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STUDIES ON STEROIDS

CCXXXXV[a]. DETERMINATION OF 5β-CHOLESTANOIC ACIDS IN HUMAN URINE BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY WITH NEGATIVE ION CHEMICAL IONIZATION DETECTION

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SUMMARY

A method for the determination of 3α,7α-dihydroxy-5β-cholestanoic acid (DHCA) and 3α,7α,12α-trihydroxy-5β-cholestanoic acid (THCA) in human urine by gas chromatography (GC) in combination with negative ion chemical ionization (NICI) mass spectrometry is described. Unconjugated, glycine- and taurine-conjugated DHCA and THCA labelled with ^{18}O and ^{2}H were used as internal standards. 5β-Cholestanoic acids in urine were extracted with a Sep-Pak C_{18} cartridge, separated into the unconjugated, glycine- and taurine-conjugated fractions by ion-exchange chromatography on piperidinohydroxypropyl Sephadex LH-20 and, following alkaline hydrolysis of conjugated forms, derivatization into the pentafluorobenzyl ester–dimethylethylsilyl ethers. Subsequent resolution of each fraction into DHCA and THCA was attained by GC on a cross-linked 5% phenylmethylsilicone fused-silica capillary column where 5β-cholestanoic acids were monitored with a characteristic carboxylate anion $[M-181]^-$ in the NICI mode using isobutane as a reagent gas. The method was applied to separation and determination of 5β-cholestanoic acids in urine from a patient with Zellweger syndrome and from healthy volunteers.

INTRODUCTION

The cerebro-hepato-renal syndrome, Zellweger syndrome, is a rare hereditary disease in which there is a complete lack of peroxisomes in hepatocites. It

[a]For Part CCXXXXIV, see J. Goto, G. Shao, H. Miura, T. Nambara, Y. Tazawa and K. Tada, J. Liq. Chromatogr., in press.

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is known that peroxisomes are important for the final step in the biosynthesis of bile acids and for oxidation of long-chain fatty acids. As a result, bile acid intermediates, 3α,7α-dihydroxy-5β-cholestanoic acid (DHCA) and 3α,7α,12α-trihydroxy-5β-cholestanoic acid (THCA), accumulate in the bile, urine and blood of patients with Zellweger syndrome [1,2]. A reliable method is therefore urgently needed for the trace determination of these 5β-cholestanoic acids in biological fluids in connection with the diagnosis of the syndrome. Among various methods, gas chromatography–mass spectrometry (GC–MS) is well recognized as a powerful tool for trace determinations and has been applied to the determination of bile acids in biological materials using the electron-impact ionization mode [3,4]. In previous studies, we found that the combined use of derivatization into the pentafluorobenzyl (PFB) ester–dimethylethylsilyl (DMES) ethers and capillary GC–MS with negative ion chemical ionization (NICI) detection is much more promising with respect to the sensitivity and versatility for the determination of bile acids [5,6]. This paper deals with the separation and determination of unconjugated and glycine- and taurine-conjugated DHCA and THCA in urine by GC–NICI-MS using stable isotope-labelled 5β-cholestanoic acids as internal standards (I.S.).

EXPERIMENTAL

Materials

DHCA and THCA were prepared from chenodeoxycholic acid and cholic acid as reported previously [7,8]. Glycine- and taurine-conjugated DHCA and THCA were also synthesized in these laboratories [9]. Cholylglycine hydrolase and DMES-imidazole were obtained from Sigma (St. Louis, MO, U.S.A.) and Tokyo Kasei Kogyo (Tokyo, Japan), respectively. A Sep-Pak C_{18} cartridge and Pre Pak-500/C_{18} were purchased from Millipore-Waters (Milford, MA, U.S.A.). All other chemicals 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) [10] and carboxymethyl-Sephadex LH-20 (CM-LH-20) (K^+ form, 1 mequiv./g) [11] were prepared as reported. All glassware was silanized with trimethylchlorosilane.

Gas chromatography–mass spectrometry

Capillary GC–MS was carried out using a VG Analytical MM12030 quadrupole mass spectrometer equipped with a Hewlett-Packard 5790A gas chromatograph. Isobutane was used as the reagent gas. A cross-linked 5% phenylmethylsilicone fused-silica capillary column (20 m×0.3 mm I.D.) (J & W Scientific, Folsom, CA, U.S.A.) was inserted into the ion source through the direct inlet. The carrier gas was helium at a linear velocity of 60 cm/s. The test samples were injected through a Van den Berg solventless injector with an inlet pressure of 0.9 kg/cm^2. The injection port, column oven and ion source were

kept at 290, 290 and 280 °C, respectively. The ionization energy was 70 eV and the emission current was 400 μA.

Preparation of $^{18}O^{2}H$-labelled 5β-cholestanoic acids

The methyl esters of DHCA and THCA were subjected to oxidation with chromium trioxide in acetic acid, followed by alkaline hydrolysis, yielding 3,7-dioxo- and 3,7,12-trioxo-5β-cholestanoic acids. In similar fashion, glycine- and taurine-conjugated oxo compounds were also prepared from the corresponding ethyl glycinate and taurine conjugates, which were synthesized according to a previous paper [9]. These structures were confirmed by ^{1}H NMR spectroscopy and their purities were over 99.5% as judged by high-performance liquid chromatography (HPLC) [9]. Dioxo- and trioxo-5β-cholestanoic acids were then converted into corresponding potassium salts using CM-LH-20 as reported previously [6]. Each 20-mg portion of the salt was dissolved in $H_2{}^{18}O$ (isotopic purity 98 atom-%, 300 μl) and heated at 90 °C for 50 h. After cooling, the solution was stirred with $NaB^{2}H_4$ (isotopic purity 97 atom-%, 4 mg) for 1 h under ice-cooling, and then poured into 5% hydrochloric acid. Unconjugated and glycine-conjugated 5β-cholestanoic acids were extracted with ethyl acetate. For taurine conjugates, the reaction mixture was poured into 5% hydrochloric acid, neutralized with 5% potassium hydroxide solution and then passed through a column (19 mm × 20 mm I.D.) of Pre Pak-500/C_{18} (2 g). After washing with water (20 ml), the desired compounds were eluted with ethanol (20 ml). The $^{18}O^{2}H$-labelled 5β-cholestanoic acids thus obtained were purified by column chromatography on silica gel and HPLC on a reversed-phase column [9].

Procedure for determination of DHCA and THCA in urine

To a urine specimen (100 μl) were added unconjugated and glycine- and taurine-conjugated $^{18}O^{2}H$-labelled DHCA and THCA (10–100 ng each) as I.S., and the mixture was diluted with 0.5 *M* phosphate buffer (pH 7.0) (1 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 a column (18 mm × 6 mm I.D.) of PHP-LH-20 (100 mg). Elution was carried out at a flow-rate of 0.3 ml/min. After washing with 90% ethanol (5 ml), unconjugated and glycine- and taurine-conjugated 5β-cholestanoic acids were fractionally separated by stepwise elution with 0.1 *M* acetic acid in 90% ethanol (8 ml), 0.2 *M* formic acid in 90% ethanol (8 ml) and 0.3 *M* acetic acid–potassium acetate in 90% ethanol (pH 6.5) (8 ml) [12]. Glycine and taurine conjugates in each fraction were then hydrolysed with 4 *M* sodium hydroxide solution (4 ml) for 12 h at 120 °C. The reaction mixture was neutralized and applied to a Sep-Pak C_{18} cartridge in the manner described above, for removal of inorganic salts. To the dried unconjugated and glycine- and taurine-conjugated fractions were added 5% (v/v)

PFB bromide in acetonitrile (60 μl) and diisopropylethylamine (10 μl), and the mixture was allowed to stand at 37°C for 45 min. The mixture was diluted with ethanol–acetonitrile (1:1, v/v) (1 ml), and then applied to a Sep-Pak C_{18} cartridge impregnated with ethanol. The PFB esters were eluted with ethanol–acetonitrile (1:1, v/v) (5 ml) and the eluate was evaporated to dryness. The residue was subjected to silylation with DMES-imidazole (50 μl) in 1% pyridine in hexane (50 μl) [13] and then injected into the GC–MS system.

Separation and characterization of THCA in urine from healthy subjects

To a urine specimen (10 ml) were added unconjugated [$^{18}O^{2}H$]DHCA and THCA (each 500 ng) and the mixture was subjected to extraction with a Sep-Pak C_{18} cartridge and deconjugation with 4 *M* sodium hydroxide solution according to the method described above. The deconjugated 5β-cholestanoic acids were purified by ion-exchange chromatography on PHP-LH-20 and then reversed-phase HPLC on a Cosmosil $5C_{18}$ (5 μm) column (15 cm × 4.6 mm I.D.; Nacalai Tesque) using 0.3% potassium phosphate buffer (pH 7.0)–acetonitrile (5:3, v/v) as the mobile phase [9]. The 5β-cholestanoic acids were monitored by UV detection at 205 nm and the eluate corresponding to DHCA and THCA on the chromatogram was collected. After removal of inorganic salts with a Sep-Pak C_{18} cartridge, each fraction was subjected to derivatization into the PFB ester–DMES ether and then injected into the GC–MS system.

RESULTS AND DISCUSSION

Gas chromatographic separation of 5β-cholestanoic acids

In the previous paper [5], we reported that the introduction of a PFB group into the carboxyl function of bile acids would be most favorable for the formation of the characteristic carboxylate anion in the NICI mode. Accordingly, a suitable derivatization for hydroxyl groups on the steroid nucleus was investigated. Initially, the PFB esters of 5β-cholestanoic acids were converted into their trimethylsilyl (TMS) ethers, but a satisfactory result could not be ob-

TABLE I

GC RETENTION TIMES OF PFB ESTER–TMS AND –DMES ETHERS OF 5β-CHOLESTANOIC ACIDS RELATIVE TO CHOLIC ACID (CA)

Bile acid	Relative retention time	
	PFB–TMS	PFB–DMES
CA	1.000	1.000
DHCA	1.511	1.216
THCA	1.513	1.508

tained for the separation of DHCA and THCA as well as chenodeoxycholic acid and cholic acid [5]. It has been demonstrated that the replacement of a methyl group with an ethyl group in TMS improves the GC separation of common bile acids on a non-polar stationary phase, depending on the number of hydroxyl groups [13,14]. Therefore, the PFB esters were derivatized into the DMES ethers. As indicated in Table I, the retention value increased with an increase in the number of hydroxyl groups, providing excellent separation of 5β-cholestanoic acids. In the NICI mode using isobutane as the reagent gas, the PFB ester–DMES ether derivatives exhibited the characteristic negative ions $[M-181]^-$ formed by elimination of the PFB group (Fig. 1).

Preparation of stable isotope-labelled 5β-cholestanoic acids

For GC–MS analysis, the use of stable isotope-labelled compounds as I.S. has been recommended for trace analysis with high accuracy, specificity and sensitivity. In previous work [6], we developed a simple method for the preparation of ^{18}O-labelled bile acids using the exchange reaction of carbonyl groups on the steroid nucleus. Hence, this method was applied to the preparation of 5β-cholestanoic acids labelled with a stable isotope. Unconjugated and glycine- and taurine-conjugated DHCA and THCA were oxidized with chromium trioxide to give the corresponding polyoxo compounds, which in turn were converted into the potassium salts by passing them through a lipophilic cation-exchange gel, CM-LH-20 (K^+ form). The potassium salt was then dissolved in $H_2{}^{18}O$ and heated at 90°C for 50 h. An aliquot of the resulting solution was reduced with sodium borohydride and, if necessary, hydrolytic cleavage of glycine and taurine conjugates with 4 *M* sodium hydroxide solution. Following derivatization to the PFB ester–DMES ether, the ^{18}O content was determined by GC–selected ion monitoring (SIM) using a characteristic ion, $[M-181]^-$. It was confirmed that the theoretical number of ^{18}O atoms were incorporated into 5β-cholestanoic acids through the oxo group at each position.

It is known that multiply labelled analogues with high isotopic purity can also be used as carriers, preventing the loss of target compounds through the clean-up procedure. Therefore, further labelling with ^2H was undertaken. The ^{18}O-labelled oxo compounds were reduced with sodium borodeuteride (NaB^2H_4) and the heavy isotope contents in the product were determined as described above. As shown in Table II, the ratios of the unlabelled fragment to the fully labelled fragment in $[^{18}O^2H]$di- and trihydroxylated 5β-cholestanoic acids were found to be 1:2000 and 1:5000, respectively. No labelled isotopes were eliminated during alkaline hydrolysis and solvolysis. A calibration graph was constructed by plotting the ratio of the peak height of each 5β-cholestanoic acid to that of the corresponding I.S. against the weight ratio of 5β-cholestanoic acid to the I.S. The calibration graphs for unconjugated DHCA and THCA were drawn using 10 ng of each of the corresponding I.S. They were linear in the range of 1:100–100:1 for DHCA ($y=1.0375x-0.0001$; $r=0.9989$, $n=10$)

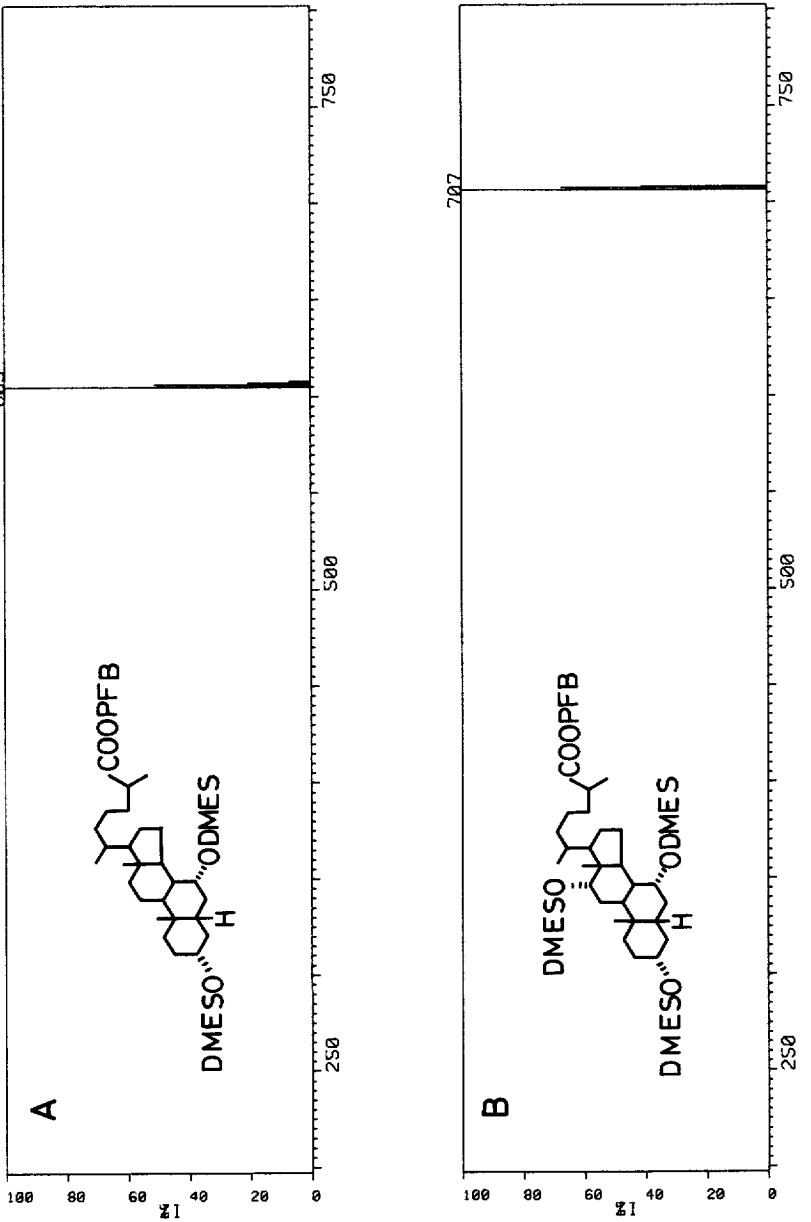

Fig. 1. Isobutane NICI mass spectra of the PFB ester–DMES ethers of (A) DHCA and (B) THCA.

TABLE II

RATIOS OF UNLABELLED TO LABELLED FRAGMENTS IN STABLE ISOTOPE-LABELLED 5β-CHOLESTANOIC ACIDS

Compound	Peak-height ratio	
	m/z 605/611	*m/z* 707/716
[3,7-($^{18}O_2{}^2H_2$)$_2$]DHCA	0.0005	
[3,7-($^{18}O_2{}^2H_2$)$_2$]GlycoDHCA	0.0006	
[3,7-($^{18}O_2{}^2H_2$)$_2$]TauroDHCA	0.0006	
[3,7,12-($^{18}O_2{}^2H_2$)$_3$]THCA		<0.0002
[3,7,12-($^{18}O_2{}^2H_2$)$_3$]GlycoTHCA		<0.0002
[3,7,12-($^{18}O_2{}^2H_2$)$_3$]TauroTHCA		<0.0002

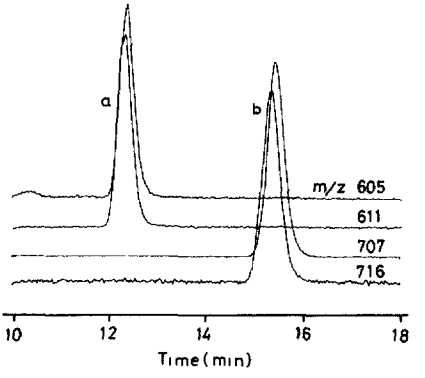

Fig. 2. NICI selected ion recording of 5β-cholestanoic acids as the PFB ester–DMES ether derivatives. (a) DHCA; (b) THCA.

and 1:200–500:1 for THCA ($y=1.1985x+0.0003$; $r=0.9995$, $n=10$). Almost the same calibration graphs were obtained for glycine and taurine conjugates. A typical selected ion recording of a synthetic mixture of unconjugated DHCA and THCA is illustrated in Fig. 2, where the monitoring ions were *m/z* 605/611 for DHCA and *m/z* 707/716 for THCA. Approximately 100 fg of each 5β-cholestanoic acid could be detected at a signal-to-noise ratio of 10.

Hydrolytic cleavage of glycine and taurine conjugates

It is known that the hydrolytic cleavage of conjugated C_{27} bile acids needs more drastic conditions than that of C_{24} bile acids [3,15,16]. Suitable conditions for hydrolysis of amide bonds with glycine and taurine were therefore investigated. Taurine-conjugated DHCA and THCA (10 ng of each) were dissolved in 4 *M* sodium hydroxide solution (4 ml) together with taurine-conjugated chenodeoxycholic acid and cholic acid (10 ng of each), and heated at

120°C at a pressure of 1.2 kg/cm^2. At a certain time, corresponding unconjugated $^{18}O^2H$-labelled bile acids were added to the reaction mixture as I.S. and, following derivatization to the PFB ester–DMES ethers, the yields of deconjugated 5β-cholestanoic acids were determined by GC–SIM. As illustrated in Fig. 3, the hydrolytic cleavage of taurine-conjugated 5β-cholestanoic acids was completed in 10 h, whereas taurine conjugates of common bile acids were quantitatively hydrolysed within 1 h. Almost the same result was obtained for glycine conjugates. When conjugated DHCA and THCA were subjected to enzymatic hydrolysis with cholylglycine hydrolase, the deconjugated C_{27} bile acids were hardly liberated [3,16].

Determination of 5β-cholestanoic acids in urine

A standard procedure for the determination of 5β-cholestanoic acids in urine is shown in Fig. 4. After addition of the I.S., a urine sample was extracted with a Sep-Pak C_{18} cartridge and then subjected to the group separation into the

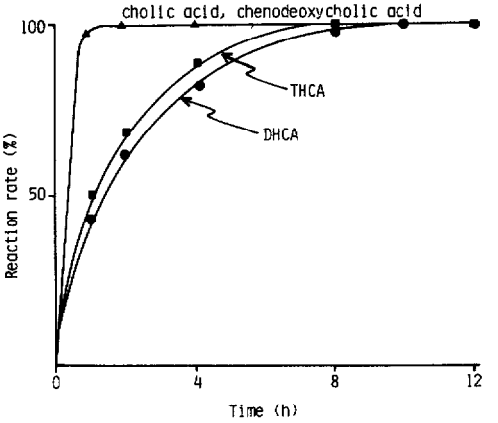

Fig. 3. Time courses for alkaline hydrolysis of taurine-conjugated bile acids.

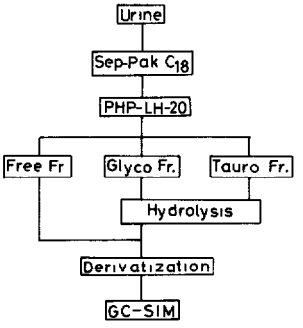

Fig. 4. Procedure for determination of 5β-cholestanoic acids in human urine.

unconjugated and glycine- and taurine-conjugated fractions by ion-exchange chromatography on PHP-LH-20 prior to deconjugation. Following hydrolysis of amide bonds, the 5β-cholestanoic acids in each fraction were derivatized into the PFB ester–DMES ethers and separated into DHCA and THCA on a capillary column coated with 5% phenylmethylsilicone, monitoring the characteristic negative ions $[M-181]^-$.

Applying the standard procedure to human urine, 5β-cholestanoic acids were determined with satisfactory reproducibility. Known amounts of unconjugated and glycine- and taurine-conjugated DHCA and THCA were added to urine from a healthy volunteer, and their recoveries were determined. As indicated in Table III, all 5β-cholestanoic acids were recovered at a rate of more than 90%. The simultaneous determination of 5β-cholestanoic acids was then carried out on a urine specimen from a patient with Zellweger syndrome. The results obtained are given in Table IV. A typical selected ion recording of the unconjugated fraction is illustrated in Fig. 5. The peaks of DHCA and THCA on the chromatogram represent approximately 30 pg and 1 ng, respectively, as amounts injected. Consisting principally of the (25R)-epimer [12], 5β-cholestanoic acids in the urine specimen show shorter retention times than the corresponding I.S.

It has been reported that a trace amount of THCA is detectable in bile specimens from healthy subjects [17]. Therefore, the separation and characterization of 5β-cholestanoic acids in urine from healthy volunteers were carried out. After addition of unconjugated [$^{18}O^2H$]DHCA and -THCA (500 ng of each) as carriers, a urine sample was subjected to extraction and deconjugation as described above. The deconjugated 5β-cholestanoic acids were purified by ion-exchange chromatography on PHP-LH-20 followed by HPLC on a reversed-phase column [9]. Subsequently, the 5β-cholestanoic acids were deri-

TABLE III

RECOVERY OF UNCONJUGATED AND CONJUGATED 5β-CHOLESTANOIC ACIDS ADDED TO URINE FROM A HEALTHY SUBJECT

5β-Cholestanoic acid	Added (ng per 100 μl)	Found (ng per 100 μl)[a]	Recovery (mean ± S.D., $n=10$) (%)
DHCA	10.0	9.67	96.7 ± 1.7
GlycoDHCA	10.0	9.84	98.4 ± 3.7
TauroDHCA	10.0	10.07	100.7 ± 2.3
THCA	100	98.8	98.8 ± 2.6
GlycoTHCA	100	93.0	93.0 ± 2.5
TauroTHCA	100	93.5	93.5 ± 1.4

[a]The total concentrations of DHCA and THCA in urine from the healthy subject were lower than 0.02 and 0.2 ng per 100 μl, respectively.

TABLE IV

AMOUNTS OF 5β-CHOLESTANOIC ACIDS IN HUMAN URINE DETERMINED BY THE PROPOSED METHOD

5β-Cholestanoic acid	Concentration (μg/ml)		
	A[a]	B[b]	C[b]
DHCA	0.23		
GlycoDHCA	0.18		
TauroDHCA	0.09		
Total	0.50	–	–
THCA	9.5		
GlycoTHCA	13.0		
TauroTHCA	6.7		
Total	29.2	0.0023	0.0012

[a]Urine specimen from a patient with Zellweger syndrome.
[b]Urine specimen from a healthy subject.

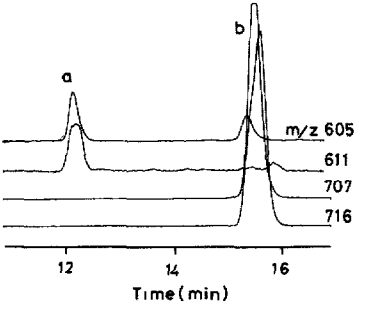

Fig. 5. NICI selected ion recording of unconjugated 5β-cholestanoic acids in urine from a patient with Zellweger syndrome as the PFB ester–DMES ether derivatives. (a) DHCA; (b) THCA.

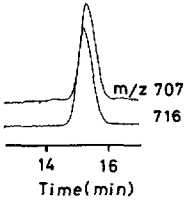

Fig. 6. NICI selected ion recording of THCA in urine from a healthy volunteer as the PFB ester–DMES ether derivative.

vatized to the PFB ester–DMES ethers and then subjected to GC–SIM. It is evident from the chromatogram in Fig. 6 that THCA is present in the urine from healthy subjects even though at an extremely low level. In contrast, the

dihydroxylated 5β-cholestanoic acid, DHCA, could not be detected (Table IV).

It is hoped that the availability of this method for the simultaneous determination of 5β-cholestanoic acids in biological fluids with satisfactory sensitivity and reliability may provide a much more precise knowledge of the metabolic profile of bile acids and be useful in the diagnosis of hepatobiliary diseases.

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