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Determination of 3-oxo-Δ^4- and 3-oxo-$\Delta^{4,6}$-bile acids and related compounds in biological fluids of infants with cholestasis by gas chromatography–mass spectrometry

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Abstract

A method has been developed for the determination of 3-oxo-Δ^4- and 3-oxo-$\Delta^{4,6}$-bile acids and related bile acids in biological fluids of infants by gas chromatography–mass spectrometry (GC–MS) of the methyl ester–dimethylethylsilyl ether–methoxime derivatives. The 7α-hydroxylated 3-oxo-Δ^4-bile acids were partially dehydrated to give the 3-oxo-$\Delta^{4,6}$-bile acids by trimethylsilyl or dimethylethylsilyl derivatization and other pretreatments under acidic or alkaline conditions for GC–MS analysis. To prevent dehydration, the 3-oxo-Δ^4-bile acids were derivatized to the oximes by treatment with O-methylhydroxylamine prior to pretreatments such as solid-phase extraction, enzymatic solvolysis and hydrolysis of the conjugates, and silylation with dimethylethylsilylimidazole. Calibration curves for the bile acids were linear over a range of 5–250 ng and the detection limit was 100 pg for each 3-oxo-Δ^4-bile acid. Recoveries of the bile acids and their glycine and taurine conjugates from bile acid-free urine and serum ranged from 94.2 to 105.9% of their added amounts. The bile acids in urine and serum of four patients with severe cholestatic liver disease were measured by the analytical method, and the 3-oxo-Δ^4-bile acids were determined to be the major bile acids (59–68%) in the urines associated with 3-oxo-Δ^4-steroid 5β-reductase deficiency or dysfunction.

Keywords: Oxobile acids; Bile acids

1. Introduction

Clayton et al. [1] found that 7α,12α-dihydroxy-3-oxo-4-cholenoic acid (CA-Δ^4-3-one) and 7α-hydroxy-3-oxo-4-cholenoic acid (CDCA-Δ^4-3-one) are the major bile acids excreted in the urine of some children with severe liver disease. Setchell et al. [2] described a newborn error in bile acid biosynthesis that caused 3-oxo-Δ^4-steroid 5β-reductase deficiency. The defect was found in identical twins with neonatal hepatitis and was characterized by increased amounts of unsaturated bile acids having a 3-oxo-Δ^4-structure and 5α-configurational allo-bile acids. Other studies suggested a relationship between the excretion of 3-oxo-Δ^4-bile acids and liver disease [3–5]. Furthermore, a 3-oxo-4,6-choladienoic acid,

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which is like the dehydrated product of the 3-oxo-Δ^4-bile acid, was detected as one of the major bile acids in urine from patients excreting a large quantity of the 3-oxo-Δ^4-bile acids [1].

It is not clear whether the 3-oxo-$\Delta^{4,6}$-bile acids are endogenous products or artifacts resulting from sample pretreatment. Gas chromatography–mass spectrometry (GC–MS) has been used for the identification and determination of 3-oxo-Δ^4-bile acids in biological fluids, and some modifications were carried out in the pretreatment steps, such as the use of enzymatic methods for the deconjugation of glycine and taurine amides instead of chemical hydrolysis, because of the lability that occurred under acidic and alkaline conditions [2]. However, the lability observed under the conditions for silyl derivatization also seems likely to disturb the quantitative determination of both 3-oxo-Δ^4- and 3-oxo-$\Delta^{4,6}$-bile acids.

In a previous paper [6], we described a highly sensitive method for the determination of fetal bile acids in biological fluids from neonates by GC–MS with negative ion chemical ionization. We now report on the development of a novel, reliable GC–MS method for the determination of the 3-oxo-Δ^4- and 3-oxo-$\Delta^{4,6}$-bile acids, together with common and fetal bile acids, in biological fluids of infants. Our new method employs stable methoxyimino derivatization prior to solid-phase extraction, enzymatic deconjugation and derivatization to the methyl ester–dimethylethylsilyl ether as pretreatments prior to analysis. The method was used to measure the urinary and serum bile acid levels, including those of 3-oxo-Δ^4- and 3-oxo-$\Delta^{4,6}$-bile acids, in normal infants and patients with cholestatic liver disease, as reported briefly in previous papers [7,8].

2. Experimental

2.1. Materials and reagents

Cholic acid (CA, **1**), chenodeoxycholic acid (CDCA, **2**), deoxycholic acid (DCA, **3**), lithocholic acid (LCA, **4**), hyocholic acid (3α,6α,7α-trihydroxy-5β-cholanoic acid, HCA, **13**) and ursodeoxycholic acid (UDCA, **17**) were purchased from Sigma (St. Louis, MO, USA). The following bile acids were synthesized in our laboratory as previously described, 1β,3α,7α,12α-tetrahydroxy-5β-cholanoic acid (CA-1β-ol, **5**) [9], 2β,3α,7α,12α-tetrahydroxy-5β-cholanoic acid (CA-2β-ol, **6**) [10], 3α,4β,7α,12α-tetrahydroxy-5β-cholanoic acid (CA-4β-ol, **7**) [11], 3α,6α,7α,12α-tetrahydroxy-5β-cholanoic acid (CA-6α-ol, **8**) [12], 3α,7α,12α,19-tetrahydroxy-5β-cholanoic acid (CA-19-ol, **9**), [13], 1β,3α,7α-trihydroxy-5β-cholanoic acid, (CDCA-1β-ol, **10**) [9], 2β,3α,7α-trihydroxy-5β-cholanoic acid, (CDCA-2β-ol, **11**) [10], 3α,4β,7α-trihydroxy-5β-cholanoic acid (CDCA-4β-ol, **12**) [11], 1β,3α,12α-trihydroxy-5β-cholanoic acid (DCA-1β-ol, **14**) [9], 3α,6α,12α-trihydroxy-5β-cholanoic acid (DCA-6α-ol, **15**) [12], 3α,7β,12α-trihydroxy-5β-cholanoic acid (UCA, **16**), 3β,7α,12α-trihydroxy-5β-cholanoic acid (CA-3β-ol, **18**), 3β,4β,7α,12α-tetrahydroxy-5β-cholanoic acid (CA-3β,4β-ol, **19**) [14], 3α,7α,12α-trihydroxy-5α-cholanoic acid (allo-CA, **20**) [15], 3α,7α-dihydroxy-5α-cholanoic acid (allo-CDCA, **21**), 3β-hydroxy-5-cholenoic acid (Δ^5-3β-ol, **22**), 3β,12α-dihydroxy-5-cholenoic acid (Δ^5-3β,12α-ol, **23**) [16], 7α,12α-dihydroxy-3-oxo-5β-chol-1-enoic acid (CA-Δ^1-3-one, **24**), 7α,12α-dihydroxy-3-oxo-4-cholenoic acid (CA-Δ^4-3-one, **25**) [17], 12α-hydroxy-3-oxo-4,6-choladienoic acid (CA-$\Delta^{4,6}$-3-one, **26**) [18], 7α-hydroxy-3-oxo-4-cholenoic acid (CDCA-Δ^4-3-one, **27**), 3-oxo-4,6-choladienoic acid (CDCA-$\Delta^{4,6}$-3-one, **28**) and 3α,7α-dihydroxy-24-nor-5β-cholan-23-oic acid (nor-CDCA, I.S.).

The silylating reagents, trimethylsilylimidazole (TMSI), dimethylethylsilylimidazole (DMESI), hexamethyldisilazane (HMDS), trimethylchlorosilane (TMCS), N,O-bis(trimethylsilyl)acetamide (BSA) and O-methylhydroxylamine hydrochloride were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Other reagents were obtained from Wako Chemicals (Osaka, Japan) unless otherwise noted, and were of analytical grade.

2.2. Collection of biological samples

The experimental groups were made up of four patients with cholestasis of unknown origin (1–2 months old) [8,9] and ten healthy infants (1–2 months old). The urine and serum samples were collected from these infants under the informed

consent of parents and stored at −25°C before analysis.

2.3. Gas chromatography–mass spectrometry (GC–MS)

GC–MS was performed on a JEOL JMS-AM150 instrument (JEOL, Tokyo, Japan). The following operating conditions were used: a gas chromatographic column DB-1 (30 m×0.2 mm I.D., fused-silica capillary column with bonded methylsilicon, J&W Scientific, Folsom, CA, USA); column temperature, programmed from 170 to 230°C at 10°C/min and 230 to 310°C at 3°C/min; the flow-rate of helium (the carrier gas) was 45 cm/s; the mass spectra were recorded at 70 eV of ionization energy with an ion source temperature of 290°C. The methyl ester–trimethylsilyl ether (Me–TMS), the methyl ester–dimethylethylsilyl ether (Me–DMES) and the Me–DMES–methoxime derivatives of bile acids were eluted between 24 and 37 min under the conditions described above.

2.4. Derivatization of bile acids for GC–MS analysis

2.4.1. Methyl ester–trimethylsilyl ether (Me–TMS) and methyl ester–dimethylethylsilyl ether (Me–DMES) derivatives

Each bile acid (2 μg) or mixture of bile acids was dissolved in 0.2 ml of methanol. The methyl ester of bile acids was prepared by reaction with diazomethane in diethyl ether at room temperature for 10 min. After removal of excess reagents, derivatization of the residue into the silyl ethers was carried out using the following silylating reagents: (1) DMESI (30 μl) at 60°C for 40 min, (2) TMSI (30 μl) at 40°C for 60 min, (3) a solution of pyridine–HMDS–TMCS (3:2:1, v/v/v; 30 μl) at 60°C for 30 min and (4) a solution of BSA–TMCS–TMSI (1:1:1, v/v/v; 30 μl) at room temperature for 10 min. Excess reagents were removed on a silica gel column (30×6 mm I.D.) equilibrated with *n*-hexane–ethyl acetate (3:1, v/v). The derivatized bile acids were recovered in the first 5 ml of effluent and the solvent was evaporated to dryness under reduced pressure. The residue was dissolved in *n*-hexane (50 μl) and an aliquot (1 μl) was injected into the GC–MS system.

2.4.2. Me–DMES–methoxime derivatives

The 3-oxo-Δ^4-bile acids were initially derivatized to the methoxime. Each bile acid (2 μg), or mixture of bile acids, was dissolved in distilled water (1.0 ml). An equal volume of pyridinium acetate buffer (1.5 *M*, pH 5) containing 1.0 *M* O-methylhydroxylamine hydrochloride was added to the solution and the mixture was kept at 37°C for 2 h. The bile acids were extracted from the solution using a Bond Elut C_{18} cartridge (3 ml, Varian, Harbor City, CA, USA). The cartridge was washed with water (5 ml) and the bile acids were eluted with ethanol (5 ml). After evaporation of the solvents, the residue was dissolved in 1 ml of 90% aqueous ethanol. The solution was applied to a piperidinohydroxypropyl Sephadex LH-20 (PHP-LH-20) [19] column (30×6 mm I.D.) that was equilibrated with 90% aqueous ethanol. The column was washed with 90% ethanol (4 ml) to remove neutral compounds and the bile acids were eluted with 0.1 *M* acetic acid in 90% ethanol (5 ml). After evaporation, the purified bile acid methoximes were derivatized to the Me–DMES for GC–MS analysis as described above.

2.5. Quantitative analysis of bile acids in biological fluids of infants

In the standard procedure, sample preparation for GC–MS analysis was performed from human biological fluids as follows. Internal standard (nor-CDCA, 2 μg) was added to an infant urine or serum sample (0.2–1.0 ml). The 3-oxo-bile acids were derivatized to the methoximes, and the bile acids were extracted from the solution using a Bond Elut C_{18} cartridge as described in Section 2.4.2. After evaporation of the solvents, the residues were subjected to simultaneous enzymatic hydrolysis and solvolysis at 37°C for 12 h, using choloylglycine hydrolase (30 U, Sigma) and sulfatase type H-1, from *Helix pomatia* (150 U, Sigma), in 0.05 *M* sodium acetate buffer, pH 5.6 (200 μl), 0.6 m*M* dithiothreitol (200 μl), 0.05 *M* EDTA (200 μl) and distilled water (100 μl). The unconjugated bile acids formed were extracted again with a Bond Elut C_{18} cartridge. After evaporation of the solvents, the residue was dissolved in 1 ml of 90% aqueous ethanol. This solution was applied to a PHP-LH-20 column as described above. After evaporation,

purified bile acids were derivatized to the Me–DMES for GC–MS analyses.

2.6. Analysis of bile acids by GC–MS using negative-ion chemical ionization

The biological samples from infants were treated with the above derivatization and quantitative procedure except for the esterification, in which the pentafluorobenzyl ester was used instead of the methyl ester in the Me–DMES–methoxime derivatives. GC-MS in the negative ion chemical ionization mode was performed by selected-ion monitoring of $[M-181]^-$ ions, m/z 604 for **25**, m/z 500 for **26**, m/z 502 for **27** and m/z 398 for **28** using the method developed previously [6].

3. Results and discussion

3.1. Derivatization of 7α,12α-dihydroxy-3-oxo-4-cholenoic (CA-Δ^4-3-one) and 7α-hydroxy-3-oxo-4-cholenoic acids (CDCA-Δ^4-3-one)

Setchell et al. [2,20] reported the analysis of 3-oxo-Δ^4-bile acids using Me–TMS derivatives for GC–MS. The TMS derivatives of the bile acids were prepared by the addition of a solution of pyridine–HMDS–TMCS (3:2:1, v/v/v). We have used the TMS and DMES derivatives prepared by the addition of TMSI and DMESI, respectively, for the analysis of fetal bile acids in previous studies [21,22]. Unfortunately, the silyl ether derivatization of CA-Δ^4-3-one and CDCA-Δ^4-3-one, with a hydroxyl group at the 7α-axial position, gave partially the silyl ethers of dehydrated CA-Δ^4,6-3-one and CDCA-$\Delta^{4,6}$-3-one, respectively, as summarized in Table 1. As the 3-oxo-Δ^4-bile acids were silylated with TMSI or DMESI, the 3-oxo-$\Delta^{4,6}$-bile acids formed significantly at a rate of 24–35%. The silylation of 3-oxo-Δ^4-bile acids with pyridine–HMDS–TMCS (3:2:1, v/v/v) or BSA–TMCS–TMSI (1:1:1, v/v/v) moderately reduced the formation of dehydrated 3-oxo-$\Delta^{4,6}$-bile acids to a rate that ranged from 6 to 20% of the 3-oxo-Δ^4-bile acids (Table 1). Since the enzymatic formation of 3-oxo-$\Delta^{4,6}$-bile acids in vivo was proposed, [23–25], it was necessary to completely suppress the formation of 3-oxo-$\Delta^{4,6}$-bile acids through silyl ether derivatization for the 7α-hydroxylated 3-oxo-Δ^4-bile acids. Oxime formation of the oxo group on steroids, combined with the silylation of hydroxyl groups, has been widely used for the analysis of steroid hormones [26] and 3-oxo-bile acids [27,28]. When the derivatization of 3-oxo-

Table 1
Effect of pretreatment of 3-oxo-Δ^4-bile acids with O-methylhydroxylamine on the formation of 3-oxo-$\Delta^{4,6}$-bile acids

Bile acid	Derivative of functional groups		Formation of $\Delta^{4,6}$-3-one (%)
	Oxo	Hydroxyl	
CA-Δ^4-3-one	None	DMES ether[a]	27.9±0.8[e]
CDCA-Δ^4-3-one	None	DMES ether[a]	24.7±1.4
CA-Δ^4-3-one	None	TMS ether[b]	34.7±2.1
CDCA-Δ^4-3-one	None	TMS ether[b]	28.6±3.4
CA-Δ^4-3-one	None	TMS ether[c]	19.2±0.2
CDCA-Δ^4-3-one	None	TMS ether[c]	15.9±1.1
CA-Δ^4-3-one	None	TMS ether[d]	6.2±1.9
CDCA-Δ^4-3-one	None	TMS ether[d]	9.2±3.0
CA-Δ^4-3-one	Methoxime	DMES ether[a]	n.d.
CDCA-Δ^4-3-one	Methoxime	DMES ether[a]	n.d.

[a] Silylation with DMESI (30 μl) at 60°C for 40 min.
[b] Silylation with TMSI (30 μl) at 40°C for 60 min.
[c] Silylation with pyridine–HMDS–TMCS (3:2:1, v/v/v; 30 μl) at 60°C for 30 min.
[d] Silylation with BSA–TMCS–TMSI (1:1:1, v/v/v; 30 μl) at room temperature for 10 min.
3α,7α-Dihydroxy-24-nor-5β-cholan-23-oic acid (1.98 μg) was added as an internal standard to each bile acid (2.00 μg) for GC-MS analysis.
n.d. = not detected.
[e] Mean±S.D. ($n=4$).

Δ^4-bile acids with O-methylhydroxylamine was performed, the oxime derivatives were not fully converted into the 3-oxo-$\Delta^{4,6}$-bile acids under dimethylethylsilylation with DMESI, as shown in Table 1. Moreover, the oxime derivatives were quite stable during various purification steps, such as the extraction using a Bond Elut C_{18} cartridge and the deconjugation with sulfatase and choloylglycine hydrolase, as described above.

Therefore, direct derivatization of the 3-oxo-Δ^4-bile acids present in samples was employed as the first step in the pretreatment prior to analysis of bile acids in biological fluids.

3.2. GC–MS analysis of the 3-oxo-Δ^4- and 3-oxo-$\Delta^{4,6}$-bile acids and related compounds

The mass spectra of the Me–DMES–methoxime derivatives of the standard CA-Δ^4-3-one and CDCA-Δ^4-3-one are shown in Fig. 1. The molecular ion peaks of these compounds are present at m/z 619 and m/z 517, and the base peaks are found at m/z 296 and m/z 382 in their spectra, respectively. The characteristic fragment ions at m/z 590 and m/z 488 indicate a facile elimination of the ethyl group from the original molecules. Further loss of dimethylethylsilanol and a methoxy group gives rise to the fragment ions at m/z 380, $[M-2x104-31]^+$, and m/z 382, $[M-104-31]^+$, respectively. The mass spectra of the Me–DMES–methoxime derivatives of CA-$\Delta^{4,6}$-3-one and CDCA-$\Delta^{4,6}$-3-one are shown in Fig. 2. The derivative of CA-$\Delta^{4,6}$-3-one gave a base peak at m/z 296, $[M-104-\text{side chain}]^+$ and other prominent ion peaks at m/z 515, $[M]^+$ and m/z 411, $[M-104]^+$, and that of CDCA-$\Delta^{4,6}$-3-one gave a molecular ion peak at m/z 413 and low intensity peaks at m/z 382, $[M-31]^+$ and m/z 298, $[M-115]^+$.

The GC–MS data from these bile acids and related compounds are summarized in Table 2, which shows the relative retention times for the internal standard and characteristic fragment ions accompanied by their relative abundances. Fig. 3 shows a chromatogram obtained by selected-ion monitoring (SIM) of the characteristic fragments in the mass spectra of the Me–DMES or Me–DMES–methoxime derivatives of the standard 3-oxo-Δ^4- and 3-oxo-$\Delta^{4,6}$-bile acids, and related compounds. It can be seen that simultaneous determination of these bile acids could be achieved by SIM in GC–MS, even if chromatographic separation is not complete.

Calibration curves for the determination of the 3-oxo-Δ^4- and 3-oxo-$\Delta^{4,6}$-bile acids and related compounds were obtained by plotting the peak-area ratios between the monitoring ions for the determination of individual bile acids and that for the internal standard versus amounts of the corresponding bile acids. A linear relationship was found over the range of 5–250 ng for each bile acid (correlation coefficient, $r>0.999$) and the detection limit of CA-Δ^4-3-one was 100 pg. Furthermore, the combination of the above pretreatment and the GC–MS with negative-ion chemical ionization developed in the preceding paper [6] using the pentafluorobenzyl ester–DMES–methoxime derivatives instead of the methyl ester–DMES–methoxime derivatives in the above electronic ionization method gave a detection limit of 50 fg for CA-Δ^4-3-one. We need to select the ionization modes according to the aim of the analysis and the amounts of biological sample available, since the chemical ionization mode is somewhat complex and troublesome although it is very sensitive for use in routine analysis.

Recoveries of bile acids and their conjugates from urine and serum, relative to the internal standard, using this analytical method ranged from 94.2 to 105.9% of the added amount of their standard samples, as shown in Table 3. In the course of our studies, the use of 2-mercaptoethanol, which was usually used in deconjugation with choloylglycine hydrolase, reduced the recoveries of 3-oxo-$\Delta^{4,6}$-bile acids. As dithiothreitol did not affect the recoveries, it was used as a thiol compound instead of 2-mercaptoethanol.

3.3. Determination of the 3-oxo-Δ^4- and 3-oxo-$\Delta^{4,6}$-bile acids in urine and serum from patients with cholestasis

The analytical method developed above was used to determine the bile acids in urine from four patients with cholestasis of unknown origin and the results are summarized in Table 4, accompanied by the composition of bile acids in the urine of normal infants. Additionally, CA-Δ^4-3-one and CDCA-Δ^4-3-one were identified in the urine of patients (Fig. 4),

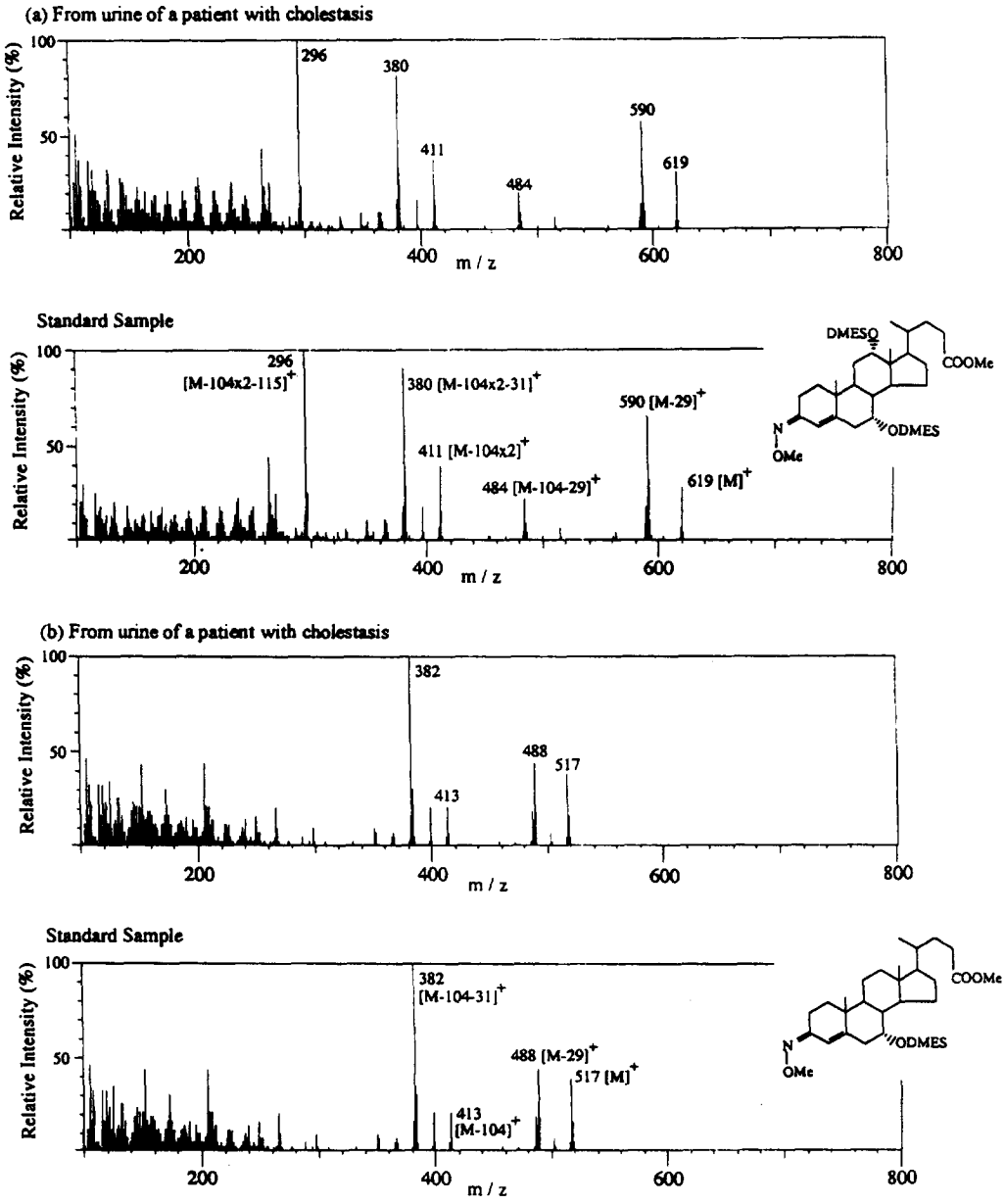

Fig. 1. Mass spectra of the Me–DMES–methoxime derivatives of 7α,12α-dihydroxy-3-oxo-4-cholenoic acid (a) and 7α-hydroxy-3-oxo-4-cholenoic acid (b) from the urine of a patient with cholestasis, and of their standard bile acids.

by comparing their mass spectra with those of standard samples, as shown in Fig. 1.

The levels of total bile acid and of the 3-oxo-Δ^4-bile acids (particularly CDCA-Δ^4-3-one) in the urine of these patients were markedly increased compared with those of normal infants. The proportion of 3-oxo-Δ^4-bile acids in the urine of patients was greater than that in the urine of normal infants (59–68% of total bile acids). Inversely, excretion of fetal bile acids (1β-, 2β-, 4β- and 6α-hydroxylated CA and CDCA) in patients decreased, in contrast to that found in normal infants. With regard to patients

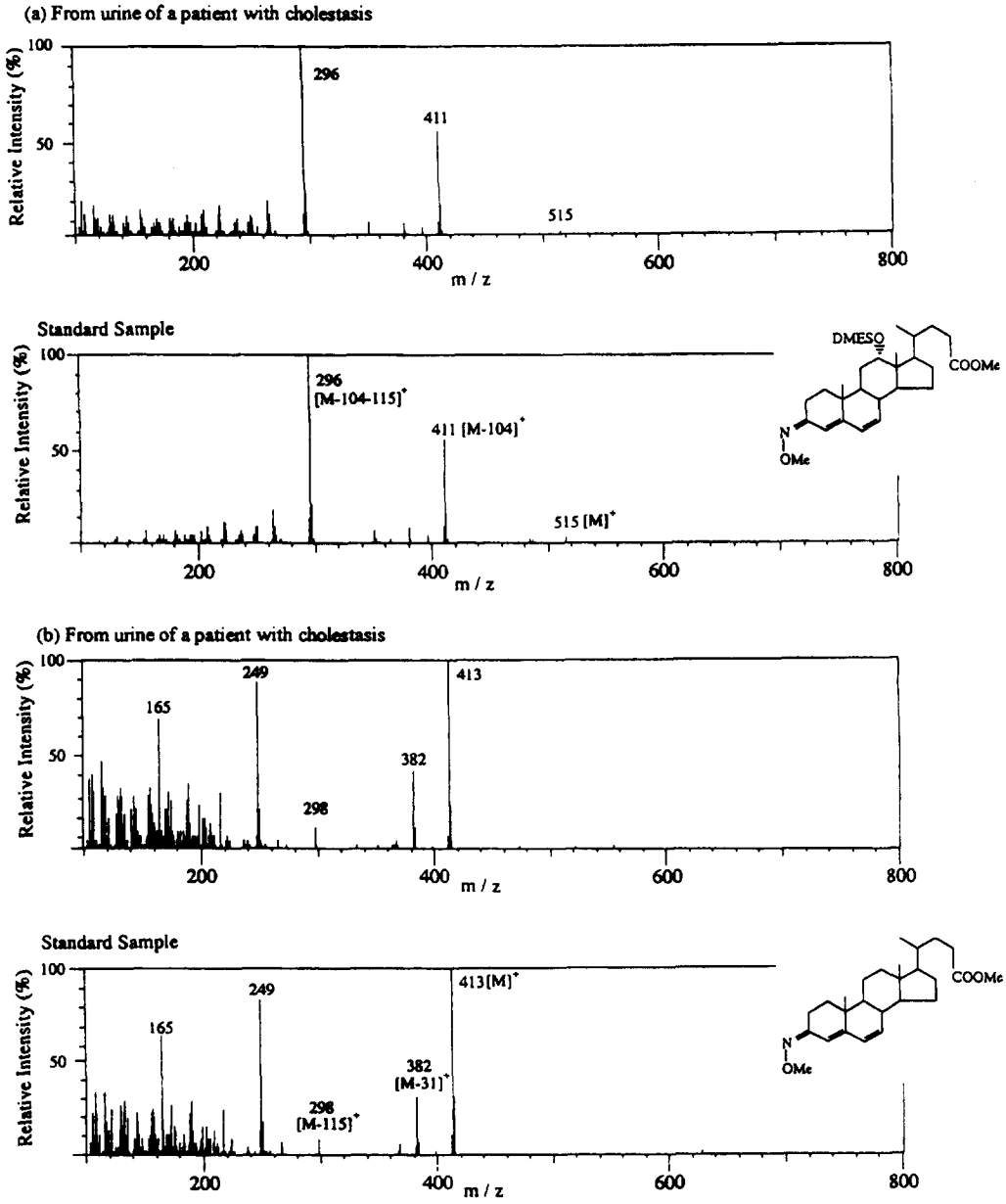

Fig. 2. Mass spectra of the Me–DMES–methoxime derivatives of 12α-dihydroxy-3-oxo-4,6-choladienoic acid (a) and 3-oxo-4,6-choladienoic acid (b) from the urine of a patient with cholestasis, and of their standard bile acids.

(patients 2 and 3) with increased excretion of CDCA, higher concentrations of CDCA-Δ^4-3-one were observed. The 3-oxo-$\Delta^{4,6}$-bile acids were also detected in the urine of patients, as shown in Figs. 2 and 4 and Table 4; however, it was not known whether these bile acids were biosynthesized in the liver or were produced artificially during the storage of urine. It should be pointed out that urine stored over a long period of time tends to show high levels of the 3-oxo-$\Delta^{4,6}$-bile acids.

Subsequently, the profiles of serum bile acids from the patients were also determined using the de-

Table 2
GC–MS data of the methyl ester-dimethylethylsilyl ether and methoxime derivatives of bile acids

No.	Bile acid	Relative retention time	Base peak *m/z*	Fragment ions, *m/z* (Relative intensity, %)
1	CA	1.19	651[a]	369(51), 253(42)
2	CDCA	1.10	445[a]	371(95), 355(10)
3	DCA	1.07	549[a]	255(87), 371(9)
4	LCA	0.95	447[a]	323(50), 372(45)
5	CA-1β-ol	1.33	245[a]	367(23), 471(10)
6	CA-2β-ol	1.44	367[a]	545(10), 357(5)
7	CA-4β-ol	1.40	367[a]	357(32), 471(15)
8	CA-6α-ol	1.31	367[a]	471(20), 545(10)
9	CA-19-ol	1.32	353[a]	457(52), 367(49)
10	CDCA-1β-ol	1.29	245[a]	369(18), 472(7)
11	CDCA-2β-ol	1.25	369[a]	337(13), 457(5)
12	CDCA-4β-ol	1.28	369[a]	329(45), 457(20)
13	HCA	1.24	369[a]	337(20), 547(7)
14	DCA-1β-ol	1.23	245[a]	651(21), 547(7)
15	DCA-6α-ol	1.22	651[a]	253(51), 369(24)
16	CA-3β-ol	1.16	651[a]	357(44), 369(30)
17	CA-3β,4β-ol	1.37	367[a]	327(44), 575(20)
18	UCA	1.21	651[a]	253(97), 357(34)
19	UDCA	1.13	549[a]	369(50), 459(30)
20	*allo*-CA	1.18	651[a]	357(31), 369(27)
21	*allo*-CDCA	1.09	371[a]	549(12), 355(10)
22	Δ^5-3β-ol	1.03	445[a]	331(88), 370(34)
23	Δ^5-3β,12α-ol	1.12	547[a]	253(69), 329(31)
24	CA-Δ^1-3-one	1.11	296[a]	380(40), 591(19)
25	CA-Δ^4-3-one	1.17	296	380[a](89), 590(68)
26	CA-$\Delta^{4,6}$-3-one	1.12	296[a]	411(59), 515(5)
27	CDCA-Δ^4-3-one	1.06	382[a]	488(47), 517(30)
28	CA-$\Delta^{4,6}$-3-one	0.98	413[a]	249(88), 382(35)
I.S.	nor-CDCA	1.00[b]	431[a]	

[a] Fragment ions used for selected-ion monitoring.
[b] Retention time is 25.56 min.

veloped GC–MS method and, in cases where there were insufficient amounts of serum, analysis was performed using the negative-ion chemical ionization mode of GC–MS that was reported previously [6]. The results are summarized in Table 5, together with the mean values of urinary bile acids found in patients. CDCA was the predominant bile acid in the serum (64.7% of total bile acids) compared with

Table 3
Recoveries of the bile acids and their conjugates from urine and serum

Bile acid	Urine		Serum	
	Added amount (μg)	Relative recovery (%)	Added amount (μg)	Relative recovery (%)
Glyco CA-Δ^4-3-one	2.01	99.9±3.5[a]	1.04	99.3±3.3[a]
Tauro CDCA-Δ^4-3-one	2.00	97.1±2.6	1.05	105.9±2.8
CA-$\Delta^{4,6}$-3-one	2.01	95.9±1.7	1.01	100.5±2.2
CDCA-Δ^4-3-one	2.05	94.2±1.5	2.05	99.7±2.7

3α,7α-Dihydroxy-24-nor-5β-cholanoic acid (1.98 μg) was added as an internal standard to urine and serum prior to GC–MS analysis.
[a] Mean±S.D. ($n=4$).

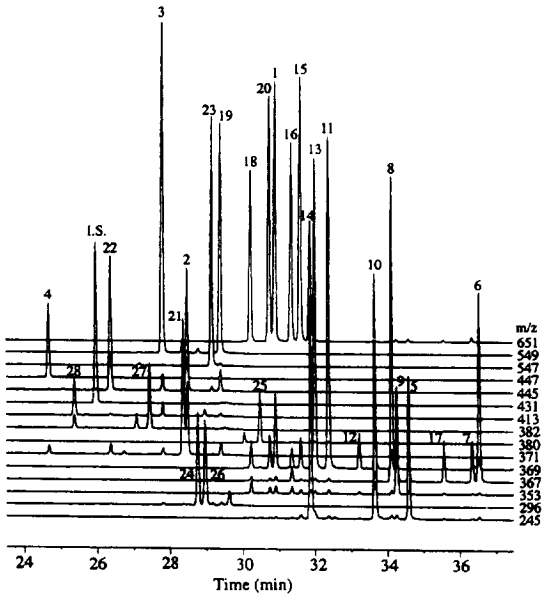

Fig. 3. Selected-ion chromatogram of standard 3-oxo-Δ^4- and 3-oxo-$\Delta^{4,6}$-bile acids and related compounds as Me–DMES–methoxime derivatives. Peak numbers and compounds are the same as in Table 2.

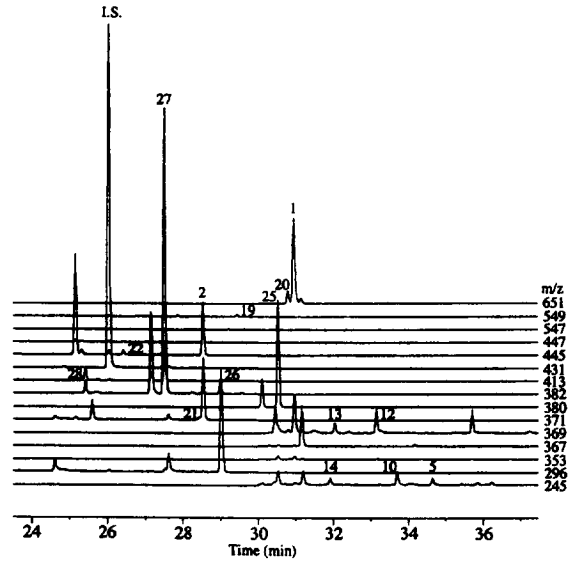

Fig. 4. Selected ion chromatogram of the Me–DMES–methoxime derivatives of bile acids in urine from a patient with cholestasis. Peak numbers and compounds as in Table 2.

Table 4
Concentration of bile acids in urine from patients with cholestasis and from normal infants

Bile acid	Patient 1		Patient 2[a]		Patient 3[a]		Patient 4		Normal infants	
	μg/ml	%	μg/ml	%	μg/ml	%	μg/ml	%	μg/ml	%
CA	28.27	30.2	1.57	5.3	0.65	4.7	0.50	6.8	0.13±0.16[b]	22.4
CDCA	1.95	2.1	9.20	31.2	2.16	15.5	0.49	6.7	0.04±0.05	6.9
DCA	0.02	0.0	0.01	0.0	tr.		tr.		0.01±0.03	1.7
LCA	n.d.		tr.		tr.		n.d.		0.01±0.02	1.7
CA-1β-ol	0.01	0.0	tr.		0.01	0.1	0.01	0.1	0.21±0.28	36.2
CA-6α-ol	tr.		tr.		0.01	0.1	tr.		0.02±0.04	3.4
CDCA-1β-ol	0.06	0.1	0.04	0.1	0.07	0.5	0.12	1.6	0.04±0.04	6.9
CDCA-2β-ol	0.01	0.0	0.01	0.0	0.03	0.2	0.02	0.3	tr.	
CDCA-4β-ol	0.02	0.0	0.18	0.6	0.13	0.9	0.06	0.8	tr.	
HCA	0.22	0.2	0.12	0.4	0.17	1.2	0.17	2.3	0.10±0.07	17.2
DCA-1β-ol	0.03	0.0	tr.		0.01	0.0	0.02	0.3	tr.	
CA-3β-ol	0.01	0.0	0.01	0.0	tr.		tr.		tr.	
allo-CA	0.29	0.3	0.06	0.2	0.10	0.7	0.09	1.3	tr.	
allo-CDCA	n.d.		0.01	0.0	0.04	0.3	0.02	0.2	tr.	
Δ^5-3β-ol	0.05	0.1	0.04	0.1	0.02	0.1	0.04	0.5	0.01±0.00	1.7
Δ^5-3β,12α-ol	0.03	0.0	tr.		tr.		n.d.		tr.	
CA-Δ^4-3-one	45.55	48.7	1.91	6.5	2.50	17.9	1.07	14.6	0.01±0.01	1.7
CA-$\Delta^{4,6}$-3-one	3.72	4.0	0.29	1.0	0.64	4.6	0.26	3.6	tr.	
CDCA-Δ^4-3-one	12.99	13.9	15.52	52.5	7.02	50.4	3.92	52.6	tr.	
CDCA-$\Delta^{4,6}$-3-one	0.39	0.4	0.56	1.9	0.38	2.7	0.52	7.1	n.d.	
Total	93.62		29.53		13.94		7.31		0.58±0.56	

n.d. = not detected, tr. = trace.
[a] Patients 2 and 3: UDCA was excluded from the profiles of bile acids, since it was administered orally for therapy.
[b] Mean±S.D. (n = 10).

Table 5
Differential composition of the bile acids in serum and urine from patients with cholestasis

Bile acid	Serum ($n=4$)		Urine ($n=4$)		U/B[a]
	μg/ml	%	μg/ml	%	
CA	0.80±0.67[b]	3.2	7.75±13.69[b]	21.5	6.72
CDCA	15.93±12.89	64.7	3.45±3.90	9.6	0.15
DCA	0.03±0.01	0.1	tr.		–
LCA	tr.		tr.		–
CA-1β-ol	tr.		tr.		–
CA-6α-ol	tr.		tr.		–
CDCA-1β-ol	0.01±0.01	0.0	0.07±0.03	0.2	–
CDCA-2β-ol	0.01±0.01	0.0	0.02±0.01	0.1	–
CDCA-4β-ol	tr.		0.10±0.07	0.3	–
CDCA-6α-ol (HCA)	0.02±0.02	0.1	0.17±0.04	0.5	5.00
DCA-1β-ol	0.17±0.20	0.9	0.02±0.01	0.1	0.11
CA-3β-ol	tr.		tr.		–
allo-CA	0.02±0.02	0.1	0.14±0.10	0.4	4.00
allo-CDCA	1.32±1.41	5.4	0.02±0.02	0.1	0.02
Δ^5-3β-ol	0.02±0.01	0.1	0.04±0.01	0.1	1.00
Δ^5-3β,12α-diol	tr.		tr.		–
CA-Δ^4-3-one	0.21±0.08	0.9	12.76±21.87	35.3	39.22
CA-$\Delta^{4,6}$-3-one	0.02±0.02	0.1	1.23±1.67	3.4	34.00
CDCA-Δ^4-3-one	4.98±6.67	20.2	9.86±5.33	27.3	1.35
CDCA-$\Delta^{4,6}$-3-one	1.20±0.98	4.9	0.46±0.09	1.3	0.27
Total±S.D.	24.62±5.89		36.10±39.46		

n.d. = not detected; tr. = trace.
[a] Ratio of bile acids (%) in urine to those in blood.
[b] Mean±S.D.

3-oxo-Δ^4-bile acids (35.3 and 27.3%) in urine. However, the amount of 3.2% of CA in serum was significantly lower than the 21.5% found in urine. Furthermore, CDCA-Δ^4-3-one was also one of the main components, at 20.2%, and CA-Δ^4-3-one found at a lower concentration of 0.9%, according to the variation of the corresponding CA bile acid.

The urinary excretion ratio (U/B) [6] for each bile acid was calculated as the ratio of the percentage in the urine (U) to that in the serum (B), and is shown in Table 5. These data suggest that CA-Δ^4-3-one and CA-$\Delta^{4,6}$-3-one, which were conjugated with glycine or taurine [2,29], were excreted predominantly in urine at very high U/B values of 39.2 and 34.0, respectively. On the other hand, U/B values of CDCA-Δ^4-3-one and CDCA-$\Delta^{4,6}$-3-one were markedly lower, at 1.35 and 0.27, respectively, compared with CA-Δ^4-3-one and CA-$\Delta^{4,6}$-3-one, suggesting that the high levels of CDCA and CDCA-Δ^4-3-one in serum were caused by their characteristics for renal excretion or severe cholestasis with liver dysfunction.

On the basis of the above data, it appears that these liver diseases might be associated with a deficiency or dysfunction of 3-oxo-Δ^4-steroid 5β-reductase. However, in these cases, excretion of large amounts of the common bile acids, CA and CDCA, was observed, which was different from the results reported by Setchell et al. [2]. Therefore, it is necessary that the enzymatic activity of 3-oxo-Δ^4-steroid 5β-reductase and immunoblot analysis for these patients is investigated.

In conclusion, this method for the analysis of 3-oxo-Δ^4- and 3-oxo-$\Delta^{4,6}$-bile acids could be used to determine accurately the bile acids present in biological fluids from infants, and analysis of the profiles of these bile acids in urine could provide useful information on the diagnosis of patients with severe cholestatic liver diseases associated with an abnormality in 3-oxo-Δ^4-steroid 5β-reductase.

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References

[1] P.T. Clayton, E. Patel, A.M. Lawson, R.A. Carruthers, M.S. Tanner, B. Strandvik, B. Egestad and J. Sjövall, Lancet, 1 (1988) 1283.
[2] K.D.R. Setchell, F.J. Suchy, M.B. Welsh, L. Zimmer-Nechemias, J. Heubi and W.F. Balistreri, J. Clin. Invest., 82 (1988) 2148.
[3] P.T. Clayton, J. Inherit. Metab. Dis., 14 (1991) 478.
[4] P.T. Clayton, J. Pediatr., 125 (1994) 845.
[5] B.L. Schneider, K.D.R. Setchell, P.F. Whitington, K.A. Neilson and F.J. Suchy, J. Pediatr., 124 (1994) 234.
[6] T. Murai, R. Mahara, T. Kurosawa, A. Kimura and M. Tohma, J. Chromatogr. B, 691 (1997) 13.
[7] A. Kimura, K. Ushijima, M. Suzuki, M. Tohma, T. Inokuchi and H. Kato, Acta Paediatr., 84 (1995) 1119.
[8] T. Inoue, A. Kimura, E. Nakashima, Y. Yamashita, T. Fujisawa, M. Suzuki, T. Kurosawa and M. Tohma, Jpn. J. Pediatr. Gastroenterol. Nutr., 9 (1995) 166.
[9] M. Tohma, R. Mahara, H. Takeshita, T. Kurosawa and S. Ikegawa, Chem. Pharm. Bull., 34 (1986) 2890.
[10] T. Takahashi, Y. Satoh and Y. Ogiya, Nippon Kagaku Zasshi, 89 (1968) 974.
[11] T. Yoshimura, R. Mahara, T. Kurosawa, S. Ikegawa and M. Tohma, Steroids, 58 (1993) 52.
[12] T. Kurosawa, R. Mahara, H. Nittono and M. Tohma, Chem. Pharm. Bull., 37 (1989) 557.
[13] T. Kurosawa, Y. Nomura, R. Mahara, T. Yoshimura, A. Kimura, S. Ikegawa and M. Tohma, Chem. Pharm. Bull., 43 (1995) 1551.
[14] T. Kurosawa, Y. Nomura, T. Yoshimura, T. Murai, R. Mahara and M. Tohma, Bunseki Kagaku, 44 (1995) 777.
[15] T. Kanazawa, A. Shimazaki, T. Sato and T. Hoshino, Proc. Jpn. Acad., 30 (1954) 391.
[16] M. Tohma, R. Mahara, H. Takeshita and T. Kurosawa, Steroids, 48 (1986) 331.
[17] A. Kallner, Acta Chem. Scand., 21 (1967) 322.
[18] R.A. Leppik, Steroids, 41 (1983) 475.
[19] J. Goto, H. Hasegawa, H. Kato and T. Nambara, Clin. Chim. Acta, 20 (1985) 187.
[20] K.D.R. Setchell and A. Matsui, Clin. Chim. Acta, 127 (1983) 1.
[21] R. Mahara, H. Takeshita, T. Kurosawa, S. Ikegawa and M. Tohma, Anal. Sci., 3 (1987) 449.
[22] J. Shoda, T. Osuga, R. Mahara, M. Tohma, K. Matsuura, N. Tanaka, Y. Matsuzaki and H. Miyazaki, J. Chromatogr., 488 (1989) 315.
[23] I. Björkhem, S. Skrede, M.S. Buchmann, C. East and S. Grundy, Hepatology, 7 (1987) 266.
[24] I. Björkhem, K. Einarsson, P. Melone and P. Hylemon, J. Lipid Res., 30 (1989) 1033.
[25] J.A. Dawson, D.H Mallonee, I. Björkhem and P.B. Hylemon, J. Lipid Res., 37 (1996) 1258.
[26] T. Fotsis and H. Adlercreutz, J. Steroid Biochem., 28 (1987) 203.
[27] R. Tandon, M. Axelson and J. Sjövall, J. Chromatogr., 302 (1984) 1.
[28] E. Wahlen, B. Egestad, B. Strandvik and J. Sjövall, J. Lipid Res., 30 (1989) 1847.
[29] T. Yoshimura, T. Taniguchi, T. Kurosawa, A. Kimura and M. Tohma, J. Liq. Chromatogr. Rel. Technol., 20 (1997) 923.