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STUDIES ON STEROIDS

CCXXVII*. SEPARATION AND DETERMINATION OF BILE ACID 7- AND 12-SULPHATES IN URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE LABELLING

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SUMMARY

A method for the characterization and determination of bile acid 7- and 12-sulphates in urine without prior deconjugation is described. The sulphate fraction was obtained from an urine specimen by passing it through a Sep-Pak C_{18} cartridge, followed by group separation by ion-exchange chromatography on a lipophilic gel, piperidinohydroxypropyl Sephadex LH-20. Bile acid 7- and 12-sulphates were derivatized quantitatively into the fluorescent compounds through the hydroxyl group at C-3 by treatment with 1-anthroyl nitrile in the presence of quinuclidine in acetonitrile. Subsequent resolution into individual 7- and 12-sulphates was attained by high-performance liquid chromatography (HPLC) on a Cosmosil $5C_{18}$ column using 0.3% potassium phosphate buffer (pH 4.0)–methanol (1:3) as a mobile phase. The 3-(1-anthroyl) derivatives of 7- and 12-sulphates were monitored by fluorescence detection. Taurochenodeoxycholate 7-sulphate in human urine was unequivocally identified on the basis of behaviour in HPLC using mobile phases of different pH values. The present method has proved to be applicable to the characterization and quantification of bile acid 7- and 12-sulphates in human urine.

INTRODUCTION

Bile acids are major metabolites of cholesterol and assist the lipolysis and absorption of fats by the formation of mixed micelles in the intestinal lumen. After conjugation with the amino acids to form glyco and tauro conjugates in the liver, bile acids are stored in the gallbladder, secreted subsequently into the duodenum via the bile duct and then return to the liver. Therefore, small amounts of bile acids are present in peripheral blood and urine in healthy subjects. However,

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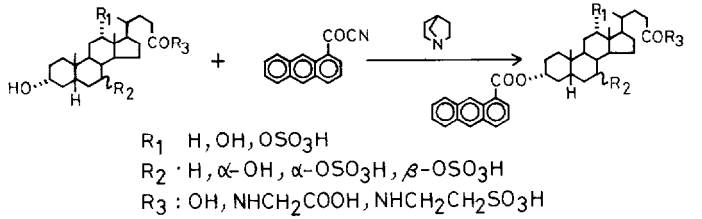

Fig. 1. Formation of 3-(1-anthroyl) derivatives of bile acid 7- and 12-sulphates.

in hepatobiliary and intestinal diseases, disturbances of synthesis and clearance by the liver and absorption by the intestine cause changes in the level and the metabolic profile of bile acids in biological fluids.

Bile acids are usually excreted into urine as their 3-sulphates. Recently, it has been suggested that bile acid 7-sulphates, which are positional isomers of 3-sulphates, would be present in human urine. The common methods for the determination of sulphated bile acids in biological fluids involve prior solvolysis or hydrolysis, followed by the chromatographic separation of deconjugated bile acids. These procedures, however, have inevitable disadvantages, such as a lack of reliability and the loss of information about the conjugated form and position. The reliable analysis of bile acid 7- and 12-sulphates needs to eliminate interferences due to isomeric 3-sulphates, which exist predominantly in urine. Bile acid 7- and 12-sulphates have the free hydroxyl function at C-3, which is more reactive toward acylation than the 7α, 7β and 12α hydroxyl groups. In the previous work, we developed a suitable precolumn method for the separation of bile acid 7- and 12-sulphates through the 3α hydroxyl group using 1-anthroyl nitrile as a labelling reagent (Fig. 1) [1]. The desired fluorescent derivatives were effectively separated into individual 7- and 12-sulphates by high-performance liquid chromatography (HPLC) on a reversed-phase column and monitored by fluorescence detection, the limit of detection being 30 fmol. The present paper deals with the application of this method to the characterization and determination of bile acid 7- and 12-sulphates in human urine.

EXPERIMENTAL

High-performance liquid chromatography

The apparatus used for this work was a 6000A solvent delivery system (Waters Assoc., Milford, MA, U.S.A.) equipped with a Uvidec-100II UV detector (205 nm) (Japan Spectroscopic, Tokyo, Japan) and a 650-10LC fluorescence spectrophotometer (excitation wavelength 370 nm; emission wavelength 470 nm) (Hitachi, Tokyo, Japan). The test samples were injected using a U6K sample loop injector (Waters Assoc.) with an effective volume of 2 ml. The Finepak SC-02 (10 μm, 25 cm$\times$4.6 mm I.D.) (Japan Spectroscopic) and Cosmosil 5C_{18} (5 μm, 15 cm$\times$4.6 mm I.D.) (Nakarai Kagaku, Kyoto, Japan) columns were used at ambient temperature.

Materials

Unconjugated, glyco- and tauro-conjugated bile acid 7- and 12-sulphates were synthesized in these laboratories by the methods previously reported [2]. Seph-

adex LH-20 was purchased from Pharmacia (Uppsala, Sweden). All 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) [3] and 1-anthroyl nitrile [4] were prepared in the manner previously reported. The Sep-Pak C_{18} cartridge (Waters Assoc.) was washed successively with ethanol and water prior to use. All glassware used was silanized with trimethylchlorosilane.

Procedure for determination of bile acid 7-sulphates in human urine

To a urine sample (500 μl), glyco- or taurodeoxycholate 12-sulphate (1 nmol) was added as an internal standard and the whole was diluted with 0.5 *M* phosphate buffer (pH 7.0) (2 ml) and applied to a Sep-Pak C_{18} cartridge. After washing with water (12 ml) the sulphate fraction was eluted with 90% ethanol (4 ml) and the eluate was applied to a column (18 mm $\times$ 6 mm I.D.) of PHP-LH-20 (100 mg). Elution was carried out at a flow-rate of 0.2 ml/min. After successive washing with 90% ethanol, 0.1 *M* acetic acid in 90% ethanol, 0.2 *M* formic acid in 90% ethanol and 0.3 *M* acetic acid–potassium acetate in 90% ethanol (pH 6.3) (each 4 ml) to remove unsulphated bile acids, the desired sulphates were eluted with 1% ammonium carbonate in 70% ethanol (4 ml). Inorganic salts in the eluate were then removed by passing it through the column (40 mm $\times$ 8.5 mm I.D.) of carboxymethyl Sephadex LH-20 (CM-LH-20) (400 mg). An aliquot of the effluent was evaporated down and, after addition of 1-anthroyl nitrile (400 μg) in acetonitrile (100 μl) and 2% quinuclidine in acetonitrile (100 μl), the wole was heated at 60°C for 20 min. The excess reagent was decomposed with methanol (50 μl) and the mixture was evaporated under nitrogen. The residue was redissolved in 90% ethanol (1 ml) and applied to a PHP-LH-20 column (100 mg, 18 mm $\times$ 6 mm I.D.). After successive washing with 90% ethanol and 0.3 *M* acetic acid–potassium acetate in 90% ethanol (pH 6.3) (each 4 ml), the sulphate fraction was eluted with 1% ammonium carbonate in 70% ethanol (4 ml). The dried eluate was redissolved in methanol (100 μl) and a 5–10 μl aliquot of the solution was injected into the HPLC system.

Recovery test for bile acid 7- and 12-sulphates

The test samples were prepared by dissolving 0.2 nmol each of 7- and 12-sulphates in human urine (500 μl) and subjected to extraction by a Sep-Pak C_{18} cartridge and group separation on PHP-LH-20. After addition of glyco- or taurodeoxycholate 12-sulphate (1 nmol) as an internal standard, the sulphate fraction was derivatized with 1-anthroyl nitrile and then subjected to determination by HPLC in the manner described above.

RESULTS AND DISCUSSION

Clean-up of bile acid sulphates in urine

Since bile acid 7- and 12-sulphates are highly polar and lack thermostability, the separation of these sulphates is markedly influenced by the clean-up procedure employed. Cartridges packed with ODS-bonded silica, e.g. Sep-Pak C_{18} and

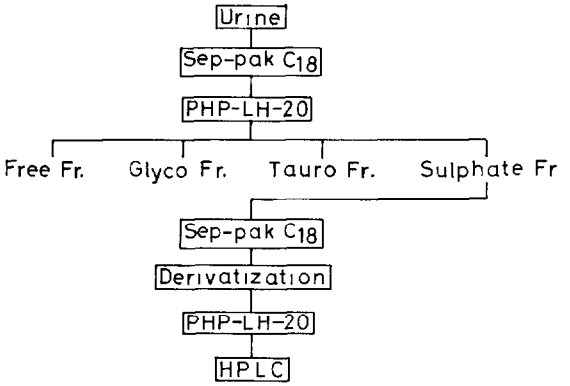

Fig. 2. Procedure for separation of bile acid 7- and 12-sulphates.

Bond Elut, are currently employed for extraction of bile acids and their 3-sulphates and 3-glucuronides in biological fluids [5–7]. In the present study, a Sep-Pak C_{18} cartridge was used for extraction of 7- and 12-sulphates in urine. A synthetic mixture of 7- and 12-sulphates dissolved in phosphate buffer (pH 7.0) was applied to the cartridge. The eluate obtained with 90% ethanol was then separated and analysed by HPLC on a Finepak SC-02 column [6]. The sulphates were recovered at a rate of more than 90% in an initial 1.5 ml of the effluent.

Previously, it has been demonstrated that bile acids are separated into the unconjugated, glyco- and tauro-conjugated and sulphated fractions on PHP-LH-20 according to their pK_a values [6]. This method is effective not only for removal of neutral and basic co-existing substances in urine but also for separation of unsulphated bile acids that are similarly derivatized with 1-anthroyl nitrile. A synthetic mixture of 7- and 12-sulphates was dissolved in 90% ethanol and applied to a column of PHP-LH-20. After removal of neutral compounds, unconjugated, glyco- and tauro-conjugated bile acids by successive washing with 90% ethanol, 0.1 *M* acetic acid in 90% ethanol, 0.2 *M* formic acid in 90% ethanol and 0.3 *M* acetic acid–potassium acetate in 90% ethanol (pH 6.3) (each 4 ml), the desired sulphate fraction was eluted with 1% ammonium carbonate in 70% ethanol and subjected to HPLC [6]. Sulphated bile acids were recovered at a rate of more than 90% in an initial 2.5 ml of the effluent.

Separation and characterization of bile acid 7- and 12-sulphates in urine

The separation and characterization of bile acid 7- and 12-sulphates in urine were carried out according to the scheme shown in Fig. 2. The urine sample from a patient with primary biliary cirrhosis (PBC) was extracted with a Sep-Pak C_{18} cartridge and then subjected to group separation on PHP-LH-20. After removal of inorganic salts by passing through a Sep-Pak C_{18} cartridge, the sulphate fraction was treated with 1-anthroyl nitrile according to the procedure described above. The 3-(1-anthroyl) derivatives obtained were subjected to HPLC on Cosmosil $5C_{18}$ using 0.3% potassium phosphate buffer (pH 4.0)–methanol (1:3) as a mobile phase [1]. As illustrated in Fig. 3, a peak having the capacity ratio (k') identical

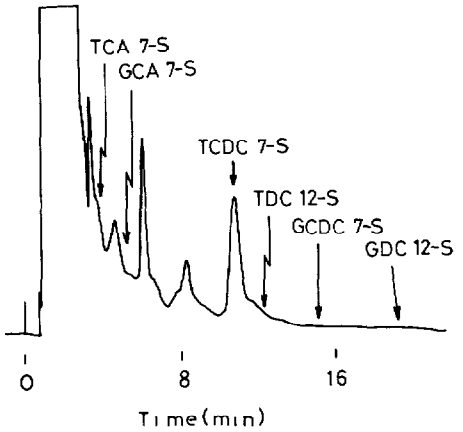

Fig. 3. Chromatogram of bile acid 7- and 12-sulphates in urine from a patient with PBC.

TABLE I

RELATIVE k' VALUES AND PEAK-AREA RATIOS OF TAUROCHENODEOXYCHOLATE 7-SULPHATE

The figures express k' values and peak-area ratios relative to glycodeoxycholate 12-sulphate. Conditions: column, Cosmosil $5C_{18}$; mobile phase, 0.3% potassium phosphate–methanol; flow-rate, 1.6 ml/min.

pH	k'		Peak-area ratio
	Standard sample	Urine sample	
4.0	0.61	0.60	4.83
5.0	0.81	0.81	4.69
6.0	0.83	0.84	4.75
7.0	0.84	0.84	4.70

TABLE II

RELATIVE k' VALUES OF TAUROCHENODEOXYCHOLATE FORMED FROM THE 7-SULPHATE BY SOLVOLYSIS

The figures express k' values relative to taurocholate. Conditions: column, Cosmosil $5C_{18}$; mobile phase, 0.3% potassium phosphate–methanol; flow-rate 1.6 ml/min.

pH	k'	
	Standard sample	Urine sample
3.0	3.26	3.28
4.0	2.84	2.87
5.0	2.65	2.67
6.0	2.54	2.56

TABLE III

RECOVERY OF BILE ACID 7- AND 12-SULPHATES ADDED TO HUMAN URINE

Bile acid★	Recovery (mean ± S.D., $n = 10$) (%)
Cholate (CA) 7-S	93.9 ± 6.8
Cholate (CA) 12-S	98.5 ± 8.0
Ursodeoxycholate (UDC) 7-S	91.9 ± 3.6
Chenodeoxycholate (CDC) 7-S	100.7 ± 8.4
Deoxycholate (DC) 12-S	97.9 ± 8.4
Glycocholate (GCA) 7-S	95.6 ± 6.5
Glycocholate (GCA) 12-S	91.2 ± 9.6
Glycoursodeoxycholate (GUDC) 7-S	88.0 ± 6.9
Glycochenodeoxycholate (GCDC) 7-S	95.3 ± 9.0
Glycodeoxycholate (GDC) 12-S	95.7 ± 6.4
Taurocholate (TCA) 7-S	94.6 ± 7.7
Taurocholate (TCA) 12-S	84.1 ± 9.3
Tauroursodeoxycholate (TUDC) 7-S	102.9 ± 6.7
Taurochenodeoxycholate (TCDC) 7-S	93.2 ± 3.0
Taurodeoxycholate (TDC) 12-S	93.8 ± 8.1

★Bile acid sulphates (each 0.2 nmol) were added to human urine (0.5 ml). S = sulphate.

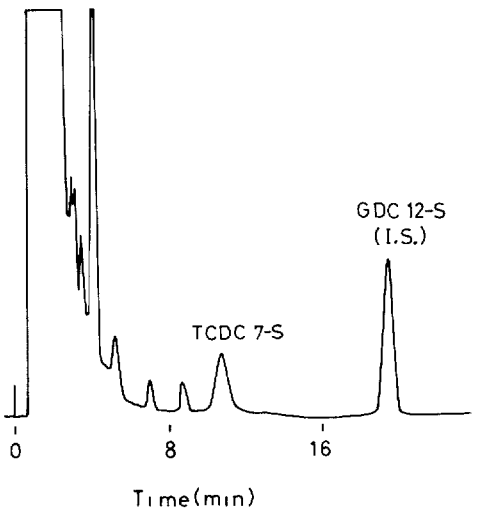

Fig. 4. Chromatogram of bile acid 7-sulphates in urine from a patient with congenital biliary atresia.

TABLE IV

AMOUNTS OF BILE ACID 7-SULPHATES IN HUMAN URINE

N.D. = not detectable; S = sulphate.

Subject	Disease	Concentration (nmol/ml)			
		TCA 7-S	GCA 7-S	TCDC 7-S	GCDC 7-S
A	Primary biliary cirrhosis	N.D.	N.D.	0.54	N.D.
B	Primary biliary cirrhosis	N.D.	N.D.	0.024	N.D.
C	Congenital biliary atresia	N.D.	N.D.	0.41	N.D.
D	Intrahepatic cholestasis	N.D.	N.D.	0.092	N.D.
E	Intrahepatic cholestasis	N.D.	N.D.	N.D.	N.D.
F	Normal	N.D.	N.D.	N.D.	N.D.
G	Normal	N.D.	N.D.	N.D.	N.D.

with that of taurochenodeoxycholate 7-sulphate was distinctly observed.

In the preceding study, we demonstrated that the chromatographic behaviour of bile acid 7- and 12-sulphates was dependent upon the number and position of the hydroyxl group on the steroid nucleus as well as the conjugated form at C-24 [1]. Therefore, inspection of chromatographic behaviour under different conditions was performed for the characterization of the 7-sulphate in urine. The eluate corresponding to the peak on the chromatogram (Fig. 3) was collected and, after addition of 3-(1-anthroyl)glycodeoxycholate 12-sulphate as an internal standard, subjected to HLPC on Cosmosil $5C_{18}$ employing four mobile phases of different pH. It is evident from the data in Table I that the relative k' value of bile acid sulphate in urine was identical with that of authentic taurochenodeoxycholate 7-sulphate. Moreover, peak-area ratios of the sulphate to the internal standard were almost constant.

The identity of the 7-sulphate was further confirmed by degradative means. The eluate corresponding to the peak on the chromatogram (Fig. 3) was subjected to solvolysis with dimethoxypropane–water–concentrated hydrochloric acid according to the procedure previously reported [8]. After addition of 3-(1-anthroyl)taurocholate as an internal standard, the 3-(1-anthroyl) bile acid liberated was subjected to HPLC on Cosmosil $5C_{18}$ with four different mobile phases. As listed in Table II, the relative k' value of the bile acid liberated was identical with that of authentic taurochenodeoxycholate. These results definitely established the presence of taurochenodeoxycholate 7-sulphate in human urine.

Determination of bile acid 7-sulphates in human urine

A standard procedure for the separation and determination of bile acid 7-sulphates in human urine is described in the Experimental section. Since no glyco- and taurodeoxycholate 12-sulphates were present in human urine, these 12-sulphates were used as internal standards. There is no doubt that for this purpose, the use of synthetic bile acid 7- or 12-sulphates with the 3α hydroxyl group is

more favourable. The calibration graph was constructed by plotting the ratio of peak area of each bile acid 7- and 12-sulphate to that of internal standard against the amount of the corresponding 7- and 12-sulphate. Known amounts of bile acid 7- and 12-sulphates were added to human urine and their recovery rates were estimated. As listed in Table III, all bile acid 7- and 12-sulphates were recovered at the satisfactory rates.

A chromatogram of bile acid 7- and 12-sulphates in urine from a patient with congenital biliary atresia is illustrated in Fig. 4. Simultaneous determination of bile acid 7- and 12-sulphates was carried out with seven urine specimens. The results obtained are listed in Table IV. In four urine specimens from patients with hepatobiliary diseases, taurochenodeoxycholate 7-sulphate was found in an amount corresponding to 1–2% of the total sulphated bile acids. Any other bile acid 7- and 12-sulphates could not be detected, even when 5 ml of human urine were subjected to analysis. Raedsch et al. [9] determined bile acid sulphates in urine from a patient with PBC by thin-layer chromatography, indicating that glyco- and tauro-conjugated cholate and chenodeoxycholate 7-sulphates were excreted in larger amounts, corresponding to 30% of the sulphate fraction. On the other hand, Almé et al. [10] developed a gas chromatographic–mass spectrometric method combined with a microchemical technique for the determination of bile acid sulphates in urine, and disclosed that no bile acid 7-sulphates were detected in urine from a patient with PBC.

It is hoped that the availability of the new method for the simultaneous determination of bile acid 7- and 12-sulphates in urine with sufficient reliability and sensitivity may provide much more precise knowledge on the metabolic profile of bile acids and may serve as a diagnosis for hepatobiliary diseases.

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