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GAS CHROMATOGRAPHIC—MASS SPECTROMETRIC PROFILE OF ORGANIC ACIDS IN URINE AND SERUM OF DIABETIC KETOTIC PATIENTS

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SUMMARY

The organic acids in the urine and serum of diabetic patients with ketoacidosis and disturbance of consciousness were studied using acidification, extraction, evaporation, methoxime formation and trimethylsilylation, gas chromatographic separation and mass spectrometric identification procedures. The organic acid profile of 1 ml of serum ultrafiltrate was obtained with good separation using a gas chromatograph equipped with a glass capillary column and a splitless injector. 5-Hydroxyhexanoic acid and 3-hydroxyvaleric acid were identified for the first time in the urine of diabetic patients with ketoacidosis. Urinary excretion and serum concentrations of 2,3-dideoxypentonic acid were increased in diabetic patients.

INTRODUCTION

The well known abnormal metabolites occurring in diabetic ketoacidosis are 3-hydroxybutyric acid, acetoacetic acid and acetone. Several studies on the organic acid profile of diabetic ketotic urine by gas chromatography—mass spectrometry (GC—MS) have been reported. Pettersen and co-workers [1, 2]

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reported that the urinary excretion of adipic acid and suberic acid in diabetic patients with ketosis was increased, and that this phenomenon was due to the increased liberation of free fatty acids from peripheral fat deposits and also to ω-oxidation of the fatty acids followed by β-oxidation. Landaas has shown that the urinary excretion of 3-hydroxyisovaleric acid [3] and of 3-hydroxyisobutyric acid and 2-methyl-3-hydroxybutyric acid [4] is increased during ketoacidosis. Landaas and co-workers reported that the urinary excretion of 2-hydroxybutyric acid [5, 6] and 2-hydroxyisovaleric acid [7] was increased in patients with lactic acidosis and ketoacidosis. Liebich and co-workers [8, 9] studied the volatile organic metabolites in the diabetic urine using GC—MS.

We describe here the organic acid profile of diabetic patients with ketoacidosis using GC—MS, which has been employed for the investigation of organic acids and phenols in uraemic blood ultrafiltrate [10—14]. By use of a GC—MS system equipped with a glass capillary column and a splitless injector, the organic acid profile of a small volume of serum ultrafiltrate can be studied with good separation and good sensitivity. About 50 compounds were identified in the urine of diabetic patients with ketoacidosis. 5-Hydroxyhexanoic acid and 3-hydroxyvaleric acid were detected in diabetic ketotic urine for the first time.

EXPERIMENTAL

Samples

Urine samples were obtained from four diabetic patients with ketoacidosis, three diabetic patients without ketosis and five healthy adults. Serum samples were obtained from four diabetic patients with ketoacidosis, six diabetic patients without ketosis and six healthy adults. The four diabetic patients with ketoacidosis had suffered from disturbance of consciousness, coma or precoma, hyperglycaemia (400—600 mg/dl), glycosuria, ketonuria and dehydration.

The urine samples and the serum samples were kept at −20°C prior to analysis.

Sample preparation

Serum was ultrafiltered using a CF25 Amicon Filter. A 1-ml volume of serum ultrafiltrate or urine was acidified to pH 1 with 6 *N* hydrochloric acid and saturated with sodium chloride. After the addition of *p*-(*n*-amyl)benzoic acid as an internal standard (10 μg in serum ultrafiltrate or 50 μg in urine), the organic acids were extracted three times with 3 ml of ethyl acetate. The organic phase was dehydrated over anhydrous sodium sulphate and evaporated to dryness under a stream of nitrogen. A 1-mg amount of methoxylamine hydrochloride in 50 μl of ethyl acetate was added to the extract and allowed to react for 30 min at 60°C. The extract was concentrated to dryness under a stream of nitrogen and trimethylsilylated with N,O-bis(trimethylsilyl)trifluoroacetamide (Pierce Chemical, Rockford, IL, U.S.A.; 20 μl for serum samples, 100 μl for urine samples).

A 3-μl volume of the sample was subjected to GC—MS.

Gas chromatograph—mass spectrometer—computer system

Mass spectral data were obtained by directly coupling a Hewlett-Packard

5710A gas chromatograph to the source of a JMS D-300 double-focusing mass spectrometer (JEOL, Tokyo, Japan). The data were stored and processed by a JMA 2000 data system (JEOL). The gas chromatograph was equipped with a 30 m × 0.25 mm I.D. OV-101 open-tubular glass capillary column and a splitless injector. The injection temperature was 250°C and the column temperature was programmed from 70 to 250°C at 3°C/min.

Low-resolution electron impact (EI) spectra were obtained under the following conditions: ionizing energy, 70 eV; ionization current, 300 μA; accelerating voltage, 3kV; and ion source temperature 210°C; scanning was over the range *m/z* 40—650 in 1 sec. Chemical ionization (CI) spectra were obtained at an ionizing energy of 260 eV by using methane as a reactant gas. High-resolution spectra were obtained by scanning and peak matching with a resolution of 5000.

RESULTS

Fig. 1 shows typical profiles of organic acids in the urine and serum of a diabetic patient with ketoacidosis and a healthy control. There are several high

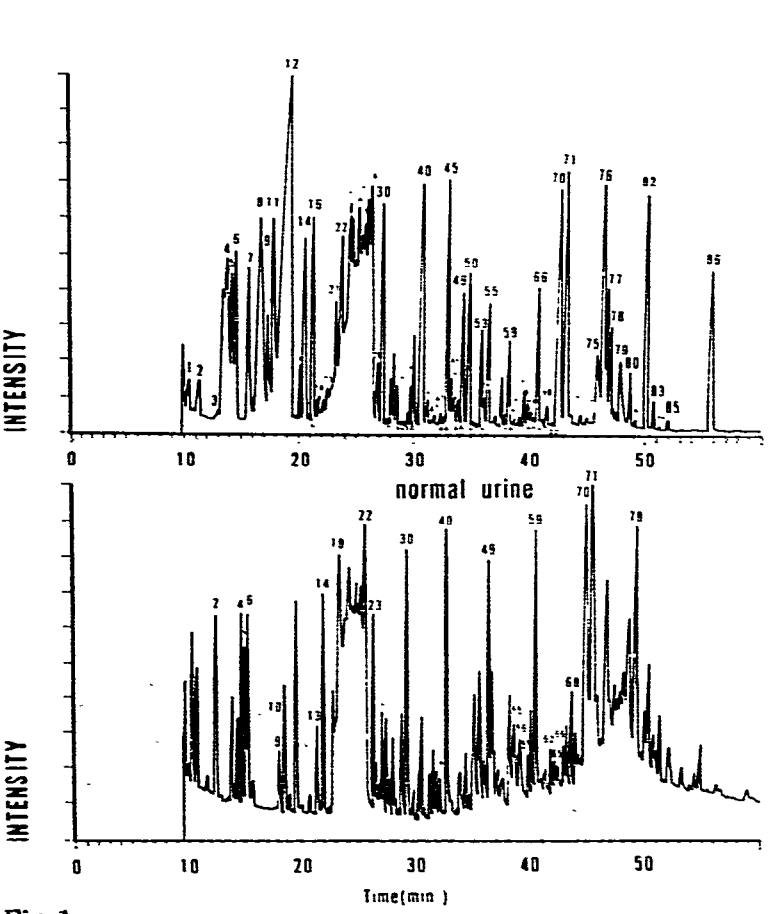

Fig. 1.

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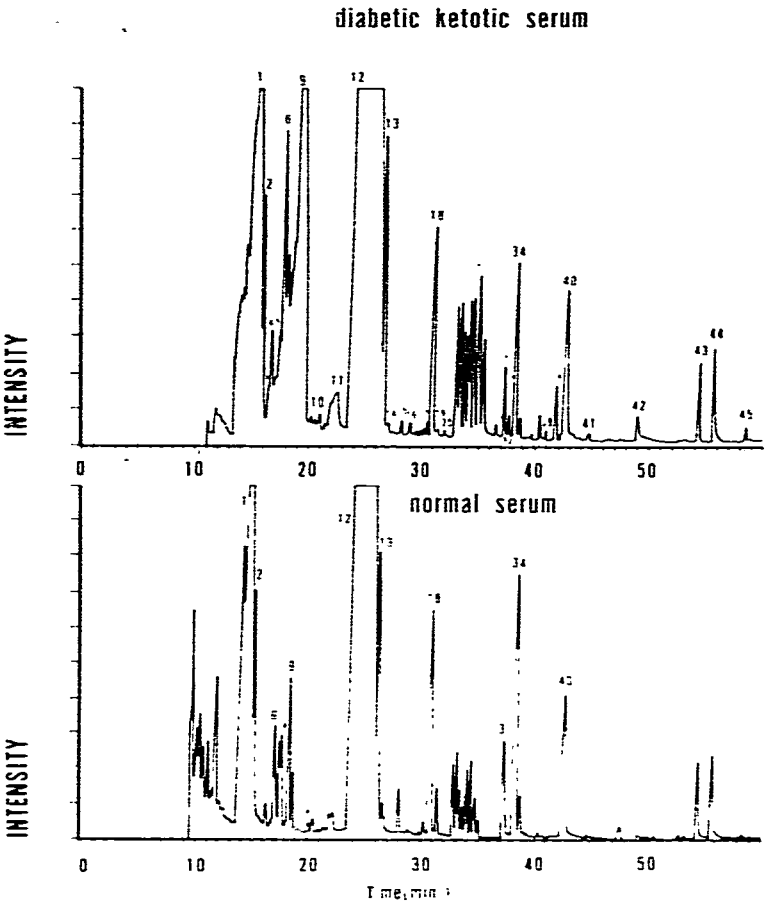

Fig. 1. Gas chromatographic profiles of organic acids in the urine and serum of a diabetic ketotic patient and a healthy subject. The extract was subjected to methoxime formation and trimethylsilylation and separated on an OV-101 open-tubular glass capillary column (30 m × 0.25 mm I.D.). The column temperature was programmed from 70° to 250°C at 3°C/min. The peaks that differed in the normal and ketoacidotic urine were identified as follows: 4, lactic acid; 7 and 8, acetoacetic acid; 9, 2-hydroxybutyric acid; 12, 3-hydroxybutyric acid and 2-hydroxyisovaleric acid (minor component); 14, 3-hydroxyisovaleric acid; 15, $C_3H_4O_3$; 17, 3-hydroxyvaleric acid; 28, fumaric acid; 29, 5-hydroxyhexanoic acid (one of two components of peak 29); 39, $C_6H_{10}O_4$; 45, adipic acid; 50, 2,3-dideoxypentonic acid. The peaks that differed in the normal and ketoacidotic serum were identified as follows: 1, lactic acid; 4, acetoacetic acid; 6, 2-hydroxybutyric acid; 9, 3-hydroxybutyric acid; 27, 2,3-dideoxypentonic acid.

peaks in the profiles of the diabetic ketotic patient compared with the healthy subject. The occurrence of acetoacetic acid and the high peak of 3-hydroxybutyric acid indicate the ketotic state of the patient. Each component of the profile was identified by EI and CI mass spectra, and high-resolution data in conjunction with its GC relative retention time. The mass spectra of the unknown compounds were compared with the published mass spectra and the mass spectra obtained in our laboratory from the derivatives.

Peak 29 in Fig. 1 (diabetic ketotic urine) was composed of two compounds.

The mass spectrum of a component of the peak is presented in Fig. 2. The molecular ion was found to be *m/z* 276 by recording the CI spectrum. High-resolution data revealed that the molecular formula was $C_6H_{12}O_3$. The compound was then identified as 5-hydroxyhexanoic acid by comparison with its published mass spectrum [15]. 5-Hydroxyhexanoic acid was detected in the urine from three out of four diabetic ketotic patients, but could not be detected in the patients' sera. 5-Hydroxyhexanoic acid was not detectable in the urine of the patients up to day 4 after admission, when the patients became non-ketotic with insulin therapy. The compound was not detected in the urine and the serum of the other non-ketotic diabetic patients or healthy subjects.

The EI mass spectrum of peak 17 in Fig. 1 (diabetic ketotic urine) is presented in Fig. 3. The CI mass spectrum revealed that the molecular ion of the compound was *m/z* 262. The molecular composition of the compound was found to be $C_5H_{10}O_3$. A fragment due to a loss of 29 a.m.u. at *m/z* 233 suggests the presence of an ethyl group. A fragment ion at *m/z* 205 due to a loss of CH_2CO group from the $(M-CH_3)^+$ ion suggests the presence of a hydroxyl group at the C_3 position, not at the C_2 position. The relatively high peak at *m/z* 131 also suggests 3-hydroxyvaleric acid. The compound was finally identified as 3-hydroxyvaleric acid by comparison with its published mass spectrum [16]. 3-Hydroxyvaleric acid was detected in the urine of three out of four diabetic patients with ketosis, but was not detected in the patients' sera. 3-Hydroxyvaleric acid was not detected in the urine and serum of the other non-ketotic diabetic patients or healthy subjects.

The diabetic ketotic patients showed a large urinary excretion of lactic acid, 2-hydroxybutyric acid, 2-hydroxyisovaleric acid, 3-hydroxyisovaleric acid, adipic acid and 2,3-dideoxypentonic acid compared with the normal adults. The excretion of 2,3-dideoxypentonic acid in four diabetic ketotic patients was 1.4 ± 0.75 [peak-height ratio with respect to the internal standard, 50 μg of *p*-(*n*-amyl)benzoic acid per mg of creatinine] compared with 0.23 ± 0.38 in five healthy adults. The serum concentration of 2,3-dideoxypentonic acid in the patients, i.e., 7.0 ± 13 [peak-height ratio with respect to the internal standard,

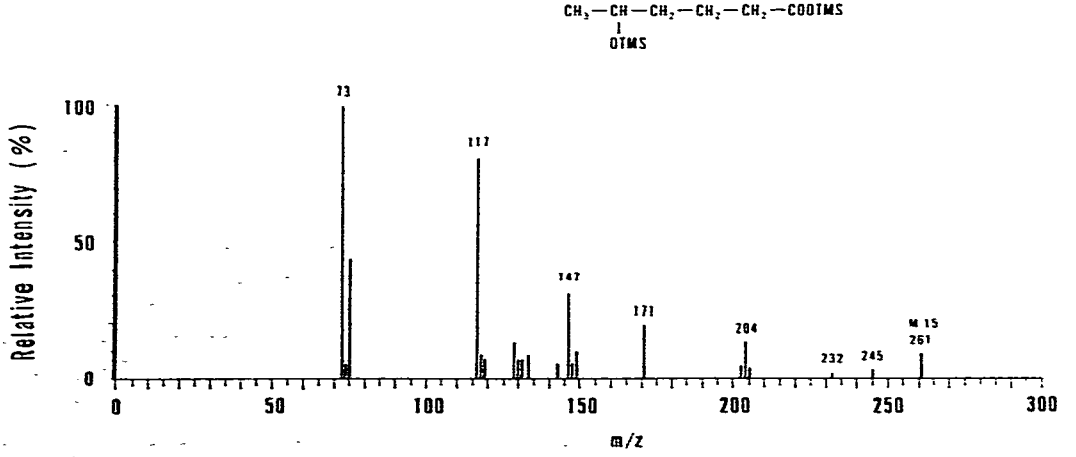

Fig. 2. EI mass spectrum of a component of peak 29 in Fig. 1 (diabetic ketotic urine). Ionizing energy, 70 eV; ionization current, 300 μA.

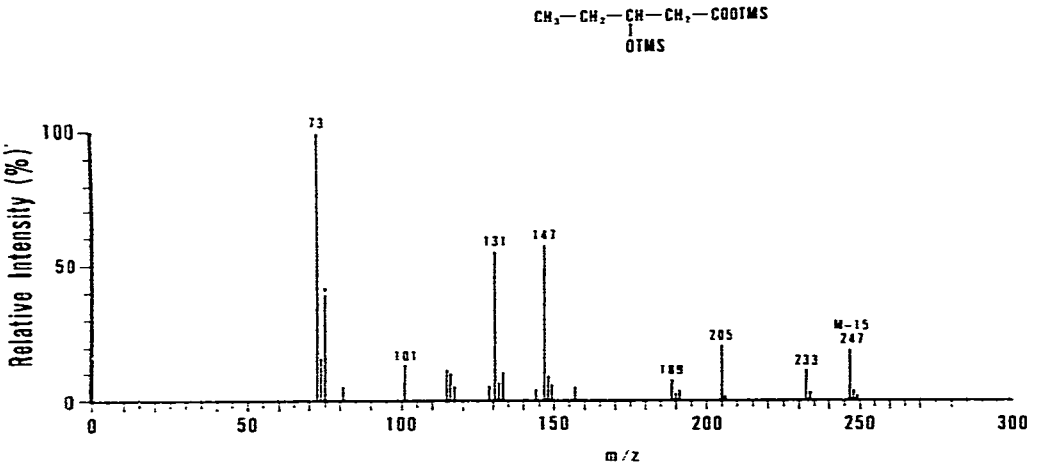

Fig. 3. EI mass spectrum of peak 17 in Fig. 1 (diabetic ketotic urine). Ionizing energy, 70 eV; ionization current, 300 μA.

10 μg of *p*-(*n*-amyl)benzoic acid] was markedly increased compared with that of the healthy subjects (0.084 ± 0.04). The diabetic ketotic patients showed only a slight increase in urinary excretion of 2-methyl-3-hydroxybutyric acid compared with the healthy controls.

DISCUSSION

5-Hydroxyhexanoic acid was first reported to be detectable in the urine of twins presenting an unusual Reye's-like syndrome characterized by severe hypoglycaemia, and the acid was considered to be a metabolite of hex-4-enoic acid [15]. Mamer et al. [17] also detected 5-hydroxyhexanoic acid in the urine of an infant with episodic hypoglycaemia together with a homologous series of hydroxy acids. In the present study 5-hydroxyhexanoic acid was also detectable in the urine of the diabetic patients with ketoacidosis.

The source of 5-hydroxyhexanoic acid may be ω−1 oxidation of the accumulated medium chain length fatty acids (particularly hexanoic acid). In diabetic ketoacidosis, liberation of free fatty acids from the peripheral fat deposits is increased. Moreover, ω-oxidation of the fatty acids is greater, causing increased excretion of medium chain length dicarboxylic acids. The increased urinary excretion of adipic acid in the present diabetic ketoacidosis cases is in agreement with published findings [2].

3-Hydroxyvaleric acid has not previously been reported to be present in the urine of diabetic patients with ketoacidosis, but has been found in the serum of patients with severe propionic and methylmalonic acidaemia [18]. Stokke et al. [18] considered that 3-hydroxyvaleric acid could be formed by condensation of propionyl-CoA and acetyl-CoA. Truscott et al. [19] reported 3-hydroxyvaleric acid in a case of propionic acidaemia during ketosis, but in the non-ketotic state 3-hydroxyvaleric acid could be detected only in trace amounts. In non-ketotic propionic acidaemia, self-condensation of propionyl-CoA is the major metabolic pathway. In diabetic patients with ketosis, the accumulation

of propionyl CoA may occur because of the enhanced degradation of isoleucine and odd-chain fatty acids. It is reasonable to consider that the condensation of propionyl-CoA and acetyl-CoA to form 3-hydroxyvaleric acid also occurs during diabetic ketoacidosis.

The up to 14-fold increase in urinary excretion of 3-hydroxyisovaleric acid in our diabetic ketotic patients was in agreement with a previous report [3]. Contrary to the latter findings, however, we also observed up to a 100-fold increase in the serum concentration of 3-hydroxyisovaleric acid during ketosis. In normal serum the compound was detectable in trace amounts. The increased urinary excretion of 3-hydroxyisobutyric acid as reported by Landaas [4] was not confirmed by us, because the peak of 3-hydroxyisobutyric acid could not be separated from the large peak of 3-hydroxybutyric acid in our GC profiles. The urinary excretion of 2-methyl-3-hydroxybutyric acid was only slightly increased in our diabetic patients with ketosis.

Ketoacidosis is usually accompanied by enhanced protein catabolism. 3-Hydroxyisovaleric acid, 3-hydroxyisobutyric acid and 2-methyl-3-hydroxybutyric acid are known to be the intermediates of the metabolism of the branched-chain amino acids leucine, valine and isoleucine, respectively. The increase in the urinary excretion of these acids during ketoacidosis is considered to be due to the inhibition of their further metabolic breakdown by the accumulated 3-hydroxybutyric acid and acetoacetic acid [20].

Increased urinary excretion of 2-hydroxybutyric acid during ketoacidosis was also observed in our profiling analysis. The serum concentration of 2-hydroxybutyric acid in the diabetic ketotic patients, however, varied anywhere from a trace amount to a 20-fold increase in relation to the normal concentration. Pettersen et al. [5] suggested that the excretion of the compound is correlated only with lactic acidosis and not ketosis. All of the diabetic ketotic patients in the present study showed 3—50 times the control urinary excretion of lactic acid. 2-Hydroxybutyric acid is derived from 2-ketobutyric acid by action of lactate dehydrogenase. 2-Ketobutyric acid is an intermediate of the metabolism of several amino acids. An increased $NADH_2/NAD$ ratio, which is often present in lactic acidosis, is considered to be the most important factor for the accumulation of 2-hydroxybutyric acid [6].

Our diabetic patients with ketosis also showed increased urinary excretion of 2-hydroxyisovaleric acid. This finding agrees well with a previous report [7]. The urinary excretion and the serum concentration of 2,3-dideoxypentonic acid were increased during diabetic ketoacidosis. The metabolic origin of the compound, however, is not yet known.

The profiling analysis of the organic acids in 1 ml of serum ultrafiltrate became feasible by use of GC—MS with a glass capillary column and a splitless injector. As this method yielded better separation with greater sensitivity than a packed column, it may prove useful for routine clinical examinations.

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