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CCLIV. Gas chromatographic–mass spectrometric determination of 4- and 6-hydroxylated bile acids in human urine with negative ion chemical ionization detection

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ABSTRACT

A method for the determination of 4- and 6-hydroxylated bile acids with a *vicinal* glycol moiety in human urine by gas chromatography–mass spectrometry with negative ion chemical ionization detection is described. The 4β-hydroxylated bile acids labelled with ^{18}O and ^{2}H were prepared as internal standards by the exchange reaction of the carbonyl group on the steroid nucleus with $H_2{}^{18}O$, followed by metal deuteride reduction. Bile acids in urine were extracted with a Sep-Pak C_{18} cartridge and, after solvolysis and hydrolysis of the conjugated forms, transformed into pentafluorobenzyl ester diethylhydrogensilyl ether diethylsilylene derivatives. Subsequent resolution into individual 4- and 6-hydroxylated bile acids was carried out on a cross-linked fused-silica capillary column; a characteristic carboxylate anion, $[M-PFB]^-$, was used to monitor each bile acid in the negative ion mode. The newly developed method was applied to the separation and determination of 4- and 6-hydroxylated bile acids in a urine specimen from a newborn infant.

INTRODUCTION

Bile acids, synthesized from cholesterol in the liver, are excreted into the duodenum via the bile duct as their glycine and taurine conjugates, and converted in part into secondary bile acids by intestinal bacteria. Bile acids assist the lipolysis and absorption of fats by the formation of mixed micelles in the intestinal lumen. In recent years, further metabolized bile acids with a *vicinal* glycol structure at C-3,4 or C-6,7 have been found in patients with liver diseases and in newborn infants and fetuses [1,2]. A reliable method is, therefore, urgently required for the determination of these unusual bile acids in biological fluids in connection with the diagnosis of hepatobiliary diseases.

Among various methods, gas chromatography–mass spectrometry (GC–MS) is well recognized as a powerful tool for the profile analysis of trace compounds in biological materials. Bile acids are usually converted into methyl ester trimethylsilyl (TMS) [3,4] or dimethylethylsilyl (DMES) [5,6] ether derivatives prior to the GC separation and then detected by MS in the electron impact ionization mode. However, the com-

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plete separation of bile acids, including unusual ones, with satisfactory sensitivity is still unavailable. In the previous studies, we demonstrated that the introduction of a pentafluorobenzyl (PFB) group into the carboxyl function would be most favourable for the formation of a characteristic carboxylate anion, $[M-PFB]^-$, in the negative ion chemical ionization (NICI) mode, permitting the highly sensitive detection of trace compounds [7,8]. It was also clarified that on treatment with a novel silylating agent, N,O-bis-(diethylhydrogensilyl)trifluoroacetamide(DEHS-BSTFA), bile acids with an isolated hydroxyl group were readily converted into the corresponding diethylhydrogensilyl (DEHS) ethers, whereas those with a *vicinal* glycol (except for the diaxial one) were transformed into cyclic diethylsilylene (DES) derivatives, leading to the efficient GC separation of these bile acids [9].

This paper deals with the separation and determination of 4- and 6-hydroxylated bile acids with a 1,2-glycol moiety in biological fluids by GC–NICI–MS using stable-isotope-labelled bile acids as internal standards.

EXPERIMENTAL

Gas chromatography–mass spectrometry

Capillary GC–MS was carried out using an MM12030 quadrupole mass spectrometer (VG Analytical, Manchester, UK) interfaced to an HP 5790A gas chromatograph (Hewlett-Packard, Avondale, PA, USA). Isobutane was used as a reagent gas. A cross-linked 5% phenylmethyl silicone fused-silica capillary column (20 m × 0.3 mm I.D.) (J & W Scientific, Folsom, CA, USA) was inserted into the ion source through the direct inlet. The carrier gas was helium at a linear velocity of 70 cm/s. The test samples were injected through a Van den Berg solventless injector with an inlet pressure of 0.8 kg/cm^2. The injection port, column oven and ion source were kept at 280, 285 and 270°C, respectively. The ionization energy was 70 eV, and the emission current was 400 μA.

Materials

Cholic, chenodeoxycholic, deoxycholic and lithocholic acids (CA, CDCA, DCA and LCA, respectively) were purchased from Sigma (St. Louis, MO, USA) and ursodeoxycholic acid (UDCA) was kindly donated by Tokyo Tanabe (Tokyo, Japan). Other bile acids [10–12] and ^{18}O,^2H-labelled CA, CDCA, DCA and LCA [8] were prepared in these laboratories by the methods previously reported. The Sep-Pak C_{18} cartridge was supplied by Waters Chromatography Div., Millipore (Milford, MA, USA) and washed successively with ethanol, water, 5% (w/v) aqueous bovine serum albumin solution and then water prior to use. N,O-Bis(diethylhydrogensilyl)trifluoroacetamide (DEHS-BSTFA) [13], piperidinohydroxypropyl Sephadex LH-20 (PHP-LH-20) (acetate form, 0.6 mequiv./g) [14] and carboxymethyl Sephadex LH-20 (CM-LH-20) (K^+ form, 1.0 mequiv./g) [15] were prepared by the known methods. Dimethylethylsilyl (DMES) imidazole was obtained from Tokyo Kasei Kogyo (Tokyo, Japan). All other chemicals were of analytical-reagent grade. All glassware used was silanized with trimethylchlorosilane.

Preparation of ^{18}O,^2H-labelled 4β-hydroxylated bile acids

To a solution of methyl 3α,4β,7α-trihydroxy-5β-cholanoate [11] (50 mg) in acetone (5 ml) were added *p*-toluenesulphonic acid (15 mg) and molecular sieves 4Å (1 g), and the mixture was refluxed for 4 h. After removal of the precipitate by filtration, the filtrate was evaporated to dryness under vacuum. The residue obtained was redissolved in methylene chloride and washed successively with 5% $NaHCO_3$ and water. The organic layer was concentrated and the resulting oily product was subjected to column chromatography on silica gel (2 g). To a solution of the 3,4-acetonide (20 mg) in pyridine (0.5 ml) was added pyridine (0.5 ml)–chromium trioxide (500 mg) complex, and the whole was stirred for 1 h. After extraction with ethyl acetate, the organic layer was washed successively with 5% $NaHSO_3$, 5% H_2SO_4, 5% $NaHCO_3$ and water, and evaporated down to give the 7-oxo compound. The product was further subjected to acidic and then alkaline hydrolysis to remove the protecting groups at C-3,4 and C-24 in the usual manner, yielding 3α,4β-dihydroxy-7-oxo-5β-cholanoic

acid (10 mg). In similar fashion, 3α,4β-dihydroxy-7,12-dioxo-5β-cholanoic acid was also prepared from methyl 3α,4β,7α,12α-tetrahydroxy-5β-cholanoate [11]. Their structures were confirmed by means of ^1H NMR spectroscopy.

Mono- and dioxo-5β-cholanoic acids thus obtained were then transformed into the corresponding potassium salts by using a lipophilic ion-exchange gel, CM-LH-20, in the manner previously reported [8]. Each 6-mg portion of the salt was dissolved in $H_2{}^{18}O$ (isotopic purity of 97 atom%, 100 μl) and heated at 90°C for 50 h. The resulting solution was stirred with NaB^2H_4 (isotopic purity of 97 atom%, 2 mg) for 30 min under ice-cooling and then poured into 5% HCl. Bile acids were extracted with ethyl acetate, and the organic layer was washed with water. The ^{18}O,^2H-labelled bile acids obtained were purified by high-performance liquid chromatography (HPLC) on a reversed-phase column, Cosmosil $5C_{18}$ (5 μm, 150 × 4 mm I.D.) (Nacalai Tesque, Kyoto, Japan), employing 0.3% potassium phosphate buffer (pH 7.0)–acetonitrile (11:4, v/v) as the mobile phase.

Procedure for the determination of 4- and 6-hydroxylated bile acids in urine

To a urine specimen (100 μl) were added [^{18}O, ^2H]-labelled CA, CDCA, DCA, LCA and 4β-hydroxylated bile acids (each 10–100 ng) as internal standards, and the whole was diluted with 4 ml of 0.5 *M* sodium phosphate buffer (pH 7.0) and applied to a Sep-Pak C_{18} cartridge. The cartridge was washed with water (5 ml), and the bile acids were eluted with 90% ethanol (5 ml). The dried eluate was subjected to solvolysis with ethanol–acetone (1:9, v/v, adjusted to pH 1 with conc. HCl) at 37°C for 3 h [16], and then alkaline hydrolysis with 4 *M* NaOH at 120°C for 3 h in the usual manner. The reaction mixture was neutralized and applied to a Sep-Pak C_{18} cartridge in the manner described above for removal of inorganic salts. The eluate was passed through a column (18 × 6 mm I.D.) of PHP-LH-20 (100 mg) and, after a washing step with 90% ethanol (5 ml), bile acids were eluted with 0.1 *M* acetic acid in 90% ethanol (5 ml) [17]. To the dried residue 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 previously washed with ethanol. The PFB esters were eluted with ethanol–acetonitrile (1:1, v/v) (5 ml). The dried eluate was subjected to silylation with DEHS-BSTFA (50 μl) in pyridine (50 μl) at room temperature for 1 h. After evaporation under a stream of nitrogen, the residue was dissolved in hexane (200–500 μl) and a 1-μl aliquot of the solution was injected into the GC–MS system.

RESULTS AND DISCUSSION

Gas chromatographic separation of 4- and 6-hydroxylated bile acids

A reliable method for the determination of unusual bile acids with a *vicinal* glycol structure at C-3,4 or C-6,7 should avoid interferences due to common bile acids which exist predominantly in urine. In the previous paper, we reported that, on treatment with the novel silylating agent DEHS-BSTFA, isolated hydroxyl groups of bile acids were readily converted into diethylhydrogensilyl (DEHS) ethers, whereas *vicinal* glycols (except for those of diaxial nature) were transformed into

TABLE I

RETENTION TIMES OF PFB ESTER DEHS ETHER AND/OR DES DERIVATIVES OF BILE ACIDS RELATIVE TO CHOLIC ACID (CA)

Bile acid	RRT	Monitoring ion (*m/z*)
3α(LCA)	0.492	461
3α,4β	0.548	475
3α,12α(DCA)	0.662	563
3α,7α(CDCA)	0.736	563
3α,7β(UDCA)	0.820	563
3α,4β,12α	0.699	577
3α,4β,7α,	0.876	577
3α,6α,7β	0.953	577
3α,6β,7β	1.061	577
3α,6α,7α	1.164	577
3α,6β,7α	0.954	665
3α,7α,12α(CA)	1.000 (15.3 min)	665
3α,4β,7α,12α	1.192	679
3α,6α,7α,12α	1.423	679
3α,6β,7β,12α	1.325	679

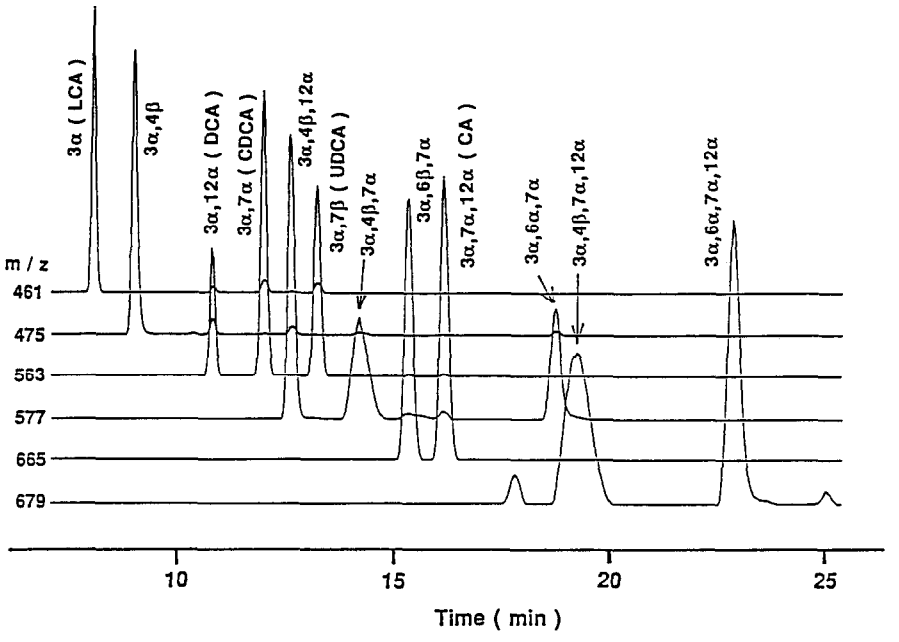

Fig. 1. NICI selected ion-recording of PFB ester DEHS ether and/or DES derivatives of bile acids.

cyclic diethylsilylene (DES) derivatives [9]. It is evident from the data listed in Table I that the PFB ester DEHS ether and/or DES derivatives of bile acids provide an efficient GC separation. In addition, these derivatives exhibited the characteristic carboxylate anion, $[M-PFB]^-$, as a base peak in the NICI mode, facilitating the highly sensitive detection of bile acids. A typical selected-ion recording of a synthetic mixture of representative bile acids is illustrated in Fig. 1. The monitoring ions were at *m/z* 475, 577 and 679 for di-, tri- and tetrahydroxylated bile acids with a vicinal glycol moiety, whereas those for common bile acids were at *m/z* 461, 563 and 665 for mono-, di- and trihydroxylated ones, respectively. Approximately 50 fg of 3α,4β,7α-trihydroxy-5β-cholanoic acid could be detected at a signal-to-noise ratio of 10.

Preparation of stable-isotope-labelled bile acids with the 4β-hydroxyl group

For trace analysis with high accuracy, specificity and sensitivity, the use of the stable-isotope-labelled analogue as an internal standard is favourable. In previous work, 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, and their use for the trace analysis of common bile acids in liver tissue [8] and 5β-cholestanoic acids in urine [18]. Accordingly, the present method was applied to the preparation of 4β-hydroxylated bile acids labelled with stable isotopes as shown in Fig. 2. The *vicinal* glycol group of 4β-hydroxylated bile acids [11] was converted into the corresponding acetonide, and intact hydroxyl groups at C-7 and C-12 were then oxidized with the pyridine–chromium trioxide complex. After removal of the protecting groups, oxo bile acids were transformed into potassium salts, dissolved in $H_2{}^{18}O$, and heated at 90°C for 50 h. Further labelling with ^2H was then performed. After derivatization to the PFB ester DMES ethers [8], the heavy isotopes in the substrates were determined by GC with selected-ion monitoring (SIM) using a characteristic ion, $[M-PFB]^-$. The ratios of the unlabelled to the fully labelled fragments formed from [^{18}O,^2H]tri- and tetrahydroxylated bile acids were found to be 1/300 and 1/2000, respectively (Table II). No loss of labelled isotopes occurred during alkaline hydrolysis or solvolysis.

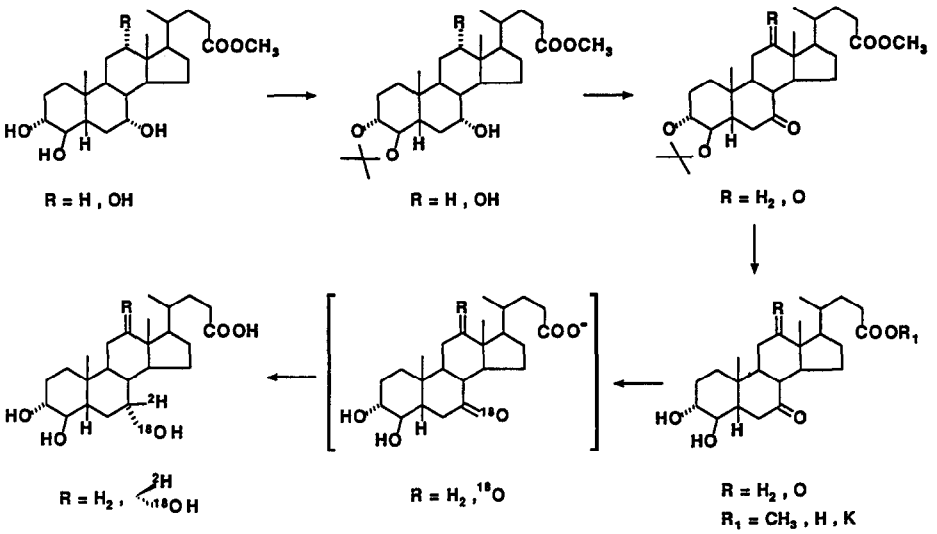

Fig. 2. Preparation of [7-^{18}O,^{2}H]3α,4β,7α-trihydroxy- and [7,12-$^{18}O_2$,$^{2}H_2$]3α,4β,7α,12α,-tetrahydroxy-5β-cholanoic acids.

Determination of 4- and 6-hydroxylated bile acids in urine

A standard procedure for the determination of 4- and 6-hydroxylated bile acids in urine is shown in Fig. 3. After addition of the internal standard, the urine specimen was extracted with a Sep-Pak C_{18} cartridge and then subjected to solvolysis. The deconjugated bile acids were purified by passing through a colum of lipophilic ion-exchange gel, PHP-LH-20 [17], and then derivatized into PFB ester DEHS ether DES derivatives. Subsequent resolution into individual bile acids was carried out by GC on a capillary column coated with 5% phenylmethyl silicone, and a characteristic carboxylate ion, $[M-PFB]^-$, was monitored. By applying the standard procedure to human urine, bile acids were determined with satisfactory reproducibility. Known amounts of 4- and 6-hydroxylated bile acids were added to human urine from a healthy volunteer, and their recoveries were estimated. As shown in Table III, the recoveries of almost all bile acids were more than 90%. Simultaneous determination of 4- and 6-hydroxylated bile acids was then carried out with a urine specimen from a newborn infant. A typical selected-ion recording is illustrated in Fig. 4. The peaks of 3α,4β,7α-trihydroxy-5β-cholanoic acid, 3α,6α,7α,12α-tetrahydroxy-5β-cholanoic acid and cholic acid on the chromatogram are equivalent to *ca.* 2 pg, 20 pg, and 1 ng injected, respectively.

TABLE II

RATIOS OF UNLABELLED TO LABELLED FRAGMENTS FORMED FROM STABLE-ISOTOPE-LABELLED BILE ACIDS

Bile acid	Peak-height ratio	
	m/z 665/668	*m/z* 767/773
[7-^{18}O,^{2}H]3α,4β,7α-$(OH)_3$	0.003	
[7,12-$^{18}O_2$,$^{2}H_2$]3α,4β,7α,12α-$(OH)_4$		<0.0005

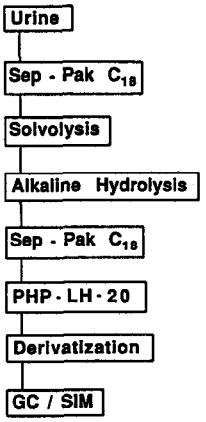

Fig. 3. Procedure for the determination of 4- and 6-hydroxylated bile acids in human urine.

TABLE III

RECOVERY OF UNCONJUGATED BILE ACIDS ADDED TO HUMAN URINE

Bile acid	Urine (ng/100 μl)	Added (ng/100 μl)	Found (ng/100 μl)	Recovery (% ± S.D.)[a]
3α,4β	–	1.90	1.69	88.9 ± 7.5
3α,4β,7α	–	2.05	2.01	98.0 ± 4.3
3α,4β,12α	–	2.00	1.88	94.0 ± 6.3
3α,6α,7α	<0.01	2.14	2.01	93.9 ± 5.8
3α,6α,7β	–	1.95	1.76	90.3 ± 6.4
3α,6β,7β	–	2.04	1.85	90.7 ± 6.7
3α,4β,7α,12α	–	1.89	1.80	96.8 ± 4.2
3α,6α,7α,12α	<0.01	1.97	1.81	91.9 ± 6.9
3α,6β,7β,12α	–	2.10	1.90	90.5 ± 7.3

[a] $n = 8$.

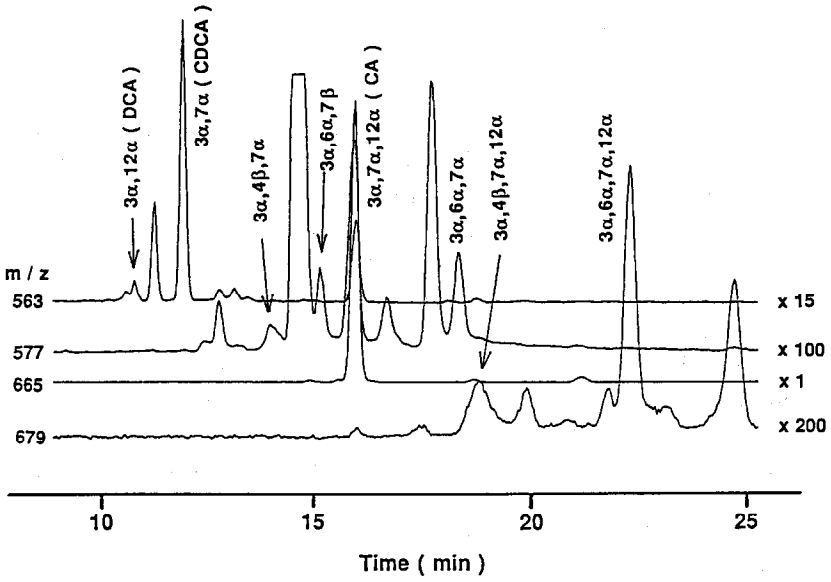

Fig. 4. NICI selected-ion recording of bile acids in human urine as PFB ester DEHS ether and/or DES derivatives.

It is hoped that the availability of an excellent method for the simultaneous determination of bile acids with a *vicinal* glycol in biological fluids with satisfactory sensitivity and reliability may provide much more precise knowledge on the metabolic profile of bile acids and serve as a diagnosis for hepatobiliary diseases.

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