Occurrence of 3β-hydroxy-5-cholestenoic acid, 3β,7α-dihydroxy-5-cholestenoic acid, and 7α-hydroxy-3-oxo-4-cholestenoic acid as normal constituents in human blood

Magnus Axelson,* Birgitta Mörk,† and Jan Sjövall†

Department of Clinical Chemistry,* Karolinska Hospital and Department of Physiological Chemistry,† Karolinska Institutet, 104 01 Stockholm 60, Sweden

Abstract Three unconjugated C_{27} bile acids were found in plasma from healthy humans. They were isolated by liquid–solid extraction and anion-exchange chromatography and were identified by gas–liquid chromatography–mass spectrometry, microchemical reactions, and ultraviolet spectroscopy as 3β-hydroxy-5-cholestenoic, 3β,7α-dihydroxy-5-cholestenoic, and 7α-hydroxy-3-oxo-4-cholestenoic acids. Their levels often exceeded those of the unconjugated C_{24} bile acids and the variations between individuals were smaller than for the C_{24} acids. The concentrations in plasma from 11 healthy subjects were 67.2 ± 27.9 ng/ml (mean ± SD) for 3β-hydroxy-5-cholestenoic acid, 38.9 ± 25.6 ng/ml for 3β,7α-dihydroxy-5-cholestenoic acid, and 81.7 ± 27.9 ng/ml for 7α-hydroxy-3-oxo-4-cholestenoic acid. The levels of the individual acids were positively correlated to each other and not to the levels of the C_{24} acids. The cholestenoic acids were below the detection limit (20–50 ng/ml) in bile and C_{27} bile acids present in bile were not detected in plasma. —**Axelson, M., B. Mörk, and J. Sjövall.** Occurrence of 3β-hydroxy-5-cholestenoic acid, 3β,7α-dihydroxy-5-cholestenoic acid, and 7α-hydroxy-3-oxo-4-cholestenoic acid as normal constituents in human blood. *J. Lipid Res.* 1988. **29:** 629–641.

Supplementary key words C_{24} bile acids • C_{27} bile acids

Cholesterol is the precursor of many biologically important steroids. For a number of years attention has been focused on the association between cholesterol metabolism and the development of atherosclerosis. In the course of our studies on steroid hormones and bile acids, methods for screening of metabolites of cholesterol in various biological materials have been developed (1–3). Using these methods, a group of cholestenoic acids was discovered as normal constituents in human blood. The compounds were identified as 3β-hydroxy-5-cholestenoic acid, 3β,7α-dihydroxy-5-cholestenoic acid, and 7α-hydroxy-3-oxo-4-cholestenoic acid. The former acid has previously been found in blood from infants with severe liver disease (4, 5), whereas the latter two acids do not seem to have been detected in biological material. This report describes the isolation of these compounds by chromatography on lipophilic Sephadex derivatives and their identification by gas–liquid chromatography–mass spectrometry, microchemical reactions, and ultraviolet spectroscopy.

MATERIALS AND METHODS

Glassware and chemicals

All glassware except round-bottomed flasks was silanized, and cleaning was carried out in an ultrasonic bath. Solvents were of analytical reagent grade and were redistilled. Hexamethyldisilazane and trimethylchlorosilane (Fluka, Buchs, Switzerland) were redistilled. [^{2}H]Trimethylchlorosilane (Merck Sharp & Dohme, Montreal, Canada) was used as supplied. Methoxyamine hydrochloride (Eastman Organic Chemicals, Rochester, NY) and hydroxylamine hydrochloride (Fluka) were recrystallized. Lithium aluminium hydride was obtained from Merck (Darmstadt, FRG). Diazomethane was freshly prepared from N-methyl-N-nitroso-*p*-toluene sulfonamide (Merck).

Column packing materials

Octadecylsilane-bonded silica (preparative C18, Waters Associates Inc., Milford, MA), about 0.4 g, was packed in jacketed glass columns (1 × 0.8 cm) and was then washed with 5 ml each of methanol, methanol–chloroform 1:1 (v/v), methanol, and water prior to use. Triethylaminohydroxypropyl Sephadex LH-20 (TEAP-LH-20) and sulfohydroxypropyl Sephadex LH-20 (SP-LH-20) were synthesized (6, 7) and Lipidex 5000 was from Packard Instrument Co. (Downers Grove, IL). The Sephadex derivatives were washed extensively prior to use (6). Suitable flow rates were obtained by application of nitrogen pressure.

Reference compounds

Most of the unlabeled bile acids were those used in previous studies or were purchased from Steraloids (Wilton, NH). Labeled bile acids were from the Radiochemical Centre (Amersham, England) and NEN Research Products (Dreieich, FRG). 3-Oxo-5β-[4-^{14}C]cholanoic acid was prepared by oxidation of [4-^{14}C]lithocholic acid with chromic acid in acetone and was purified by reversed-phase high-performance chromatography on a μBondapak C18 column using 80% aqueous methanol with 1% acetic acid as mobile phase. The following compounds were kind gifts from a number of colleagues: 7α- and 26-hydroxycholesterols from Prof. P. Eneroth, Stockholm; 3-oxo-C_{27} steroids from Prof. I. Björkhem, Huddinge; methyl 3β,7α-dihydroxy-5-cholenoate from Dr. K. Uchida (Shionogi Research Laboratories, Osaka); and methyl 3-oxo-4,6-choladienoate from Prof. D. N. Kirk, MRC Steroid Reference Collection. 3-Oxo-4-cholesten-26-oic acid was obtained by oxidation of 26-hydroxycholesterol with chromic acid in acetone.

Subjects and samples

Blood from apparently healthy human subjects (age 23–35 years) was collected in tubes with or without heparin. Following centrifugation, plasma/serum was separated and stored at −20°C until analyzed. Storage of samples for longer periods (months) at this temperature appeared to result in decreased concentrations of the 7-hydroxylated cholestenoic acids. The subjects were not fasting prior to sampling, but most samples were collected in the morning. In a separate experiment, sampling was standardized and blood from four female subjects (age 27–31 years) was drawn at 8:20 AM (after fasting over night) and then at 12:30 PM (1.5 hr after the beginning of the lunch meal).

Bile fistula bile was obtained from patients undergoing gallstone surgery at the Karolinska Hospital. The samples were collected in connection with other research projects, which had been approved by the local Ethics Committee.

Analytical procedure

Following addition of ^{14}C-labeled 3-oxocholanoic acid, plasma or serum (1–5 ml) was diluted with one volume of 0.5 M aqueous triethylamine sulfate. The solution was then heated to 64°C for 5 min and extracted on a column (1 × 0.8 cm) of ODS-bonded silica at the same temperature (flow rate about 1 ml × min^{-1}) (8). The column was washed with 5 ml each of water (at 64°C) and 10% aqueous methanol (at room temperature) and steroids were eluted with 10 ml of 95% aqueous methanol.

The eluate was passed through a column (6 × 0.4 cm) of TEAP-LH-20 in HCO_3^--form, packed in 95% aqueous methanol. This column retains acids (1), and following a wash with 5 ml each of 95% aqueous methanol and methanol–chloroform 1:1 (v/v), unconjugated steroids with one carboxyl group were eluted with 4 ml of 0.15 M acetic acid in 95% aqueous methanol (3). This fraction was taken to dryness in vacuo and the residue was dissolved in methanol and transferred to a stoppered tube. Hexatriacontane (Fluka), 1.2 μg, was added as internal standard prior to methylation with diazomethane and trimethylsilylation (see below). An aliquot (1/20) was taken for determination of the recovery of added radioactivity.

For analysis of bile, 0.5-ml samples were diluted with 5 ml of aqueous triethylamine sulfate, and the extraction and isolation of unconjugated bile acids were then performed essentially as described for plasma. Tetratriacontane (Fluka) was used as internal standard for quantitative analysis.

Preparation of derivatives

Methylation. Samples were methylated by one of two procedures. *a*) Methylation with freshly prepared diazomethane at 0°C, using 2 ml of methanol–diethyl ether 1:9 as solvent. The sample was evaporated to dryness after 15–30 min. This procedure was used for quantitative analysis of cholestenoic acids in plasma. *b*) Methylation on a column (4 × 0.4 cm) of SP-LH-20 (H^+-form) in methanol (3). The sample was dissolved in 0.2 ml of methanol and allowed to enter the column. After 45 min the column was rinsed with 4 ml of methanol, which was collected in a stoppered tube and taken to dryness under a stream of nitrogen. This procedure resulted in chemical changes of the 7-hydroxylated cholestenoic acids.

Oximes and O-methyloximes. Unsubstituted oximes (or O-methyloximes (MO)) were prepared by addition of 5 mg of hydroxylamine hydrochloride (or methoxyamine hydrochloride) and 50 μl of pyridine to the dried sample and heating at 60°C for 30 min (9, 10). The pyridine was removed under a stream of nitrogen, 1 ml of water was added, and the products were extracted with 3 × 1 ml of ethyl acetate, which was washed with 1 ml of water. The extract was taken to dryness under a stream of nitrogen.

Trimethylsilyl (TMS) and [$^2H_9$]TMS derivatives. Unlabeled TMS derivatives were prepared by addition of about 0.1 ml of pyridine–hexamethyldisilazane–trimethylchlorosilane 3:2:1 (by vol), and heating at 60°C for 30 min. The reagents were removed under a stream of nitrogen and the derivatives were redissolved in hexane. [$^2H_9$]TMS derivatives were prepared by reaction with 0.2 ml of [$^2H_9$]trimethylchlorosilane–pyridine 1:20 (v/v), for 15 min at room temperature. The reagents were removed under a stream of nitrogen.

Reduction with $LiAlH_4$. The sample was dissolved in 1 ml of dry diethyl ether and 1 mg of $LiAlH_4$ was added. After 1 hr at room temperature, the reaction was stopped by addition of ethyl acetate in diethyl ether, 1:10 (v/v). Water, 2 ml, was added and the products were extracted twice

with ethyl acetate. The extract was taken to dryness under a stream of nitrogen.

Subfractionation of methylated steroid acids on Lipidex 5000

In some experiments the methylated steroid acids were separated according to polarity on a column (7 × 0.4 cm) of Lipidex 5000 packed in hexane (11). The sample was dissolved in hexane and the following fractions were collected: *1*) 3 ml of hexane, *2*) 4 ml of hexane, *3*) 4 ml of hexane–chloroform 9:1 (v/v), *4*) 4 ml of hexane–chloroform 8:2, *5*) 4 ml of hexane–chloroform 7:3, and *6*) 4 ml of hexane–chloroform 1:1. The fractions were taken to dryness, trimethylsilylated, and analyzed by gas–liquid chromatography–mass spectrometry.

Gas–liquid chromatography (GLC)

GLC was carried out using a Carlo Erba HRGC 5300 gas chromatograph connected to a Spectra-Physics SP 4270 integrator. An on-column injector system and a fused silica column (25 × 0.32 mm) coated with a 0.25-μm layer of cross-linked methyl silicone (Quadrex Corp., New Haven, CT) were used with a flame ionization detector. The temperature of the oven was 80°C during the injection, and was then programmed from 80°C to 280°C at a rate of 25°C × min^{-1}. The amounts of C_{24} and C_{27} bile acids were calculated by comparisons of the peak areas with those given by known amounts of the corresponding reference compounds. When the latter were not available, comparisons were made with peak areas given by compounds with similar structures in the A- and B-rings. The internal standard, hexatriacontane, was used to normalize injection volumes. The responses relative to this hydrocarbon of the TMS ethers of methyl 3β-hydroxy-5-cholestenoate and 3β,7α-dihydroxy-5-cholestenoate were estimated to be about 83%. The corresponding value for the derivative of 7α-hydroxy-3-oxo-4-cholestenoic acid was about 67%.

Gas–liquid chromatography–mass spectrometry (GLC/MS)

GLC/MS analyses were carried out using a Finnigan 1020 instrument housing a fused-silica column (30 m × 0.32 mm) coated with a 0.25-μm layer of SE-30 DB-1 (J & W Scientific, Inc., Rancho, Cordova, CA) ending in the ion source. An on-column injection device was used. The oven temperature was about 50°C during the injection and, after 6 min, was rapidly increased to 190°C and was then programmed from 190 to 285°C at a rate of 5°C × min^{-1}. Retention indices were calculated by comparison of retention times with those of the normal C_{30}–C_{38} hydrocarbons analyzed under the same conditions. The temperature of the ion source was 290°C and the electron energy was 40 eV. Repetitive scanning (20 scans × min^{-1}) over the *m/z* range 50–800 was started after a suitable delay. The instrument was tuned so that intensities of fragment ions with *m/z* values above 200–300 were enhanced relative to those of lighter fragment ions.

High-performance liquid chromatography (HPLC)

HPLC of the methylated and trimethylsilylated acids was carried out on a column (250 × 4.5 mm) of LiChrospher (Hibar, Si 100, 5 μm) (Merck, Darmstadt, FRG) connected to a pump (Constametric III) and a fixed wavelength (254 nm) detector (LDC/Milton Roy, Riviera Beach, FL). The mobile phase was hexane–isopropanol 98:2 (v/v). This method could be used for quantitative analysis of 7α-hydroxy-3-oxo-4-cholestenoic acid in plasma.

UV spectroscopy

UV spectra were recorded over the range 210–350 nm on a UV/VIS Spectrophotometer 8450 (Hewlett-Packard) using solutions of 0.5–10 μg/ml in ethanol or hexane and a 1-cm light path.

RESULTS

Identification of cholestenoic acids in plasma

Initially, a simple GLC/MS method was developed for the analysis of metabolic profiles of unconjugated bile acids in human plasma. Following extraction and isolation of steroids possessing one carboxyl group, methylation was carried out on the lipophilic strong cation exchanger SP-LH-20 (H^+-form) in methanol (3). When this fraction was analyzed by GLC and GLC/MS, two quantitatively important compounds with relatively long retention times were found in addition to the common bile acids. Their mass spectra indicated that they were steroidal C_{27} acids. Following studies of their properties using liquid chromatography, GLC/MS, and UV spectroscopy, they were identified as 3β-hydroxy-5-cholestenoic acid and 3-oxo-4,6-cholestadienoic acid. The latter acid was not detected when treatment with SP-LH-20 was omitted and diazomethane was used for methylation. 3-Oxo-4,6-cholestadienoic acid was subsequently shown to be formed on the acidic SP-LH-20 column from 7α-hydroxy-3-oxo-4-cholestenoic acid present in plasma. Another C_{27} steroid acid, 3β,7α-dihydroxy-5-cholestenoic acid, was also detected when diazomethane was used for methylation (see below).

3β-Hydroxy-5-cholestenoic acid. The chromatographic behavior of this compound on TEAP-LH-20 indicated that it had one free carboxyl group. Following methylation with diazomethane or SP-LH-20 in methanol, the compound was eluted before methyl dihydroxycholanoates on a Lipidex 5000 column in a straight-phase system (fraction 2) suggesting that it had one hydroxyl function. This was confirmed by GLC/MS analysis of the TMS and [$^2H_9$]TMS derivatives. Strong evidence for the compound being 3β-hydroxy-5-cholestenoic acid was obtained by

comparing the GLC retention time (**Table 1**) and mass spectrum of the derivative (**Fig. 1**) with those of the TMS ethers of cholesterol and methyl 3β-hydroxy-5-cholenoate. The expected similarities and differences were observed. In addition to the molecular ion (*m/z* 502), the mass spectrum showed intense fragment ions at *m/z* 412 (M–90), *m/z* 373 (M–129) and *m/z* 129, the latter two ions indicative of a 3-trimethylsiloxy-Δ^5 structure. The ion at *m/z* 291 represents the C and D-ring of the steroid skeleton with the intact side chain and corresponds to the ions at *m/z* 247 and *m/z* 249, respectively, in the mass spectra of the derivatives of cholesterol and 3β-hydroxy-5-cholenoic acid. The mass spectrum was also similar to that previously published of the TMS ether of methyl 3β-hydroxy-5-cholestenoate (4). Since the reference compound was not available to us, definitive evidence for the structure was obtained by reduction with $LiAlH_4$. This yielded a compound, the TMS ether of which had the same retention index of 3460 and mass spectrum as synthetic 26-hydroxycholesterol TMS ether. The configuration at C-25 remains unknown.

3β,7α-Dihydroxy-5-cholestenoic acid. The identification of this compound was indirect since the reference compound was not available to us. The mobility on TEAP-LH-20 indicated the presence of one carboxyl group. Following methylation with diazomethane, the mobility on Lipidex 5000 in a straight-phase system was similar to that of common dihydroxycholanoates, suggesting the presence of two hydroxyl groups. This was confirmed by GLC/MS analyses of the TMS and [$^2H_9$]TMS derivatives. The mass spectrum of the TMS ether showed a very intense peak at *m/z* 500 (M–90), as expected from a TMS ether of a dihydroxycholestenoate with an allylic hydroxyl group (Fig. 1). The ions at *m/z* 590 (M^+) and *m/z* 575 (M–15) were only of low intensity. Indirect evidence for the structure was the lability of the compound on the acidic SP-LH-20 column. In methanol, this ion exchanger catalyzed methylation and subsequent degradation, analogous to reactions undergone by 3β,7α-dihydroxy-5-cholenoic acid (12). The stereochemistry of the hydroxyl groups was deduced from the retention time of the methyl ester TMS ether (Table 1). A 3β-hydroxy configuration is expected for biological reasons. If this is the case, a 7β configuration can be excluded, since the 3β,7β isomer would have a considerably longer retention time (13). The retention index was 235 units higher than that of the TMS ether of methyl 3β,7α-dihydroxy-5-cholenoate. Comparisons of derivatives of the other couples of C_{27} and C_{24} bile acids gave values between 240 and 260. The slightly lower value for the 3β,7α-dihydroxy-Δ^5 structure appears to be due to the structure of the side chain. Thus, the addition of a 7α-trimethylsiloxy group to steroids with a 3β-trimethylsiloxy-Δ^5 structure resulted in a ΔRI (change of retention index) of −15 for neutral C_{27} steroids and ± 0 for C_{24} bile acids. The value for the C_{27} acid from plasma was −25 (Table 1).

TABLE 1. Retention indices of derivatives of reference C_{27} steroids and bile acid methyl esters and of derivatives of methyl cholestenoates isolated from human blood

	Neutral C_{27} Steroids		C_{27} Acids from Plasma		C_{24} Bile Acids	
Derivative[a]	Structure[b]	RI[c]	Structure[b]	RI[c]	Structure[b]	RI[c]
TMS	C^5-3β-ol	3130	C^5-3β-ol-26-oate	3445	B^5-3β-ol-24-oate	3185
TMS	C^5-3β,7α-ol	3115	C^5-3β,7α-ol-26-oate	3420	B^5-3β,7α-ol-24-oate	3185
TMS	C^4-7α-ol-3-one	3233	C^4-7α-ol-3-one-26-oate	3550	B^4-7α-ol-3-one-24-oate	3295
MO-TMS	C^4-7α-ol-3-one	3197 3213[d]	C^4-7α-ol-3-one-26-oate	3490 3505[d]	B^4-7α-ol-3-one-24-oate	3250 3265[d]
Oxime-TMS	C^4-7α-ol-3-one	3280	C^4-7α-ol-3-one-26-oate	3582	B^4-7α-ol-3-one-24-oate	3340
None	$C^{4,6}$-3-one	3210	$C^{4,6}$-3-one-26-oate[e]	3527	$B^{4,6}$-3-one-24-oate	3270
MO	$C^{4,6}$-3-one	3232[d] 3247	$C^{4,6}$-3-one-26-oate[e]	3537[d] 3552	$B^{4,6}$-3-one-24-oate	3290[d] 3505
Oxime-TMS	$C^{4,6}$-3-one	3339	$C^{4,6}$-3-one-26-oate[e]	3640	$B^{4,6}$-3-one-24-oate	3400
None	C^4-3-one	3192	C^4-3-one-26-oate[f]	3503	B^4-3-one-24-oate	3248
MO	C^4-3-one	3215			B^4-3-one-24-oate	3268
Oxime-TMS	C^4-3-one	3285 3306[d]			B^4-3-one-24-oate	3347 3362[d]
TMS					B^4-7α,12α-ol-3-one-24-oate	3360
MO					B^4-7α,12α-ol-3-one-24-oate	3285 3307[d]
Oxime-TMS					B^4-7α,12α-ol-3-one-24-oate	3355

[a] TMS, trimethylsilyl; MO, O-methyloxime.
[b] C, cholestane; B, cholane; superscripts indicate position of double bonds; Greek letters denote configuration of hydroxyl groups.
[c] Retention index (Kovats) on a fused silica capillary column coated with a 0.25-μm layer of cross-linked SE-30.
[d] Major isomer, constituting 70–85% of the total.
[e] From plasma following treatment with SP-LH-20 in methanol.
[f] Obtained by oxidation of authentic 26-hydroxycholesterol.

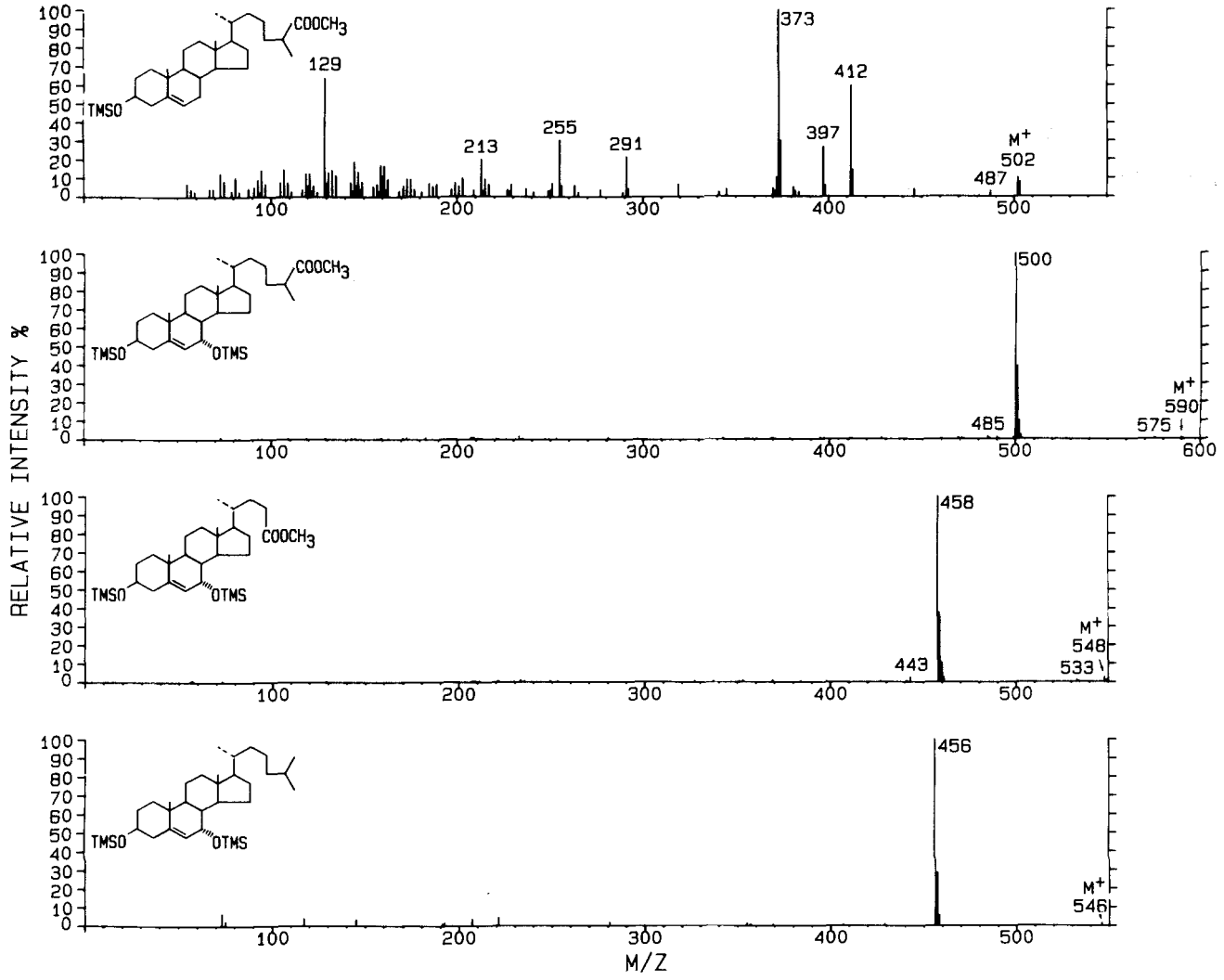

Fig. 1. Mass spectra of the methyl ester TMS ether derivatives of the two acids in plasma identified as 3β-hydroxy-5-cholestenoic acid and 3β,7α-dihydroxy-5-cholestenoic acid. Mass spectra of structures analogous to the latter compound are shown for comparison.

7α-Hydroxy-3-oxo-4-cholestenoic acid. As was the case with 3β,7α-dihydroxy-5-cholestenoic acid, the identification of 7α-hydroxy-3-oxo-4-cholestenoic acid was based on comparisons with compounds having analogous structures. The mobility on TEAP-LH-20 indicated the presence of one carboxyl group. The polarity of the methyl ester, as determined by straight-phase chromatography on Lipidex 5000, was higher than that of methyl 3β-hydroxy-5-cholestenoate but lower or similar to the polarity of methyl dihydroxycholanoates. The mass spectrum of the TMS (and [$^2H_9$]TMS) ether derivative suggested the presence of one hydroxyl group, one oxo function, and a double bond, giving a molecular ion at *m/z* 516 (**Fig. 2**). The oxo group was definitively established by preparation of the oxime derivatives. The unsubstituted oxime was retained on a column of SP-LH-20 (H^+) in methanol indicating that the oxime was in the 3-position (3, 7). This was supported by the UV spectrum of the underivatized compound in hexane which showed a peak at 230 nm, as expected for a steroid with a 3-oxo-Δ^4 structure.

The position and configuration of the hydroxyl group was deduced from the chemical transformation of the compound on SP-LH-20 and from comparisons, after methylation, of GLC retention times and mass spectra of TMS, MO-TMS, and oxime-TMS derivatives with those of corresponding derivatives of analogous compounds (Table 1). The introduction of a 7α-trimethylsiloxy group into steroids with a 3-oxo-Δ^4 structure gave a ΔRI of +41 for C_{27} steroids and +47 for bile acids. The value for the compound from plasma was +47. The formation (or separation) of *syn/anti* isomers of unsubstituted oximes and O-methyloximes depended on the structure of the parent compound (Table 1). A characteristic feature of the 7α-hydroxy-3-oxo-Δ^4 steroids was that two GLC peaks

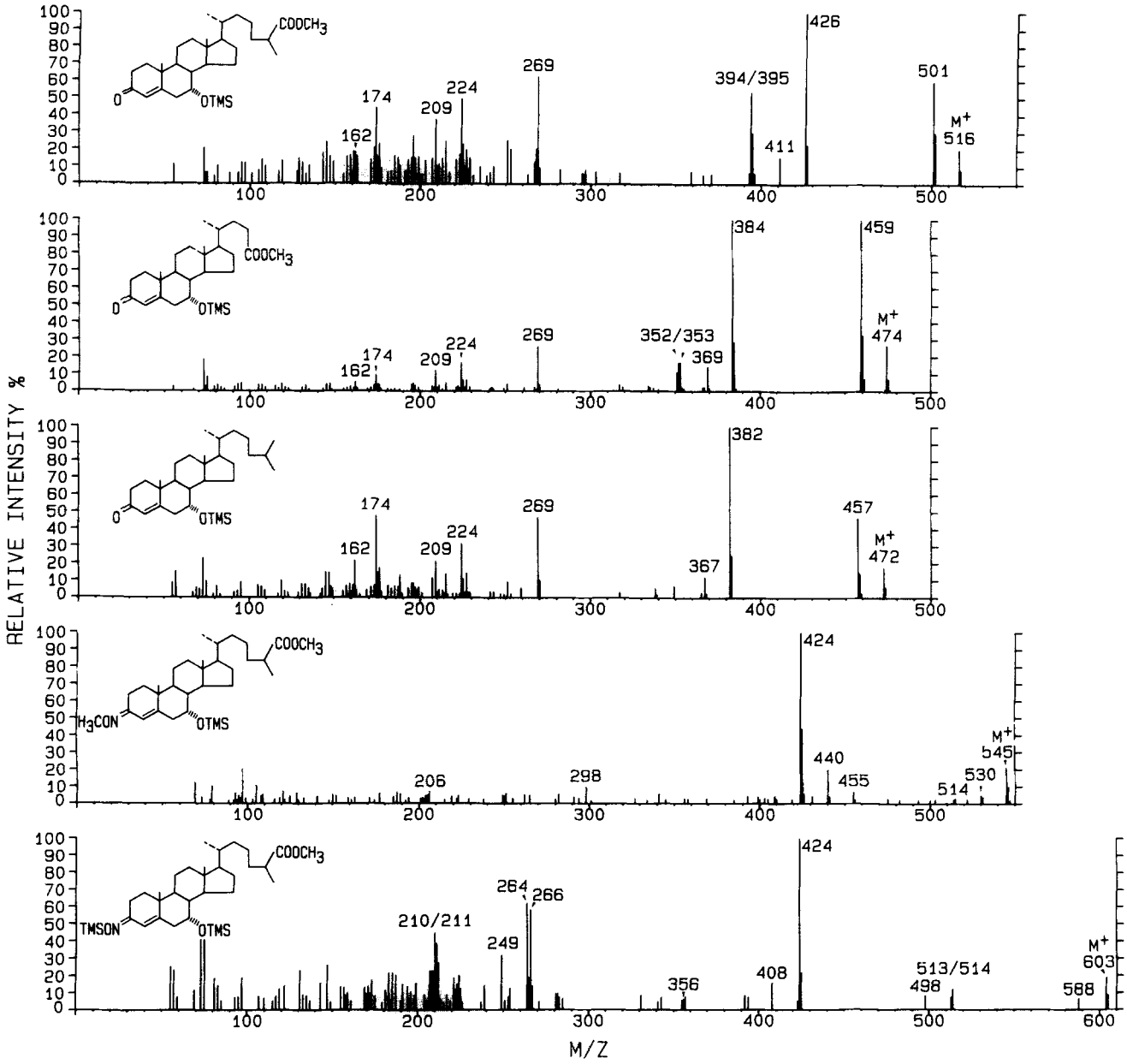

Fig. 2. Mass spectra of derivatives of the bile acid in plasma identified as 7α-hydroxy-3-oxo-4-cholestenoic acid. Mass spectra of analogous compounds differing by the side chain structure are shown for comparison.

were obtained from the MO-TMS derivative, the second isomer being predominant, whereas the oxime-TMS derivative only gave rise to one peak. For simple 3-oxo-Δ^4 steroids (e.g., 4-cholesten-3-one) the opposite is usually the case (3, 9).

The mass spectrum of the methyl ester TMS ether of the acid from plasma showed fragment ions at *m/z* 501 (M-15), *m/z* 426 (M-90, base peak), and *m/z* 411 (M-90-15) whose intensities were similar to those of analogous ions given by the TMS ethers of methyl 7α-hydroxy-3-oxo-4-cholenoate and 7α-hydroxy-4-cholesten-3-one (Fig. 2). Peaks at M-121 and M-122 given by the two methyl esters probably represent loss of trimethylsilanol and the methoxy group from the ester. The ion at *m/z* 269 given by all three compounds is due to the steroid skeleton after loss of the side chain and trimethylsilanol. The ion at *m/z* 224 shifted to *m/z* 233 in the spectrum of the d_9-TMS derivative. This fact and its presence in the spectra of both reference compounds strongly indicates that this ion contains the A-ring, C-6, C-7, and the substituents. The ions at *m/z* 161/162 and 174 probably arise by fragmentation through the C-ring after prior elimination of trimethylsilanol from C-7 (cf. mass spectra shown in **Fig. 3**).

The methyl ester MO-TMS derivative yielded major

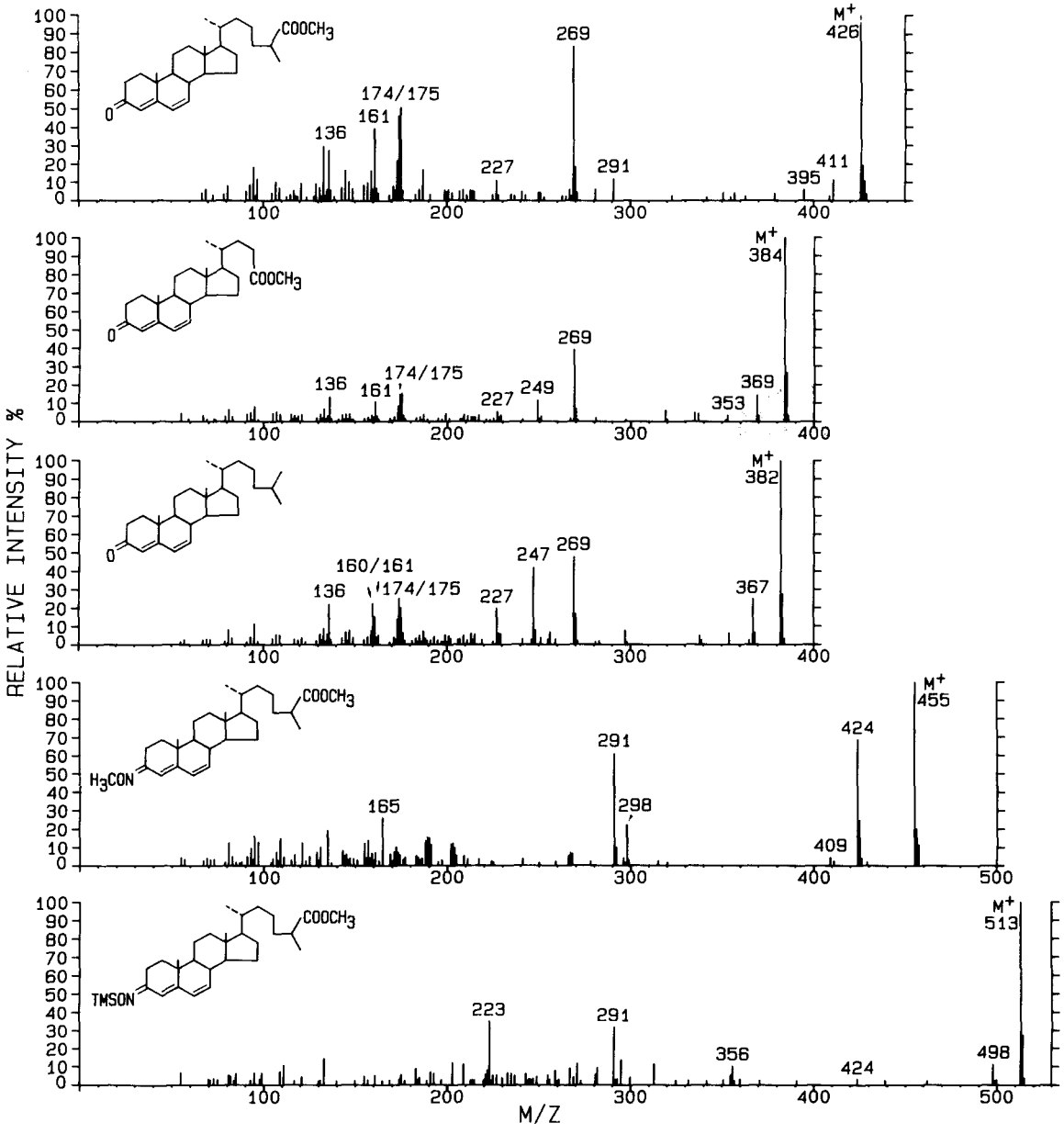

Fig. 3. Mass spectra of methyl 3-oxo-4,6-cholestadienoate and its O-methyloxime and O-trimethylsilyloxime isolated from plasma after methylation with SP-LH-20 in methanol. Mass spectra of methyl 3-oxo-4,6-choladienoate and 4,6-cholestadien-3-one are shown for comparison.

ions at *m/z* 545 (M^+), 455 (M-90), 440 (M-90-15), and 424 (M-90-31), the latter being the base peak due to loss of trimethylsilanol and the methoxy group characteristic of O-methyloxime derivatives (Fig. 2). The ion at *m/z* 298 represents the steroid nucleus with loss of the side chain and trimethylsilanol.

The methyl ester oxime-TMS derivative gave a molecular ion at *m/z* 603, fragments at M-89/90 and a base peak at *m/z* 424 (M-89-90) (Fig. 2). Corresponding ions were seen in the spectra of the reference compounds. The ion at *m/z* 356 represents the steroid skeleton after loss of trimethylsilanol and the ions at *m/z* 264/266 are most likely formed by loss of the trimethylsiloxy group on the oxime. The ions at *m/z* 210/211 were given by all three compounds, *m/z* 211 being characteristic for a 3-trimethylsilyloxime-4-ene structure (3).

The retention time data and the fragmentation patterns of the TMS, MO-TMS, and oxime-TMS derivatives, which were analogous to those of the corresponding reference compounds, strongly indicated that the structure of the compound in plasma was 7α-hydroxy-3-oxo-4-cholestenoic acid. Additional evidence was obtained by conversion of the 7α-hydroxy-3-oxo-Δ^4 into a 3-oxo-$\Delta^{4,6}$ structure. Following treatment with SP-LH-20 in metha-

nol, 7α-hydroxy-3-oxo-4-cholestenoic acid disappeared and a less polar compound (eluted prior to methyl 3β-hydroxy-5-cholestenoate on a column of Lipidex 5000 in the straight-phase system) appeared. This was identified as methyl 3-oxo-4,6-cholestadienoate, based on the GLC retention times of the oxo compound and the *syn* and *anti* isomers of the O-methyloxime and TMS-oxime (Table 1), and on the characteristic mass spectra (Fig. 3).

The molecular ion was the base peak in the mass spectra of the product from plasma and the reference compounds with a 3-oxo-$\Delta^{4,6}$ structure (Fig. 3). The two methyl esters gave an ion at *m/z* M-31, due to loss of the methoxy group. The ion at *m/z* 269 is formed by loss of the side chain, and losses of the D and A ring give rise to the ions at *m/z* 227 and 199, respectively (14). The ion at *m/z* 136 probably represents the A-ring, C-6, and C-7. The product from plasma gives an ion at *m/z* 291 corresponding to *m/z* 249 and 247 in the spectra of methyl 3-oxo-4,6-choladienoate and 4,6-cholestadien-3-one, respectively. These ions represent the C and D ring with the attached side chain (14).

The O-methyloxime of the compound from plasma gave a base peak and molecular ion at *m/z* 455 and an intense fragment ion at M-31 (*m/z* 424) characteristic of the O-methyloxime (Fig. 3). The ion at *m/z* 298 represents the steroid nucleus after loss of the side chain. Cleavage through the B ring probably gives rise to the ion at *m/z* 165, analogous to *m/z* 136 in the spectrum of the oxo compound and to *m/z* 223 in the spectrum of the trimethylsilyloxime (Fig. 3). The ion at *m/z* 291, representing the C and D-rings with the attached side chain, was intense as were the corresponding ions at *m/z* 249 and 247, respectively, in the spectra of the O-methyloximes of the C_{24} and C_{27} reference compounds.

The UV spectra of the underivatized and derivatized plasma compound were similar to those of methyl 3-oxo-4,6-choladienoate and its derivatives. Thus, the absorption maxima in ethanol were at 285, 281, and 275 nm for the free ketone, the O-methyloxime and the oxime, respectively. Corresponding values for the reference compound were 284, 281, and 277. The absorption maximum in hexane of the underivatized compound from plasma and the reference compound was at 271 nm. The difference between the spectra in ethanol and hexane is expected for an α,β-unsaturated oxo group (15).

Concentrations of cholestenoic acids in plasma

The concentrations of the identified cholestenoic acids and the major unconjugated C_{24} bile acids in plasma were determined by GLC after trimethylsilylation. Examples of chromatograms are shown in **Fig. 4.** As an alternative to GLC, the concentration of 7α-hydroxy-3-oxo-4-cholestenoic acid could be determined by HPLC with UV-detection (**Fig. 5**). The results with the two methods were similar. The concentrations of the cholestenoic acids in plasma

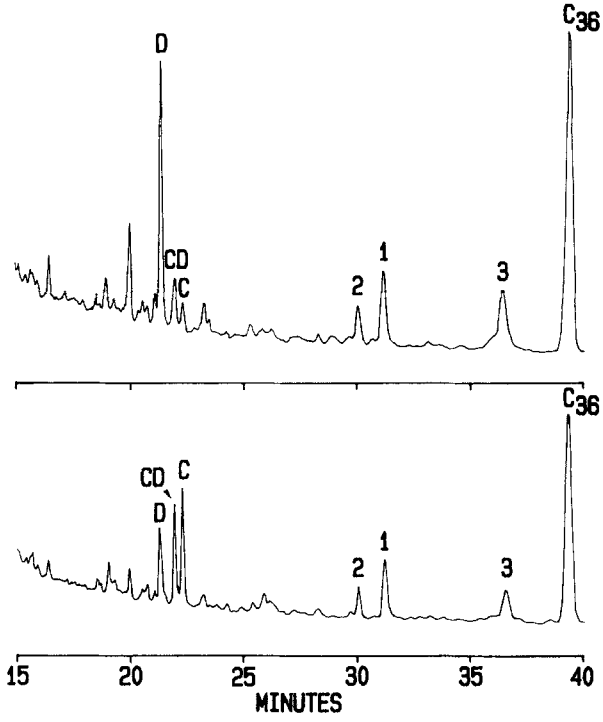

Fig. 4. Gas chromatograms obtained in the analyses of unconjugated bile acids in plasma from two healthy women (subjects B, top and A, bottom; cf. Table 2). The peaks of the derivatized deoxycholic (D), chenodeoxycholic (CD), cholic (C), 3β,7α-dihydroxy-5-cholestenoic (2), 3β-hydroxy-5-cholestenoic (1), and 7α-hydroxy-3-oxo-4-cholestenoic (3) acids are indicated. C_{36} is the peak of the internal standard, hexatriacontane. The equivalent of about 80 μl of plasma was injected.

from 11 apparently healthy human subjects and in a pool of plasma samples from 50 patients with various diseases were in the same range as those of common unconjugated C_{24} bile acids (**Table 2**). The mean concentration was highest for 7α-hydroxy-3-oxo-4-cholestenoic acid and the level of 3β,7α-dihydroxy-5-cholestenoic acid was significantly lower than those of the other C_{27} acids. When diazomethane was used for methylation, 3-oxo-4,6-cholestadienoic acid could not be detected (< 10 ng/ml of plasma). Statistical evaluation of the concentrations in plasma showed significant positive correlations between 3β-hydroxy-5-cholestenoic and 3β,7α-dihydroxy-5-cholestenoic acids (correlation coefficient $r = 0.76$, $P < 0.0001$), between the latter and 7α-hydroxy-3-oxo-4-cholestenoic acid ($r = 0.64$, $P < 0.01$) and between 3β-hydroxy-5-cholestenoic and 7α-hydroxy-3-oxo-4-cholestenoic acids ($r = 0.59$, $P < 0.01$).

The concentrations of the C_{27} acids, particularly 7α-hydroxy-3-oxo-4-cholestenoic acid, appeared to be less variable between individuals than those of the C_{24} acids. Thus, the coefficient of variation for 7α-hydroxy-3-oxo-4-cholestenoic acid was about 35% while the values for C_{24} acids were between 56–159%. Results from ongoing studies of patients with various diseases, particularly liver

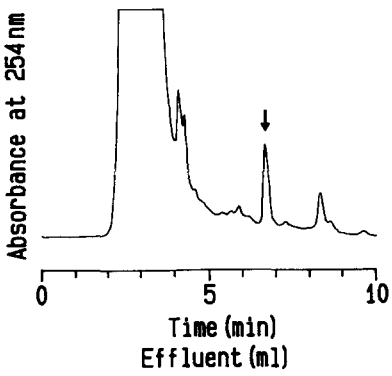

Fig. 5. Chromatogram obtained in the HPLC analysis of the methyl ester TMS ether derivative of 7α-hydroxy-3-oxo-4-cholestenoic acid in plasma from a healthy woman (subject B). The equivalent of 0.3 ml plasma was injected and the peak indicated represents about 35 ng.

diseases, also indicate that the plasma levels of the cholestenoic acids are relatively constant. However, a few patients with very high or low (but detectable) levels have been found. A patient with primary biliary cirrhosis had the highest concentration of 7α-hydroxy-3-oxo-4-cholestenoic acid so far observed (372 ng/ml). Low levels (< 20 ng/ml) have often been seen in patients with uremia before and after dialysis. The significance of these results is not known and the results of these studies will be published elsewhere.

A variation in bile acid concentrations was observed when samples collected from the same subjects in the morning after fasting and at 1 PM, 1.5 hr after lunch, were analyzed. The levels of C_{27} acids were increased in the second sample from three of the four subjects (**Table 3**). The concentrations of unconjugated C_{24} bile acids showed inconsistent variations, not related to the changes of the C_{27} acids.

Unconjugated C_{27} bile acids in bile

The possible occurrence in bile of the cholestenoic acids found in plasma was investigated by the same analytical method. A gas–liquid chromatographic analysis of unconjugated bile acids isolated by ion exchange chromatography of an extract of bile is shown in **Fig. 6.** GLC–MS analyses confirmed the presence of 3α,7α,12α-trihydroxy-5β-cholestanoic, 3α,7α,12α-trihydroxy-5β-cholest-24-enoic, 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic, and 3α,7α,12α,26-tetrahydroxy-5β-cholestan-27-oic acids recently reported by Matoba, Une, and Hoshita (16). Significant amounts of 3α,7α,12α,25-tetrahydroxy-5β-cholestanoic acid were also found (Fig. 6). However, the cholestenoic acids present in plasma could not be detected in bile fistula bile (< 10–50 ng/ml). Details of the results from the study on C_{27} bile acids in bile will be reported separately.

DISCUSSION

The structures of the cholestenoic acids identified in this study are shown in **Fig. 7.** While 3β-hydroxy-5-cholestenoic acid has previously been found in blood from patients with liver disease (4, 5), 3β,7α-dihydroxy-5-cholestenoic and 7α-hydroxy-3-oxo-4-cholestenoic acids have not been detected until now. This may be because most methods for trace analysis of bile acids use conditions which produce artefacts from these acids. Our methods for isolation and group separation using solid sorbents and inert ion exchangers (1, 2) make it possible to obtain a fraction of unconjugated bile acids which can be derivatized with mild methods prior to analysis by GLC–MS. Similar methods were used by Setchell et al. (17) in analyses of unconjugated bile acids in

TABLE 2. Concentrations of unconjugated bile acids in blood from healthy adult humans and in a pool of plasma containing samples from 50 patients

			Bile Acid[a] Concentration, ng/ml plasma					
Subject	Sex	Age	C_{27}-I	C_{27}-II	C_{27}-III	C	CD	D
A	F	23	74	35	50	76	73	59
B	F	25	62	32	102	13	33	146
C	F	26	95	43	74	94	234	178
D	F	27	50	19	45	42	136	284
E	F	27	55	37	63	205	97	100
F	F	29	77	41	125	610	207	82
G	M	25	61	35	83	29	32	108
H	M	27	134	112	128	15	51	137
I	M	32	41	22	80	26	57	97
J	M	34	56	32	90	62	99	89
K	M	35	34	20	59	37	70	301
Mean ± SD			67.2 ± 27.9	38.9 ± 25.6	81.7 ± 27.9	109.9 ± 174.6	99.0 ± 67.6	143.7 ± 80.6
Plasma pool[b]			66	58	78	86	117	158

[a] C_{27}-I, 3β-hydroxy-5-cholestenoic acid; C_{27}-II, 3β,7α-dihydroxy-5-cholestenoic acid; C_{27}-III, 7α-hydroxy-3-oxo-4-cholestenoic acid; C, cholic acid; CD, chenodeoxycholic acid; D, deoxycholic acid.
[b] See text.

TABLE 3. Concentrations of unconjugated bile acids in blood collected from women at 8:20 AM after fasting overnight and at 12:30 PM, 1.5 hr after the lunch meal

Subject	Age	Sampling Time	Bile Acid[a] Concentration, ng/ml plasma					
			C_{27}-I	C_{27}-II	C_{27}-III	C	CD	D
L	27	8:20 AM	26	13	63	11	52	118
		12:30 PM	49	30	77	31	213	485
M	30	8:20 AM	42	55	75	156	101	162
		12:30 PM	59	77	117	61	28	128
N	31	8:20 AM	32	26	15	15	31	74
		12:30 PM	50	36	68	24	26	85
O	31	8:20 AM	74	49	92	201	121	161
		12:30 PM	68	47	75	7	12	96
Mean		8:20 AM	43.5	35.8	61.3	95.8	76.3	128.8
		12:30 PM	56.5	47.5	84.3	30.8	69.8	198.5

[a] Abbreviations, see Table 2.

plasma, but the cholestenoic acids were not observed. Analogous methods were used by Matoba et al. (16) to study the unconjugated bile acids in bile. While a number of C_{27} acids were described, the cholestenoic acids now found in plasma were not detected. Our analyses confirm these findings. All the unconjugated C_{27} acids described by Matoba et al. (16) were found in bile, but the cholestenoic acids present in plasma were not detected. If present at all, the concentrations of the latter acids would be at least 2–5 times lower in hepatic bile than in plasma. Thus, if formed in the liver, the C_{27} bile acids in plasma may be secreted directly into the blood and not undergo an enterohepatic circulation. Their concentration in plasma increased after a meal in three of four subjects, indicating some relationship to the passage of bile acids through the liver. Their clearance from plasma may be slow, supported by the absence of detectable amounts also in urine (Mörk, B., G. Edstrom, M. Axelson, and J. Sjövall, unpublished results).

The C_{27} acids present in bile were not above the GLC detection limits in plasma (< 5 ng/ml). This constitutes an interesting case of compartmentation, and indicates that different aspects of bile acid biosynthesis and excretion are reflected by the C_{27} acids in plasma and bile.

The data do not permit conclusions regarding the pathways of formation of the C_{27} acids in plasma. An involvement of extrahepatic enzymes cannot be excluded. The configuration at C-25 may be the same or different from that of the C-26 oxygenated intermediates in the normal bile acid biosynthesis. Regardless of these uncertainties, three metabolic reactions are required: 26-hydroxylation

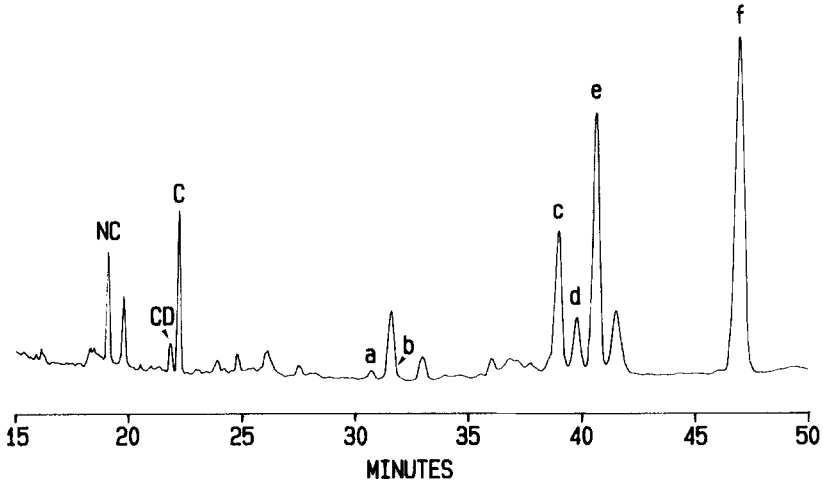

Fig. 6. Gas chromatogram obtained in the analysis of unconjugated bile acids in bile fistula bile from a 69-year-old woman operated for gallstones. The peaks of derivatized norcholic (NC), chenodeoxycholic (CD), cholic (C), 3α,7α,12α-trihydroxy-5β-cholestanoic (a), 3α,7α,12α-trihydroxy-5β-cholest-24-enoic (b), 3α,7α,12α,24ζ(25S)-tetrahydroxy-5β-cholestanoic (c), 3α,7α,12α,24ζ(25R)-tetrahydroxy-5β-cholestanoic (d), 3α,7α,12α,25-tetrahydroxy-5β-cholestanoic (e), and 3α,7α,12α,26-tetrahydroxy-5β-cholestan-27-oic (f) acids are indicated. The equivalent of about 10 μl of bile was injected and peak f represents about 40 ng. Tetratriacontane, not added to this sample, had a retention time of about 28.2 min.

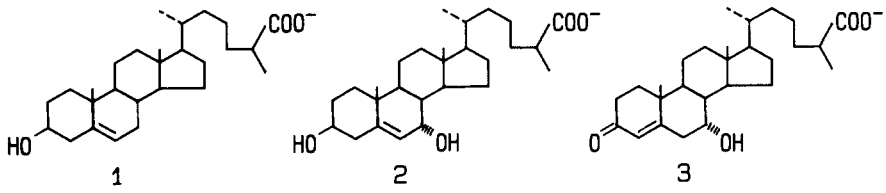

Fig. 7. Structures of unconjugated cholestenoic acids identified in plasma.

(with subsequent oxidation), 7α-hydroxylation, and oxidation by a 3β-hydroxy-Δ^5-steroid oxidoreductase (with isomerization of the 5,6 double bond). In the major pathway from cholesterol to cholic acid, the metabolic changes of the nucleus are generally believed to be completed before the oxidation of the side chain (18). However, 26-hydroxylation may occur at an earlier stage, and cholesterol, 7α-hydroxycholesterol, 7α-hydroxy-4-cholesten-3-one can all serve as substrates of the mitochondrial 26-hydroxylase in the human liver (19). The alternative pathway to chenodeoxycholic acid, first demonstrated by Mitropoulos and Myant in the rat (20, 21) and by Javitt and coworkers in hamster and man (22, 23), starts with 26-hydroxylation of cholesterol while 7α-hydroxycholesterol seems to be 26-hydroxylated in the pathway studied by Yamasaki (24). All of these pathways provide opportunities for the formation of the unconjugated cholestenoic acids in plasma. 7α-Hydroxycholesterol and 7α-hydroxy-4-cholesten-3-one are better substrates of the mitochondrial 26-hydroxylase than cholesterol (18, 25) and both are present in measurable concentrations in the human liver, although their subcellular distribution in vivo is not known (26). They might be released from the chain of reactions in the endoplasmic reticulum to be 26-hydroxylated in the mitochondria. Microsomal 26-hydroxylation is not believed to occur in man (19) but cannot be discounted in a formation of cholestenoic acids as side products from neutral intermediates in the bile acid biosynthesis.

Potential precursors also occur in blood. Thus, 26-hydroxycholesterol occurs both in free and esterified form at a total concentration of 92–256 ng/ml (27). The level of the free sterol is similar to that of 3β-hydroxy-5-cholestenoic acid. The levels of 7α-hydroxycholesterol (28), reported to be 20–180 ng/ml (29), are difficult to determine due to the ease of formation of autooxidation products of cholesterol during the analysis (30). The concentration of 7α-hydroxy-4-cholesten-3-one in plasma is about 50 ng/ml (31). If the intermediates in the major pathway are not involved, 26-hydroxycholesterol is a potential precursor of all three cholestenoic acids in plasma. The significant correlations between their plasma levels indicate a close metabolic relationship between the acids.

Regardless of the order of the reactions, an oxidation of the 26-hydroxyl group is required. The cytosolic liver alcohol dehydrogenase (EC 1.1.1.1) efficiently oxidizes 5β-cholestane-3α,7α,12α,26-tetrol at C-26 and is believed to be important for bile acid biosynthesis in vivo (32). However, 26-hydroxycholesterol is not oxidized (33), and bile acid formation appears to be normal in deermice genetically lacking alcohol dehydrogenase (34). Thus, another dehydrogenase may be active in the formation of the C_{27} acids present in plasma.

The metabolic fate of the C_{27} acids is unknown. If taken up by the liver, the 7α-hydroxylated ones are likely to be converted to normal bile acids (18). The β-oxidation is considered to be peroxisomal (35–37) and this is the reaction that has originally not taken place. The 3β-hydroxy-5-cholestenoic acid might be an intermediate in a conversion of 26-hydroxycholesterol into 3β-hydroxy-5-cholenoic acid (20–23, 38). The latter is a normal constituent in bile, urine, plasma, amniotic fluid, and meconium (see ref. 39) and it occurs mainly in a conjugated and sulfated form (see refs. 40–42). The total levels in plasma from healthy subjects have been determined by GLC–MS and found to be 70 ± 20 ng/ml (expressed as unconjugated compound). About 60% of this was in sulfated and 20% in glucuronidated form. Since hydrolysis with cholylglycine hydrolase preceded the analysis, it is not known whether any unconjugated acid was present. Even if this were the case, the levels would be lower than those of unconjugated 3β-hydroxy-5-cholestenoic acid.

The high concentrations of unconjugated cholestenoic acids relative to unconjugated C_{24} bile acids may be due to low rates of conjugation of bile acids with a long side chain (43, 44). Although we have not found any conjugates of these acids in plasma, this does not exclude their presence. The unsaturated 7α-hydroxy acids are chemically unstable and mild methods are required for their detection in unchanged form (cf. refs. 12, 42). Neutral steroids and C_{24} bile acids containing a 3β-hydroxy-Δ^5 structure are usually esterified with sulfuric acid in man (see refs. 12, 39–42). Further studies are needed to establish whether this is also the case with the analogous C_{27} bile acids.

Since the cholestenoic acids are potential intermediates/side products in the biosynthesis of bile acids, their analysis in plasma could possibly provide information about cholesterol metabolism under physiological and/or pathological conditions. Further studies will reveal the possible correlations between cholestenoic acids in plasma and disorders of cholesterol metabolism

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