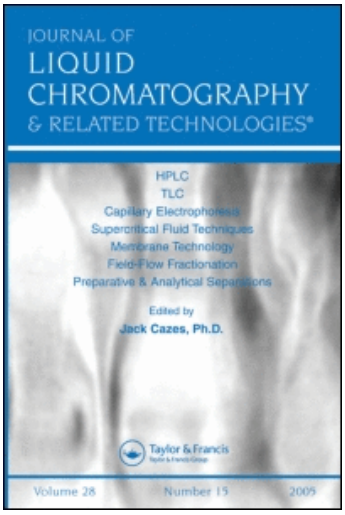

Journal of Liquid Chromatography & Related Technologies

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 Goto, Junichi , Gang, Shao , Miura, Hiroya , Nambara, Toshio , Tazawa, Yusaku and Tada, Keiya(1989) 'Studies on Steroids CCXXXXIV. Separation and Characterization of C-25 Epimers of Unconjugated and Conjugated Trihydroxycholestanoic Acids in Urine from a Patient with Zell Weger Syndrome by High-Performance Liquid Chromatography', Journal of Liquid Chromatography & Related Technologies, 12: 6, 1075 — 1084

To link to this Article: DOI: 10.1080/01483918908051780

URL: http://dx.doi.org/10.1080/01483918908051780

JOURNAL OF LIQUID CHROMATOGRAPHY, 12(6), 1075-1084 (1989)

STUDIES ON STEROIDS CCXXXXIV. SEPARATION AND CHARACTERIZATION OF C-25 EPIMERS OF UNCONJUGATED AND CONJUGATED TRIHYDROXYCHOLESTANOIC ACIDS IN URINE FROM A PATIENT WITH ZELLWEGER SYNDROME BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

The separation and characterization of C-25 epimers of unconjugated and glycine- and taurine-conjugated 3α,7α,12α-trihydroxy-5β-cholestanoic acid (THCA) in biological fluids by high-performance liquid chromatography (HPLC) are described. The 5β-cholestanoic acid fraction was obtained from a urine specimen from a patient with Zellweger syndrome by passing it through a Sep-pak C_{18} cartridge. Bile acids were derivatized quantitatively into the fluorescent compounds through the hydroxyl group at C-3 by treatment with 1-anthroyl nitrile. The derivatives were separated into the unconjugated, glycine- and

taurine-conjugated fractions by ion-exchange chromatography on a lipophilic gel, piperidinohydroxypropyl Sephadex LH-20. Subsequent resolution of each fraction into (25S)- and (25R)-THCA was attained by HPLC on a Cosmosil $5C_{18}$ column. The C-25 epimers of unconjugated and conjugated THCA were unequivocally identified on the basis of their behaviors in HPLC using mobile phases of different pHs. The ratios of the unconjugated, glycine- and taurine-conjugated (25R)-THCA to the corresponding (25S)-epimers were 16:1, 5:4 and 3:2, respectively.

INTRODUCTION

Bile acids are synthesized from cholesterol in the liver and assist the lipolysis and absorption of fats by the formation of mixed micells in the intestinal lumen. Considerable attention has recently been focused on the biodynamics of bile acids in man in connection with the diagnosis of hepatobiliary diseases. It is generally accepted that the degradation of the side chain of cholesterol in the biosynthesis of bile acids is initiated with a mitochondrial 26-hydroxylation followed by oxidation, yielding 3α,7α-dihydroxy- and 3α,7α,12α-trihydroxy-5β-cholestanoic acids (DHCA and THCA). Further conversion into chenodeoxycholic acid and cholic acid takes place efficiently in liver peroxisomes. In connection with the biosynthetic mechanism of bile acids, a particular interest has recently been directed to

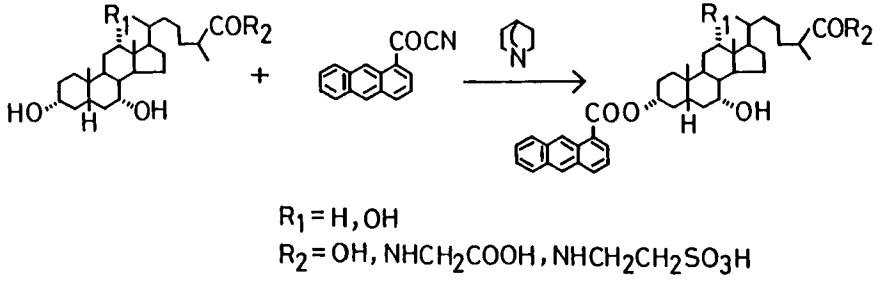

FIGURE 1 Derivatization of 5β-Cholestanoic Acids with 1-Anthroyl Nitrile.

the stereochemistry at C-25 of these 5β-cholestanoic acids. In the previous paper, we developed a method for the resolution of C-25 epimers of unconjugated, glycine- and taurine-conjugated DHCA and THCA by high-performance liquid chromatography (HPLC) with pre-column fluorescence labeling through the 3α-hydroxyl group (Fig.1)(1). This paper describes the separation and characterization of C-25 epimers of THCA in urine from a patient with Zellweger syndrome who lacks liver peroxisomes completely.

EXPERIMENTAL

Materials

DHCA and THCA were prepared from chenodeoxycholic acid and cholic acid in the manner previously reported (2,3). Glycine- and taurine-conjugated DHCA and THCA were also synthesized in these laboratories (1). Two pairs of C-25 epimers were obtained by fractional crystallization from ethyl acetate or by preparative HPLC (1). The Sep-pak C_{18} cartridge was purchased from Millipore Co. Waters Chromatography Div. (Milford, MA, U.S.A.). All other chemicals employed were of analytical-reagent grade. Solvents were purified by distillation prior to use. Piperidinohydroxypropyl Sephadex LH-20 (PHP-LH-20)(acetate form, 0.6 mequiv./g)(4) and 1-anthroyl nitrile (5) were prepared in the manner previously reported. All glassware used was silanized with trimethylchlorosilane.

High-Performance Liquid Chromatography

The apparatus used for this work was a 510 solvent delivery system (Millipore Co. Waters Chromatography Div.) equipped with a 875-UV detector (205 nm)(Japan Spectroscopic Co., Tokyo) and a 650-10LC fluorescence spectrophotometer (excitation wavelength 370 nm; emission wavelength 470 nm)(Hitachi Ltd., Tokyo). A Cosmosil $5C_{18}$ (5 μm, 15 cm X 4.6 mm I.D)(Nacalai Tesque Inc., Kyoto) column was used at ambient temperature.

Procedure for Separation and Characterization of C-25 Epimers of THCA

A urine specimen (500 µl) from a patient with Zellweger syndrome was diluted with 0.5M phosphate buffer (pH 7.0)(4 ml) and applied to a Sep-pak C_{18} cartridge. After successive washing with water (4 ml) and 1.5% ethanol (4 ml), 5β-cholestanoic acids were eluted with 90% ethanol (5 ml), and the eluate was applied to columns (18 mm X 6 mm I.D. X 3) of PHP-LH-20 (each 100 mg). Elution was carried out at a flow rate of 0.3 ml/min. After washing with 90% ethanol (5 ml), unconjugated, glycine- and taurine-conjugated THCA were fractionally separated by stepwise elution with 0.1M acetic acid in 90% ethanol (8 ml), 0.2M formic acid in 90% ethanol (8 ml) and 0.3M acetic acid-potassium acetate in 90% ethanol (pH 6.5)(8 ml). The taurine-conjugated fraction was concentrated and subjected to a Sep-pak C_{18} cartridge to remove inorganic salts. Each fraction was then evaporated down, added with 1-anthroyl nitrile (400 µg) in acetonitrile (100 µl) and 0.16% quinuclidine in acetonitrile (100 µl), and the whole was heated at 60°C for 20 min. After addition of methanol (50 µl) to decompose the excess reagent, the mixture was evaporated down under nitrogen and the residue was subjected to purification with PHP-LH-20 according to the procedure described above. The residue obtained was redissolved in methanol (100-200 µl) and a 5-10 µl aliquot of the solution was injected into the HPLC system.

RESULTS AND DISCUSSION

Clean-up of Unconjugated and Conjugated THCA in Urine

The separation of trace compounds in biological fluids is markedly influenced by the clean-up procedure employed. The cartridge packed with ODS bonded silica is currently used for extraction of bile acids, their sulfates and glucuronides in

biological fluids (6). In the present study, a Sep-pak C_{18} cartridge was used for extraction of unconjugated and conjugated THCA in urine. A synthetic mixture of 10 nmol each of unconjugated, glycine- and taurine-conjugated THCA dissolved in phosphate buffer (pH 7.0) was applied to the cartridge. The eluate obtained with 90% ethanol was then separated and determined by HPLC on a Cosmosil $5C_{18}$ column. The C-25 epimers were recovered at a rate of more than 90% in an initial 2 ml of the effluent.

Previously, we demonstrated that C-25 epimers of 3-(1-anthroyl) THCA were effectively resolved on a Cosmosil $5C_{18}$ column with a neutral mobile phase (1). Under this condition, little difference in the capacity ratio (k') was observed among unconjugated, glycine- and taurine-conjugated THCA. Therefore, the group separation into the three forms on a lipophilic ion-exchange gel became prerequisite. This procedure was also useful for removal of urinary neutral and basic co-existing substances which interfere with the derivatization reaction and separation of C-25 epimers. A synthetic mixture of 5 nmol each of unconjugated and conjugated THCA was dissolved in 90% ethanol and applied to a column of PHP-LH-20. After washing with 90% ethanol, the group separation was carried out by stepwise elution with 0.1M acetic acid in 90% ethanol, 0.2M formic acid in 90% ethanol and 0.3M acetic acid-potassium acetate in 90% ethanol (pH 6.5). Unconjugated, glycine- and taurine-conjugated THCA were completely separated into the three groups.

Separation and Characterization of C-25 Epimers of THCA in Urine

The separation and characterization of C-25 epimers of unconjugated, glycine- and taurine-conjugated THCA in urine were carried out according to the scheme shown in Fig.2. A urine sample from a patient with Zellweger syndrome was extracted with a Sep-pak C_{18} cartridge and then subjected to group separation

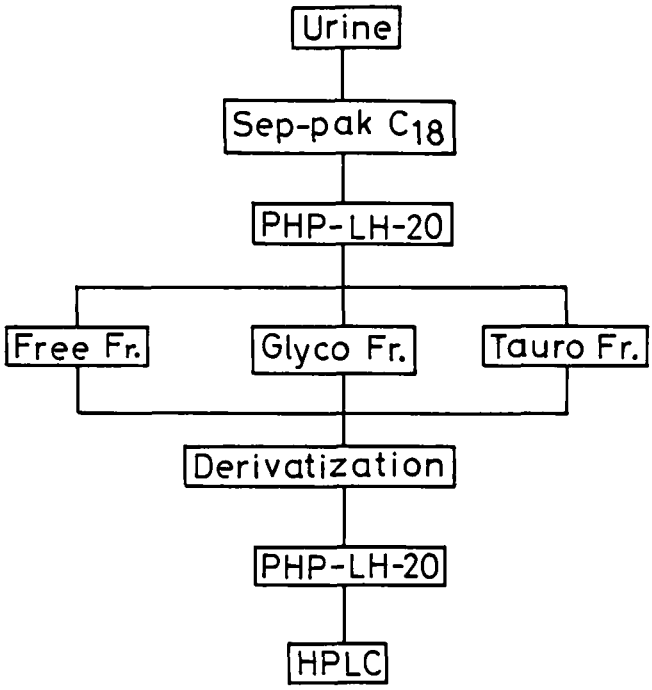

FIGURE 2 Procedure for Determination of C-25 Epimers of 5β-Cholestanoic Acids in Human Urine.

on PHP-LH-20. Each fracion was treated with 1-anthroyl nitrile in the presence of quinuclidine in acetonitrile according to the procedure described above. After purification with PHP-LH-20, the 3-(1-anthroyl) derivatives obtained were subjected to HPLC on a Cosmosil $5C_{18}$ column using 0.3% potassium phosphate buffer (pH 7.0)-methanol (1:4) as a mobile phase. No epimerizaton occurred at C-25 in this procedure. As illustrated in Fig.3, the peaks having the k' values identical with those of C-25 epimers of unconjugated, glycine- and taurine-conjugated THCA were distinctly observed.

It has been pointed out that the HPLC method has a disadvantage in structural elucidation. In this method, however,

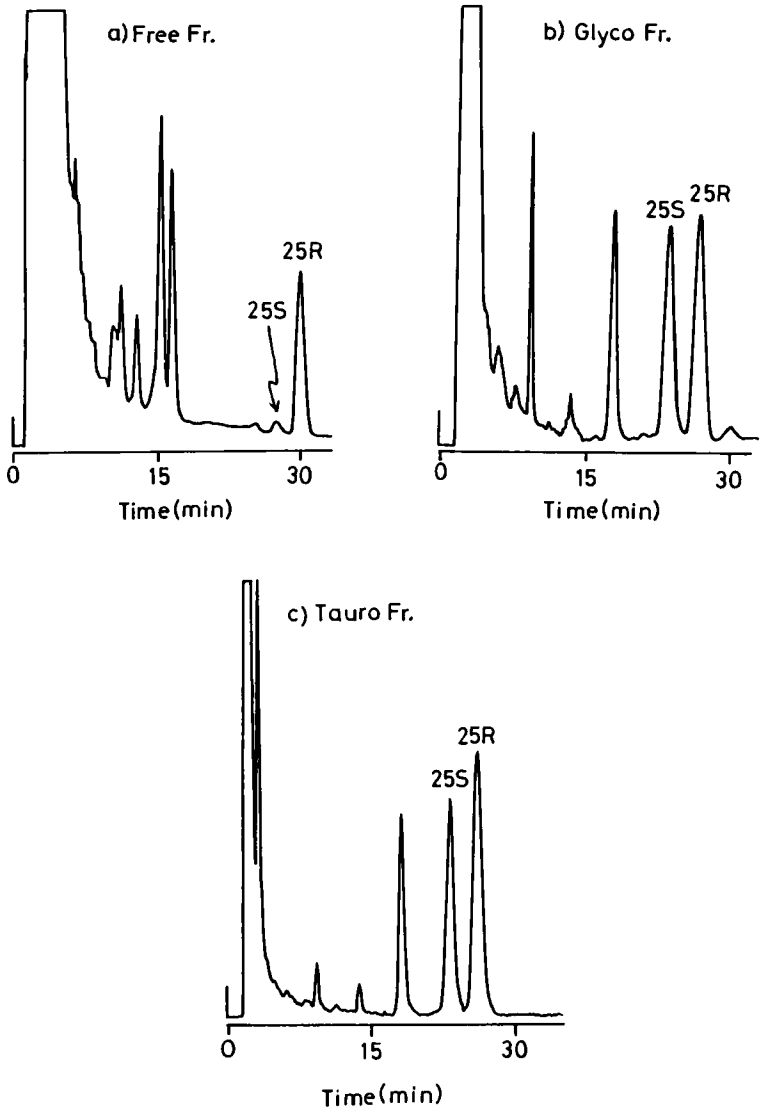

FIGURE 3 Separation of C-25 Epimers of 3α,7α,12α-Trihydroxy-5β-cholestanoic Acids in Urine from a Patient with Zellweger Syndrome.
Conditions: column, Cosmosil $5C_{18}$; mobile phase, 0.3% potassium phosphate buffer (pH 7.0)/methanol (1:4), 1.5 ml/min.

TABLE 1

Capacity Ratios and Peak Area Ratios (25R/25S) of C-25 Epimers of 3α,7α,12α-Trihydroxy-5β-cholestanoic Acids (THCA) in Urine from a Patient with Zellweger Syndrome

THCA		pH 4.0[a] k'	pH 4.0[a] 25R/25S	pH 5.5[b] k'	pH 5.5[b] 25R/25S	pH 7.0[c] k'	pH 7.0[c] 25R/25S
Unconjugated							
Standard	S	5.84		2.98		2.67	
	R	6.48		3.20		2.97	
Urine	S	5.82	16.0	2.97	16.5	2.67	16.2 (9.5)*
	R	6.46		3.19		2.96	
Glycine-conjugated							
Standard	S	3.67		2.84		2.73	
	R	4.11		3.11		3.03	
Urine	S	3.68	1.26	2.84	1.25	2.72	1.22 (13.0)*
	R	4.12		3.12		3.03	
Taurine-conjugated							
Standard	S	5.67		5.04		5.16	
	R	6.33		6.00		5.78	
Urine	S	5.67	1.60	5.41	1.65	5.14	1.60 (6.7)*
	R	6.33		6.00		5.77	

Conditions: column, Cosmosil $5C_{18}$; mobile phase, 0.3% potassium phosphate buffer/methanol, a) 1:11, b) 2:17, c) 1:5.

*Total concentration (μg/ml) determined by gas chromatography-mass spectrometry with negative ion chemical ionization detection (8).

various combinations of stationary and mobile phases are available. Therefore, inspection of chromatographic behavior under different conditions was performed for the unequivocal characterization of C-25 epimers in urine. The eluates corresponding to the peaks on the chromatogram (Fig.3) were collected and subjected to HPLC on Cosmosil $5C_{18}$ employing three mobile phases of different pHs. It is evident from the data in Table 1 that the k' values of C-25 epimers in urine were identical with those of authentic specimens.

It is of interest that the ratios (25R/25S) of (25R)-THCA to the corresponding (25S)-epimer are remarkably different between unconjugated and conjugated THCA. In the unconjugated fraction (25R)-THCA was predominant while in the glycine- and taurine-conjugated fractions the amounts of the (25S)- and (25R)-epimers were nearly equal. It has been demonstrated that the (R)-epimer of α-arylpropionic acids is selectively transformed into the CoA thioester which undergoes epimerization enzymatically to the (S)-form (7). The result listed in Table 1 suggests that the similar epimerization mechanism may be operative for the formation of glycine- and taurine-conjugated THCA. Further detailed studies are needed for clarifying these findings.

It is hoped that the availability of an excellent method for the separation and characterization of C-25 epimers of THCA in urine may provide much more precise knowledge on the biosynthesis of bile acids.

ACKNOWLEDGEMENT

This work was supported in part by grants from the Tokyo Biochemical Research Foundation and the Ministry of Education, Science and Culture of Japan.

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