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GAS CHROMATOGRAPHY–MASS SPECTROMETRY OF SATURATED AND UNSATURATED DICARBOXYLIC ACIDS IN URINE

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SUMMARY

Saturated and unsaturated dicarboxylic acids in urine are analyzed within the total profile of organic acids, using the methyl ester derivatives. Twenty-three acids with two carboxyl groups were identified. The method is employed for comparative studies of the excretion of dicarboxylic acids by individuals with normal and with increased fatty acid oxidation. In the group of the unsaturated acids, the *cis–trans* isomers mesaconic acid and citraconic acid, the two isomers of 3-methylglutaconic acid and muconic acid are characterized by the mass spectra of their methyl esters. The saturated unbranched and even-numbered dicarboxylic acids are elevated during fasting and diabetic ketoacidosis. In the total profile of the organic acids, succinic and adipic acid are indicators for ketoacidotic states.

INTRODUCTION

Among the aliphatic organic acids excreted in urine, dicarboxylic acids are predominant. This group of excretory products comprises acids with and without additional functional groups.

Adipic and suberic acid in urine from ketotic patients were described by Pettersen *et al.*[1]. These acids as well as higher-molecular-weight straight-chain and even-numbered dicarboxylic acids were found by Lindstedt *et al.*[2] in urine in cases of congenital lactic acidosis. A large number of acids with two carboxylic groups were identified in the urine of normal individuals by Spiteller and Spiteller[3]. It has been shown that the dicarboxylic acids are formed from long-chain monocarboxylic acids by ω-oxidation followed by β-oxidation[4]. Branched-chain dicarboxylic acids in urine were described by Pettersen and Stokke[5] and explained as products of the ω-oxidation of long-chain 3-methyl-substituted or *ante-iso* monocarboxylic acids formed by the microorganisms of the intestinal tract.

The hydroxyl group is the major additional functional group in dicarboxylic acids. Spiteller and Spiteller[6] identified a number of α-alkyl-substituted malic acids and β-hydroxy-β-alkyl-substituted acids. Medium-chain 3-hydroxydicarboxylic acids in urine during ketoacidosis were found by Greter *et al.*[7].

The object of this work was to identify and study the behaviour of saturated

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and unsaturated dicarboxylic acids during fasting and diabetic ketoacidosis in comparison with normal fatty acid metabolism, and to provide an analytical procedure suitable for comparative investigations as well as for control of the course of ketoacidosis.

EXPERIMENTAL

Extraction procedure

Urine was collected either for 12 h or for 24 h. It was either processed within 5 h after collection or stored deep-frozen until it was analyzed.

An aliquot of 1% of the urine was acidified to pH 1 with concentrated HCl and mixed with 35 g of NaCl per 100 ml of urine, to increase the extraction yield. The sample was extracted twice with ethyl acetate and once with diethyl ether, using a urine–solvent ratio of 1:1. After drying the combined extracts for 30 min over anhydrous sodium sulphate, the solvents were evaporated.

Derivatization

A solution of diazomethane in diethyl ether was added to the residue containing the acids dropwise until the reaction mixture developed a yellow colour and no more nitrogen bubbles were formed. The resulting sample of the methyl esters was immediately analyzed by gas chromatography (GC) or GC–mass spectrometry (MS).

GC analysis

The samples were concentrated to a volume of 10 μl in a stream of nitrogen and separated on a Model 3700 gas chromatograph with flame-ionization detector (Varian, Darmstadt, G.F.R.) under the following conditions: 25-m glass capillary column coated with OV-17 (Bodenseewerk Perkin-Elmer, Überlingen, G.F.R.); carrier gas, nitrogen at 5 ml/min; column temperature, 30°C for 5 min, then programmed to 230°C at 2°C/min; injector block temperature, 250°C; attenuation, 256; sample size, 1 μl at a split ratio of 1:20.

GC–MS analysis

The samples concentrated to a volume of 10 μl, were analyzed on the combination of a Model 2700 gas chromatograph, CH 5 mass spectrometer and Spectrosystem 100 MS computer (Varian, Bremen, G.F.R.). The gas chromatograph and the mass spectrometer were interfaced by a 30 cm × 0.01 mm I.D. platinum capillary allowing the total effluent of the GC column to enter into the ion source of the mass spectrometer. By automatic repetitive scanning, the mass spectra were recorded over the mass range *m/e* 15–380 and stored on magnetic tape. Helium was used as the carrier gas at a flow-rate of 4 ml/min. Otherwise, the same GC conditions were used as described under *GC analysis*.

The MS conditions were as follows: ionization by electron impact; ionization energy for the mass spectra, 70 eV; ionization energy for the total ion current, 20 eV; accelerating voltage, 3 kV; multiplier voltage, 2.75 kV; emission current, 300 μA; ion source temperature, 220°C; interface temperature, 220°C; resolution, 700.

Reference substances

Mesaconic acid, muconic acid and the methyl ester of 3-methylglutaconic acid were purchased from EGA-Chemie (Steinheim, G.F.R.), citraconic acid from Fluka (Buchs, Switzerland). The saturated dicarboxylic acids came from Fluka or Merck (Darmstadt, G.F.R.).

RESULTS

The identification of the dicarboxylic acids and the investigation of their behaviour during ketoacidosis were undertaken with the total profile of the organic acids in urine. Any fractionation into groups of acids was omitted.

Because of its simplicity and speed, this method is suitable for comparative studies of the urinary excretion of acids in individuals with normal fatty acid metabolism or with increased fatty acid oxidation leading to ketoacidosis. It allows comparisons of the amounts of the excreted substances from sample to sample, but not from compound to compound within one sample. Quantifications of the concentrations of the acids were not made.

In the total profile of the organic acids in urine of normal subjects (Fig. 1), 23 dicarboxylic acids were identified. The majority of these acids are either saturated or unsaturated compounds without additional functional groups; some are hydroxydicarboxylic acids. Table I lists the diarboxylic acids among some of the other organic acids.

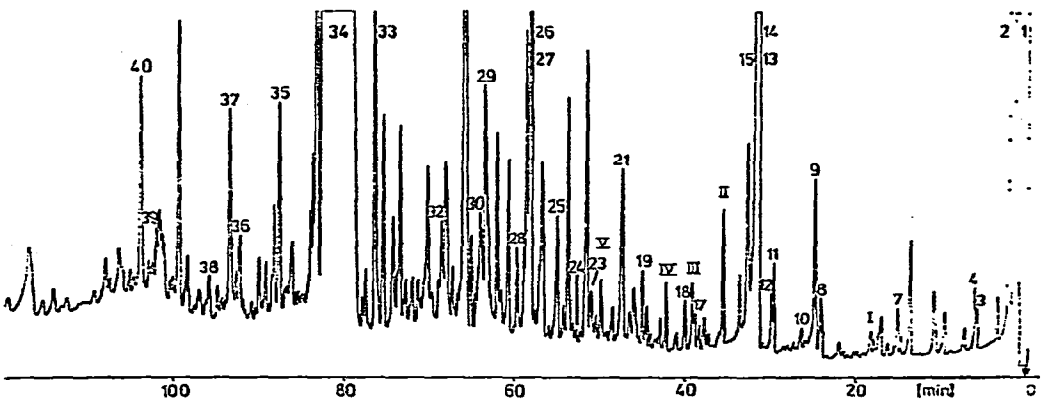

Fig. 1. Gas chromatographic profile of the methyl esters of the urinary acids of a normal individual.

The methyl esters of saturated dicarboxylic acids are recognized mass spectrometrically by their fragments M — 31 and M — 59, corresponding to the loss of OCH_3 and $COOCH_3$, respectively. The mass spectra were described by Spiteller and Spiteller[3].

From the group of the unsaturated dicarboxylic acids with one double bond, the *cis–trans* isomeric C_5 acids, mesaconic acid and citraconic acid, and two isomeric 3-methylglutaconic acids, are consistently found in urine. In addition, further unsaturated C_6 acids and in smaller amounts higher-molecular-weight unsaturated dicarboxylic acids are identified.

TABLE I

DICARBOXYLIC (×) AND OTHER ORGANIC ACIDS IN URINE IDENTIFIED BY MS

The peak numbers refer to the chromatograms, numbered and unnumbered (—) substances are listed in the order of increasing retention times.

Peak number	*Substance*	*Peak number*	*Substance*
1	Solvent	19	× Adipic acid
2	Solvent	—	× 2-Isopropylmalic acid
3	2-Hydroxyisobutyric acid	20	3-Phenylpropionic acid
4	2-Oxopropionic acid	21	× 3-Methyladipic acid
5	3-Hydroxybutyric acid	V	× Muconic acid
6	Acetoacetic acid	22	× Pimelic acid
7	× Oxalic acid	23	3-Hydroxybenzoic acid
I	2-Methyl-3-hydroxybutyric acid	24	× 3-Methylpimelic acid
8	× Methylmalonic acid	25	4-Hydroxybenzoic acid
9	Cresol (derivatized)	26	2,6-Di-*tert.*-butylphenol (from diethyl ether)
10	Phenol (not derivatized)		
11	× Ethylmalonic acid	27	Suberic acid
12	× Succinic acid	28	× 4-Hydroxyphenylacetic acid
13	× Methylsuccinic acid	29	Azelaic acid
14	Cresol (not derivatized)	30	× Citric acid
15	Benzoic acid	—	3-Methylazelaic acid
—	× Mesaconic acid	32	× Furoyl glycine
II	× Citraconic acid	33	Saccharine (from diet)
16	× Glutaric acid	—	Sebacic acid
17	× 3-Methylglutaric acid	34	× Hippuric acid
III	× Malic acid	35	Indolacetic acid
18	Phenylacetic acid	36	3-Hydroxybenzoyl glycine
IV	× 3-Methylglutaconic acid	37	4-Hydroxybenzoyl glycine
—	× 3-Ethylglutaric acid	39	5-Hydroxyindolacetic acid
—	× 3-Methylglutaconic acid	40	N-Phenylacetylglutamic acid

MS fragmentation of the unsaturated dicarboxylic acid methyl esters is also characterized by M — 31 and M — 59. More important than in the case of saturated acids, are the additional fragments, M — 32 and M — 60. The differences in the intensity ratios of M — 31 and M — 32 as well as M — 59 and M — 60 can be used for differentiation between the isomers. In the mass spectrum of the methyl ester of mesaconic acid (*trans*-methylbutendioic acid) the fragments *m/e* 98 and *m/e* 99 (M — 60 and M — 59) and *m/e* 126 and *m/e* 127 (M — 32 and M — 31) are similar in size (Fig. 2). In the spectrum of the methyl ester of citraconic acid (*cis*-methylbutendioic acid) *m/e* 99 and *m/e* 127 are dominant (Fig. 3). The spectra of the two isomers are further well distinguishable in so far as citraconic acid methyl ester forms a molecular peak and a peak at M — 15 which are both absent in the spectrum of mesaconic acid methyl ester.

The differences between the spectra of the two isomers of the methyl esters of 3-methylglutaconic acid (3-methyl-pentendioic acid) are much less pronounced. The characteristic fragments of both acids are *m/e* 112 and *m/e* 113 as well as *m/e* 140 and *m/e* 141. In analogy with the C_5 acids, however much less obvious, the ratio of M — 59 to M — 60 is larger in the isomer with the longer GC retention time (Figs. 4 and 5). No difference occurs in the ratio of M — 31 to M — 32. In both isomers no

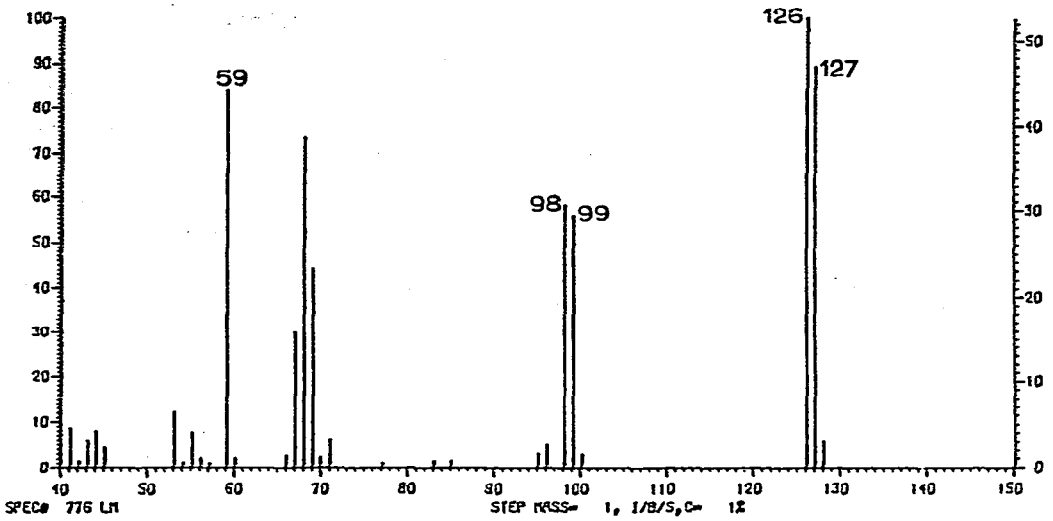

Fig. 2. Mass spectrum of the methyl ester of mesaconic acid from urine.

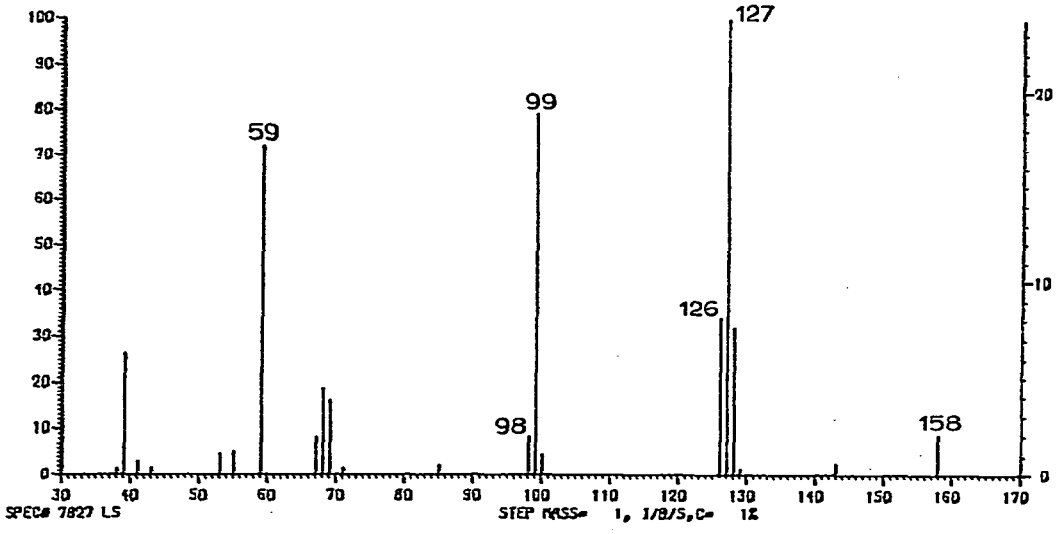

Fig. 3. Mass spectrum of the methyl ester of citraconic acid from urine (peak II in Fig. 1).

molecular peak (*m/e* 172) and no M — 15 are observed. However, the two isomers can be clearly distinguished by the fragments *m/e* 108 and *m/e* 109 (M — 74 and M — 73, respectively). In the isomer with the shorter retention time *m/e* 109 is higher than *m/e* 108, in the isomer with the longer retention time the two fragments are equal in size.

The identities of the two pairs of isomers were confirmed by good agreement of both the mass spectra and the retention data of reference compounds with those of the urinary substances. Mesaconic acid and citraconic acid were available as free acids and were methylated with diazomethane, 3-methylglutaconic acid could be used as methyl ester and contained both isomers (Fig. 6).

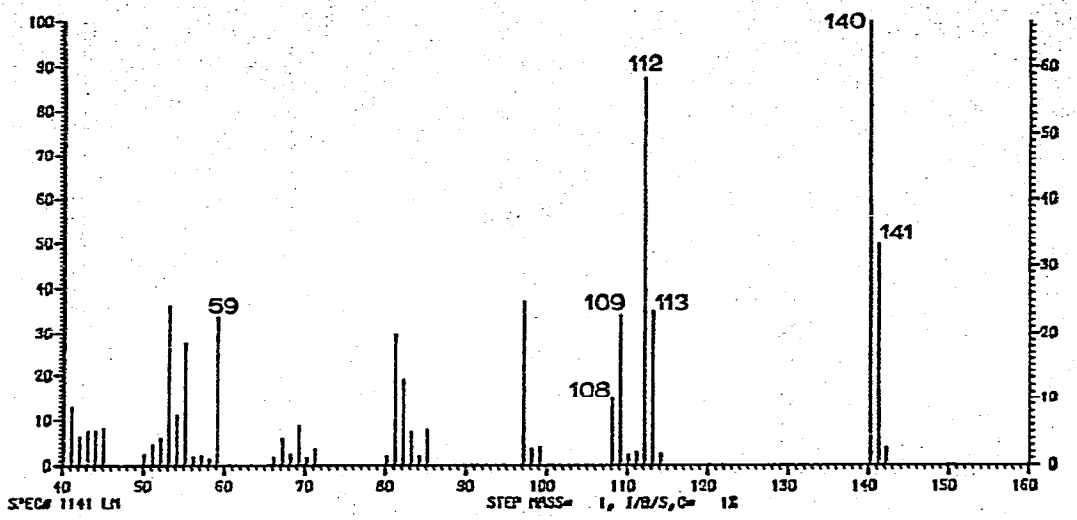

Fig. 4. Mass spectrum of the methyl ester of the first isomer of 3-methylglutaconic acid from urine (peak IV in Fig. 1).

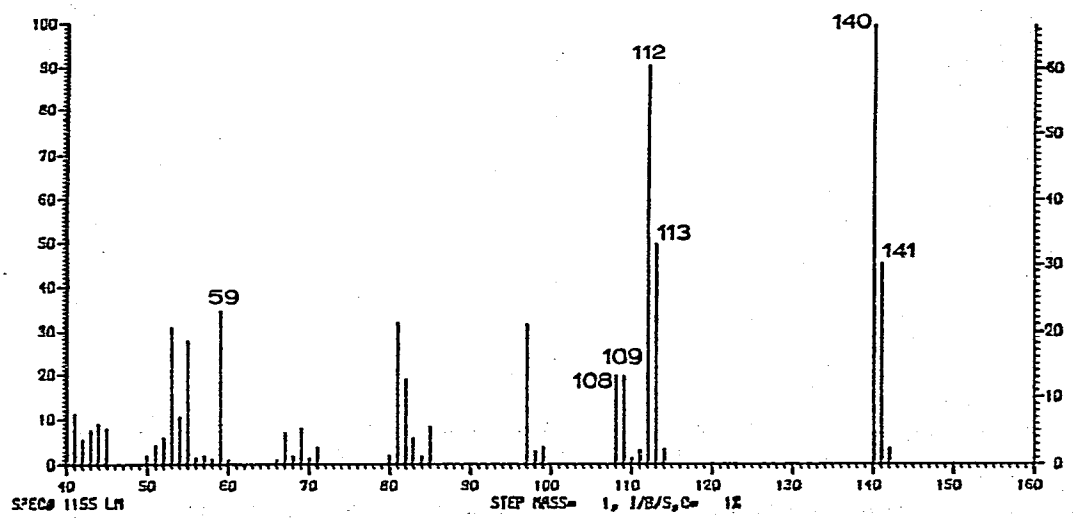

Fig. 5. Mass spectrum of the methyl ester of the second isomer of 3-methylglutaconic acid from urine.

Using diazomethane as methylating agent for the acids, it is necessary to analyze the sample directly after derivatization. In samples which are kept for some hours before GC or GC–MS analysis, diazomethane may produce artifacts from unsaturated dicarboxylic acids by reacting at the double bond. With fumaric acid the production of three artifacts is so rapid that methylation with diazomethane is not suitable for analysis of fumaric acid.

The major unsaturated dicarboxylic acid with two double bonds is muconic acid (2,4-hexadiendioic acid). Its mass spectrum (Fig. 7) shows mainly the molecular peak (*m/e* 170) and the fragments M — 31 (*m/e* 139) and M — 59 (*m/e* 111).

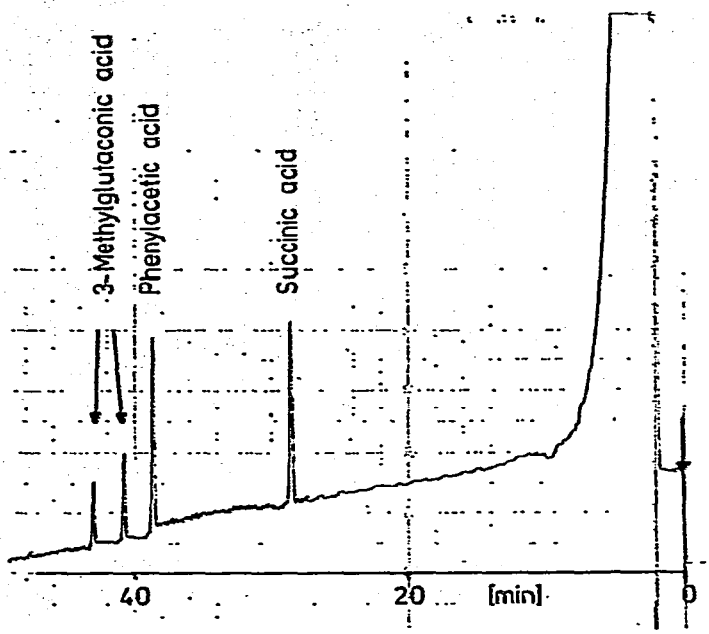

Fig. 6. Total ion current chromatogram of a standard mixture of the methyl esters of succinic and phenylacetic acid with the reference substance 3-methylglutaconic acid methyl ester.

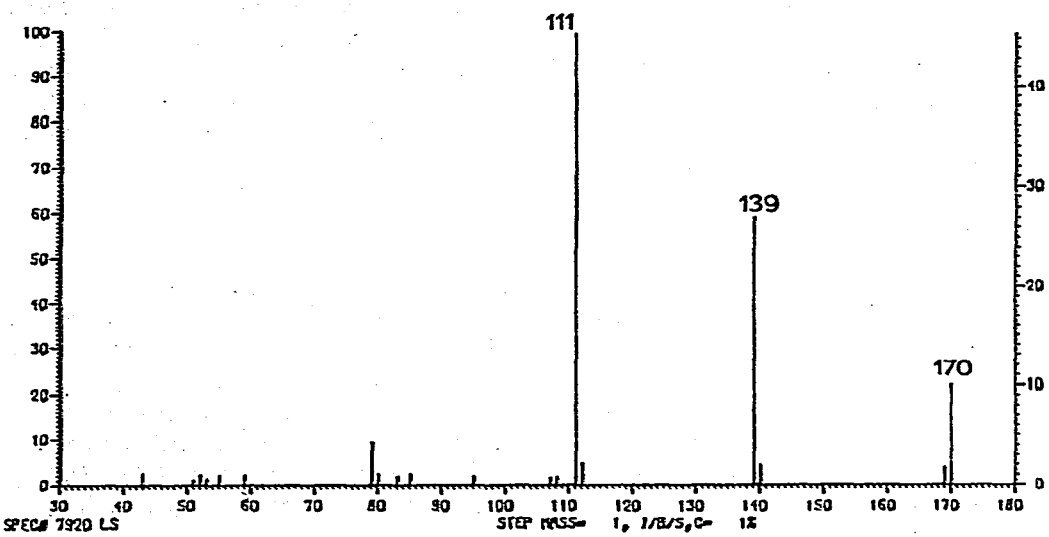

Fig. 7. Mass spectrum of the methyl ester of muconic acid from urine (peak V in Fig. 1).

Fig. 8 demonstrates a chromatogram of the organic acids in urine of a patient under fasting conditions. The urinary excretion of saturated unbranched and even-numbered dicarboxylic acids increases during all forms of ketoacidosis. In the total profile of urinary acids this behaviour of the dicarboxylic acids can be recognized by the change of succinic acid (peak 12) and adipic acid (peak 19). The

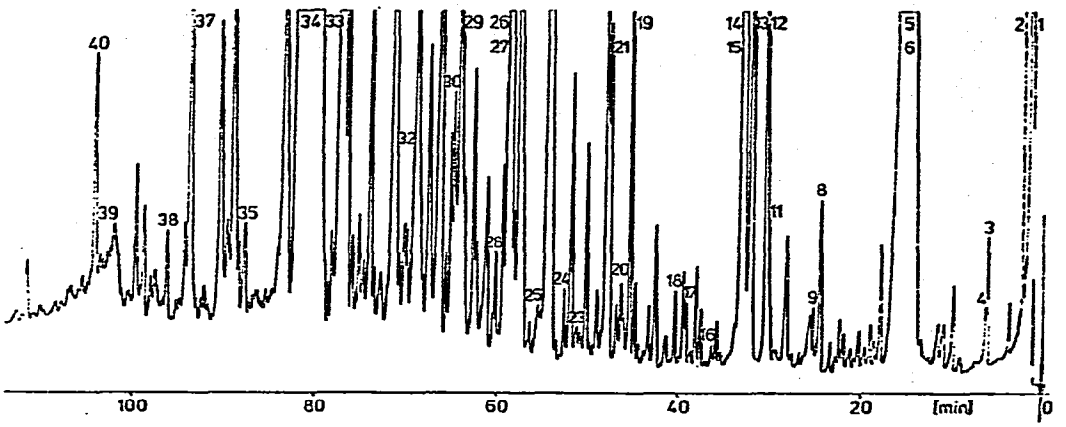

Fig. 8. Gas chromatographic profile of the methyl esters of the urinary acids of a fasting patient with ketoacidosis.

homologous acids suberic and sebacic acid are also elevated. Because of interfering peaks from aromatic acids in the chromatogram, this change cannot be easily detected in the total profile.

The diabetic ketoacidosis is accompanied by the same changes in the excretion of dicarboxylic acids as the ketoacidosis under fasting conditions. It was found that in fasting patients succinic acid reaches its maximum of excretion approximately 2 to 4 days later than the other dicarboxylic acids. For the methyl-branched dicarboxylic acids and for the hydroxydicarboxylic acids no increased excretion during ketoacidotic states could be proved. The unsaturated dicarboxylic acids, especially 3-methylglutaconic acid, were elevated in some of the patients with ketoacidosis. However, in other cases studied, this elevation was not observed.

DISCUSSION

The saturated dicarboxylic acids are formed by ω-oxidation and subsequent β-oxidation of long-chain monocarboxylic acids. The possibility of oxidation of fatty acids at the last carbon atom has been known for a long time and was called ω-oxidation by Verkade and Van der Lee[8]. In normal metabolism, the proportion of ω-oxidation in fatty acid oxidation is small. However, it gains importance in situations of increased fatty acid oxidation and ketogenesis. Björkhem[9] and Kam *et al.*[10] showed that in fasted rats, 5–11% of palmitic acid were metabolized by initial ω-oxidation. With shorter-chain fatty acids the percentage was even higher (17–21% for lauric acid).

Mainly medium-chain saturated dicarboxylic acids were detected in urine. This is in accordance with the findings of Pettersen[11] that long-chain dicarboxylic acids are metabolized to medium-chain dicarboxylic acids. Since succinic acid reaches its maximum of excretion later than the other dicarboxylic acids, it may originate from precursors other than long-chain even-numbered fatty acids, such as from amino acids.

The formation of the unsaturated acids excreted in urine is not completely

clear. However, one biochemical pathway can be the metabolizing of amino acids. It is known that 3-methylglutaconic acid is formed in the degradation process of leucine. Mesaconic acid and citraconic acid may originate from a side reaction of the metabolism of valine.

Using the simple procedure of the total profile of organic acids in urine instead of fractionating the acids, succinic acid and adipic acid are suitable indicators for the development and the course of a ketoacidosis. They complement the ketone bodies 3-hydroxybutyric acid and acetoacetic acid which are the conventional indicators for ketoacidosis.

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