[Chem. Pharm. Bull.
29(1) 137—145 (1981)]

Quantitative Determination of Ursodeoxycholic Acid and Its Deuterated Derivative in Human Bile by Gas Chromatography-Mass Fragmentography

MASAHIKO TOHMA,*,[a] YUKO NAKATA,[a] HIROMI YAMADA,[a] TAKAO KUROSAWA,[a]
ISAO MAKINO,[b] and SHOICHI NAKAGAWA[b]

Faculty of Pharmaceutical Sciences, Higashi Nippon Gakuen University,[a] Ishikari-Tobetsu, Hokkaido 061-02, Japan and Hokkaido University School of Medicine,[b] Kita-15, Nishi-5, Kita-ku, Sapporo 060, Japan

(Received April 28, 1980)

Deuterium-labeled ursodeoxycholic acid (UDCA-d_2) was synthesized from cholic acid by heterogeneous catalytic reduction of the 11,12-unsaturated bile acid with deuterium gas. A new method for the simultaneous determination of UDCA, UDCA-d_2, related bile acids and cholesterol in human bile has been developed by gas chromatography-mass fragmentography of the methyl ester trimethylsilyl ether derivatives. The glycine conjugate of 5β-chol-3-en-24-oic acid was prepared from lithocholic acid and used as an internal standard. Calibration plots gave a good linear relationship over the range of 100—500 ng of each compound, and their recoveries (95—99%) relative to the internal standard suggested that the present method is sufficiently accurate for the practical analysis of these bile acids in human bile. The method was applied to investigate the fate of UDCA-d_2 orally administered to a patient with complete bile fistula.

Keywords——bile acid; conjugated bile acid; ursodeoxycholic acid; deuterium labeling; quantitative determination; methyl ester-trimethylsilyl ether derivative; internal standard; mass chromatography; mass fragmentography; human bile

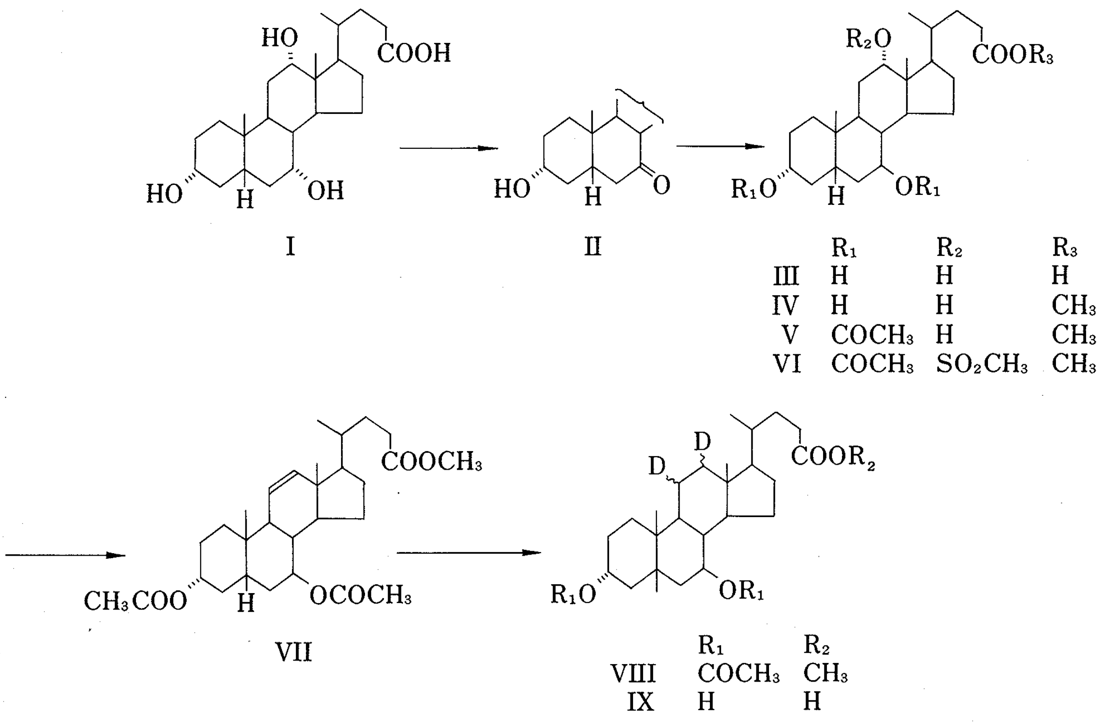
Recently, considerable attention has been focused on the metabolism of bile acids in connection with hepatic diseases[1] and the dissolution of cholesterol gallstones.[2] Nakagawa and co-workers reported that ursodeoxycholic acid (UDCA), the 7β- epimer of chenodeoxycholic acid (CDCA), induced desaturation of bile acids and gallstone dissolution in patients with radiolucent gallstones.[3] This bile acid has been widely used for a long time as a cholagogue in Japan, and is present in human bile in a small amount, but the absorption and metabolism of this bile acid in man have not been studied due to lack of a specific and sensitive method for the determination of the bile acids.

It is now generally recognized that a convenient way of studying the metabolism of steroids is to employ stable isotope-labeled compounds, because these are safer than radioactive ones and are distinguishable from the parent compounds by the difference of their fragment ions in mass spectrometry.[4]

In order to investigate the effect of UDCA on biliary lithogenesis and bile acid metabolism, deuterium-labeled UDCA (UDCA-d_2) was synthesized by heterogeneous catalytic reduction of the 11,12-unsaturated bile acid prepared from cholic acid, and a new gas chromatography-mass fragmentographic method for simultaneous determination of UDCA, UDCA-d_2, related bile acids and cholesterol was established. The method was applied to the analysis of these bile acids in human biles.

Synthesis of [11,12-$^2H_2$]-Labeled Ursodeoxycholic Acid (UDCA-d_2)

Some work has been reported on the synthesis of deuterium-labeled bile acids,[5] but these products had low isotopic purity and the label was partially removed by enteric flora in the biological pathway.[6] Cowen *et al.* have studied the deuterium labeling of CDCA and lithocholic acid at C-11 and C-12 in high isotopic purity by heterogeneous catalytic reduction, and found that these compounds are stable during enterohepatic cycling.[7] We prepared 11,12-deuterium-labeled ursodeoxycholic acid (UDCA-d_2) from cholic acid by the process shown in Chart 1. Cholic acid (I) was oxidized with N-bromosuccinimide in aqueous acetone to give the 7-oxo compound (II), which was reduced stereoselectively to the 3α,7β,12α-triol (III) with sodium in 1-propanol.[8] Methylation and partial acetylation of the triol (III) afforded methyl 3α,7β-diacetoxy-12α-hydroxy-5β-cholanoate (V) as a key intermediate for the synthesis of the Δ^{11}-olefin (VII), and treatment of the hydroxy triester (V) with methanesulfonyl chloride in pyridine gave the 12-mesyloxy compound (VI). Dehydromesylation was effected under mild conditions by warming a mixture of the mesylate (VI) in hexamethylphosphoric triamide containing an excess of sodium acetate to give the Δ^{11}-olefin (VII) without detectable formation of rearrangement products.[9] The labeling of the olefin (VII) was accomplished by reduction with deuterium and platinum oxide in dioxane containing deuterated perchloric acid. The product was saponified in methanolic potassium hydroxide to give the desired [11,12-$^2H_2$]-ursodeoxycholic acid (IX) with high isotopic purity; the content of dideutero species was 80.7%.



	R_1	R_2	R_3
III	H	H	H
IV	H	H	CH_3
V	$COCH_3$	H	CH_3
VI	$COCH_3$	SO_2CH_3	CH_3

	R_1	R_2
VIII	$COCH_3$	CH_3
IX	H	H

Chart 1

Fig. 1 shows the mass spectra in the region above *m/e* 200 of UDCA-d_2 (IX), the unlabeled UDCA and CDCA as their methyl ester bis-trimethylsilyl derivatives. The spectrum of UDCA-d_2 is essentially superimposable upon that of the parent compound by shifting it by two mass units, except for the fragment ion at *m/e* 243 generated from rings A and B.[10] These results are compatible with the fact that the hydroxyl group at C-7 of the synthesized UDCA-d_2 has an equatorial 7β-configuration and that the deuteriums were incorporated preferentially at C-11 and C-12; it seems likely that the prominent ion at *m/e* 372 would permit a sensitive determination of UDCA-d_2 by mass spectrometry.

Gas Chromatography-Mass Spectrometry

Table I summarizes the mass numbers of the base peaks and relative intensities of the fragment ions near *m/e* 370 in the mass spectra of the methyl ester and/or trimethylsilyl ether derivatives of bile acids, cholesterol and 5β-chol-3-en-24-oic acid (internal standard). On the basis of these data, mass chromatography or mass fragmentography was carried out by monitoring the selected ions listed in Table I.

It is hard to achieve gas chromatographic separation of these bile acids on an SE-30 column in combination with total ion monitoring. Mass chromatography, however, permits the individual determination of the standard bile acids by selected ion monitoring of their characteristic fragments without complete chromatographic separation, as shown in Fig. 2.

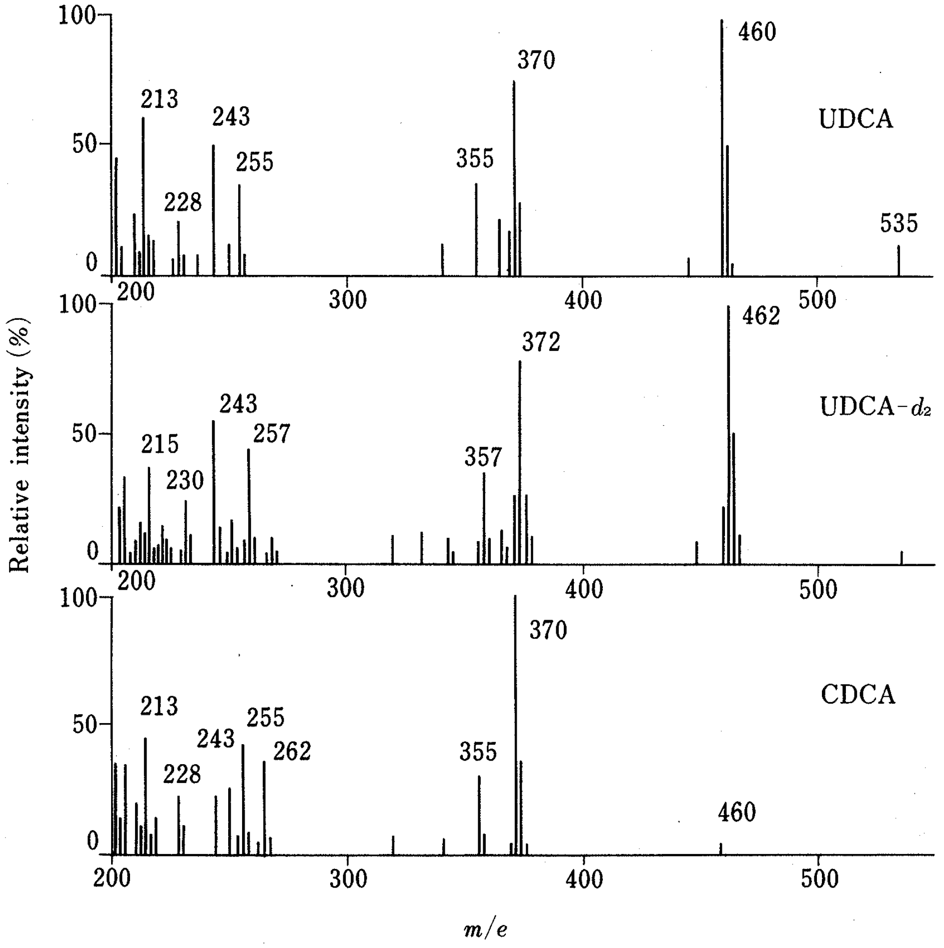


Fig. 1. Mass Spectra of the Methyl Ester Trimethylsilyl Ether Derivatives of Ursodeoxycholic Acid (UDCA), its Deuterate (UDCA-d_2) and Chenodeoxycholic Acid (CDCA)

TABLE I. Relative Intensities of Fragment Ion Peaks of the Methyl Ester Trimethylsilyl Ether Derivatives of Bile Acids and Cholesterol

Bile acid	Base peak (*m/e*)	Relative intensity *m/e* 368	370	372	374
Lithocholic acid	215	—	—	49.8*	—
Deoxycholic acid	255	—	10.1*	—	—
Chenodeoxycholic acid	370*	—	100*	3.5	—
Chenodeoxycholic acid-d_2	372*	—	—	100*	15.3
Ursodeoxycholic acid	460	—	74.6*	2.4	—
Ursodeoxycholic acid-d_2	462	—	—	76.7*	6.3
Cholic acid	253	85.1*	—	—	—
7-Epicholic acid	253	26.2*	—	—	—
7-Oxolithocholic acid	386*	63.8*	—	—	—
7-Oxodeoxycholic acid	253, 341*	—	—	—	—
Cholesterol	329	63.6*	—	—	—
5β-Chol-3-enoic acid	372*	—	—	100*	—

*) These fragment ions were used as monitoring ions in mass fragmentography.

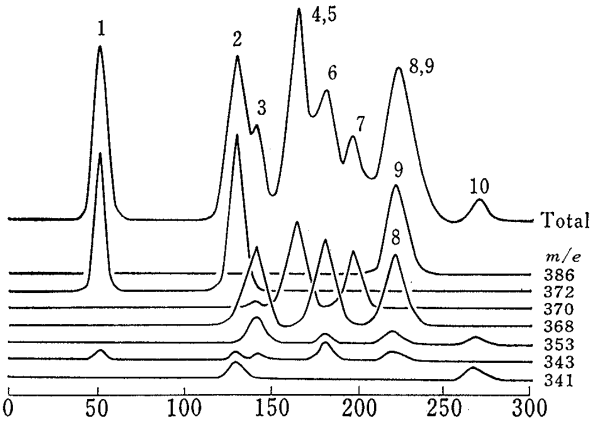


Fig. 2. Mass Chromatogram of the Methyl Ester Trimethylsilyl Ether Derivatives of the Standard Bile Acids on a 1.5% SE-30 Column at 250°

1, 5β-chol-3-enoic acid; 2, lithocholic acid; 3, cholesterol; 4, chenodeoxycholic acid; 5, deoxycholic acid; 6, cholic acid; 7, ursodeoxycholic acid; 8, 7-epicholic acid; 9, 7-oxo-lithocholic acid; 10, 7-oxo-deoxycholic acid.

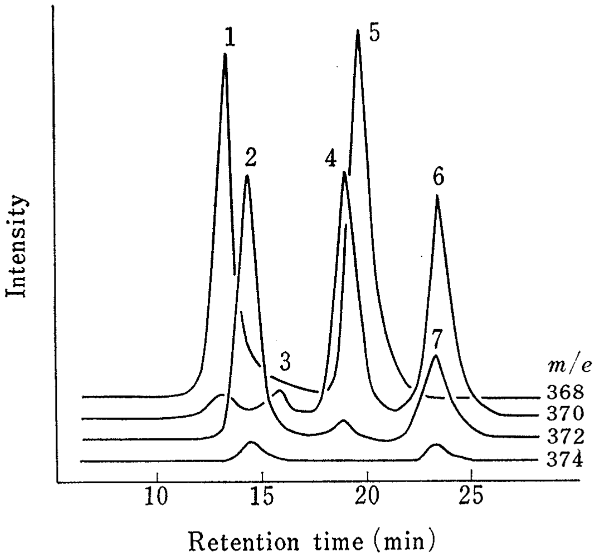


Fig. 3. Mass Fragmentogram of the Methyl Ester Trimethylsilyl Ether Derivatives of Bile Acids from Human Bile on a 1.5% Poly I-110 Column at 270°

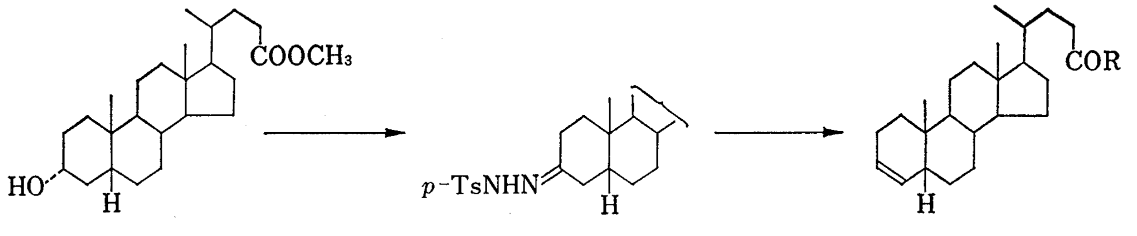
1, cholic acid; 2, 5β-chol-3-enoic acid; 3, deoxycholic acid; 4, chenodeoxycholic acid; 5, cholesterol; 6, ursodeoxycholic acid; 7, ursodeoxycholic acid-d_2.

The trimethylsilyl ethers of CDCA and deoxycholic acid methyl esters could not be determined by mass chromatography with an SE-30 column, since they have similar retention times and the same mass number at *m/e* 370. An improved gas chromatographic separation of these compounds was achieved by the use of Poly I-110 as a stationary phase.11)

Further, the individual determination of these bile acids could be performed more sensitively and easily from the mass fragmentogram obtained by means of a gas chromatograph–mass spectrometer equipped with a multiple ion detector system, by measurement of the peak area of the focused ion for each compound. Fig. 3 shows that the simultaneous determination of UDCA, UDCA-d_2 and related bile acids is possible.

Quantitative Analysis of UDCA and UDCA-d_2

The determination of bile acids in human bile was usually carried out after several pretreatments involving extraction and alkaline hydrolysis of bile acid conjugates.12) The treatments reduce the precision of the analysis, and inaccuracy in the sampling for gas chromatographic injection created further problems. Thus, we synthesized the glycine conjugate of 5β-chol-3-en-24-oic acid (XIV) as an internal standard (Chart 2), to be added to the bile prior to the pretreatments.



X XI

XII : $R=OCH_3$
XIII : $R=OH$
XIV : $R=NHCH_2COOH$

Chart 2.

The tosylhydrazone of methyl 3-oxo-5β-cholanoate (XI) prepared from lithocholic acid methyl ester (X) afforded the 3-ene (XII) accompanied by a trace amount of the isomeric 2-ene upon refluxing with potassium hydride in toluene under an argon atmosphere. After hydrolysis of the ester (XII) in methanolic potassium hydroxide, the desired conjugate (XIV) was obtained by coupling of the bile acid (XIII) with glycine ethyl ester in the presence of N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinolin in ethyl acetate.[13] The mass spectrum of methyl 5β-chol-3-en-24-oate (XII) revealed the expected molecular ion and base peak at *m/e* 372, and gave satisfactory mass spectral characteristics, as shown in Fig. 4. On the basis of these results, the glycine conjugate (XIV) was added to the bile as an internal standard prior to the hydrolysis and derivatization to the methyl ester (XII) for gas chromatography-mass spectrometry in the biliary bile acid analysis.

The calibration curves for the standard UDCA-d_2 and related bile acids were obtained by plotting peak area ratios at the monitoring ions (listed in Table I) for mixtures of various amounts of the methyl ester trimethylsilyl ether derivative of each bile acid with a fixed amount of methyl 5β-chol-3-en-24-oate (XII). A good linear relationship was found over the range of 100—500 ng of each compound, as shown in Fig. 5.

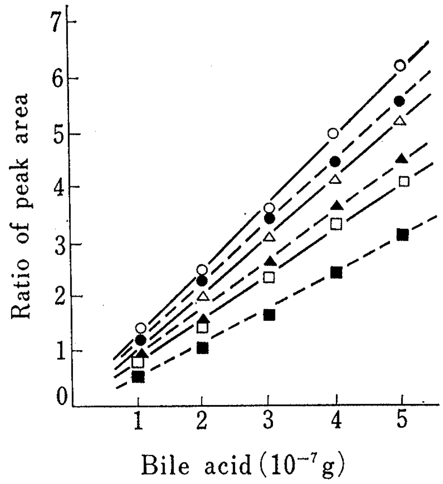


Fig. 5. Calibration Curves for Bile Acids and Cholesterol obtained by Mass Fragmentography with a 1.5% SE-30 Column at 250°

○—○, chenodeoxycholic acid-d_2 (*m/e* 372); ●···●, ursodeoxycholic acid-d_2 (*m/e*372); △—△, cholic acid (*m/e* 368); ▲···▲, cholesterol (*m/e* 368); □—□, deoxycholic acid (*m/e* 370); ■···■, 7-oxo-lithocholic acid (*m/e* 386).

The ratio of peak areas was obtained from the peak area of the monitoring ion for the bile acid or cholesterol and that of the internal standard (*m/e* 372).

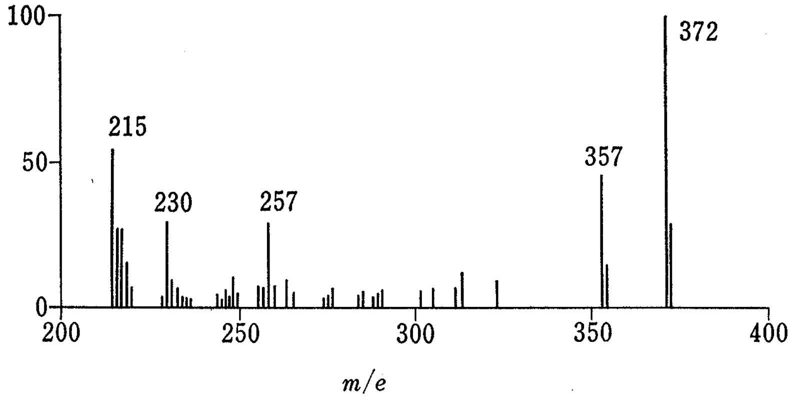


Fig. 4. Mass Spectrum of Methyl 5β-Chol-3-enoate (XII)

Next, the accuracy of this method was investigated for the quantitation of UDCA and related bile acids in human bile. Appropriate amounts of the glycine-conjugated bile acids and the internal standard (XIV) were added to human bile, and these compounds were extracted with ethanol and saponified with sodium hydroxide, followed by extraction with ethyl acetate. After derivatization to their methyl ester–trimethylsilyl ethers, mass fragmentography was carried out for analysis of the bile acids as described in the experimental section. Fig. 3 depicts a typical fragmentogram without interference from other bile components in this analysis.

The analytical data and recoveries relative to the internal standard are given in Table II; the proposed method appears to be sufficiently accurate for the quantitative determination of these bile acids in human bile. The recoveries were calculated on the basis of the amount of the free bile acid (XIII) derived from the added internal standard (XIV), and the resulting values were used for calculation in the following bile acid analysis. The low recovery of cholesterol may have been due to an error in the extraction from biles.

The mass fragmentographic analysis was applied to the quantitative determination of UDCA-d_2 and related bile acids in the bile from a patient with complete bile fistula.[14] After oral administration of UDCA-d_2, bile samples were collected through a drain. The results

Table II. Recoveries of Glycine–Conjugated Bile Acids from Human Bile

Bile acid	Endogeneous (mg/ml)	Added a) (mg)	Recovered (mg)	Recovery b) Mean ± S.D. (%, $n=3$)
Ursodeoxycholic acid	—	0.50	0.49 ± 0.02	98 ± 4
	—	1.04	1.01 ± 0.05	97 ± 4
	—	1.56	1.49 ± 0.04	96 ± 3
Chenodeoxycholic acid	1.57	2.89	2.87 ± 0.19	99 ± 7
Cholic acid	1.33	2.02	1.91 ± 0.09	95 ± 3
Cholesterol	0.52	0.61	0.50 ± 0.01	82 ± 2
5β-Chol-3-enoic acid	—	1.74	1.68 ± 0.04	97 ± 2 c)

a) Amounts of free bile acids were calculated from the amounts of added glycine conjugates.

b) Recoveries were calculated on the basis of the amount of 5β-chol-3-enoic acid derived from the glycine conjugate added as an internal standard.

c) Estradiol was used as a secondary internal standard in the GC–MS analysis.

Table III. Time Courses of Biliary Excretions of Ursodeoxycholic Acid-d_2, Cholesterol and Total Bile Acids after Oral Administration of UDCA-d_2 (33 mg) to a Complete Bile Fistula Patient

Time	(min)	Bile (ml)	Recovered UDCA-d_2 (mg)	(%)	Total bile acids (mg/ml) a)	Cholesterol (%) b)
Before	0—30	24.2	0	0	10.7	11.0
After	0—30	8.8	1.8	5.5	12.8	6.7
	30—60	14.0	19.4	58.8	33.7	4.6
	60—90	16.6	7.6	23.0	19.7	5.2
	90—120	13.2	0.3	0.9	29.7	5.8
	120—180	26.2	0.8	2.4	12.6	7.9
	180—240	6.0	0.3	0.9	12.3	10.3
	240—300	16.0	0.6	1.8	12.7	10.5
	300—360	22.0	0.6	1.8	15.9	11.1

a) Total amounts of biliary bile acids were determined from the peak areas of the detectable acids on the mass fragmentograms.

b) Ratio (%) of cholesterol to the total amount of bile acids and cholesterol.

of analysis of biliary bile acids are shown in Table III. The recovery rate of UDCA-d_2 was 88.2% within the first 2 hr and 95.1% until 6 hr, without significant biotransformation into other bile acids, while the cholesterol levels were decreased in contrast to the increased total amounts of biliary bile acids after administration of UDCA-d_2.

On the basis of these data, this method is currently being applied in a study of the pharmacokinetics of UDCA in normal men and patients with hepatocellular damage. Details of these investigations will be reported in another paper.

Experimental

Melting points were determined on a Mitamura micro hot-stage apparatus and are uncorrected. Optical rotations were taken with a Union Giken PM-201 polarimeter and IR spectra were recorded on a JASCO IR A-102 spectrometer. NMR spectra were obtained at 90 MHz on a Hitachi NMR R-40 spectrometer and chemical shifts are given in ppm relative to tetramethylsilane as an internal standard.

Methyl 3α,7β,12α-Trihydroxy-5β-cholan-24-oate (IV)——The 3α,7β,12α-trihydroxy acid (III) was prepared through N-bromosuccinimide oxidation of cholic acid (I, 21.4 g) in aqueous acetone, followed by reduction with sodium in 1-propanol.[8] The crude product was methylated with HCl–MeOH by refluxing for 1.5 hr, and purified by chromatography on silica gel to give the ester (IV) as colorless needles (14.0 g), mp 95—97°. $[\alpha]_D^{21}$ +75.9° ($c=0.405$, dioxane). IR $\nu_{\max}^{\text{KBr}}$ cm^{-1}: 3370 (OH), 1735 ($COOCH_3$). NMR ($CDCl_3$) δ: 0.70 (3H, s, 18-CH_3), 0.93 (3H, s, 19-CH_3), 3.67 (3H, s, $COOCH_3$), 3.97 (1H, t, 12-H), 3.4—3.8 (2H, m, 3,7-H). *Anal.* Calcd for $C_{25}H_{42}O_5$: C, 71.05; H, 10.02. Found: C, 70.85; H, 9.92.

Methyl 3α,7β-Diacetoxy-12α-hydroxy-5β-cholan-24-oate (V)——A mixture of IV (9.5 g), acetic anhydride (6.4 ml), pyridine (6.4 ml) and benzene (55 ml) was allowed to stand at room temperature overnight. The reaction mixture was then poured into ice water and extracted with ethyl acetate. The extract was washed with 2 N HCl, 5% $NaHCO_3$ and saturated aqueous NaCl, then evaporated to dryness *in vacuo*. The residue was subjected to chromatography on silica gel, and the eluate with *n*-hexane–ethyl acetate (8: 2) gave the diacetate (V) as colorless needles (5.7 g), mp 164—165.5°. $[\alpha]^{21}_{\mathrm{D}}$ +41.9° (c=0.569, dioxane). IR $\nu^{\mathrm{Nujol}}_{\mathrm{max}}$ cm^{-1}: 3545 (OH), 1740, 1735, 1720 ($COOCH_3$, $OCOCH_3$). NMR ($CDCl_3$) δ: 0.71 (3H, s, 18-CH_3), 0.95 (3H, s, 19-CH_3), 1.98 (3H, s, 7-$OCOCH_3$), 2.02 (3H, s, 3-$OCOCH_3$), 3.68 (3H, s, $COOCH_3$), 4.00 (1H, dd, 12-H), 4.5—5.0 (2H, m, 3,7-H). *Anal.* Calcd for $C_{29}H_{46}O_7$: C, 68.74; H, 9.15. Found: C, 68.82; H, 9.01.

Methyl 3α,7β-Diacetoxy-12α-methanesulfonyloxy-5β-cholan-24-oate (VI)——Methanesulfonyl chloride (1.8 g) was added dropwise to an ice-cooled, stirred solution of V (5.3 g) in dry pyridine (20 ml). The reaction mixture was stirred at 0° for 2 days under a nitrogen atmosphere, and then poured into ice water and extracted with ether. The organic layer was washed with 2 N HCl and saturated NaCl, dried over anhydrous $MgSO_4$ and evaporated to dryness *in vacuo*. The residue was crystallized from *n*-hexane–isopropyl ether to give the mesylate (VI) as colorless needles (6.0 g), mp 106—108°. $[\alpha]^{21}_{\mathrm{D}}$ +24.8° (c=0.404, dioxane). IR $\nu^{\mathrm{Nujol}}_{\mathrm{max}}$ cm^{-1}: 1720 ($COOCH_3$, $OCOCH_3$), 1160 (OSO_2CH_3). NMR ($CDCl_3$) δ: 0.80 (3H, s, 18-CH_3), 0.87 (3H, s, 19-CH_3), 2.00, 2.03 (3H, s, $OCOCH_3$), 3.06 (3H, s, OSO_2CH_3), 3.68 (3H, s, $COOCH_3$), 3.5—5.0 (2H, m, 3,7-H), 5.13 (1H, dd, 12-H). *Anal.* Calcd for $C_{30}H_{48}O_9S$: C, 61.62; H, 8.27; S, 5.48. Found: C, 61.68; H, 8.27; S, 5.32.

Methyl 3α,7β-Diacetoxy-5β-chol-11-en-24-oate (VII)——A solution of VI (4.5 g) and sodium acetate (16 g) in hexamethylphosphoric triamide (50 ml) was stirred at *ca.* 100° for 28 hr under a nitrogen atmosphere. The solvent was distilled off *in vacuo*, and the oily product was extracted with ethyl acetate. The organic layer was washed with 2 N HCl and saturated NaCl, dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure. The residue was subjected to chromatography on silica gel (150 g) to give the olefin (VII) as a colorless oil (3.0 g). $[\alpha]^{21}_{\mathrm{D}}$ +36.9° (c=0.623, dioxane). IR $\nu^{\mathrm{Nujol}}_{\mathrm{max}}$ cm^{-1}: 3040 (CH=CH), 1740 ($COOCH_3$, $OCOCH_3$). NMR ($CDCl_3$) δ: 0.78 (3H, s, 18-CH_3), 0.93 (3H, s, 19-CH_3), 2.00 (6H, s, 3,7-$OCOCH_3$), 3.68 (3H, s, $COOCH_3$), 5.29 (1H, d, J=12 Hz, 12-H), 6.07 (1H, dd, J_1=12 Hz, J_2=5 Hz, 11-H). *Anal.* Calcd for $C_{29}H_{44}O_6$: C, 71.28; H, 9.08. Found: C, 71.13; H, 9.00.

Methyl 3α,7β-Diacetoxy-5β-cholan-24-oate-11,12-*d*$_2$ (VIII)——A solution of VII (2.23 g) in dioxane (60 ml) was shaken with platinum oxide (253 mg) and $DClO_4$ (10 drops) under deuterium gas (5 kg/cm^2) for 2 days. After filtration, the solvent was evaporated off and the residue was extracted with ethyl acetate. The organic layer was washed with saturated $NaHCO_3$ and saturated NaCl, dried over anhydrous Na_2SO_4 and evaporated to dryness *in vacuo*. The residue (1.85 g) was chromatographed on silica gel (60 g), and the eluate with *n*-hexane–ethyl acetate (9: 1) gave the deuterated compound (VIII) as colorless needles (1.35 g). mp 99.5—102.5°. $[\alpha]^{20}_{\mathrm{D}}$ +43.5° (c=0.690, $CHCl_3$). NMR ($CDCl_3$) δ: 0.69 (3H, s, 18-CH_3), 0.98 (3H, s, 19-CH_3), 2.04, 2.06 (3H, s, $OCOCH_3$), 3.74 (3H, s, $COOCH_3$), 4.5—5.0 (2H, m, 3,7-H).

3α,7β-Dihydroxy-5β-cholan-24-oic Acid-11,12-*d*$_2$ (UDCA-*d*$_2$, IX)——A solution of VIII (500 mg) in 1 N KOH–MeOH (10 ml) was refluxed for 4 hr. After cooling, the reaction mixture was concentrated under reduced pressure, acidified with 2 N HCl and extracted with ethyl acetate. The organic layer was washed with saturated NaCl, dried over anhydrous Na_2SO_4 and evaporated to dryness *in vacuo*. The residue was recrystallised from ethyl acetate to give UDCA-d_2 (IX) as colorless needles (266 mg). mp 208—210°. $[\alpha]^{20}_{\mathrm{D}}$ +55.8° (c=0.628, dioxane). IR $\nu^{\mathrm{Nujol}}_{\mathrm{max}}$ cm^{-1}: 3480 (OH), 1701 (COOH). This product showed high isotopic purity (2.9% $^2H_0$, 16.3% $^2H_1$, 80.7% $^2H_2$) on analysis by mass fragmentography.

The Tosylhydrazone of Methyl 3-Oxo-5β-cholan-24-oate (XI)——*p*-Toluenesulfonyl hydrazine (6.8 g) and 1-propanol (3 ml) were added to a solution of methyl 3-oxo-5β-cholan-24-oate (4.11 g) [prepared from lithiocholic acid[15)]] in methanol, and the whole was refluxed for 5 hr. The precipitated product was separated by filtration and recrystallized from $CHCl_3$–MeOH to give the tosylhydrazone (XI) as colorless needles (5.15 g). mp 196—200°. IR $\nu^{\mathrm{Nujol}}_{\mathrm{max}}$ cm^{-1}: 3200, 1640 (N–H), 1710 ($COOCH_3$), 1595 (aromatic). NMR ($CDCl_3$) δ: 0.67 (3H, s, 18-CH_3), 0.93 (3H, s, 19-CH_3), 3.54 (3H, s, $COOCH_3$), 7.03—7.58 (4H, m, aromatic-H).

Methyl 5β-Chol-3-en-24-oate (XII)——A mixture of XI (2.93 g) and potassium hydride (0.6 g) in toluene (50 ml) was refluxed for 3 hr under an argon atmosphere. The reflux was interrupted by dropwise addition of acetic acid in benzene, and the reaction mixture was extracted with ether. The organic layer was washed with 5% $NaHCO_3$ and saturated NaCl, dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure. The residue was chromatographed on silica gel. Elution with *n*-hexane–benzene (95: 5) and crystallization from methanol gave the 3-ene (XII) as colorless needles (1.09 g). mp 74.5—75.5° (lit.[15)] mp 73.5—75°, $[\alpha]_{\mathrm{D}}$ +17°). $[\alpha]^{20}_{\mathrm{D}}$ +17.8° (c=0.483, $CHCl_3$). IR $\nu^{\mathrm{Nujol}}_{\mathrm{max}}$ cm^{-1}: 3025 (CH=CH), 1730 ($COOCH_3$). NMR ($CDCl_3$) δ: 0.68 (3H, s, 18-CH_3), 0.96 (3H, s, 19-CH_3), 3.66 (3H, s, $COOCH_3$), 5.36 (1H, d, J=10 Hz, 4-H), 5.70 (1H, dd, J_1=10 Hz, J_2=4 Hz, 3-H). *Anal.* Calcd for $C_{25}H_{40}O_2$: C, 80.54; H, 10.82. Found: C, 80.40; H, 11.06.

5β-Chol-3-en-24-oic Acid (XIII)——A solution of XII (797 mg) in 1 N KOH–MeOH (11 ml) was refluxed for 4 hr. After cooling, the reaction mixture was acidified to pH 3 with 2 N HCl and extracted with ethyl acetate. The organic layer was washed with saturated NaCl, dried over anhydrous Na_2SO_4 and evaporated to dryness *in vacuo*. The residue was recrystallized from methanol to give 5β-chol-3-en-24-oic acid (XIII) as colorless needles (718 mg). mp 144—145°, $[\alpha]_D^{20}$ +21.5° (c=0.506, $CHCl_3$). (lit.[16] mp 155—156°, $[\alpha]_D$ +18°). IR ν_{max}^{Nujol} cm^{-1}: 3050, 1620 (CH=CH), 1730 (COOH). NMR ($CDCl_3$) δ: 0.66 (3H, s, 18-CH_3), 0.95 (3H, s, 19-CH_3), 5.30 (1H, d, J=11 Hz, 4-H), 5.65 (1H, dd, J_1=11 Hz, J_2=4 Hz, 3-H). *Anal.* Calcd for $C_{24}H_{38}O_3$: C, 80.39; H, 10.68. Found: C, 80.41; H, 10.48.

The Glycine Conjugate of 5β-Chol-3-en-24-oic Acid (XIV)——Anhydrous K_2CO_3 was added with shaking to a solution of glycine ethyl ester hydrochloride (638 mg) in ethyl acetate (65 ml) and water (1.6 ml). The organic layer was separated by centrifugation, N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (845 mg) and XIII (1 g) were added to it, and the whole was refluxed overnight. The reaction mixture was washed successively with 0.1 N HCl, 5% $NaHCO_3$, 0.1 N NaOH and saturated NaCl. The organic layer was evaporated to dryness *in vacuo*, and the residue was dissolved in ethanol (100 ml). After addition of 2 N NaOH (100 ml), the solution was permitted to stand at room temperature for 3 hr. Water (20 ml) was added to the reaction mixture, and the whole was concentrated to about 100 ml under reduced pressure at 25°, acidified with concentrated HCl and extracted with ethyl acetate. The organic layer was washed with saturated NaCl, dried over anhydrous Na_2SO_4 and concentrated to give crystals of the glycine conjugate (XIV) as colorless needles (822 mg). mp 191.5—192.5°. $[\alpha]_D^{20}$ +14.5° (c=0.508, MeOH). IR ν_{max}^{Nujol} cm^{-1}: 3325, 1580, 1540 (CONH), 3000 (CH=CH), 1715 (COOH). *Anal.* Calcd for $C_{26}H_{41}O_3N$: C, 75.13; H, 9.94; N, 3.37. Found: C, 75.16; H, 9.84; N, 3.39.

Gas Chromatography-Mass Spectrometry (GC-MS)——GC–MS was performed on a Shimadzu-LKB 9000 system equipped with a multiple-ion detector and a data-processing system (Shimadzu GC–MS PAC 300M). Gas chromatographic columns (2 m × 3 mm I.D. glass coil) packed with 1.5% SE-30 on Chromosorb W (60—80 mesh) and 1.5% Poly I-110 on Gas Chrom Q (80—100 mesh) were used at 250° and 270°, respectively. The flow rate of helium carrier gas was 30 ml/min. The temperature of the flash heater was 280°, and that of the separator was 300°. The mass spectra were measured at 70 eV with an ion source temperature of 290°.

Derivatization——A single bile acid or a mixture of bile acids was derivatized to the methyl ester with diazomethane–ether solution at room temperature. After removal of the solvent, the trimethylsilyl ethers of hydroxyl groups were prepared by heating the residue with trimethylsilyl imidazole in acetonitrile at 60° for 30 min. Excess reagents were evaporated off in a nitrogen stream and the residue was redissolved in *n*-hexane prior to gas chromatographic analysis.

Quantitative Analysis of Bile Acids in Human Bile——A suspension of UDCA-d_2 (33 mg) and UDCA (77 mg) in water containing equimolar $NaHCO_3$ was administered to a patient with complete bile fistula. Bile samples were collected from a drain at 30 min intervals during 6 hr in a fasting state. XIV (1 mg) was added as an internal standard to a bile sample (1 ml) in ethanol (10 ml) with agitation in an ultrasonic bath. After centrifugation and removal of the solvent, the residue was hydrolyzed with 15% NaOH (10 ml) at 120° for 3 hr in an autoclave. The hydrolysate was acidified with 6 N HCl, and the bile acids were extracted with ethyl acetate (50 ml). The organic layer was washed with saturated NaCl, dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure. An appropriate amount of the residue was converted to the methyl ester trimethylsilyl ether derivative, and subjected to GC–MS analysis described above.

Acknowledgement The authors are indebted to Mrs. H. Matsumoto and Miss A. Maeda of the Analytical Center of Hokkaido University for the elemental analyses, and to Mr. A. Sakushima of this faculty for his kind help in operating the GC–MS.

References and Notes

1) I. Makino, H. Hashimoto, K. Shinozaki, K. Yoshino, and S. Nakagawa, *Gastroenterology*, **68**, 545 (1975); G. Paumgartner and A. Stiehl (ed.), "Bile Acid Metabolism in Health and Disease," MTP Press Ltd., Lancaster, 1977.
2) R.C. Danzinger, A.F. Hofmann, L.J. Shoenfield, and J.L. Thistle, *New Eng. J. Med.*, **286**, 1 (1972); C.D. Bell, B. Whitney, and R.H. Dowling, *Lancet*, *II*, 1213 (1972); A.F. Hofmann and G. Paumgartner (ed.), "Chenodeoxycholic Acid Therapy of Gallstones," F.K. Schattauer Verlag, Stuttgart, 1975.
3) S. Nakagawa, I. Makino, T. Ishizaki, and I. Dohi, *Lancet*, *II*, 367 (1977).
4) M. Tohma, Y. Nakata, and T. Kurosawa, *J. Chromatogr.*, **171**, 469 (1979); W.F. Balistreri, A.E. Cowen, A.F. Hofmann, P.A. Szczepanik, and P.D. Klein, *Pediat. Res.*, **9**, 757 (1975); M. Shino, Y. Nezu, T. Terayama, K. Sakaguchi, K. Katayama, J. Tsutsumi, and K. Kawabe, *Yakugaku Zasshi*, **99**, 421 (1979).
5) J.B. Watkins, D. Ingall, P. Szczepanik, and R. Lester, *New Eng. J. Med.*, **288**, 431 (1973); D.L. Hachey, P.A. Szczepanik, O.W. Berngruber, and P.D. Klein, *J. Labelled Compd.*, **9**, 703 (1973).
6) N.F. LaRussi, N.F. Hoffman, and A.F. Hofmann, *J. Lab. Clin. Med.*, **84**, 769 (1974); D.K. Panveliwalla, D. Pertsemlidis, and E.H. Ahrens, Jr., *J. Lipid Res.*, **15**, 530 (1974).
7) A.E. Cowen, A.F. Hofmann, D.L. Hachey, P.J. Thomas, D.T.E. Belobaba, P.D. Klein, and L. Tökes, *J. Lipid Res.*, **17**, 231 (1976).
8) B. Samuelsson, *Acta Chem. Scand.*, **14**, 17 (1960).
9) C.H. Chen, *Synthesis*, **1976**, 125.

10) J. Sjövall, "Bile Salt Metabolism," ed. by L. Schiff, J. Care, and J. Dietschy, Thomas, Springfield, Ill., 1969, p. 205.
11) H. Miyazaki, M. Ishibashi, M. Inoue, M. Itoh, and T. Kubodera, *J. Chromatogr.*, **99**, 553 (1974).
12) W.T. Beher, S. Stradnieks, G.R. Beher, and G.L. Lin, *Steroids*, **32**, 355 (1978); G.P. van Berge-Henegouwen, R.N. Allan, A.F. Hofmann, and P.Y.S. Yu, *J. Lipid Res.*, **18**, 118 (1977).
13) L. Lack, F.O. Dorrity, Jr., T. Walker, and G.D. Sinletary, *J. Lipid Res.*, **14**, 367 (1973).
14) The patient gave his informed consent.
15) L.F. Fieser and R. Ettorre, *J. Am. Chem. Soc.*, **75**, 1700 (1953).
16) F.C. Chang, A. Feldstein, J.R. Gray, G.S. McCaleb, and D.H. Sprunt, *J. Am. Chem. Soc.*, **79**, 2167 (1957).