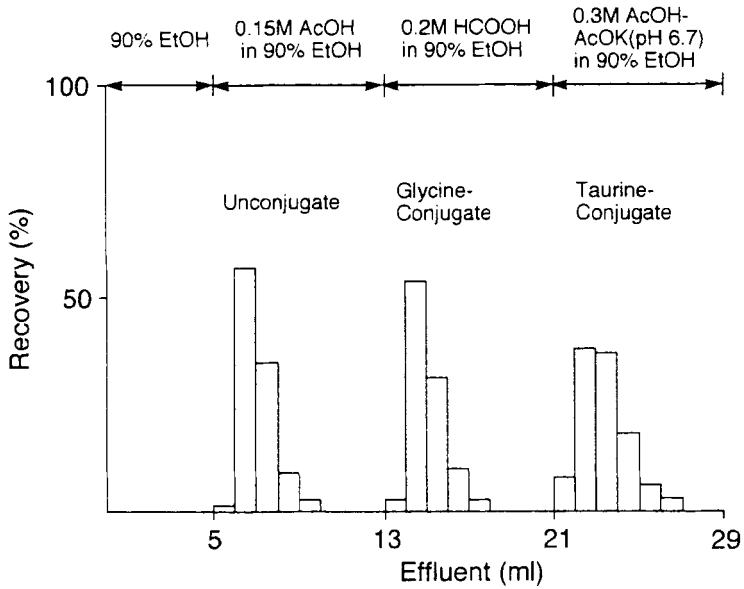

FIGURE 1 Group separation of 9-anthroyl derivatives of bile acid N-acetylglucosaminides on PHP-LH-20.

tives of bile acid N-acetylglucosaminides were separated into unconjugated, glycine- and taurine-conjugated fractions on PHP-LH-20, and each fraction obtained was subjected to HPLC on a Cosmosil $5C_{18}$ column. As illustrated in Fig. 3, three peaks showed k' values identical with those of unconjugated, glycine- and taurine-conjugated ursodeoxycholate 7-N-acetylglucosaminides. The same results were obtained even when the purification prior to derivatization was performed by HPLC (12). When 500 ng each of N-acetylglucosaminides were added to urine from a healthy volunteer and determined by the present method, the recovery rates were estimated over 90%.

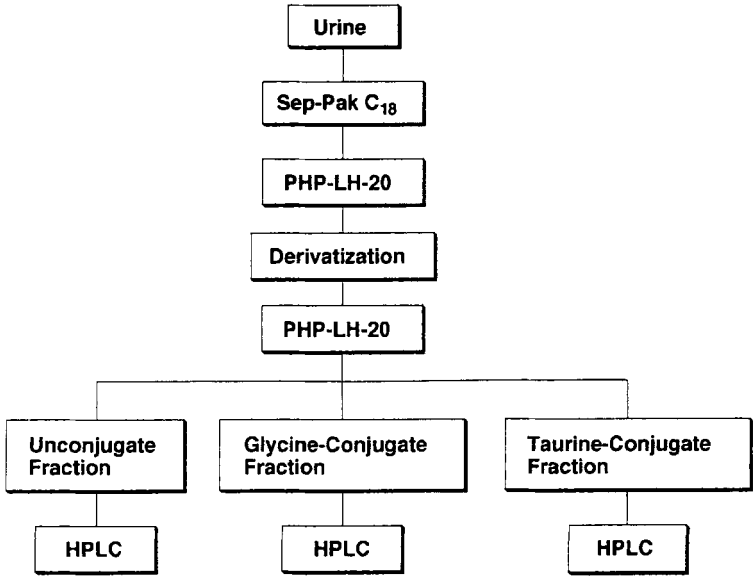

FIGURE 2 General scheme for separation and characterization of ursodeoxycholate 7-N-acetylglucosaminides.

In the preceding study, we investigated the chromatographic behavior of these derivatives and disclosed that retention values were markedly influenced by pH of the mobile phase, according to the conjugated form at C-24 as well as the number and position(s) of hydroxyl group(s) on the steroid nucleus. This finding was applied to structural elucidation of ursodeoxycholate 7-N-acetylglucosaminides in urine. The eluate corresponding to the peak on the chromatogram (Fig. 3) was collected and, after addition of an internal standard, subjected to HPLC on a Cosmosil $5C_{18}$ column with mobile phases of various pHs and organic modifiers. It is evident from

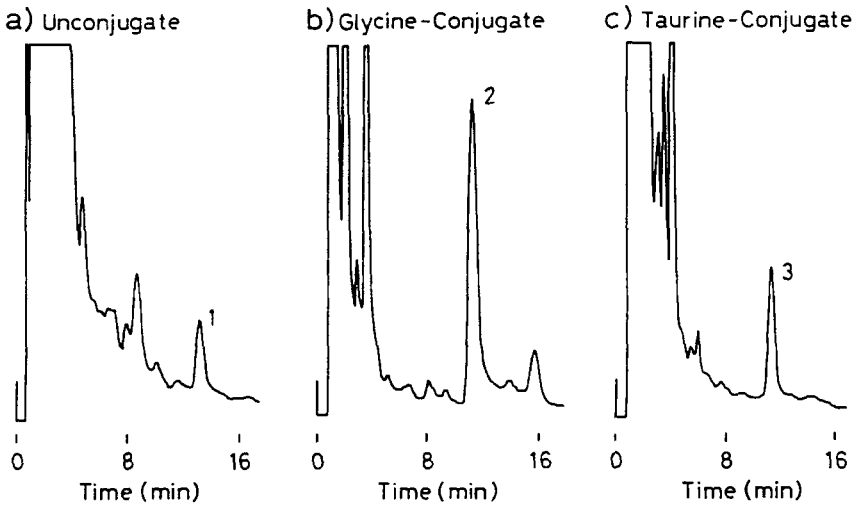

FIGURE 3 Separation of ursodeoxycholate 7-N-acetylglucosaminides in human urine by HPLC.
Conditions: column, Cosmosil $5C_{18}$; mobile phase, 0.3% potassium phosphate buffer (pH 3.5)-methanol (a) 1:5, (v/v), (b) 1:4, (v/v), (c) 1:3.5, (v/v), flow rate, 1.8 ml/min. 1=Ursodeoxycholate 7-N-acetylglucosaminide, 2=glycoursodeoxycholate 7-N-acetylglucosaminide, 3=tauroursodeoxycholate 7-N-acetylglucosaminide.

the data in TABLE 1 that the relative k' values of unconjugated, glycine- and taurine-conjugated ursodeoxycholate 7-N-acetylglucosaminides in urine are identical with those of the authentic samples.

It is obvious from these results that ursodeoxycholate 7-N-acetylglucosaminides are excreted in human urine. The principal component of the N-acetylglucosaminide fraction was identified as glycoursodeoxycholate 7-N-acetylglucosaminide

TABLE 1

Relative k' Values of Ursodeoxycholate 7-N-Acetylglucosaminides in Human Urine. Conditions: column, Cosmosil $5C_{18}$; mobile phase, 0.3% potassium phosphate buffer-methanol or -acetonitrile, 1.8 ml/min.

Bile acid N-acetylglucosaminide	relative k'*			
	methanol			acetonitrile
	pH 3.5	pH 5.0	pH 7.0	pH 7.0
Ursodeoxycholic acid 7-N-acetylglucosaminide	2.0	1.1	0.81	0.40**
Urine	2.0	1.1	0.81	0.39**
Glycoursodeoxycholic acid 7-N-acetylglucosaminide	0.98	0.78	0.75	0.50
Urine	0.98	0.78	0.75	0.50
Tauroursodeoxycholic acid 7-N-acetylglucosaminide	0.73	0.73	0.72	0.52
Urine	0.73	0.72	0.72	0.52

* relative to taurochenodeoxycholic acid 3-N-acetylglucosaminide
** relative to chenodeoxycholic acid 3-N-acetylglucosaminide

(ca. 56 μg/ml). The ratio of unconjugated, glycine- and taurine-conjugated ursodeoxycholate 7-N-acetylglucosaminides was estimated to be approximately 1: 28: 7. Any other N-acetylglucosaminides of common bile acids could not be detected. These results are in good accord with the previous results by Matern and his co-workers demonstrating the substrate specificity of N-acetylglucosaminyltransferase from human kidney and liver towards 6α- or 7β-hydroxylated bile acids (13,14). It has also been reported that amidation of ursodeoxycholic acid with glycine or taurine resulted in a marked decrease in the rate of N-acetylglucosaminidation. This finding appears to be incompatible with the present result that glycoursodeoxycholate 7-N-acetylglucosaminide is predominant.

In 1964 Layne et al. first isolated 17α-estradiol 17-N-acetylglucosaminide 3-glucuronide from urine of rabbits administered estrone benzoate (3). Subsequently, *in vivo* formation of steroid N-acetylglucosaminides, was also recognized with the cases of pregnenolone (4) and 15α-hydroxyestrone (5) in human urine. These N-acetylglucosaminides occurred in the double conjugate forms simultaneously conjugated with sulfuric acid or glucuronic acid. Furthermore, it has been demonstrated that the substrates for N-acetylglucosaminyltransferase necessitate pre-conjugation with sulfuric acid or glucuronic acid (15-17). The present results are fairly consistent with the previous findings,

considering that ursodeoxycholic acids are conjugated also with amino acids.

Further studies on the metabolism of bile acid N-acetylglucosaminides in hepatobiliary diseases are being conducted in these laboratories, and the details will be reported elsewhere.

ACKNOWLEDGEMENTS

The authors express their sincere thanks to Dr. Naoaki Tanno (School of Medicine, Tohoku University) for providing the urine specimen. 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: November 4, 1992
Accepted: December 23, 1992