Detection of 3-hydroxy-etianic and 3-hydroxy-bisnorcholanoic acids in human serum

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Abstract Capillary GLC–MS analysis of the free/sulfate/glucuronide bile acid fraction obtained from human cholestatic serum demonstrated the presence of 3-hydroxyandrostan-17β-carboxylic (etianic) and 3-hydroxybisnorcholanoic acids.—**St. Pyrek, J., J. M. Little, and R. Lester.** Detection of 3-hydroxy-etianic and 3-hydroxy-bisnorcholanoic acids in human serum. *J. Lipid Res.* 1984. **25:** 1324–1329.

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Routine methods of GLC separation of bile acids (BA) are optimized for di- and tri-hydroxylated C_{24} acids and do not permit easy detection of C_{20}–C_{22} components. This is reflected in recent reviews of BA analysis in which the possible presence of these lower homologues is not considered (1–3). Apart from C_{24} BA and their C_{27} precursors, however, steroidal acids with shorter and longer side chains are present in biological fluids. Thus C_{21} and C_{20} acids are known urinary catabolites formed from corticosteroids (4) and progesterone (5). C_{22} acids are intermediates in the microbiological degradation of sterols and BA (6) and their formation in the colon is conceivable. Dietary sterols may also serve as precursors of C_{28} and C_{29} acids (7–9). Other, more obscure, metabolic processes may also be involved as indicated by the recent determination of the structure of the C_{29} dicarboxylic acid isolated from patients with coprostanic acidemia (10, 11).

The present preliminary study of the monohydroxylated fraction of human serum demonstrates the occurrence of minor C_{20} and C_{22} components. This study originates from the previous investigation of human meconium in which four 3-hydroxy-androstane-17β-carboxylic (C_{20}, etianic) (12) and three bisnorcholanoic (C_{22}) acids were identified (13).

METHODS

Separation of monohydroxylated bile acid fraction

The sample of serum (5 ml, patient with portacaval shunt, bilirubin 48 mg/dl) was treated overnight with a solution of sodium borohydride (100 mg in 0.5 ml of water, pH 12). Subsequently 6 N HCl was added to decompose the excess of borohydride and the pH was adjusted to 4.5 with 1 ml of 5 M acetate buffer. *Helix pomatia* intestinal juice (0.1 ml) was then added and the mixture was incubated for 24 hr at 37°C. This solution (10 ml) was treated with dimethoxypropane (69 ml) and 12 N HCl (0.5 ml) for 30 min. After dilution with acetone (190 ml), solvolysis was performed for 72 hr at room temperature (according to ref. 14). The precipitate was removed by centrifugation and washed with methanol–acetone 1:1. The combined supernatant was evaporated in vacuo and the residue was dissolved in 1 N NaOH in 50% methanol. The mild alkaline hydrolysis was performed for 20 hr at 75°C. The hydrolysate was acidified with 6 N HCl and extracted with freshly distilled ethyl ether (4 × 25 ml). The extract was subsequently washed with water (2 × 10 ml), evaporated in vacuo, and redissolved in 75% methanol. A trace of [^{3}H]lithocholic acid (10^4 dpm) was added at this stage. The solution was applied on a Dowex 1 × 2 column and eluted as described earlier (12, 13). The acid fraction eluted with 1 N acetic acid in 75% methanol was evaporated and treated with an excess of diazomethane in methanol–ethyl ether. After evaporation the residue was separated on a small column prepared from silica gel prewashed with acetone and benzene. The column was eluted with benzene followed by the increasing concentrations of acetone in benzene. Two and 3% acetone in benzene eluted monohydroxylated BA methyl esters.

Capillary GLC–MS analysis

GLC–MS analysis was performed on 15 m SE-54 fused silica capillary, 0.25 mm i.d., directly introduced

Abbreviations: BA, bile acid; C_{20}, androstan-17β-carboxylic acid; C_{21}, pregnan-21-oic acid; C_{22}, bisnorcholanoic acid; C_{24}, cholanoic acid; C_{27}, cholestanoic acid; GLC, gas–liquid chromatography; MS, mass spectrometry; R_t, retention time; TLC, thin-layer chromatography; TMS, trimethylsilyl; TMSOH, trimethylsilanol; TIC, total ion current.

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into the ion source of the Finnigan (Sunnyvale, CA) quadrupole mass spectrometer, model 3200, with a modified interface and INCOS data system (12). Solvent-free samples of methyl ester-TMS ethers, prepared as described before (13), were injected using a falling needle injector (290°C) the oven temperature was programmed from 160 to 260°C, 10° per min. Low resolution electron impact mass spectra were measured at 20 eV in the range 100–600 atomic mass units, one spectrum per second. The separation was monitored using the total ion current (TIC) and the intensity of selected fragment ions (mass chromatograms). Additionally, for partially resolved GLC peaks, resolution enhancement was obtained using the modified Biller-Biemann approach available in the INCOS software (MSDS).

RESULTS

Capillary GLC and GLC–MS analyses of monohydroxylated BA fraction (methyl ester, acetates, broad temperature program) obtained from samples of normal (three) and cholestatic (three) serum demonstrated the presence of 3α-hydroxy-5β-cholan-24-oic acid (lithocholic, >50%) and a trace of 3β-hydroxy-5β-cholan-24-oic acid (isolithocholic, 2%), while 3β-hydroxychol-5-en-24-oic acid was barely detectable in five of the samples. In the range of retention time (R_t)) expected for derivatives of C_{20} and C_{22} acids, varying amounts of saturated and unsaturated components (0–10% of the total fraction) were clearly observed.

Fig. 1 and **Fig. 2** show two fragments of capillary

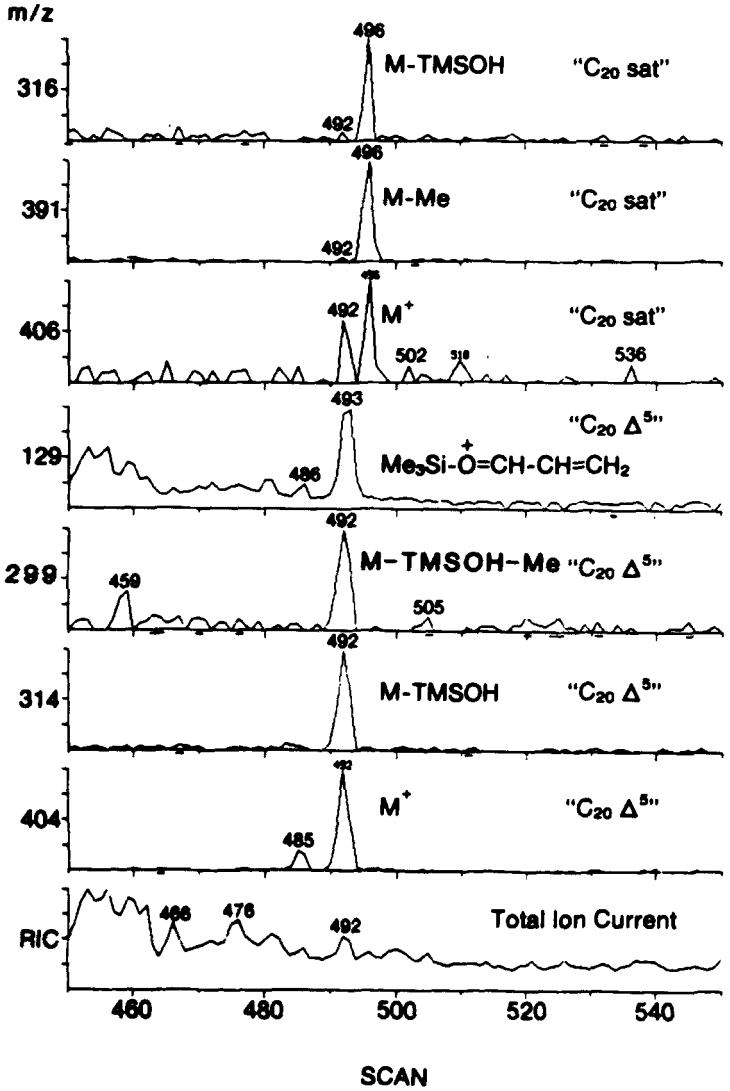

Fig. 1. Mass chromatograms corresponding to saturated and unsaturated C_{20} components detected in the monohydroxylated fraction of human serum; the separation of methyl ester trimethylsilylether and GLC–MS conditions are described in the text.

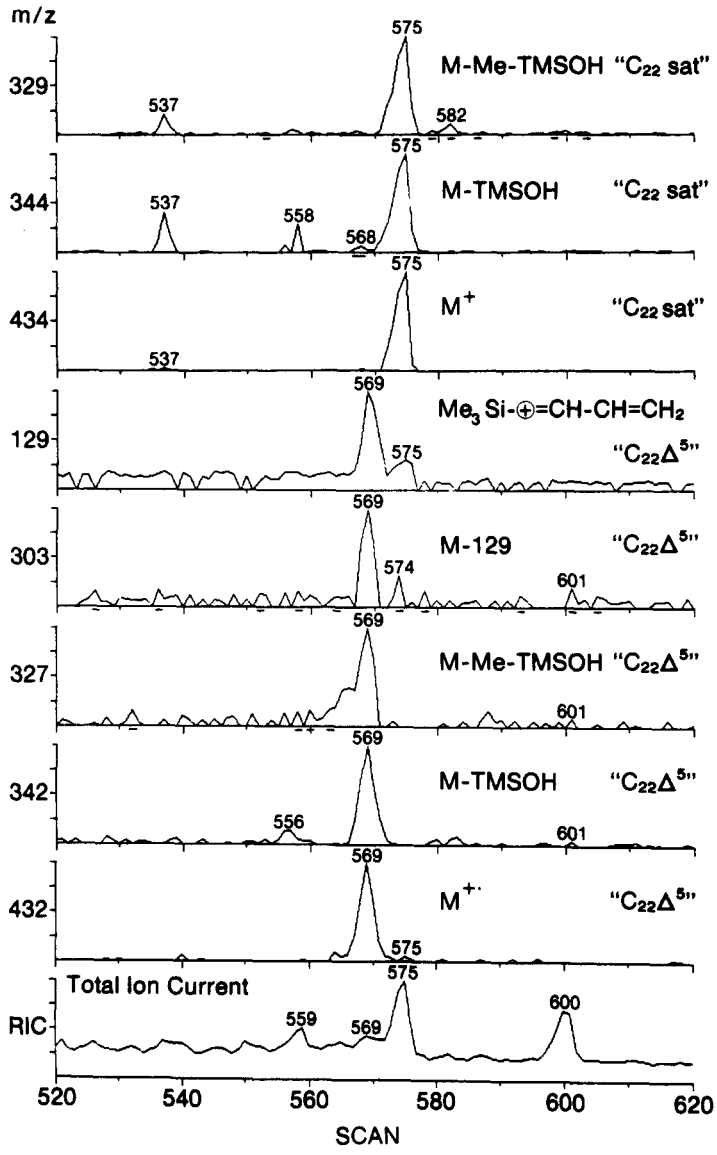

Fig. 2. Mass chromatograms corresponding to saturated and unsaturated C_{22} components detected in the monohydroxylated fraction of human serum; conditions as in Fig. 1.

GLC–MS analysis of the monohydroxylated BA isolated from serum of a patient with portacaval shunt and a highly elevated level of 3β-hydroxychol-5-en-24-oic acid. Borohydride reduction, hydrolysis with *Helix pomatia* intestinal juice, acid solvolysis, and mild alkaline hydrolysis were successively applied and the fraction, corresponding to free acids, sulfates, and glucuronides, was analyzed in the form of methyl ester TMS ethers. This GLC separation resolves all 3,5-diastereomers of C_{20} and C_{22} BA (13).

In the region of R_t corresponding to C_{20}–C_{27} compounds, the computer-enhanced mass chromatograms revealed the presence of 15 major and at least 50 minor components. Several unidentified components with the value of R_t extremely close to those expected for the C_{20} and C_{22} BA derivatives were also observed (**Table 1**). Some of these components displayed fragment ions diagnostic for BA derivatives; however, their mass spectral fragmentation precluded any structural relationship with BA.

The part of the GLC separation between scan 450 and 550 was analyzed for the possible presence of C_{20} components. Fig. 1 shows the fragment of TIC trace and mass chromatograms corresponding to major fragment ions in the spectra of saturated and 5-monounsaturated methyl ester-TMS ethers of C_{20} acids including ions at m/z 129 and 275 (M–129) prominent in mass spectra of TMS ether of 3β-hydroxy-5-unsaturated steroids (15). Two close GLC peaks were detected at scan 492 and 496. Mass spectra obtained by computer en-

TABLE 1. Identification of major components of the monohydroxylated bile acid fraction of human cholestatic serum by capillary GLC–MS

Scan #	Mass Spectral Data: m/z (% Intensity, at 20 eV)	Identification
492	404 (20, M^+), 389 (5, M–Me), 348 (11, M–56), 314 (25, M–TMSOH), 299 (20, M–TMSOH–Me), 275 (35, M–129), 215 (40, ABC ion), 129 (100) (Fig. 3)	The spectrum was identical with that of methyl 3β-trimethylsilyloxyandrost-5-ene-17β-carboxylate
496	406 (6, M^+), 391 (100, M–Me), 316 (30, M–TMSOH), 301 (12, M–TMSOH–Me) (Fig. 3)	The spectrum was identical with that of methyl 3β-trimethylsilyloxy-5α-androstane-17β-carboxylate
504	410 (100), 395 (10), 342 (20), 292 (40), 277 (40), 259 (25), 251 (25), 215 (60)	Compound not identified
537	434 (5, M^+), 419 (15, M–Me), 344 (100, M–TMSOH), 329 (42, M–TMSOH–Me), 215 (60) (Fig. 3)	Identified as methyl 3ξ-trimethylsilyloxybisnor-5ξ-chol-22-oate, $\alpha\alpha$ or $\beta\beta$ (less likely) (Fig. 3)
540	410 (85), 259 (100), 213 (60), 215 (80)	Compound not identified
559	424 (10), 409 (3), 357 (70), 343 (48), 229 (60), 215 (100), 157 (60)	Compound not identified
569	432 (30, M^+), 376 (20, M–56), 342 (65, M–TMSOH), 327 (30, M–TMSOH–Me), 303 (45, M–129), 129 (100) (Fig. 3)	The spectrum was identical with that of methyl 3β-trimethylsilyloxybisnorchol-5-en-22-oate
570	494 (50), 479 (10), 377 (35), 160 (100)	Compound not identified
575	434 (32, M^+), 419 (100, M–Me), 377 (15, M–57), 344 (40, M–TMSOH), 329 (32, M–TMSOH–Me), 230 (20, ABC fragment), 215 (90, ABC fragment) (Fig. 3)	The spectrum was identical with that of methyl 3β-trimethylsilyloxybisnor-5α-cholan-22-oate
600	494 (0.5, M^+), 404 (4, M–TMSOH), 389 (10, M–TMSOH–Me), 346 (10), 331 (100), 313 (15)	The spectrum may indicate the structure of methyl ester, bistrimethylsilyl ether of a dihydroxylated, saturated etianic acid
635	423 (10, M–Me?), 388 (15), 185 (100)	Compound not identified
662	458 (30, M^+), 443 (5, M–Me), 368 (M–TMSOH), 353 (M–TMSOH–Me), 129 (100)	Trace of trimethylsilyl-cholesterol
665	462 (10, M^+), 372 (100, M–TMSOH), 357 (45, M–TMSOH–Me), 230 (20, ABC fragment), 215 (65, ABC fragment)	Identified as methyl 3β-trimethylsilyloxy-5β-cholan-24-oate (isolithocholic acid)
670	462 (2, M^+), 372 (65, M–TMSOH), 357 (32, M–TMSOH–Me), 230 (15, ABC fragment), 229 (15), 215 (100, ABC fragment)	Identified as methyl 3α-trimethylsilyloxy-5β-cholan-24-oate (lithocholic acid)
704	460 (1, M^+), 370 (12, M–TMSOH), 355 (10, M–TMSOH–Me), 331 (100, M–129), 199 (40), 185 (25)	Identified as methyl ester, trimethylsilyl ether of a monounsaturated hydroxycholanoic acid
734	460 (50, M^+), 445 (10, M–Me), 404 (20, M–56), 383 (12), 370 (M–TMSOH), 355 (40, M–TMSOH–Me), 331 (80, M–129), 255 (20), 249 (20), 129 (100)	Identified as methyl 3β-trimethylsilyloxychol-5-en-24-oate
738		Trace of methyl 3β-trimethylsilyloxy-5α-cholan-24-oate
816	502 (12, M^+), 446 (10, M–56), 412 (65, M–TMSOH), 397 (25, M–TMSOH), 373 (45, M–129), 213 (60, ABC fragment), 129 (100)	Tentatively identified as an unsaturated C_{27} BA
964	502 (20, M^+), 446 (10, M–56), 412 (65, M–TMSOH), 397 (30, M–TMSOH-Me), 373 (95, M–129), 291 (20), 255 (30), 129 (100)	Tentatively identified as an unsaturated C_{27} BA

hancement (**Fig. 3**, ion assignment in Table 1) as well as relative R_t values indicated that parent acids are 3β-hydroxyandrost-5-ene-17β-carboxylic and 3β-hydroxy-5α-androstane-17β-carboxylic acids, respectively.

Mass chromatograms corresponding to fragment ions characteristic for methyl ester-TMS ethers of saturated and unsaturated C_{22} acids (Fig. 2) demonstrated the possible presence of the two saturated components (scans 537 and 575) and one 5-unsaturated component (scan 569). Mass spectra (Fig. 3, Table 1) confirmed the identification of parent acids as (20S)-3ξ-hydroxybisnor-5ξ-cholan-22-oic (537), (20S)-3β-hydroxybisnorchol-5-en-

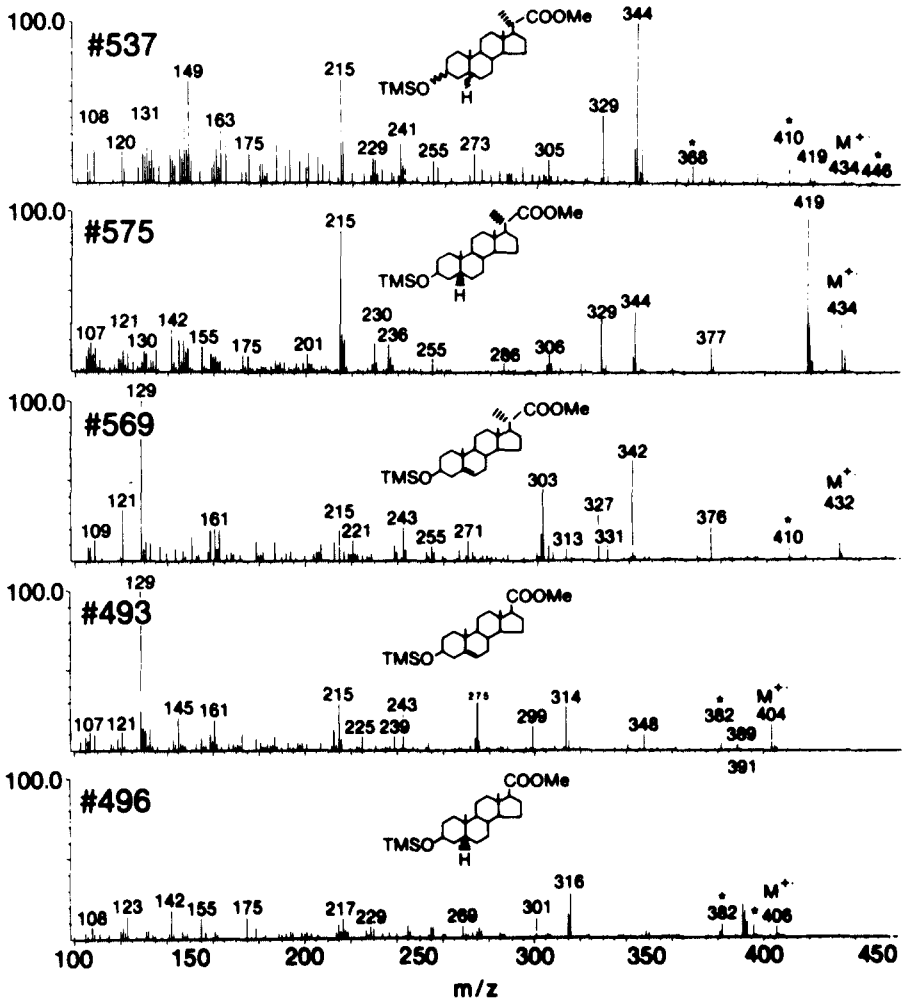

Fig. 3. Electron impact mass spectra (at 20 eV) of short chain bile acid components detected in the sample of human cholestatic serum. Scan numbers refer to Figs. 1 and 2. Fragment ions (*) are due to interfering substances; major fragment ions are assigned in Table 1.

22-oic (569), and (20S)-3β-hydroxybisnor-5α-cholan-22-oic (575). Methyl ester-TMS ethers of 3β-hydroxychol-5-enoic acid (major component, scan 734), 3β- and 3α-hydroxy-5β-cholanoic acids (iso- and lithocholic, scans 665 and 670, respectively) were identified by the direct comparison with standards. In addition, two unsaturated C_{24} and two unsaturated C_{27} components were tentatively identified based on their mass spectra (Table 1).

DISCUSSION

The analysis of human serum was performed in order to investigate the possible presence of short chain BA components and is illustrated for the sample obtained from a cholestatic patient. The exhaustive borohydride reduction of the whole material prior to the extraction and hydrolysis is the key step in the detection of C_{20} acids, since it prevents the oxidative degradation of certain neutral pregnanes leading to the artifactual formation of C_{20} acids. This method was introduced in the previous analysis of meconium components (12).

The final GLC–MS analysis on fused silica capillary column, in a broad temperature program, was limited only to the monohydroxylated fraction and the precise quantitative measurement was not attempted. Furthermore, the analysis was limited to those bile acids which were either free or conjugated with either sulfuric or glucuronic acids. The formation of glucuronides and their efficient secretion in bile was indicated in metabolic experiments in which 3α-hydroxy-5β-androstan-17β-carboxylic acid (C_{20}) was injected into rats with a biliary fistula (16).

It should be noted that saturated short chain BA identified in serum may be at least partially produced by the borohydride reduction of respective 3-ketones in the introductory extraction stage. Note that equatorial

isomers, the major product of hydride reduction, predominate and such an alternative cannot be excluded. The native occurrence, however, is certain for the two 3β-hydroxy-5-enes, as their formation by the borohydride reduction would require rather unlikely 3-oxo-5-ene precursor.

The above results support our former conclusion (12, 13, 16, 17) that short chain steroidal acids are present as components of the heterogeneous BA pool. The heterogeneity of this pool is not only structural, e.g., structure of side chain, hydroxylation pattern, and form of conjugation, but also biogenetic. Thus, apart from the preponderant C_{24} BA, formed directly from cholesterol, products of the indirect degradation of cholesterol can also be detected and included C_{20} and C_{21} acids (catabolites of C_{21} steroids) and C_{22} acids (evident microbial products). The detection of the latter group, both in meconium and serum, is especially significant and indicates that the degradation of side chain beyond C-24 does occur in the intestine and the corresponding products are accepted into the enterohepatic circulation. Therefore C_{22} acids ought to be included in the class of secondary BA.

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