5-Decynedioic Acid, an Acetylenic Compound in Human Urine

Sven Lindstedt and Göran Steen

An acetylenic dicarboxylic acid, 5-decynedioic acid, has been isolated from human urine. The structure was determined by mass spectrometry of several derivatives, and by infrared spectroscopy. On osmium tetroxide oxidation, a vic-diketone was formed, the mass spectrum of which established the position of the triple bond between carbon atoms 5 and 6. Mass spectrometric evidence for the presence of a small amount of the isomeric 4-decynedioic acid was also obtained. The 24-h excretion of these acids is 0.01–0.1 mmol for healthy adults on a regular diet.

Additional Keyphrases: normal values • ketosis • 4-decynedioic acid

Normal human urine contains a number of aliphatic dicarboxylic acids (1-5). In the last few years several conditions have been described in which there occurs an abnormally large excretion of some of these acids. Thus, in ketosis caused by diabetes mellitus or starvation, up to 5 mmol of n-hexanedioic and n-octanedioic acid may be excreted per 24 h (4). Inborn errors of metabolism may give rise to a characteristic pattern in the urinary dicarboxylic acids. We have for instance observed a large excretion of C-6 to C-14 dicarboxylic acids in a patient with severe neonatal acidosi (6). Another recently described metabolic disease is glutaric aciduria, a neurodegenerative disorder (7).

During systematic studies of the urinary acids it has become evident that a number of them originate from unusual long-chain fatty acids, which have been ingested or have been formed in the gut. These acids give rise to dicarboxylic acids by a combination of β-oxidation and ω-oxidation. Examples of these types are 3-methyl-branched acids (2), and acids containing a cyclopropane ring (3).

Here, we describe the identification of a new type of dicarboxylic acid in urine, a C-10 dicarboxylic acid containing a triple bond.

Materials

Diethyl ether, petroleum ether (bp 40–60 °C), chloroform, and tert-butanol of analytical grade, and platinum oxide and osmium tetroxide were all obtained from BDH Chemicals, Poole, England. Silicic acid (100 mesh) was obtained from Mallinckrodt Chemical Works, St. Louis, Mo. 63160. To remove fines, we suspended it in water and allowed it to sediment for 10 min before the supernatant fluid was decanted. This procedure was repeated three times and the silicic acid was then dried at 120 °C for 40 h. Silicic acid/silver nitrate was prepared by mixing 90 g of silicic acid with 10 g of silver nitrate (E. Merck AG, Darmstadt, West Germany) dissolved in 200 ml of water. The mixture was dried at 120 °C for 24 h. Silica gel G for thin-layer chromatography was from E. Merck AG and column packing for gas chromatography, 3% OV-17 on 100–120 mesh GasChrom P, was from Applied Science Labs, Inc., State College, Pa. 16801. N,O-Bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) was bought from Pierce Chem. Co., Rockford, Ill. 61015. Sulfur oxide was generated from sodium hydrogen sulfite and concd sulfuric acid. The gas was dissolved in methanol. Diazomethane was prepared from N-nitroso-toluene-4-sulfomethylamide (8). n-Decanedioic acid was obtained from Fluka AG, Buchs, Switzerland. 5-Decynedioic acid was synthesized as described elsewhere (Lindstedt, S., Norberg, K., and Steen, G., in preparation).

Methods

Urine Collection

Human urine was collected from healthy adults on a regular diet. The urine was immediately frozen to -20 °C and was processed within a few weeks.

Analytical Procedures

Extraction, esterification, and saponification. After acidification to pH 1 with 6 mol/liter hydrochloric acid, the urine was extracted three times with equal volumes of diethyl ether. The combined extracts were concentrated under reduced pressure, dried over sodium sulfate, and then treated with diazomethane in ethereal solution until no more reaction was noted. Free acids were prepared from methyl esters by leaving them in 0.1 mol/liter potassium hydroxide in water at 60 °C for 4 h, followed by acidification and three successive extractions with diethyl ether.

Column chromatography. Silicic Acid. Samples of esterified material were dissolved in diethyl ether/petroleum ether (15/85 by vol), and applied onto columns of silicic acid prepared in the same solvent. About 20 g of silicic acid were used per gram of sample. The columns were eluted with increasing concentrations of the diethyl ether/petroleum ether (15, 20, 25, and 30%) and finally with diethyl ether. Two frac-

Department of Clinical Chemistry, University of Gothenburg, Sahlgren's Hospital, S-413 45 Gothenburg, Sweden.
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tions, each corresponding to 2.5 ml/g of silicic acid, were collected in each step. The fractions were analyzed by gas chromatography.

Silicic Acid/Silver Nitrate. The columns were prepared in diethyl ether/petroleum ether (20/80 by vol), and the samples applied in the same solvent, 10–20 mg of sample per gram of column packing. The columns were eluted with increasing concentrations of diethyl ether in petroleum ether (20, 21, 22, 23, 24, 25, 28, 30, 35, and 40 volumes per 100 volumes) and finally with diethyl ether. Ten fractions, 0.5 ml/g of column packing, were collected in each step.

Partition Chromatography. Partition chromatography of free acids was performed according to Bové and Raveaux (9). The stationary phase was 0.25 mol/liter sulfuric acid on a supporting medium of silicic acid (5 ml on 8 g), and the mobile phase was tert-butanol/chloroform (0, 2, 4, and 8 vol per 100 vol) that had been equilibrated against 0.25 mol/liter sulfuric acid. For each step, 100 ml of eluate was collected in 4-ml fractions.

Thin layer chromatography. Glass plates, 20 × 20 cm, were coated with a 0.25-mm thickness of silica gel G. The solvent used was diethyl ether/petroleum ether (30/60 by vol). Spots were detected with iodine vapor or with a reagent composed of copper acetate and orthophosphoric acid (10).

Gas chromatography. The stationary phase, 3% OV-17 on 100–120 mesh GasChrom P, was packed into glass columns of 2 m × 4 mm (i.d.). The carrier gas was helium at a flow rate of 30 ml/min. The instrument was a Model 5750 (Hewlett-Packard Instruments, Avondale, Pa. 19311), equipped with a flame-ionization detector. The oven temperature was programmed from 120 to 250 °C at a rate of 10 °C/min. For quantitative determinations, n-eicosane was used as internal standard.

Mass spectrometry. Mass spectra were recorded with an LKB 9000 combined gas chromatography/mass spectrometer (LKB-Produkter, Solna, Sweden), with use of the same kind of column as for gas chromatography. The acceleration voltage was 3.5 kV, electron energy 70 eV, and ion-source temperature 270 °C.

Infrared spectrometry. Infrared spectra were recorded with a Model 257 spectrometer (Perkin-Elmer Corp., Norwalk, Conn. 06856) with use of 100-μl sodium chloride cuvettes. The sample, 0.5 mg, was dissolved in carbon tetrachloride.

Trimethylsilylation. The sample, 0.1–0.5 mg, was dissolved in 100 μl of N,O-bis(trimethylsilyl)trifluoroacetamide/acetonitrile (1/1 by vol) and the solution was kept in the dark at room temperature for 30 min.

Catalytic hydrogenation. Platinum oxide, 5 mg, suspended in 0.5 ml of methanol was reduced by flushing with hydrogen gas. The sample, 0.5 mg dissolved in 0.5 ml of methanol, was hydrogenated for 90 min at room temperature. The catalyst was removed by filtration and the solvent evaporated.

Osmium tetroxide oxidation. Freshly prepared osmium tetroxide in dioxane (0.1 ml of a solution containing 50 mg/ml) was added to 0.2–0.5 mg of the methyl ester dissolved in 0.3 ml of dioxane/pyridine (8/1 by vol) (11). The mixture was left in the dark at room temperature for 2 h. Then, 1 ml of a solution of sulfur dioxide in methanol (100 g/liter) was added, followed by 0.5 ml of water (12), and the sample was left in the dark at room temperature for another 2 h. It was then evaporated under nitrogen, and dissolved in 2 ml of chloroform/methanol (1/1 by vol). The solution was passed through a small column containing 1 g of silicic acid, which was washed with a further 10 ml of the same solvent. After evaporation, the sample was briefly treated with diazomethane, for re-esterification of carboxyl groups. The sample was used for gas chromatography/mass spectrometry, with or without prior trimethylsilylation.

Results

Gas Chromatography of Urinary Extracts

On gas chromatography of a methylated diethyl ether extract, the present compound could be observed as a peak of intermediary size, eluting somewhat later than n-decanedioic acid, but earlier than hippuric acid, which is usually the largest peak of the chromatogram.

Isolation

First chromatography. Material (9.62 g) obtained by methylation of a diethyl ether extract of 10 liters of human urine was fractionated on a column (33 × 4 cm) containing silicic acid (200 g). Diethyl ether/petroleum ether (25/75 by vol), eluted a fraction (0.47 g) containing most of the aliphatic dicarboxylic acid esters.

Second chromatography. The above material was put onto a column (23 × 2 cm) containing silicic acid/silver nitrate (40 g). Saturated esters of dicarboxylic acids were eluted with diethyl ether/petroleum ether (21 to 23 per 100, by vol). The desired compound was then eluted with diethyl ether/petroleum ether (24/76 by vol) together with several minor components, mainly isomeroc octenedioic acid esters. The weight of the fraction was 51 mg.

Third chromatography. Part of the above fraction was saponified and 6 mg of the resulting free acids were subjected to partition chromatography on silicic acid with sulfuric acid as the stationary phase and increasing concentrations of tert-butanol in chloroform as eluent. The first few fractions (tert-butanol/chloroform 2/98 by vol) eluted 3.9 mg of crystalline material, mp 105–108 °C, which gave one peak on gas chromatography and one spot on a thin-layer plate.

Determination of Structure

The mass spectrum of the above material after treatment with diazomethane is shown in Figure 1 (top spectrum). The spectrum has several features characteristic of a methyl ester of an aliphatic carboxylic acid. If we assume that the small ion at m/e
Fig. 1. Mass spectrum of the methyl ester of urinary (top) and synthetic (bottom) 5-decynedioic acid

Fig. 2. Infrared spectra of the methyl esters of urinary (top) and synthetic (bottom) 5-decynedioic acid
226 represents the molecular ion, there are ions resulting from the loss of: (a) one methoxy group (M-31 at m/e 195), (b) one molecule of methanol (M-32 at m/e 194), (c) one carboxymethyl group (M-59 at m/e 167), and (d) one carboxymethyl group and one hydrogen atom (M-60 at m/e 166). The large ion at m/e 152 (M-74) could originate from the loss of one carboxymethyl group plus one methylene group and one hydrogen. Another series of fragments indicate the presence of two carboxymethyl groups. These are: m/e 163 (M-31–32), m/e 162 (M-32–32), m/e 135 (M-60–31 or M-59–32), m/e 93 (M-59–74), m/e 92 (M-60–74), and m/e 91 (M-60–74–1). The base peak at m/e 79 is probably M-74–73.

When the methyl ester was treated with silylating reagent, there was no change of retention time on gas chromatography, and the same mass spectrum was obtained. These findings exclude the presence of hydroxyl groups, and most likely also keto groups, which usually give rise to silyloxy groups after enolization. Furthermore, the infrared spectrum (Figure 2) shows no absorbance in the interval 3200–3600 cm⁻¹, where OH-stretching vibration should be seen, and there is a single ester-carbonyl band at 1740 cm⁻¹.

The analysis so far indicated that the parent acid had the formula C₁₂H₂₀(OOH)₉, i.e., with four hydrogens less than in decanedioic acid. On catalytic hydrogenation of the methyl ester, four hydrogens were added to yield a compound of molecular weight 230. The mass spectrum and the gas-chromatographic retention time were in perfect agreement with those of authentic n-decanedioic acid. This excluded the presence of a cyclopropane or cyclopropene ring, in the original compound, because such rings are slowly opened by catalytic hydrogenation with the formation of methyl-branched compounds (13).

The main alternatives to be considered were the presence of two double bonds or one triple bond. To decide between these structures, we treated the methyl ester of the original compound with osmium tetroxide and trimethylsilylated. The resulting spectrum (Figure 3, top spectrum) was not of the type one would have expected if a compound containing vicinal trimethylsilyloxy groups had been formed from a parent diene. The increment in mass after oxidation and trimethylsilylation was 104 m.u., indicating the introduction of one trimethylsilyloxy group and one oxygen. This is compatible with the following reaction sequence:

\[ \text{R}_1\text{C} = \text{C} - \text{R}_2 \xrightarrow{\text{OmO}_2} \]

(I)

\[ \text{R}_1\text{C} - \text{C} - \text{R}_2 \xrightarrow{\text{BSTFA}} \]

(II)

(trimethylsilyl)

(III)

The oxidation of I to II would be in analogy with what is known to occur on oxidation of triple bonds with potassium permanganate at neutral pH (14),
and compound III would be formed after enolization of one ketone function. Ions expected for compound III are found at m/e 330 (M), m/e 315 (M-15, methyl from TMS group), and m/e 298 (M-32). There are, however, no conspicuous ions resulting from fragmentation around the original centre of unsaturation, which would have defined the position in the chain. The intense ions seen at m/e 181 and m/e 167 have been attributed to M-15–60–74 and M-15–74–74, respectively.

The mass spectrum was then recorded after treatment with osmium tetroxide but without silylation. A simple spectrum was obtained, which is easily interpreted as shown in Figure 4, i.e., as representing a symmetrical diketone. The evidence presented establishes that the original acid is 5-decynedioic acid. A sample of the authentic acid [mp 109 °C, lit. 109 °C (15)] was available in the laboratory and was subjected to the same procedures as described above. Figure 1 shows the mass spectrum of the methyl ester (bottom spectrum) and those of the two oxidized derivatives are shown in Figures 3 and 4. These spectra are essentially identical with those of the urinary compound. Minor differences in relative intensity can be attributed to the lack of correction for differences in total ionization current, because the spectra have been recorded from gas-chromatography peaks. A qualitative difference is, however, observed when the spectra for the diketones are compared (Figure 4). The urinary compound contains small ions at m/e 87, 115, and 143, which are absent in the synthetic compound. This indicates the presence of a small amount of an isomer, 4-decynedioic acid, because fragmentations at the keto groups of the oxidized derivative should give the above-mentioned ions. The infrared spectrum of the synthetic compound is shown in Figure 2; it is identical to the spectrum of the urinary compound. The melting point of the isolated urinary compound was 105–108 °C, i.e., slightly lower than expected, probably due to the presence of some 4-decynedioic acid.

**Amount Excreted**

The urinary excretion of 5-decynedioic plus 4-decynedioic acids by eight adults on a regular diet was 0.04 (0.01–0.1) mmol per 24 h.

**Discussion**

The present acetylenic compounds, 5-decynedioic acid and 4-decynedioic acid, have not been isolated before from human urine. They have, however, been identified as metabolites of acetylenic fatty acids.
(15–18). When very large amounts of stearolic acid (9-octadecenoic acid) or behenolic acid (13-docosynoic acid) were fed to rats, in the form of triglycerides, 5-decynedioic acid was found in the urine (15, 16). Dogs did not seem to metabolize acetylenic fatty acids in the same way, as no 5-decynedioic acid at all could be found in the urine after an oral load of behenolic acid (16). Instead, the excretion of azelaic (n-nonanedicic) acid and succinic acid increased slightly (16). When rats were given tariric acid (6-octadecynoic acid), another urinary metabolite, dec-4-ene-6-ynedioic acid was identified (17). Crepenyonic acid (octadec-9-ene-12-ynoic acid), given to rats, gave 4-decynedioic acid as the urinary metabolite (18). Of the four fatty acids examined, all except crepenyonic acid were easily incorporated into depot fats. Stearolic and tariric acids were also incorporated to some extent into liver lipids.

A large number of naturally occurring acetylenic compounds are known, from higher plants, fungi and bacteria (19–21). They include aliphatic compounds, such as fatty acids, as well as heterocyclic compounds, with oxygen or sulfur. The great number of acetylenic fatty acids from seed oils contain several unsaturation centres, usually two or three triple bonds in combination. The metabolic fate of this kind of acetylenic fatty acids in higher animals seems to be totally unknown.

It seems likely that the two present acetylenic compounds of human urine reflect an exogeneous exposure to acetylenic fatty acids, such as stearolic, behenolic, and crepenyonic acids. When the total aliphatic dicarboxylic acid fraction of human urine is analyzed by gas chromatography, 5-decynedioic and 4-decynedioic acids together make up one of the major peaks. Among the very large number of minor aliphatic dicarboxylic acids seen, other acetylenic metabolites are likely to be present. This is under study in our laboratory.

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References