

Glucuronic Acid-conjugated Dihydroxy Fatty Acids in the Urine of Patients with Generalized Peroxisomal Disorders*

(Received for publication, October 5, 1995, and in revised form, November 15, 1995)

Jacqueline M. Street^{‡§¶}, James E. Evans^{‡§¶}, and Marvin R. Natowicz^{‡§¶*}

From the [‡]Division of Medical Genetics and Mass Spectrometry Facility, E. K. Shriver Center for Mental Retardation, Waltham, Massachusetts 02254, Departments of [§]Neurology and [¶]Pathology, Massachusetts General Hospital, Boston, Massachusetts 02114, and Departments of [§]Neurology and [¶]Pathology, Harvard Medical School, Boston, Massachusetts 02115

Urine extracts from children diagnosed with generalized peroxisomal disorders were screened by continuous flow-negative ion fast atom bombardment-mass spectrometry. In 45 of 60 children with generalized peroxisomal disorders, we observed one or more intense ions (m/z 489, 505, 461, and others) that are infrequently found in children with cholestatic liver disease or normal children. Compounds giving rise to these ions were isolated using reverse phase and anion exchange chromatography. After appropriate derivatization and/or methanolysis the compounds were analyzed using capillary gas chromatography-mass spectrometry. The major compounds were found to be 12,13-dihydroxy-9-octadecenoic acid and 9,10-dihydroxy-12-octadecenoic acid, with one of the hydroxyl groups in glycosidic linkage with glucuronic acid. Minor compounds were glucuronic acid conjugates of 9,10-dihydroxy-octadecanoic acid, and 12,13-dihydroxy-6,9-, 15,16-dihydroxy-9,12-, and 9,10-dihydroxy-12,15-octadecadienoic acids. A series of hexadecanoic, hexadecenoic, and hexadecadienoic acid glucuronides which appear to be β -oxidation products of the C18 fatty acids were also observed, with the major species being 10,11-dihydroxy-7-hexadecenoic acid glucuronide. In all, 16 C16 and C18 dihydroxy fatty acids were identified by gas chromatography-mass spectrometry. A series of at least 11 trihydroxy fatty acids was also observed but not fully characterized. Measurement of these compounds may prove to be useful in the diagnosis of some peroxisomal disorders.

Since the first demonstration of the presence of a fatty acid β -oxidation system in rat liver peroxisomes by Lazarow and de Duve (1), it has become increasingly apparent that peroxisomes play an important role in a number of lipid metabolic pathways. The peroxisomal disorders, a group of inherited metabolic disorders (2), derive from defects of peroxisomal biogenesis and/or dysfunction of peroxisomal enzymes. Numerous biochemical abnormalities may result: decreased red blood cell plasmalogens (3), increased plasma phytanic acid (4), pristanic acid (5), pipecolic acid (6), and very long chain fatty acids (7), and increased urinary medium and long chain dicarboxylic acids (8), cholestanoic acids (9), eicosanoids (10), epoxy-dicarboxylic acids (11), and 2-hydroxy acids (12). The abnormal

metabolism of these compounds provides evidence for the importance of peroxisomes in many catabolic and biosynthetic lipid pathways. In addition, analyses of these compounds may serve as diagnostic tools for the detection of peroxisomal disorders. Fast atom bombardment mass spectrometric (FAB-MS)¹ analysis of cholestanoic acids in urine is useful in the diagnosis of many peroxisomal β -oxidation defects (13–15). In evaluating more than 100 urine samples from patients with peroxisomal disorders, we noted several ions in the FAB-MS spectra that appear to be associated with generalized peroxisomal disorders and have not been previously reported. The ions were rarely observed in spectra obtained from normal or cholestatic liver disease control samples. Our experience suggested that ions at m/z 489 and 505 represent novel compounds accumulating in peroxisomal disease, characterization of which may be useful both in understanding of the pathogenesis of these diseases and as a diagnostic tool. In this study we are reporting the structures of the compounds represented by these ions.

EXPERIMENTAL PROCEDURES

Materials

All solvents were obtained from Curtin Matheson, Wilmington, MA, and were Omnisolv or HPLC grade. All glassware was silanized with 5% dimethylchlorosilane (Sigma) and washed thoroughly with toluene and methanol before use. Extract Clean/RC C18 cartridges (0.1 and 0.5 g) (Alltech Associates, Deerfield, IL) were washed and used as described previously (15). Octadecylsilane chromatography material, Sephalyte (Analytichem International, Harbor City, CA), was similarly prepared. The lipophilic anion exchange gel diethylaminohydroxypropyl (DEAP)-Sephadex LH-20 was synthesized from Sephadex LH-20 using the method described by Alme *et al.* (16). For this synthesis epichlorohydrin and diethylamine were obtained from Sigma. Titration of the prepared gel with 0.1 M HCl in methanol demonstrated it had an exchange capacity of 1.42 mEq/g. The gel was converted to the acetate form by washing with 1 M acetic acid in 72% ethanol followed by 72% ethanol to neutrality. Volume requirements for elution of compounds of interest were determined by the use of appropriate bile acid standards. Type HP-2 β -glucuronidase (crude solution from Helix Pomatia), anhydrous tetrahydrofuran, acetyl chloride, Sigma Sil A, acetic anhydride ACS, hexamethyldisilazane (HMDS); trimethylchlorosilane (TMCS); *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA); pyridine ACS, bile acid standards, and *erthro*- and *threo*-9,10-dihydroxy stearic acid were obtained from Sigma. Econo columns for liquid chromatography were obtained from Bio-Rad. The DB-17 fused silica GC capillary column (30 m \times 0.25 mm inside diameter \times 0.25 μ m phase thickness) was from J & W Scientific, Chicago, IL.

* This work was supported by National Institutes of Health NICHD Grant HD05515. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: E. K. Shriver Center, 200 Trapelo Rd., Waltham, MA 02254. Tel.: 617-642-0139; Fax: 617-893-4018.

¹ The abbreviations used are: FAB, fast atom bombardment; MS, mass spectrometry; GC, gas chromatography; DEAP, diethylaminohydroxypropyl; HMDS, hexamethyldisilazane; TMCS, trimethylchlorosilane; BSTFA, bis(trimethylsilyl)trifluoroacetamide; CF, continuous flow; HPLC, high pressure liquid chromatography; PM, permethylated; TMS, trimethylsilyl; HOTMS, trimethylsilylanol; OTMS, trimethylsilyloxyl; CI, chemical ionization; EI, electron impact.

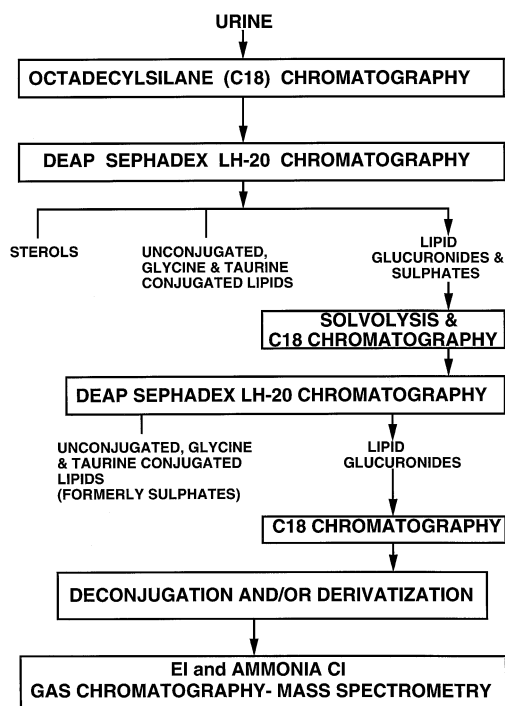


FIG. 1. Schematic representation of the procedures used in the isolation and analysis of the hydroxylated fatty acids.

Methods

Continuous Flow (CF)/FAB-MS

Urine samples (2 ml) from normal children, children with cholestatic liver disease, and patients with established and suspected peroxisomal disease were extracted and analyzed by CF/FAB-MS (15). Direct injection-CF/FAB-MS and micro high performance liquid chromatography CF/FAB-MS were carried out as described previously (15) using a VG70–250 S.E. double focusing mass spectrometry system linked to a Brownlee Labs Microgradient solvent delivery system and incorporating a 1-mm inside diameter \times 100-mm column packed with 3 μ m ODS Hypersil (Keystone Scientific, Bellafont, PA) for HPLC.

Sodium Borodeuteride Reduction of Ketones

Urine extracts were reduced by the method of Bjorkhem *et al.* (17).

Extraction Procedures

A schematic of the isolation procedure is shown in Fig. 1. Urine was collected from patients with m/z 489 as an intense ion in the FAB-MS spectra, and an aliquot of up to 500 ml was extracted. Columns (0.7–1.5 cm inside diameter, dependent on sample size) were packed with octadecylsilane bonded phase (1 g/20 ml of urine) and washed with methanol (10 ml/g) and distilled water (10 ml/g) prior to application of sample. Nitrogen gas pressure was applied to obtain a flow rate which did not exceed 10 ml/min. The column was washed several times with water (60 ml/g of column packing) and eluted with methanol (10 ml/g of column packing).

Chromatographic Conditions

DEAP-Sephadex LH-20 (acetate form) was packed (0.7 \times 10 cm) in Bio-Rad Econo columns under nitrogen gas pressure (0.3–0.5 kg/cm²) and the column equilibrated in 70% methanol. The methanol eluate from the C18 extraction step was diluted with water to 70% methanol and passed through the column. Buffer systems described by Alme *et al.* (16) were used to elute the compounds of interest and separate them from the bulk of other compounds in the extract. Three fractions were collected, corresponding to those described by Alme *et al.* (16) for the (a) sterols, (b) unconjugated and glycine/taurine-conjugated bile acids, and (c) sulfate/glucuronide-conjugated bile acids. The columns were successively washed with 10 ml of 70% ethanol (fraction a), followed by 10 ml of 0.15 M acetic acid in 70% ethanol (pH adjusted to 6.6 with ammonium hydroxide) (fraction b). The compounds of interest were then eluted along with other sulfated and glucuronic acid conjugates with 10 ml of 0.3 M acetic acid in 70% ethanol (pH adjusted to 9.6 with ammonium hydroxide) (fraction c). This fraction was evaporated to a small volume,

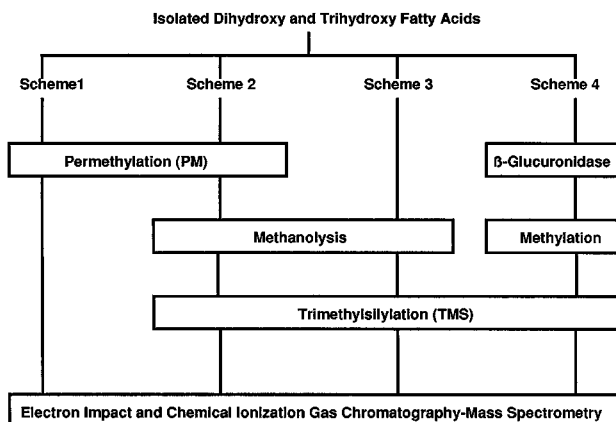


FIG. 2. Schematic representation of the derivatization methods used in the analysis of the hydroxylated fatty acids by GC-MS.

diluted with water to less than 5% methanol, and desalted on a C18 column (1 g/50 ml of urine extract).

Solvolysis

Solvolysis was carried out in 20 ml of freshly prepared tetrahydrofuran-4 M H₂SO₄ (100:0.15, v/v) at 50°C for 3.5 h. Products were recovered by diluting the solvolysis solution to less than 5% tetrahydrofuran with water and desalted on a C18 column. The methanol eluate was evaporated, redissolved in a small volume of 70% methanol, and chromatographed again on a DEAP-Sephadex LH-20 column. Compounds conjugated to glucuronic acid were now concentrated in this final fraction.

Glucuronide Fraction: Purification and Derivatization for Gas Chromatography-Mass Spectrometry

The glucuronide fraction was evaporated and subjected to enzyme hydrolysis with β -glucuronidase or further purified as follows. The sample was redissolved in a small volume of water and applied to a 0.5 g of Extract Clean/RC C18 cartridge. The cartridge was washed with 30 ml of water and eluted with 4 ml of each of 10, 20, 40, 50, 60, 70, 80, and 100% methanol in water. The elution profile was monitored using direct injection CF/FAB-MS. The compounds of interest eluted in the 50 and 60% methanol fractions. These fractions were then evaporated and derivatized for GC-MS analysis.

In order to provide comprehensive characterization of the glucuronide fatty acids four derivatization procedures were used as shown in Fig. 2. Samples were derivatized as follows: scheme 1, permethylated (PM) (18), providing molecular weight data and information on the number of derivatization sites; scheme 2, permethylated (18) followed by methanolysis and trimethylsilylation (PM-methanolysis-TMS), providing information about the site of glucuronide conjugation; scheme 3, subjected to methanolysis before trimethylsilylation (methanolysis-TMS), providing high mass fragment ions useful in the characterization of the aglycone structure; and scheme 4, subjected to treatment with β -glucuronidase prior to methylation of the carboxyl group and trimethylsilylation of the hydroxyl groups, providing confirmatory evidence of the glucuronic acid conjugation.

Methanolysis—Methanolysis was carried out using an adaptation of the method of Wiesner and Sweeley (19). The methanolysis reagent was freshly prepared by slowly adding acetyl chloride (50 μ l) to methanol (1 ml) (20). The samples were transferred to silanized microcapillary tubes (approximately 75 mm \times 1.6 mm) with one end sealed. The tubes were placed in plastic cups and the solvent evaporated in a Speed-Vac under vacuum. After methanolysis reagent (30 μ l) was added, the tubes were sealed in a flame and placed in an oven at 90°C overnight. After cooling the capillaries were scored and broken, and the reagent was evaporated under vacuum.

TMS—The methylated samples were trimethylsilylated in sealed microcapillary tubes with 10 μ l of Sigma Sil-A at 60°C for 30 min. Deuterated derivatives were formed using 10 μ l of a mixture of d_{18} -HMDS, d_9 -TMCS, and d_{18} -BSTFA in dry pyridine (5:1:1, v/v/v) at 60°C for 30 min.

Glucuronidase Treatment—The fractions were dissolved in a minimum of 5 ml 0.2 M acetate buffer, pH 4.5, and 100 μ l of β -glucuronidase was added (approximately 1000 IU). After shaking overnight at 37°C,

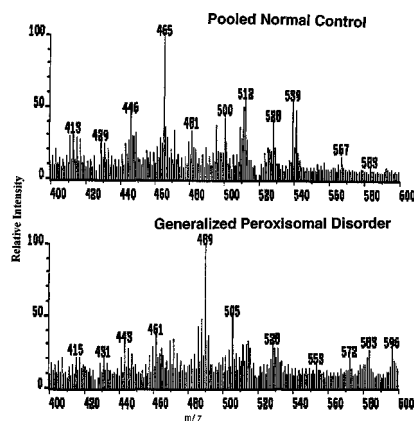


FIG. 3. Negative ion CF/FAB mass spectra of a pooled control urine and a urine sample from a patient with generalized peroxisomal disorder.

an additional aliquot of β -glucuronidase was added and the incubation repeated. In order to extract the aglycone, the hydrolysis solution was diluted with water and twice applied to a prepared C18 column (2 g). The column was washed with water (120 ml) and eluted with methanol (30 ml).

Methylation—The aglycone was methylated in 30 μ l of methanolysis reagent, prepared as described above, heated at 60°C for 30 min.

GC-MS

A Finnigan 4500 quadrupole GC-MS system equipped with a Teknivent Vector/Two data system and Hewlett Packard 5890 GC was used for these studies. A fused silica DB-17 capillary column was used. The oven temperature was held at 180°C for 2 min and then programmed at 10°C/min to 330°C, where it was held for 2 min. Chemical ionization (CI) spectra were obtained using ammonia gas (0.7 Torr) with source temperature at 180 °C. Electron impact (EI) spectra were obtained at 70 eV with the source temperature at 190 °C.

RESULTS

FAB-MS

FAB-MS spectra from pooled normal control urine and urine from a patient with generalized peroxisomal disease are shown in Fig. 3. The m/z 489 ion was one of the 10 most intense ions in the FAB-MS spectra of urine from 45 of 60 well characterized generalized peroxisomal disorder patients studied. It was also observed as one of the 10 most intense ions in the FAB-MS spectra of 1 of 12 patients with bifunctional protein enzyme deficiency,² a single enzyme defect in the peroxisomal β -oxidation pathway. Preliminary results indicated that the ion at m/z 489 was not observed as one of the 10 most intense ions in FAB-MS spectra of 15 normal children or in 15 children with cholestatic liver disease or hepatic failure. Less intense ions which also appeared in some peroxisomal patients' urine samples included m/z 491, 487, 463, 461, 459, 507, 505, and 596. Compounds which correspond to these ions are described in this report.

The major FAB-MS ions of interest at m/z 489 and 505, which represent $(M - H)^-$ ions for compounds of M_r 490 and 506, respectively, do not shift after reduction with sodium borodeuteride, suggesting these compounds do not contain a reducible ketone group. They consistently elute from DEAP-Sephadex LH-20 in the fraction containing sulfate and glucuronic acid conjugates. Monitoring of anion exchange chromatography by CF/FAB-MS showed that sulfated bile acids are successfully solvolysed using the solvolysis conditions described. In contrast, the ions of interest at m/z 489 and 505 are

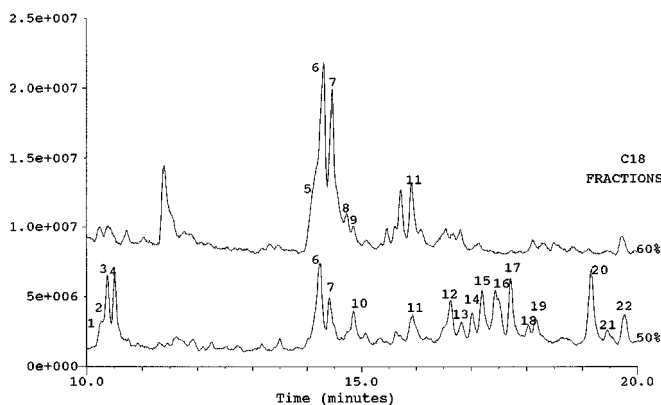


FIG. 4. TIC plot of a CI/GC-MS analysis of dihydroxy and trihydroxy fatty acids. 50 and 60% methanol fractions from octadecylsilane chromatography are indicated. The peaks are identified in Table I.

TABLE I
List of identified compounds

| Peak no. | Compound | Glucuronide position |
|----------|--|----------------------|
| 1 | 7,8-Dihydroxy-4,10-hexadecadienoic acid | 7 |
| 2 | 7,8-Dihydroxyhexadecanoic acid | 7 |
| | 7,8-Dihydroxyhexadecanoic acid | 8 |
| 3 | 7,8-Dihydroxy-10-hexadecenoic acid | 7 |
| | 10,11-Dihydroxy-7-hexadecenoic acid | 10 |
| 4 | 10,11-Dihydroxy-7-hexadecenoic acid | 11 |
| 5 | 9,10-Dihydroxy-octadecanoic acid | 9 |
| | 9,10-Dihydroxy-octadecanoic acid | 10 |
| 6 | 9,10-Dihydroxy-12-octadecenoic acid | 10 |
| | 9,10-Dihydroxy-12-octadecenoic acid | 9 |
| | 12,13-Dihydroxy-9-octadecenoic acid | 12 |
| 7 | 12,13-Dihydroxy-9-octadecenoic acid | 13 |
| 8 | 12,13-Dihydroxy-9-octadecadienoic acid | 13 |
| 9 | 12,13-Dihydroxy-6,9-octadecadienoic acid | |
| 10 | 9,10-Dihydroxy-12,15-octadecadienoic acid | 9 |
| 11 | 15,16-Dihydroxy-9,12-octadecadienoic acid | 15 |
| 12-18 | tentative identification as trihydroxy fatty acids | |
| 19 | 9,10,?-Trihydroxyoctadecenoic acid | 9 |
| 20 | 9,10,?-Trihydroxyoctadecenoic acid | 9 |
| | 9,10,?-Trihydroxyoctadecenoic acid | 9 |
| 21, 22 | Trihydroxy fatty acids | |

unaffected by solvolysis but disappear with glucuronidase treatment.

Characterization of Hydroxylated Fatty Acids

Two purified fractions, the 50 and 60% methanol elution bands from C18 chromatography, were found to contain the bulk of the compounds of interest. Fig. 4 shows TIC chromatograms from CI/GC-MS of these fractions after PM-methanolysis-TMS treatment. The compounds identified are listed in Table I. Characterization of the major species is discussed below. Mass spectral data used in the characterization of the major compound, 12,13-dihydroxy-9-octadecenoic acid, are shown in Fig. 5. Similar data were obtained and utilized in the characterization of the other compounds listed in Table I, although only the EI spectra of the methanolysis-TMS derivatives are shown (Figs. 6 and 7). Specification of the site of the double bonds is tentative, although there are strong indications in the mass spectra obtained for the assignments given.

Permethylation

Ammonia CI mass spectra of permethylated dihydroxy C18 fatty acid glucuronides give prominent $(M + NH_4)^+$ ions at m/z 594, 592 (Fig. 5A), and 590 for the saturated, monounsaturated, and diunsaturated forms, respectively. Similar ions are seen at m/z 464, 462, and 460 in spectra from dihydroxy C16

² M. R. Natowicz, J. E. Evans, R. I. Kelley, A. B. Moser, P. A. Watkins, and H. W. Moser, manuscript submitted for publication.

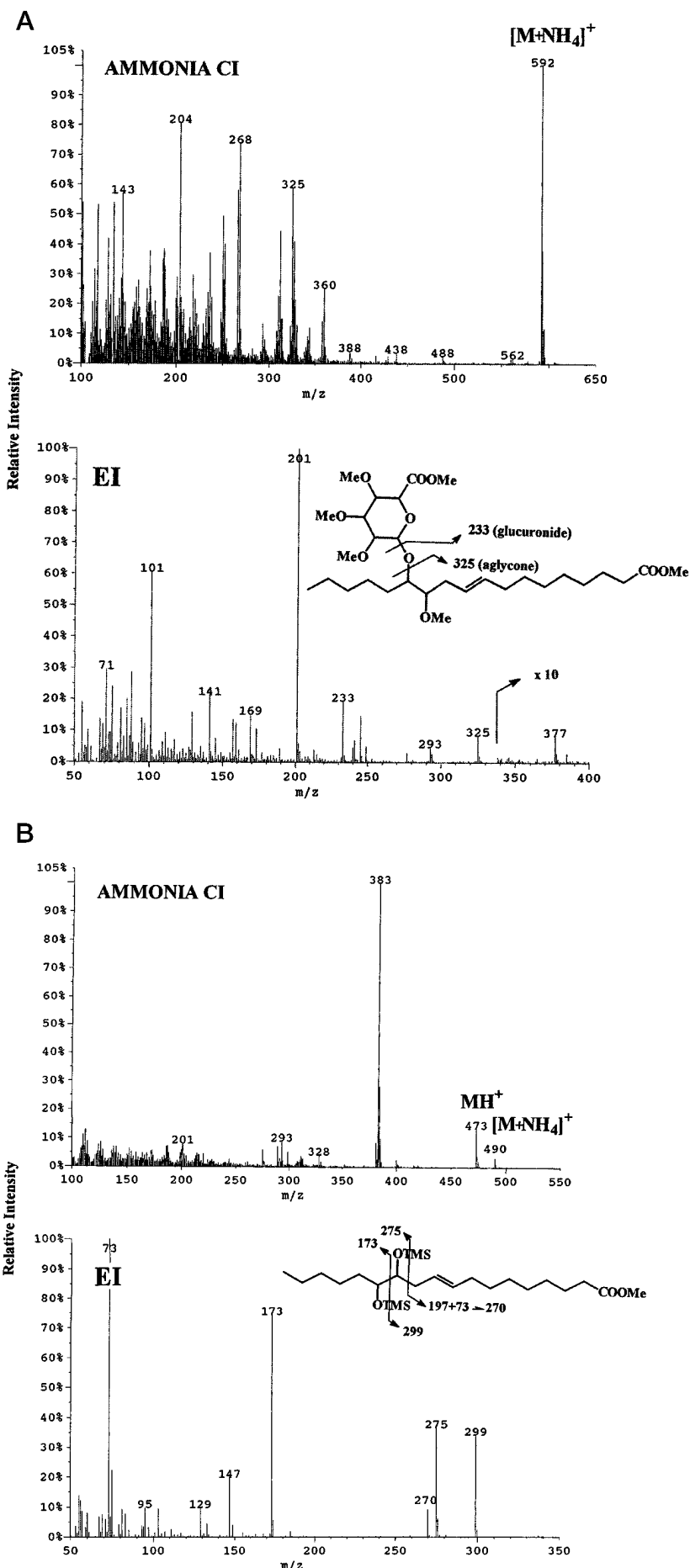


FIG. 5. EI and ammonia CI mass spectra of the glucuronide conjugated 12,13-dihydroxy-9-octadecenoic acid **A**, as a permethylated derivative; **B**, after methanolysis and trimethylsilylation; and **C**, after permethylation, methanolysis, and trimethylsilylation.

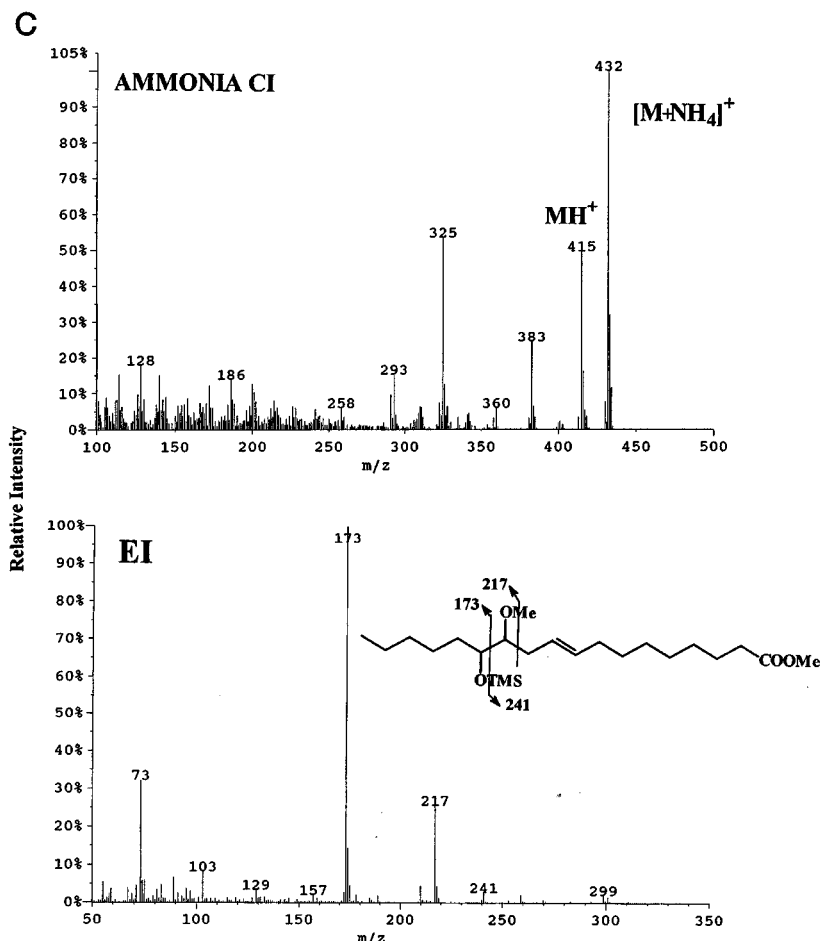


FIG. 5—continued

fatty acids. The EI spectra are dominated by ions arising from the glucuronide moiety, although ions corresponding to aglycone fragments are also observed.

Deuterium-labeled TMS Derivatives

Derivatization with d_9 -TMS reagents produces incremental mass changes dependent on the number of TMS groups in a fragment ion and are useful in assigning fragment ion structures. As an example the fragment ion seen at m/z 155 in methanolysis-TMS EI spectra of 9-hydroxylated fatty acids (Fig. 6B) did not change with deuterium labeling of the TMS derivative. This is consistent with the proposed ion structure of $(O-CH-(CH_2)_7-C=O)^+$. In contrast, after d_9 -TMS derivatization, the ions m/z 173, 299, and 270 in the EI spectra shown in Fig. 5B all shifted 9 mass units, consistent with a single TMS group in the ions, and m/z 275 shifted 18 mass units, consistent with the presence of two TMS groups. Similar assessments were made in our analysis of the data for all the hydroxy fatty acids and in each case the incremental change after d_9 -TMS derivatization is consistent with the proposed structure.

Glucuronidase Treatment

Spectra obtained following glucuronidase treatment are similar to those found with methanolysis-TMS analysis, although recoveries were poor and only the major compounds are observed.

GC-MS Analysis: Characterization of Major Species

12,13-Dihydroxy-9-octadecenoic Acid (2 Isomers)

PM—The CI spectra of the permethylated derivative of the major isomer as its intact glucuronide has as base ion m/z 592 $((M + NH_4)^+)$ (Fig. 5A). The ion at m/z 325 corresponds to a

loss of glucuronic acid. The EI spectrum is dominated by ions arising from the glucuronide moiety but also shows ions due to the aglycone fragment and fragmentation between the glucuronic acid and methyl substituted hydroxyl groups at m/z 377.

Methanolysis-TMS—The CI spectra obtained following methanolysis and trimethylsilylation show an intense ion at m/z 383 ($MH^+ - 90$), with ions at m/z 473 (MH^+), 293 ($MH^+ - (2 \times 90)$), and 490 ($(M + NH_4)^+$), confirming the number of OTMS groups in the compound (Fig. 5B). In the EI spectra, abundant fragment ions at m/z 173 and 299 arise from fragmentation between the vicinal TMS hydroxyl groups. The position of the double bond at C-9 would direct fragmentation adjacent to the oxygenated carbon atom closest to the double bond (22). This is consistent with the formation of the ion at m/z 275. The ion at m/z 270 is due to the olefinic fragment from this cleavage with transfer of a TMS group to the carboxylate radical site.

PM-Methanolysis-TMS—Evidence for glucuronide linkage at the C-12 and C-13 positions is apparent in the PM-methanolysis-TMS products, although the major species appears to be the C-13-glucuronide. The CI and EI spectra from the 13-glucuronide product (*i.e.* the 12-*O*-methyl, 13-OTMS-9-octadecenoic methyl ester) and proposed fragmentation are shown in Fig. 5C.

GC-MS Analysis: Characterization of Minor C18 Dihydroxy Fatty Acids

9,10-Dihydroxyoctadecanoic Acid

Methanolysis-TMS—The CI spectrum of this compound contains major ions at m/z 475 (MH^+) and 385 ($MH^+ - 90$). In the EI spectrum (Fig. 6A) the abundant ions at m/z 259 and 215 result from cleavage between the two OTMS substituted car-

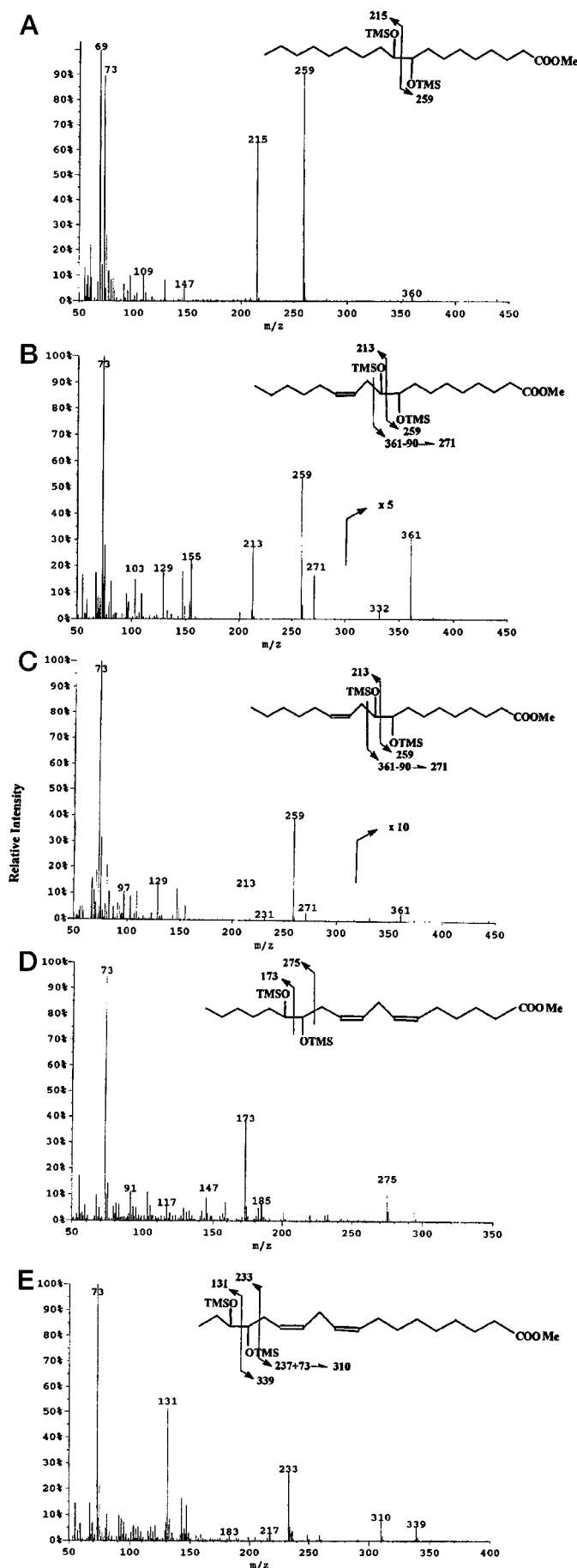


FIG. 6.

bonds with little other fragmentation. The spectra and retention times obtained are identical to those obtained from the methyl ester-TMS ether derivative of standard threo-9,10-dihydroxyoctadecanoate and well separated from the standard erythro isomer.

PM-Methanolysis-TMS—The EI spectra indicate that the glucuronide is principally found at C-10. With a TMS group substituted for the original glucuronide, the most intense ion in the spectra is at m/z 215 which is the ω -end fragment ion following cleavage between the vicinal methyl and TMS ethers.

9,10-Dihydroxy-12-octadecenoic Acid (2 Isomers)

Methanolysis-TMS—Derivatives of the two isomers give very similar mass spectra. In the CI spectra ions are observed at m/z 473 (MH^+) and m/z 490 ($(M + NH_4)^+$). An intense ion at m/z 383 and a smaller one at m/z 293 correspond to the loss of one and two trimethylsilanols, respectively. The EI spectra of both isomers are also similar (Fig. 6, B and C) with prominent ions at m/z 259 and 213 due to cleavage between the vicinal trimethylsilyl ethers. A weak ion at m/z 332 arises by migration of a OTMS group to the carboxylate radical site. Fragmentation, to give ions at m/z 361 and 271 (Fig. 6B), is probably directed by the presence of the double bond located one methylene unit away from the oxygenated carbon atom (22), as are the ions at m/z 103 and 129 which correspond to $(CH_2=O-Si(CH_3)_3)^+$ and $(CH_2=CH-CH=O-Si(CH_3)_3)^+$. The lack of these ions in the second isomer may indicate the double bond is in a different position.

PM-Methanolysis-TMS—Evidence for substitution with the glucuronic acid on both the 9 and 10 hydroxyl groups is observed in the mass spectra of PM-methanolysis-TMS derivatives. The first is characterized by an intense ion at m/z 259, consistent with the glucuronide at C-9, and the second by an ion at m/z 213, consistent with the glucuronide at C-10 (not shown).

12,13-Dihydroxy-6,9-octadecadienoic, 15,16-Dihydroxy-9,12-octadecadienoic, and 9,10-Dihydroxy-12,15-octadecadienoic Acids

Methanolysis-TMS—The EI spectra of the diunsaturated dihydroxy C18 fatty acids are shown in Figs. 6, D and E. Fragmentation is consistent with the structures of 12,13-dihydroxy-6,9-octadecadienoic acid and 15,16-dihydroxy-9,12-octadecadienoic acid. In each case fragmentation occurred between the vicinal TMS ethers with the saturated fragment giving the most intense signal. Saturated fragments that include both vicinal TMS ethers also occur, presumably because fragmentation is directed by the presence of a double bond located one methylene unit away. Good spectra of the 9,10-dihydroxy-12,15-octadecadienoic acid isomer could not be obtained due to interfering ions from other chromatographic peaks. Identification of this compound was principally made on the basis of the PM-methanolysis-TMS data (not shown).

PM-Methanolysis-TMS—Data from these derivatives support the observation that at least three species of diunsaturated dihydroxy fatty acids are present. In CI spectra, base ions are seen at m/z 430 ($(M + NH_4)^+$), along with ions at m/z 413 (MH^+), 323 ($MH^+ - HOTMS$), 381 ($MH^+ - \cdot OCH_3$), and 291 ($MH^+ - \cdot OCH_3 - HOTMS$). The EI spectra in each case show an intense ion which is attributable to a saturated frag-

FIG. 6. EI mass spectra of methyl trimethylsilyl derivatives of A, 9,10-dihydroxyoctadecanoic acid; B, 9,10-dihydroxy-12-octadecenoic acid (major isomer); C, 9,10-dihydroxy-12-octadecenoic acid (minor isomer); D, 12,13-dihydroxy-6,9-octadecadienoic acid; and E, 15,16-dihydroxy-9,12-octadecadienoic acid.

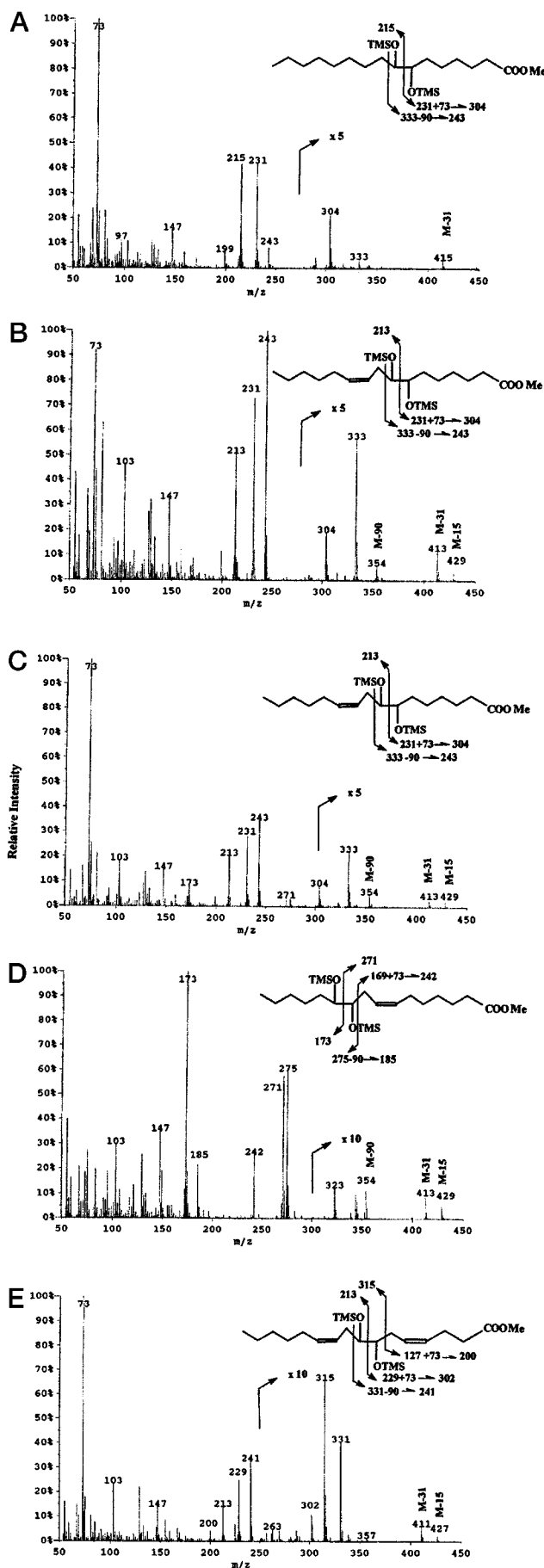


FIG. 7. EI mass spectra of methyl trimethylsilyl derivatives of **A**, 7,8-dihydroxyhexadecanoic acid; **B**, 7,8-dihydroxy-10-hexade-

ment ion containing the TMS ether and suggest that the glucuronide is located on C-15 of 15,16-dihydroxy-9,12-octadecadienoic acid and on C-9 of the 9,10-dihydroxy-12,15-octadecadienoic acid. The data obtained do not permit us to ascertain the position of the glucuronide on 12,13-dihydroxy-6,9-octadecadienoic acid.

GC-MS Analysis: Characterization of Dihydroxy C16 Fatty Acids

7,8-Dihydroxyhexadecanoic Acid

Methanolysis-TMS—The CI spectrum shows prominent ions at m/z 447 (MH^+) and 357 ($MH^+ - HOTMS$). The EI spectrum contains ions arising from cleavage between the vicinal TMS ethers, m/z 215 and 231, with both saturated ions having similar intensity (Fig. 7A).

PM-Methanolysis-TMS—The EI spectra of this derivative suggests that either of the hydroxyl groups can be glucuronidated giving rise to two spectra containing intense ions at m/z 231 or 215. These correspond to a glucuronide at C-7 and C-8, respectively.

7,8-Dihydroxy-10-hexadecenoic Acid (2 Isomers)

Methanolysis-TMS—The CI spectra of these derivatized compounds show abundant ions at m/z 445 (MH^+) and 355 ($MH^+ - HOTMS$). The EI spectra of the major isomer shows fragment ions arising from cleavage between the vicinal TMS ethers, with the saturated fragment ion at m/z 231 providing a more intense ion than the olefinic fragment ion at m/z 213 (Fig. 7B). The weak ion at m/z 333 is presumably due to cleavage between C-8 and C-9. Loss of a HOTMS group gives rise to the ion at m/z 243. The abundance of these two ions, in comparison to similar ions seen in the saturated 7,8-dihydroxyhexadecanoic acid, strongly suggests the double bond is located at C-10, one methylene unit from the hydroxylated carbon. A minor isomer of this compound was also observed and gave a similar spectrum (Fig. 7C), although high mass fragment ions were less intense relative to the ion at m/z 73.

PM-Methanolysis-TMS—The EI spectrum shows a base peak at m/z 231 and ions at m/z 275 and 185 arising from double bond-directed cleavage between C-8 and C-9. This is consistent with the glucuronide at C-7.

10,11-Dihydroxy-7-hexadecenoic Acid

Methanolysis-TMS—The CI spectrum is similar to those seen for 7,8-dihydroxy-10-hexadecenoic acid isomers, with the inclusion of a weak ($M + NH_4$) $^+$ ion at m/z 462. The EI spectrum (Fig. 7D) shows ions arising from cleavage between vicinal TMS ethers with the saturated fragment ion at m/z 173 more abundant than the olefinic fragment ion at m/z 271. Abundant ions at m/z 275 and 185 arise from cleavage between C-9 and C-10, probably directed by a double bond at C-7. An intense rearrangement ion, m/z 242, also arises from cleavage at this position with migration of a TMS group to the carboxyl carbonyl.

PM-Methanolysis-TMS—Two compounds are seen with spectra consistent with a glucuronide at C-10 and C-11, respectively. In the former, ions at m/z 217 and 271, arise from cleavage between C-9 and C-10 (ω -fragment) and C-10 and C-11 (olefinic fragment), respectively. In the latter the ω -fragment ion, m/z 217, is also apparent but base peak is m/z 173 due to the glucuronide at C-11.

cenoic acid (major isomer); **C**, 7,8-dihydroxy-10-hexadecenoic (minor isomer); **D**, 10,11-dihydroxy-7-hexadecenoic acid; and **E**, 7,8-dihydroxy-4,10-hexadecadienoic acid.

7,8-Dihydroxy-4,10-hexadecadienoic Acid

Methanolysis-TMS—Only one diunsaturated dihydroxy C16 fatty acid is observed. The CI spectrum shows prominent ions at m/z 443 (MH^+), 353 ($MH^+ - HOTMS$) and 263 ($MH^+ - (2 \times HOTMS)$), with a weak ion at m/z 460 ($(M + NH_4)^+$). The EI spectrum obtained from the methanolysis-TMS derivative is shown in Fig. 7E. This spectrum contains fragment ions arising from cleavage between the vicinal OTMS groups, at m/z 229 and 213, and on either side of them, at m/z 315 and 331. Loss of a HOTMS group from each of the latter fragment ions, gives rise to ions at m/z 225 and 241, respectively. The observed cleavage on both sides of the vicinal OTMS groups suggests the double bonds may occur at C-4 and C-10, one methylene unit away from C-7 and C-8, respectively.

PM-Methanolysis-TMS—An abundant ion at m/z 229, consistent with fragmentation of the substituted vicinal diol with the OTMS group at C-7, was seen in the EI spectrum of this derivative. Other ions presumably arise from fragmentation directed by the double bonds at C-4 and C-10. Ions due to cleavage between C-6 and C-7, at m/z 257 (ω -fragment) and 200 (rearrangement ion of α -fragment with transfer of a TMS group to the carboxylate carbonyl), and between C-8 and C-9, m/z 273 (α -fragment) are seen. This spectrum is consistent with the glucuronide at C-7.

GC-MS Analysis: Trihydroxy C18 Fatty Acids

CF/FAB-MS analyses of patients' urines show that the ion m/z 505 is often associated with m/z 489, suggesting that monounsaturated trihydroxy C18 fatty acids also occur.

PM—CI/GC-MS analysis of the PM derivatives of the intact glucuronides support this observation with the expected $(M + NH_4)^+$ ion, m/z 622, observed in the spectra of numerous compounds. These CI spectra also contain ions at m/z 268 and 204, characteristic of a glucuronide. Also observed are fragment ions arising from the aglycone at m/z 355 (aglycone). The EI spectra of these compounds show many ions arising from the glucuronic acid component.

PM-Methanolysis-TMS—The CI spectra of at least eleven compounds show ions at m/z 445 (MH^+) and 462 ($(M + NH_4)^+$). The EI spectrum of the major compound shows an intense ion at m/z 259 and ions at m/z 303 and 213 consistent with a 9,10,?-trihydroxyoctadecenoic acid. However, many of the compounds observed provide CI spectra that exhibit only weak ions at m/z 462 ($(M + NH_4)^+$). Their EI spectra contain many of the ions that are seen in the spectra obtained from the dihydroxy fatty acids, in particular m/z 173 and 259, indicating ω - and α -end fragments, respectively (see Figs. 5 and 6). Although there is evidence of at least one saturated trihydroxy fatty acid in the urine from patients, there is no indication of diunsaturated species. Further characterization of this group of hydroxylated fatty acids is ongoing.

GC-MS Analysis: Glycine and Taurine Conjugation of Dihydroxy Fatty Acids

There is some evidence to suggest that the glucuronic acid conjugated dihydroxy monounsaturated fatty acids, may also be conjugated at the carboxyl group with glycine or taurine. These species would give ions by negative ion FAB-MS at m/z 546 and 596, respectively. Such ions are observed and the ion at m/z 596 is prominent in the spectra of some patients (Fig. 3). Glucuronidase treatment of these compounds, isolation by HPLC and FAB-MS analysis, gave ions consistent with the glycine and taurine conjugated aglycone. Methyl esterification of the compound represented by m/z 596 gave rise to an intense ion at m/z 610. This is consistent with the presence of a single unesterified carboxyl group on the glucuronic acid. The

other on the fatty acid component is presumably blocked by taurine.

DISCUSSION

We observed that the FAB-MS spectra obtained by analysis of urine from children with generalized peroxisomal disorders show several unusual ions which had not been described previously. These ions were among the ten most intense ions in spectra from the majority of children with these disorders but have thus far not been observed in the ten most intense ions in spectra obtained from normal children or children with cholestatic liver disease. Therefore we postulated that these ions represented an accumulation of novel compounds in the urine of patients with peroxisomal disorders and that characterization of these compounds would enhance our understanding of the pathophysiology of peroxisomal disease. Our preliminary results suggested analysis of these compounds may be useful as a diagnostic test. Using CI and EI GC-MS analyses we established the structure of many of these compounds as glucuronic acid conjugated, dihydroxy and trihydroxy, C16 and C18 fatty acids. The major components were shown to be the glucuronic acid conjugated, monounsaturated dihydroxy fatty acids, 12,13-dihydroxy-9-octadecenoic acid and 9,10-dihydroxy-12-octadecenoic acid. These compounds correspond to the major $(M - H)^-$ ion at m/z 489, observed in the FAB-MS spectra shown in Fig. 3. Similarly $(M - H)^-$ ions observed in the FAB-MS spectra at m/z 491 and 487 correspond to the glucuronide conjugated saturated and diunsaturated C18 dihydroxy fatty acids described. A significant group of compounds that elute early in the GC program (Fig. 4, *Peaks 1-4*) were identified as dihydroxy saturated, monounsaturated and diunsaturated C16 fatty acids. The glucuronic acid conjugates of these compounds are consistent with $(M - H)^-$ ions at m/z 463, 461, and 459 in the FAB-MS spectra. Numerous compounds eluting later than the dihydroxy fatty acids (Fig. 3, *Peaks 12-22*) are tentatively identified as trihydroxy C18 fatty acids. It is of interest that other ions in the FAB-MS spectra obtained with extracts of patients' urines, may correspond to C12 and C14 hydroxylated fatty acids (e.g. m/z 433, 417, and 415).

Several mechanisms exist which may explain the formation of the dihydroxy fatty acids. Dihydroxy C18 fatty acids have been described previously as chemical hydrolysis products formed in the characterization of epoxy fatty acids (22, 23) but have also been shown to arise by autooxidation *in vitro* (24). It would be reasonable to suppose that dihydroxy fatty acids could be formed from unsaturated fatty acids during storage of samples. However, the presence of a glucuronide moiety on one of the hydroxyl groups indicates that the compounds we have observed were formed *in vivo*, since conjugation with glucuronic acid implies hepatic processing. Epoxy fatty acids, which can be precursors of fatty acids with vicinal hydroxyl groups, may be derived from the diet (25-27) or synthesized *in vitro*. The epoxigenase pathway appears to be a major microsomal oxidation route for polyunsaturated fatty acids in a number of tissues (23, 28-33); however, nonenzymatic autooxidation probably also occurs *in vivo* (29, 34). Vicinal dihydroxy C18 fatty acids have also been observed as the products of microsomal metabolism *in vitro* (35, 36). The major metabolites from linoleic and linolenic acids were the same as compounds 6, 7, 10, and 11 identified in Fig. 3. 12,13-Dihydroxy-9,15-octadecadienoic acid was also observed (35).

We postulate that 12,13-dihydroxy-9-octadecenoic acid and 9,10-dihydroxy-12-octadecenoic acid are derived from linoleic acid (18:2($n-6$)), an abundant fatty acid in the body and that the saturated analogue, 9,10-dihydroxyoctadecanoic acid, may be derived from oleic acid (18:1($n-9$)). Similarly 12,13-dihydroxy-6,9-octadecadienoic acid may be derived from γ -linolenic acid

(18:3(*n*-6)), whereas oxidation of α -linolenic (18:3(*n*-9)) may give rise to 9,10-dihydroxy-12,15-octadecadienoic acid and 15,16-dihydroxy-9,12-octadecadienoic acid. The direct relationship between the position of the hydroxyl groups in the characterized compounds and the double bond position in the C18 unsaturated fatty acids is supportive of this contention, as is the threo configuration demonstrated in the 9,10-dihydroxyoctadecanoic acid. This configuration must result from a *cis* double bond as found in the unsaturated fatty acids described above.

The unsaturated fatty acids, oleic, linoleic, and α -linolenic acids, are readily β -oxidized in the mitochondria and do not appear to accumulate in peroxisomal disorders (37, 38). In contrast γ -linolenic and docosahexanoic acid are poorly oxidized in the mitochondria (38). Accumulation of these and other unsaturated fatty acids may inhibit mitochondrial β -oxidation (39) possibly channeling the fatty acids into pathways leading to hydroxy compounds. It is unknown whether the C16 and C18 dihydroxy and/or epoxy fatty acids are predominantly β -oxidized in the peroxisome, such that dysfunction of this organelle leads to their accumulation. However, our findings are consistent with this theory. β -Oxidation of numerous compounds has been localized to the peroxisome (for review, see van den Bosch *et al.* (40) and Brown *et al.* (41)) with accumulation of these compounds in peroxisomal disease (4–12). It is of interest that only one patient with a well characterized single enzyme defect in fatty acid β -oxidation has been observed to have prominent *m/z* 489 and 505 ions in the urine by FAB-MS analysis.

The major C16 fatty acids observed, in particular the monounsaturated dihydroxy C16 fatty acids, are the expected β -oxidation products of the major C18 dihydroxy species described above. Hence, 7,8-dihydroxy-10-hexadecenoic acid and 10,11-dihydroxy-7-hexadecenoic acid are the expected products from a single cycle of the β -oxidation pathway from 9,10-dihydroxy-12-octadecenoic acid and 12,13-dihydroxy-9-octadecenoic acid, respectively. This does not preclude that β -oxidation has occurred prior to hydroxylation. Hence 7,8-dihydroxy-10-hexadecenoic acid could be derived from 16:2(*n*-6) although unsaturated C16 fatty acids do not appear to accumulate in generalized peroxisomal disorders. Our findings suggest that the dihydroxy and/or epoxy fatty acids are β -oxidized to a limited extent in these patients. It is also conceivable that secondary inhibition of mitochondrial β -oxidation (42) may account for the accumulation of these compounds.

Hydroxylation at the ω or ω -1 position or hydroxylation allylic to the double bond and vicinal to an epoxide would give rise to a trihydroxy species (30, 43, 44) and may channel through a hydroperoxy-epoxy octadecenoate intermediate, which has been reported as an autooxidation product of linoleic acid (45).

At this stage it is premature to suggest the mechanism by which the dihydroxy and trihydroxy fatty acids accumulate in generalized peroxisomal diseases. Although defective β -oxidation may be the underlying defect, it is also possible that disruption of enzyme compartments and/or the peroxisomal scavenging system for free radicals may lead to accumulation of reactive oxygen species and oxidative attack on cell lipids. The peroxisomes are potent sources of hydrogen peroxide because of their high concentration of oxidases (41) and generate superoxide radicals (46, 47). Mechanisms in peroxisomes counteract the production of these reactive oxygen species by enzymes catalyzing the conversion of the superoxide radical ion (O_2^-) to hydrogen peroxide and then to H_2O and O_2 (48). Failure to form the intact peroxisome in disorders of peroxisomal biogenesis may perturb the balance of formation and catabolism of these reactive oxygen species and lead to lipid peroxidation. We

would expect that autooxidation *in vivo* would lead to the formation of other oxidized metabolites such as monohydroxy fatty acids. These have not been detected in our samples but such compounds have not been looked for systematically. Finally other peroxisomal enzymes which are involved in the conversion of unsaturated fatty acids to dihydroxy fatty acids are also impaired (*e.g.* peroxisomal epoxide hydrolase) (49). The significance of this can not be assessed at this point.

Polyunsaturated fatty acids in the phospholipids of biological membranes are important in increasing membrane fluidity, compressibility and permeability (21). These in turn will affect activity of membrane enzymes, cell-cell interaction, receptor interactions and membrane transport. Accumulation of oxidized metabolites of unsaturated fatty acids may therefore disrupt the normal functioning of cells by this mechanism or by their own biological activity. Further investigation of oxygenated fatty acids in normal metabolism and peroxisomal disease will be useful in extending our knowledge of the role of peroxisomes in fatty acid metabolism and of the pathophysiology of peroxisomal disease.

Acknowledgments—We thank Dr. Robert H. McCluer. Without his valuable help and advice, this work would not have been completed. We also thank Denise Brescia for editorial assistance, Barbara A. Evans for superb technical support, and Drs. Mia MacCollin, Hugo Moser, Richard Kelley, and Ann Moser for providing samples from patients.

REFERENCES

- Lazarow, P. B., and de Duve, C. (1976) *Proc. Natl. Acad. Sci. U. S. A.* **73**, 2043–2046
- Moser, H. W. (1991) *Clin. Biochem.* **24**, 343–351
- Heymans, H. S. A., Schutgens, R. B. H., Tan, R., van den Bosch, H., and Borst, P. (1983) *Nature* **306**, 69–70
- Poulos, A., Sharp, P., and Whiting, M. (1984) *Clin. Genet.* **26**, 579–586
- Poulos, A., Johnson, D., and Singh, H. (1990) *Clin. Genet.* **37**, 106–110
- Danks, D. M., Tippet, P., Adams, C., and Campbell, P. (1975) *J. Pediatr.* **86**, 382–387
- Moser, A. B., Singh, I., Brown, F. R., III, Solish, G. I., Kelley, R. I., Benke, P. J., and Moser, H. W. (1984) *N. Engl. J. Med.* **310**, 1141–1145
- Bjorkhem, I., Blomstrand, S., Haga, P., Kase, B. F., Palonek, E., Pedersen, J. I., Strandvik, B., and Wikstrom, S. A. (1984) *Biochim. Biophys. Acta* **795**, 15–19
- Hanson, R. F., Szepanik-van Leeuwen, P., Williams, G. C., Grabowski, G., and Sharp, H. L. (1979) *Science* **203**, 1107–1108
- Diczfalussy, U. (1994) *Prog. Lipid Res.* **33**, 403–428
- Pitt, J. J., and Poulos, A. (1993) *Clin. Chim. Acta* **223**, 23–29
- Rocchiccioli, F., Cartier, P. H., Aubourg, P., and Bougnères, P. F. (1986) *Biomed. Environ. Mass Spectrom.* **13**, 315–318
- Setchell, K. D. R., and Street, J. M. (1987) *Semin. Liver Dis.* **7**, 85–99
- Clayton, P. T., Lake, B. D., Hall, N. A., Shortland, D. B., Carruthers, R. A., and Lawson, A. M. (1987) *Eur. J. Pediatr.* **146**, 166–173
- Evans, J. E., Ghosh, A., Evans B. A., and Natowicz, M. R. (1993) *Biol. Mass Spectrom.* **22**, 331–337
- Alme, B., Bremmelgaard, A., Sjøvall, J., and Thomassen, P. (1977) *J. Lipid Res.* **18**, 339–362
- Bjorkhem, I., Angelin, B., Einarsson, K., and Ewerth, S. (1982) *J. Lipid Res.* **23**, 1020–1025
- Larson, G., Karlsson, H., Hansson, G. C., and Pimlott, W. (1987) *Carbohydr. Res.* **161**, 281–290
- Wiesner, D. A., and Sweeley, C. C. (1994) *Anal. Biochem.* **217**, 316–322
- Lillingston, J. M., Trafford, D. J. H., and Makin, H. L. J. (1981) *Clin. Chim. Acta* **111**, 91–98
- Spector, A. A., and Yorek, M. A. (1985) *J. Lipid Res.* **26**, 1015–1035
- Kleiman, R., and Spencer, G. F. (1972) *J. Am. Oil Chem. Soc.* **50**, 31–38
- Fahlstadius, P. (1988) *Lipids* **23**, 1015–1018
- Maker, A., and Spittler, G. (1994) *Biochim. Biophys. Acta* **1214**, 209–220
- Kato, T., Yamaguchi, Y., Ueyhara, T., and Yokoyama, T. (1983) *Tetrahedron. Lett.* **24**, 4715–4718
- Ulsaker, A., and Teien, G. (1984) *Analyst* **109**, 967–968
- Hamberg, M., and Hamberg, G. (1990) *Arch. Biochem. Biophys.* **283**, 409–416
- Hayakawa, M., Sugiyama, S., Takamura, T., Yokoo, K., Iwata, M., Suzuki, K., Taki, F., Takahashi, S., and Ozawa, T. (1986) *Biochem. Biophys. Res. Commun.* **137**, 424–430
- Ozawa, T., Hayakawa, M., Takamura, T., Sugiyama, S., Suzuki, K., Iwata, M., Taki, F., and Tomita, T. (1986) *Biochem. Biophys. Res. Commun.* **134**, 1071–1078
- Oliv, E. H., Guengerich, P., and Oates, J. A. (1982) *J. Biol. Chem.* **257**, 3771–3781
- Pritchard, K. A., Jr., Toto, R. R., Stemerman, M. B., and Wong, P. Y.-K. (1990) *Biochem. Biophys. Res. Commun.* **167**, 137–142
- Sawazaki, S., Salem, N., and Kim, H.-Y. (1994) *J. Neurochem.* **62**, 2437–2447
- Amruthesh, S. C., Boerschel, M. F., McKinney, J. S., Willoughby, K. A., and Ellis, E. F. (1993) *J. Neurochem.* **61**, 150–159
- Mead, J. F., Alfin-Slater, R. B., Howton, D. R., and Popjak, G. (1986) *Lipids*:

- Chemistry, Biochemistry and Nutrition*, pp. 83–100, Plenum Press, New York
35. Oliw, E. H. (1983) *Biochim. Biophys. Res. Commun.* **111**, 644–651
36. Oliw, E. H. (1983) *J. Chromatogr.* **275**, 245–259
37. Lazarow, P. B. and Moser, H. W. (1989) in *Metabolic Basis of Inherited Disease* (Scriver, C. R., Beaudet, A. L., Sly, W. S., Valle, D., eds) 6th Ed., pp. 1479–1509, McGraw-Hill, New York
38. Osmundsen, H., Thomassen, M. S., Hiltunen, J. K., and Berge, R. K. (1987) in *Peroxisomes in Biology and Medicine* (Fahimi, H. D., and Sies, H. eds) pp. 152–165, Springer-Verlag, Berlin
39. Osmundsen, H., and Bjornstad, K. (1985) *Biochem. J.* **230**, 329–337
40. van den Bosch, H., Schutgens, R. B. H., Wanders, R. J. A., and Tager, J. M. (1992) *Annu. Rev. Biochem.* **61**, 157–197
41. Brown, F. R., Voight, R., Singh, A. K., and Singh, I. (1993) *Am. J. Dis. Child.* **147**, 617–626
42. Roels, F., Espeel, M., and De Craemer, D. (1991) *J. Inherited Metab. Dis.* **14**, 853–875
43. VanRollins, M., Baker, R. C., Sprecher, H. W., and Murphy, R. C. (1984) *J. Biol. Chem.* **259**, 5776–5783
44. Weiss, R. H., Arnold, J. L., and Estabrook, R. W. (1987) *Arch. Biochem. Biophys.* **252**, 334–338
45. Imigawa, T., Kasai, S., Matsui, K., and Nakamura, T. (1982) *J. Biochem. (Tokyo)* **92**, 1109–1121
46. Angermuller, S., Bruder, G., Volkl, A., Wesch, H., and Fahimi, H. (1987) *Eur. J. Cell Biol.* **45**, 137–144
47. Gutierrez, G., Okita, R., and Krisans, S. (1988) *J. Lipid Res.* **29**, 613–628
48. Dhaunsi, G. S., Singh, I., and Hanevold, C. D. (1993) *Mol. Cell. Biochem.* **126**, 25–35, and references therein
49. Chang, C., and Gill, S. S. (1991) *Arch. Biochem. Biophys.* **285**, 276–284