3-Hydroxydecanedioic Acid and Related Homologues: Urinary Metabolites in Ketoacidosis
Joachim Greter, Sven Lindstedt, Helène Seeman, and Göran Steen

Urine from patients with ketoacidosis was found to contain a number of aliphatic 3-hydroxy dicarboxylic acids. The acids were purified by silicic acid chromatography and their structures determined by gas chromatography–mass spectrometry of different derivatives. The major compound was 3-hydroxydecanedioic acid. Minor compounds were 3-hydroxyoctanedioic acid, 3-hydroxoctenedioic acid, 3-hydroxyoctenedioic acid, 3-hydroxydodecanedioic acid, 3-hydroxydodecanedioic acid, and 3-hydroxytetradecadienedioic acid. The excretion of 3-hydroxydecanedioic acid correlated positively with the excretion of hexanoic acid, another metabolite constantly found in ketoacidosis (Pettersen et al., Clin. Chim. Acta 38: 17–24, 1972). We suggest that the 3-hydroxy dicarboxylic acids are formed from fatty acids by a combination of ω-oxidation and incomplete β-oxidation.

Additional Keyphrases: fatty acid metabolism · ω- and β-oxidation of fatty acids · mass spectrometry · gas chromatography · chromatography, adsorption

In ketoacidosis due to diabetes mellitus or of other etiology, urine does not contain merely the three classic metabolites 3-hydroxybutyric acid, 3-oxobutyric acid, and acetone. Pettersen et al. (1) identified hexanoic acid (adipic acid), octanoic acid (suberic acid), and occasionally pentanoic acid (glutaric acid) and butanoic acid (succinic acid) in urine from patients with ketoacidosis of different etiologies. Another series of new metabolites was described by Landaas (2), who identified 3-hydroxyisobutyric acid, 2-methyl-3-hydroxybutyric acid, and 3-hydroxyisovaleric acid. The increased excretion of these compounds was shown to be the result of inhibition by ketone bodies and their CoA-derivatives of enzymes involved in the degradation of branched-chain amino acids (3).

A detailed knowledge of secondary metabolites excreted in ketoacidosis is necessary for a correct interpretation of an organic acid profile in metabolic disease. In this report, we describe the identification of 3-hydroxydecanedioic acid and related homologues in urine samples from patients with ketoacidosis.

Methods and Materials

Analysis of Organic Acids in Urine

We obtained urine samples from patients with ketoacidosis due to diabetes mellitus or undefined metabolic disorders. Samples were stored at −20 °C and analyzed within one week after collection. The method for analysis of organic acids in urine has been described before (4). For quantitative determination of hexanoic acid and 3-hydroxydecanedioic acid, we used 2-hydroxy-3-methylbenzoic acid (Merck, Darmstadt, FRG) as an internal standard. To prepare a standard curve for hexanoic acid, we added the acid to urines that contained less than 0.03 mol of this acid per liter. We used the same standard curve for 3-hydroxydecanedioic acid, which was not available for standardization and thus could not be used to correct for detector response differences and extraction losses. Mass spectra were recorded on an LKB 9000 combined gas chromatograph–mass spectrometer (LKB-Clinicon, Bromma, Sweden). Electron-impact spectra were recorded at an electron energy of 70 eV, an ion source temperature of 270 °C, and an acceleration voltage of 3.5 kV.

Preparation of the Unknown Compounds from Urine

A 50-mL portion of a urine sample containing the unknown compounds in high concentration was adjusted to pH 1 and extracted twice with 100-mL portions of ethyl acetate. The combined extracts were taken to dryness under reduced pressure in a rotary evaporator, and the residue was dissolved in a small volume of diethyl ether and treated briefly with diazomethane in diethyl ether (5). The extract was again taken to dryness and dissolved in a small volume of diethyl ether/n-heptane, 20/80 by vol, and applied onto a sillicic acid column (10 g) packed in the same solvent (5). To elute the sample, we increased the proportion of diethyl ether/n-heptane step-wise: 20/80, 30/70, 40/60, 50/50, and 100/0, by vol. The volume of each fraction was 100 mL. The fraction that contained the major unknown compound (diethyl ether/n-heptane, 40/60 by vol) was chromatographed on another sillicic acid column, 4 g of sillicic acid and 40-mL fractions, with a stepwise increase in the proportion of diethyl ether/n-heptane: 25/75, 28/72, 31/69, 34/66, 37/63, 40/60, 43/57, 46/54, and 49/51, by vol. A small part of each fraction (2/100) was taken to dryness and treated with N,O-bis(trimethylsilyl)trifluoroacetamide before analysis by gas chromatography (4).

Chemical Procedures

We subjected methyl esters to alkaline hydrolysis with 1 mol/L aqueous potassium hydroxide. Free acids were extracted with ethyl acetate after acidification. Methyl esters as well as free acids were silylated with N,O-bis(trimethylsilyl)trifluoroacetamide/acetonitrile, 1/1 by vol, for 1 h at room temperature or with N,O-bis(nonadeterotrtrimethylsilyl)-acetamide (Merck, Sharp & Dohme, Montreal, Canada). Parts of the methyl ester fractions were treated with methoxyamine hydrochloride in pyridine (20 g/L) at room temperature for 1 h, followed by trimethylsilylation.

Part of the major unknown compound, as free acid (20 μg), was taken to dryness and treated with 200 μL of dimethylformamide di(trimdeuteromethyl)lacetal (Tri-Deuter 8; Pierce Chem. Co., Rockford, IL) at 60 °C for 8 h. We then added 300 μL of N,O-bis(trimethylsilyl)trifluoroacetamide for trimethylsilylation. For dehydratization, 1 mg of the methyl ester of the major unknown compound was refluxed for 3 h in 10 mL of n-heptane containing catalytic amounts of p-toluensulfonic acid (BDH, Poole, U.K.). For catalytic hydrogenation, the product of dehydratization was dissolved in 1 mL of methanol and added to a platinum catalyst suspended...
in 1 mL of methanol. The platinum catalyst was prepared by reduction of 5 mg of platinum dioxide (Adam's catalyst; BDH) with hydrogen in methanol. The metallic platinum was then washed two times each with 2 mol/L hydrochloric acid, water, and methanol.

Results

Preparation of the Major Unknown Compound

An unknown compound constantly observed in urine samples from patients with ketoacidosis (Figure 1) was purified as the methyl ester by column chromatography on silicic acid. It was eluted in diethyl ether/n-heptane, 34/66 by vol. We obtained 4.8 mg of a fraction that contained about 90% of the unknown compound, the major contaminant being a homologue.

Identification of the Major Unknown Compound

The trimethylsilyl derivative of the unknown compound (Figure 2) had a relative molecular mass of 434, as shown by an (M-CH₃) ion at m/e 419 and other ions well compatible with the molecular mass and known fragmentations of trimethylsilyl derivatives (see Table 1). The (M-CD₃) ion in the deuterotrimethylsilyl derivative was found at m/e 443, showing a relative molecular mass of 461 for this derivative. The mass difference for these two derivatives was thus 27 (3 x 9) mass units, which demonstrated the presence of three trimethylsilyl groups in the molecule.

The presence of two carboxyl groups in the molecule was shown in two ways. The relative molecular mass was 318 for the methyl ester trimethylsilyl ether derivative (Figure 3 and Table 1) and 324 for the corresponding deuteromethyl ester derivative. Because the mass difference for one methyl ester group is 3, the presence of two ester groups was indicated. The mass difference between the trimethylsilyl ester trimethylsilyl ether (434) and the ester trimethylsilyl ether (318) was 116 (2 x 58), 58 being the mass difference between a trimethylsilyl group and a methyl group.

The absence of keto groups was demonstrated by the fact that treatment of the methyl ester with methoxyamine hydrochloride in pyridine before trimethylsilylation did not change the mass spectrum.

The presence of one hydroxyl group in the molecule was shown in two ways. The trimethylsilylation with the deuterium analogue had shown the presence of three functional groups, two of them carboxyls. Treatment of the methyl ester with deuterium-labeled silylating reagent gave a derivative

```
Table 1. Interpretation of the Mass Spectrum of the Trimethylsilyl Derivative of the Unknown Compound

<table>
<thead>
<tr>
<th>Mass increment</th>
<th>Content of TMS</th>
<th>Content of DMS</th>
<th>Ion formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-CH₃</td>
<td>2</td>
<td>1</td>
<td>M-CH₃</td>
</tr>
<tr>
<td>M-CH₃-CH₂CO</td>
<td>2</td>
<td>1</td>
<td>M-CH₃-CH₂CO</td>
</tr>
<tr>
<td>M-CH₃-TMSOH</td>
<td>1</td>
<td>1</td>
<td>M-CH₃-TMSOH</td>
</tr>
<tr>
<td>M-CH₂·COOTMS</td>
<td>2</td>
<td>0</td>
<td>M-CH₂·COOTMS</td>
</tr>
<tr>
<td>M-CH₂·COOTMS-H</td>
<td>1</td>
<td>1</td>
<td>M-CH₂·COOTMS-H</td>
</tr>
<tr>
<td>CHOTMS·CH₂·COOTMS</td>
<td>2</td>
<td>0</td>
<td>CHOTMS·CH₂·COOTMS</td>
</tr>
<tr>
<td>Heterogeneous</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* After derivatization with deuterotrimethylsilyl reagent.

TMS, trimethylsilyl; DMS, dimethylsilyl.
```

```
Table 2. Interpretation of the Mass Spectrum of the Unknown Compound as Methyl Ester Trimethylsilyl Ether Derivative (cf. Fig. 3.)

<table>
<thead>
<tr>
<th>Methyl ester trimethylsilyl ether, m/e</th>
<th>Methyl ester trimethylsilyl ether, m/e</th>
<th>Mass increment</th>
<th>Content of TMS</th>
<th>Content of DMS</th>
<th>Deuteromethyl ester trimethylsilyl ether, m/e</th>
<th>Mass increment</th>
<th>Content of methyl ester CH₃</th>
<th>Ion formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>303</td>
<td>309</td>
<td>6</td>
<td>0</td>
<td>1</td>
<td>309</td>
<td>6</td>
<td>2</td>
<td>M-CH₃</td>
</tr>
<tr>
<td>271</td>
<td>277</td>
<td>6</td>
<td>0</td>
<td>1</td>
<td>274</td>
<td>3</td>
<td>1</td>
<td>M-CH₃</td>
</tr>
<tr>
<td>245</td>
<td>254</td>
<td>9</td>
<td>1</td>
<td>0</td>
<td>248</td>
<td>3</td>
<td>1</td>
<td>CH₃OOC</td>
</tr>
<tr>
<td>229</td>
<td>235</td>
<td>6</td>
<td>0</td>
<td>1</td>
<td>232</td>
<td>3</td>
<td>1</td>
<td>CH₃OOC . CH₂</td>
</tr>
<tr>
<td>175</td>
<td>184</td>
<td>9</td>
<td>1</td>
<td>0</td>
<td>178</td>
<td>3</td>
<td>1</td>
<td>CH₃OOC . CH₂</td>
</tr>
<tr>
<td>159</td>
<td>165</td>
<td>6</td>
<td>0</td>
<td>1</td>
<td>162</td>
<td>3</td>
<td>1</td>
<td>CH₃OOC . CH₂</td>
</tr>
<tr>
<td>133</td>
<td>142</td>
<td>9</td>
<td>1</td>
<td>0</td>
<td>136</td>
<td>3</td>
<td>1</td>
<td>CH₃O</td>
</tr>
<tr>
<td>133</td>
<td>142</td>
<td>9</td>
<td>1</td>
<td>0</td>
<td>136</td>
<td>3</td>
<td>1</td>
<td>CH₃O . CHOTMS</td>
</tr>
<tr>
<td>89</td>
<td>95</td>
<td>6</td>
<td>0</td>
<td>1</td>
<td>92</td>
<td>3</td>
<td>1</td>
<td>CH₃O</td>
</tr>
<tr>
<td>89</td>
<td>95</td>
<td>6</td>
<td>0</td>
<td>1</td>
<td>92</td>
<td>3</td>
<td>1</td>
<td>CH₃O . DMS</td>
</tr>
<tr>
<td>73</td>
<td>82</td>
<td>9</td>
<td>1</td>
<td>0</td>
<td>73</td>
<td>0</td>
<td>0</td>
<td>TMS</td>
</tr>
<tr>
<td>59 (part of)</td>
<td>65</td>
<td>6</td>
<td>0</td>
<td>1</td>
<td>59</td>
<td>0</td>
<td>0</td>
<td>DMS + H</td>
</tr>
<tr>
<td>59 (part of)</td>
<td>59</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>62</td>
<td>3</td>
<td>1</td>
<td>CH₃OOC</td>
</tr>
</tbody>
</table>
```

TRIMETHYLSILYL DERIVATIVE

DEUTEROTRIMETHYLSILYL DERIVATIVE

Fig. 2. Mass spectra of the major unknown compound as trimethylsilyl derivative and as deuterotrimethylsilyl derivative

with a relative molecular mass of 327 (Figure 3 and Table 2). The corresponding unlabeled derivative had a relative molecular mass of 318. The resulting mass difference of 9 is consistent with the presence of one trimethylsilyl group.

We calculated the number of methylene groups (seven) by subtracting the mass of two carboxyl groups and one hydroxymethylene group from the mass of the underivatized compound. However, the structure of the aliphatic part of the molecule was more directly shown by dehydration and catalytic hydrogenation, which yielded n-decanedioic acid, the mass spectrum of which was available for comparison (5). This result also showed that there were no branches in the carbon chain.

The position of the hydroxyl group was shown by specific fragmentations in the mass spectra (Figures 2 and 3). In the spectrum of the trimethylsilyl (TMS) derivative, there was an ion at m/e 303, i.e., M-CH₂-COOTMS, and an ion at m/e 233, CHOTMS-CH₂-COOTMS. In the spectrum of the methyl ester trimethylsilyl ether, there was an intense ion at m/e 175, CHOTMS-CH₂-COOC₂H₅, and an ion at m/e 245, CHOTMS-(CH₂)₃-COOC₂H₅. All fragments showed the expected shifts in mass for the differently labeled compounds (Figures 2 and 3). From these observations, the position of the hydroxyl group was assigned to carbon atom 3. In conclusion, the major unknown compound was identified as 3-hydroxydecanedioic acid.

Identification of Saturated and Unsaturated Homologues to 3-Hydroxydecanedioic Acid

In the course of the preparative work, we observed some homologues to 3-hydroxydecanedioic acid. They were all 3-hydroxy acids, as shown by the ion at m/e 175 in the mass spectra of the methyl ester trimethylsilyl ether derivatives. The following pairs of compounds were detected by gas chromatography–mass spectrometry in different fractions from the silicic acid column chromatography: 3-hydroxyoctanedioic acid and 3-hydroxyoctenedioic acid, 3-hydroxydecanedioic acid and 3-hydroxydecenedioic acid, 3-hydroxydodecanedioic acid and 3-hydroxydodecenedioic acid, as well as 3-hydroxytetradecenedioic acid and 3-hydroxytetradecadienedioic acid. We did not determine double-bond positions and stereochemistry.

Excreted Amounts of 3-Hydroxydecanedioic Acid

3-Hydroxydecanedioic acid is occasionally observed, in concentrations up to 20 μmol/L, in urine samples from healthy individuals. The acid was present in concentrations of 10 to 400 μmol/L in urine samples from patients with ketoacidosis. Correlation with the concentration of hexanediol acid was good, as shown in Figure 4. The excretion of 3-hydroxydecanedioic acid was about half as much as for hexanediol acid. The amount of excretion of the other homologues combined was of the same order of magnitude as for 3-hydroxydecanedioic acid.

Discussion

3-Hydroxydecanedioic acid was isolated from the urine of patients with ketoacidosis, and appears to be constantly present in ketoacidosis of different etiologies. We confirmed its structure by mass spectrometry of several derivatives, and by the fact that n-decanedioic acid was obtained after dehydration and catalytic hydrogenation of the parent compound. Saturated and unsaturated homologues with eight, 12, and 14 carbon atoms were also present, which indicates that the dicarboxylic acids found are metabolites of fatty acids.
This conclusion is further supported by the finding that the excretion of 3-hydroxydecanedioic acid is positively correlated with the excretion of hexanedioic acid, which can be formed from fatty acids by an initial ω-oxidation and subsequent β-oxidation cycles. 3-Hydroxydecanedioic acid and its homologues could be formed by such an oxidation sequence if the β-oxidation cycle is arrested at the 3-hydroxy acid or 3-oxo acid intermediate stage.

The ω-oxidation of fatty acids appears to be a minor pathway under normal conditions. In liver slices from normal rats it accounts for less than 5% of the total oxidation of stearic acid (7). This pathway seems to be quantitatively more important in ketoacidosis, at least in absolute terms. Using the 20 000 × g supernate from rat liver homogenate, Björkhem (8) has demonstrated an increased ω-oxidation of stearic acid in starvation and in alloxan-diabetes. The increased ω-oxidation could not be demonstrated with a microsomal fraction alone, but was related to factors in the 100 000 × g supernate. In a later report (9), he has shown that ATP is at suboptimal concentration in the 100 000 × g supernate from the livers of starved rats, with the result that synthesis of glycerides is low. Consequently, larger amounts of fatty acids become available for the microsomal ω-oxidation system.

The ω-oxidation of fatty acids may have an important function in ketoacidosis. In alloxan-diabetic rats, administration of long-chain dicarboxylic acids alleviated ketoacidosis (10). The β-oxidation of dicarboxylic acids may produce succinyl-CoA, but many observations demonstrate a low enzyme capacity in the mammalian degradation of medium-chain dicarboxylic acids. When, for instance, dogs were given long-chain dicarboxylic acids (11), they excreted medium-chain dicarboxylic acids, mainly decanoic acid, octanoic acid, and hexanoic acid. Decanoic acid was degraded mainly into octanoic acid and hexanoic acid, whereas...

**Fig. 3.** Mass spectra of the major unknown compound as methyl ester trimethylsilyl ether derivative, as methyl ester deuterotrimethylsilyl ether derivative, and as deuteromethyl ester trimethylsilyl ether derivative.
hexanedioic acid, given in the diet or by injection, was largely excreted unmetabolized (11, 12). In vitro experiments have shown that long-chain dicarboxylic acids are not activated and transported into the mitochondria as readily as fatty acids, and that they have to compete with fatty acids for the same enzyme systems (13, 14). In a metabolic situation with high fat utilization and formation of long-chain dicarboxylic acids, dicarboxylic acids may be to a considerable extent oxidized through the cytoplasmic β-oxidation system. The activity of these enzymes has not been studied with dicarboxylic acids as substrate, but it is known that cytoplasmic β-oxidation of long-chain fatty acids results in accumulation of fatty acids of medium chain length as well as 3-hydroxy acids (15). Cytoplasmic enzymes are also less efficient than the mitochondrial enzymes with substrates such as dodecanoyl-CoA and octanoyl-CoA (16).

An intermittent excretion of hexanedioic acid and octanedicarboxylic acid has been reported in carnitine deficiency (17, 18). We have recently observed two children who had no ketoadicosis but excreted very impressive amounts of hexanedioic acid, octanodicarboxylic acid, and the related homologues. Both have a muscular disease, one of them with a progressive encephalopathy. Therefore, it is unlikely that either the metabolites now reported or hexanedioic acid and octanedicarboxylic acid are formed as a result of an inhibition of the β-oxidation by ketone bodies; rather, a defect in transport of fatty acids into the mitochondria will result in an increase in ω-oxidation and excretion of dicarboxylic acids.

In the two metabolic situations discussed above, ω-oxidation is increased because of a high fatty acid concentration in the cytoplasm. Another role of the ω-oxidation system is in the degradation of fatty acids with structures that block the β-oxidation system. Examples of such fatty acids are cyclopropane fatty acids (19), cyclopropene fatty acids (20), methyl-branched fatty acids (21), acetylenic fatty acids (22), and possibly mycolic acids (23). By a combination of ω- and β-oxidation these acids give rise to short- or medium-chain dicarboxylic acids, which retain the structure unmodified or slightly modified that blocks the β-oxidation system. Finally, we note that dicarboxylic acids of longer chain length such as cis-5-dodecedenedioic acid and cis-5-tetradecenedenedioic acids, which we found in the urine of a fatal case of acidosis (5), have not been found in cases of ketoadicosis. The reported case possibly represented an enzyme defect in the β-oxidation system.

This work was supported by grants from the Swedish Medical Research Council (13X-585) and from the Ragnar Söderbergs Stiftelse.

References