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IDENTIFICATION OF 2-HYDROXY-2-METHYLLEVULINIC ACID IN URINE AND SERUM OF DIABETIC PATIENTS WITH KETOACIDOSIS

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SUMMARY

A new organic acid, 2-hydroxy-2-methyllevulinic acid, was identified in the urine of four diabetic patients with ketoacidosis using gas chromatography—mass spectrometry. The compound was also detected in two serum samples of the four patients. The compound became undetectable in the urine of the patients after insulin therapy and was not detected in urine and serum of healthy subjects or diabetic patients without ketosis.

2-Hydroxy-2-methyllevulinic acid was also detectable in the urine of a child with elevated blood lactate and pyruvate, and ketosis. This finding suggests that the occurrence of 2-hydroxy-2-methyllevulinic acid is not specific to "diabetic" ketosis but is correlated to ketosis itself.

INTRODUCTION

The well-established abnormal urinary metabolites described in ketosis are 3-hydroxybutyrate, acetoacetate, and acetone. In addition, in ketosis certain other metabolites have also been found to be excreted into urine in large amounts. The urinary excretion of aliphatic dicarboxylic acids, namely, adipic acid and suberic acid, was found to be increased by Pettersen et al.

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[1, 2]. 3-Hydroxyisovaleric acid [3], 3-hydroxyisobutyric acid, 2-methyl-3-hydroxybutyric acid [4], 2-hydroxybutyric acid [5, 6], and 2-hydroxyisovaleric acid [7] were also demonstrated to appear in ketotic urine in high concentrations. Recently, the present authors reported that two abnormal metabolites, 5-hydroxyhexanoic acid and 3-hydroxyvaleric acid, were found in the urine of diabetic patients with ketoacidosis [8]. In the present study 2-hydroxy-2-methyllevulinic acid was demonstrated in the urine of patients with diabetic ketoacidosis and in the urine of a patient with pyruvic acidemia and ketosis. To our knowledge this metabolite has never been reported to be present in ketotic urine thus far.

METHOD

Chemicals

2-Hydroxy-2-methyllevulinic acid was synthesized according to the method of Armengaud et al. [9].

Trimethylsilylating agent, N,O-bis(trimethylsilyl)trifluoroacetamide, was purchased from Pierce, Rockford, IL, U.S.A. Methoxylamine hydrochloride and hydroxylamine hydrochloride were purchased from Tokyo Kasei Co., Tokyo, Japan.

Samples

Urine samples were obtained from four patients with diabetic ketoacidosis, three diabetic patients without ketosis, and five healthy subjects. Serum samples were obtained from four patients with diabetic ketoacidosis, six diabetic patients without ketosis, and six healthy adults. The four patients with diabetic ketoacidosis had suffered from disturbance of consciousness, hyperglycemia, glycosuria, ketonuria, acidosis and dehydration.

Urine was obtained from a 9-year-old boy with ketosis and elevated blood lactate, pyruvate, and alanine.

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

Sample preparation

Serum was filtered through a CF-25 membrane filter (Amicon, Lexington, MA, U.S.A.).

A 1-ml volume of serum ultrafiltrate or urine was acidified to pH 1 with hydrochloric acid, and saturated with sodium chloride. As an internal standard, 10 μg or 50 μg of *p*-(*n*-amyl)benzoic acid were added to serum ultrafiltrate or urine, respectively. The organic acids were extracted three times with 3 ml of ethyl acetate. The organic phase was dehydrated over anhydrous sodium sulfate and evaporated with a nitrogen stream. Methoxylamine hydrochloride, 1 mg 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 nitrogen stream and trimethylsilylated with N,O-bis(trimethylsilyl)trifluoroacetamide (Pierce; 20 μl for serum samples, 100 μl for urine samples).

For oxime-trimethylsilylation of the extracts, 1 mg of hydroxylamine hydrochloride was used in place of methoxylamine hydrochloride. For tri-

methylsilylation without methoxime formation, the reaction with methoxylamine hydrochloride was omitted.

A 3-μl aliquot of the sample was subjected to gas chromatography—mass spectrometry (GC—MS).

Instrumentation

We used a Hewlett-Packard 5710A gas chromatograph combined with a double-focussing mass spectrometer (JMS D-300; JEOL). The data were stored and processed by a JMA 2000 data system of 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. Injection temperature was 250°C. The column temperature was programmed from 70°C to 260°C at 3°C/min.

Electron-impact (EI) ionization mass spectra were recorded at an ionizing energy of 22 eV, an ionization current of 300 μA, a separator temperature of 270°C, and an accelerating voltage of 3 kV. Chemical-ionization (CI) mass spectra were recorded with methane as a reactant gas. Ionizing energy was 260 eV. The other conditions were the same as for EI. High-resolution spectra were obtained by peak matching with a resolution of 5000.

RESULTS

Fig. 1 (upper chromatogram) shows the gas chromatogram of organic acids in the urine of a diabetic patient with ketoacidosis. Each component of the profile was identified by EI and CI mass spectra, and high-resolution data. The EI mass spectrum of peak 30 is shown in Fig. 2 (lower spectrum). The CI mass spectrum showed that the molecular ion of the compound was 319. High-resolution data of the compound are summarized in Table I. The composition of the molecular ion indicates mono-methoxime-di-trimethylsilyl (TMS) derivative of the original composition of $C_6H_{10}O_4$. The intense ion at *m/z* 202 is derived from a loss of COOTMS. The composition of the intense fragment ion of *m/z* 233 suggests the fragment structure of $C(CH_3)$ (OTMS) — COOTMS. Peak 30 and the methoxime-TMS derivative of synthesized 2-hydroxy-2-methyllevulinic acid showed identical retention times on the gas chromatograms (Fig. 1) and identical EI mass spectra (Fig. 2).

The derivatization method was changed to confirm the identification of 2-hydroxy-2-methyllevulinic acid. Trimethylsilylation of 2-hydroxy-2-methyllevulinic acid and of the ethyl acetate extract from the diabetic ketotic urine was performed. The EI mass spectrum of the di-TMS derivative of 2-hydroxy-2-methyllevulinic acid is shown in Fig. 3 (upper spectrum). An abnormal peak in the diabetic ketotic urine and trimethylsilylated 2-hydroxy-2-methyllevulinic acid showed identical retention times on the OV-101 capillary column and identical EI mass spectra (Fig. 3). Oxime-trimethylsilylation was also used to derivatize 2-hydroxy-2-methyllevulinic acid and the ethyl acetate extract from the diabetic ketotic urine. An abnormal peak in the diabetic ketotic urine and the oxime-TMS derivative of 2-hydroxy-2-methyllevulinic acid showed identical retention times on the OV-101 capillary column and identical EI mass spectra (Fig. 4). The above evidence verifies the identifica-

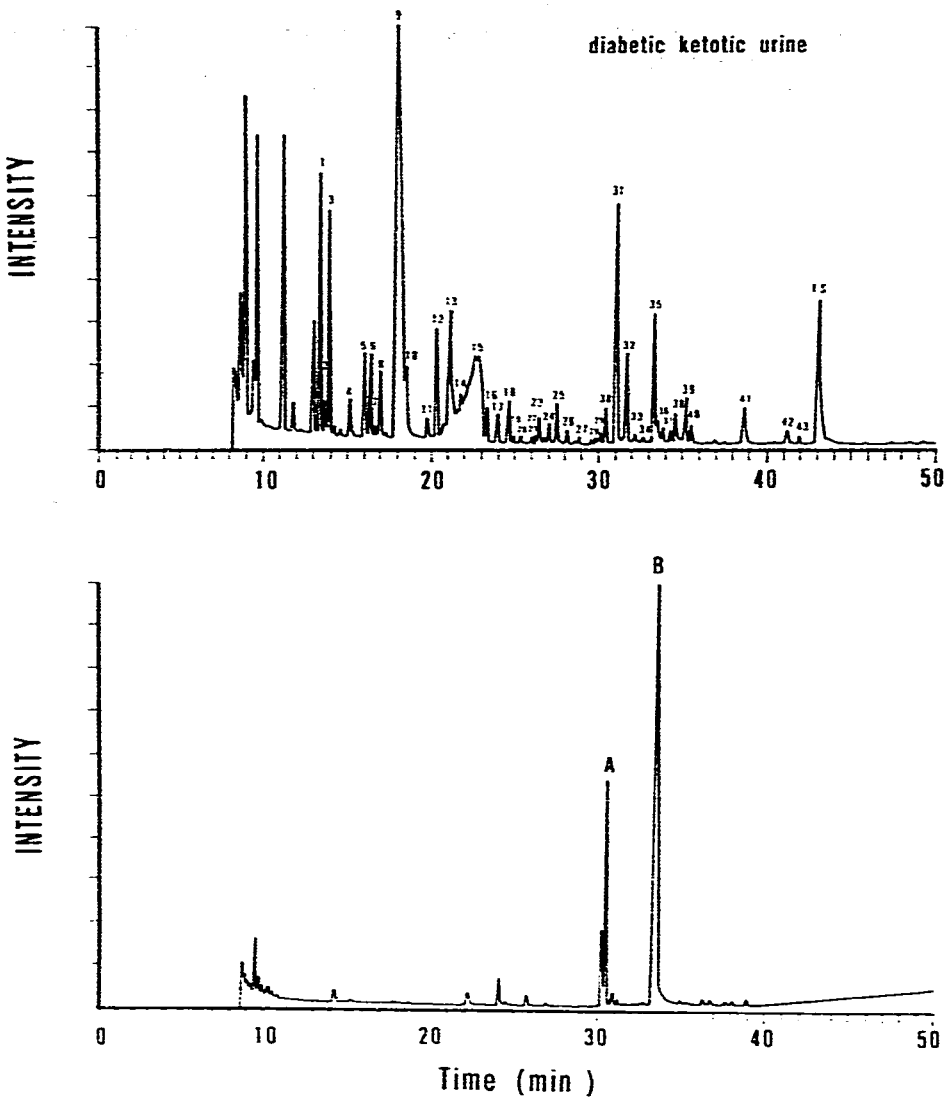

Fig. 1. Gas chromatograms of the methoxime-trimethylsilylated ethyl acetate extract from diabetic ketotic urine (upper chromatogram) and of methoxime-trimethylsilylated 2-hydroxy-2-methyllevulinic acid (peak A, lower chromatogram). Peak identifications: 1, lactic acid; 2, 2-hydroxyisobutyric acid; 3, glycolic acid; 4, 5, acetoacetic acid; 6, 2-hydroxybutyric acid; 8, 3-hydroxypropionic acid; 9, 3-hydroxybutyric acid; 10, 2-hydroxyisovaleric acid; 11, 2-methyl-3-hydroxybutyric acid; 12, 3-hydroxyisovaleric acid; 13, 2-ethylhydracrylic acid; 14, 3-hydroxyvaleric acid; 15, urea; 16, dimethylmalonic acid; 17, glycerol; 18, succinic acid; 22, glyceric acid; 23, fumaric acid; 24, 5-hydroxyhexanoic acid, 4-deoxyerythronic acid; 25, 4-deoxythreonic acid; 29, 3-deoxytetronic acid; 30, 2-hydroxy-2-methyllevulinic acid; 31, 2-deoxytetronic acid; 35, adipic acid; 38, 3-methyladipic acid; 39, 2,3-dideoxypentonic acid; 41, 4-hydroxyphenylacetic acid; 43, isosaccharino-1,4-lactone; internal standard (I.S.), *p*-(*n*-amyl)benzoic acid. A indicates 2-hydroxy-2-methyllevulinic acid, B indicates adipic acid.

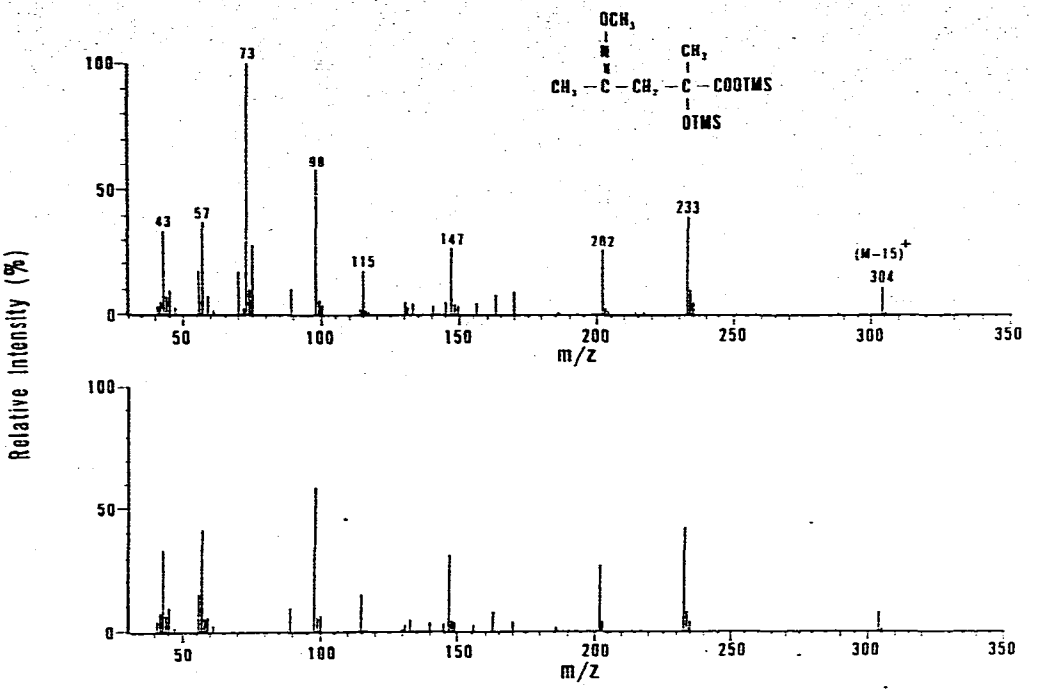

Fig. 2. EI mass spectra of methoxime-trimethylsilylated 2-hydroxy-2-methyllevulinic acid (upper spectrum) and of peak 30 (lower chromatogram) of Fig. 1. Spectra were recorded under the following conditions: ionizing energy 70 eV, ionization current 300 μA, and accelerating voltage 3 kV.

TABLE I

HIGH-RESOLUTION MASS SPECTRAL DATA

m/z	Observed *m/z*	Error (milli mass)	Unsaturation	Probable composition	Fragmentation
304	304.1386	—1.2	2.5	$C_{12}H_{26}N_1O_4Si_2$	M^+—CH_3
233	233.1035	0.5	1.5	$C_9H_{21}O_3Si_2$	[—C(CH_3) (OTMS) COOTMS]$^+$
202	202.1253	—0.7	1.5	$C_9H_{20}N_1O_2Si_1$	M^+—COOTMS
170	170.0814	5.0	3.0	$C_8H_{14}O_2Si_1$	
156	156.0599	—0.5	3.0	$C_7H_{12}O_2Si_1$	

tion of 2-hydroxy-2-methyllevulinic acid in the urine of a diabetic patient with ketoacidosis.

2-Hydroxy-2-methyllevulinic acid was detected in the urine of all the four diabetic patients with ketoacidosis, and also in two serum samples of the four diabetic patients. The compound was not detectable in the urine and the serum of the patients up to day 4 of admission, when they became non-ketotic under insulin therapy. The compound was not detected in the urine and the serum of the other non-ketotic diabetic patients or healthy adults.

2-Hydroxy-2-methyllevulinic acid was also detected in the urine of a 9-year-old boy with ketosis and elevated blood pyruvate and lactate. This find-

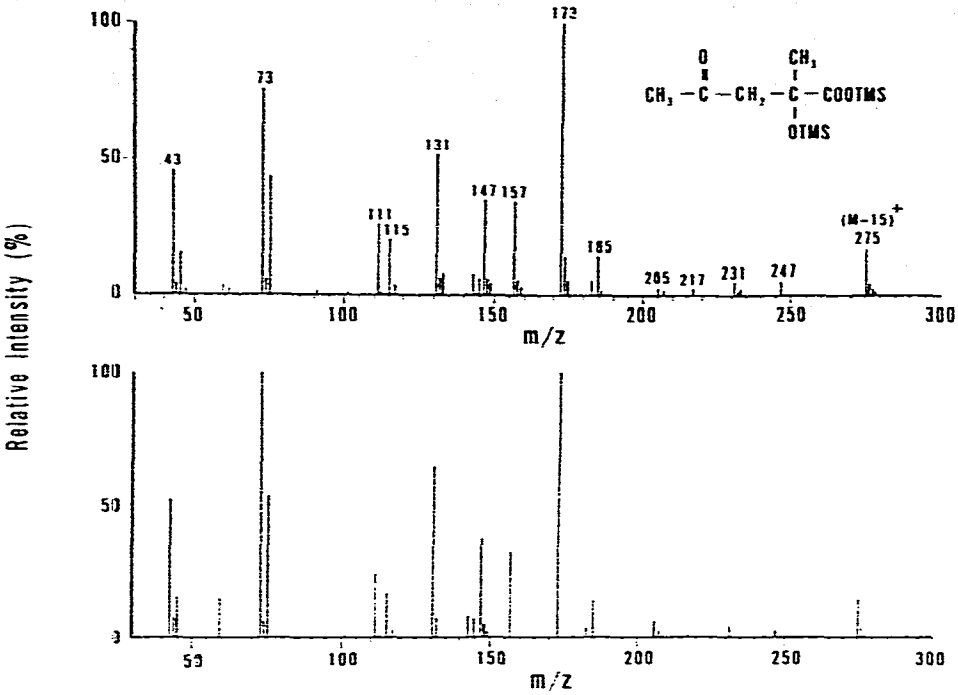

Fig. 3. EI mass spectra of trimethylsilylated 2-hydroxy-2-methyllevulinic acid (upper spectrum) and of a peak (lower spectrum) in the gas chromatogram of the trimethylsilylated ethyl acetate extract from diabetic ketotic urine.

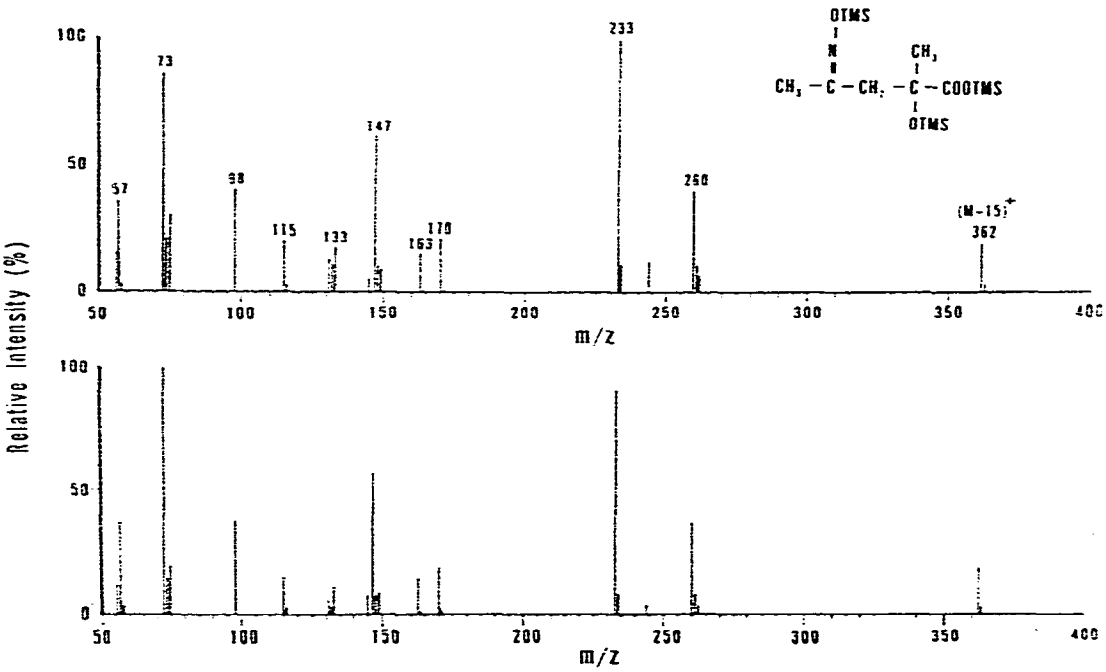

Fig. 4. EI mass spectra of oxime-trimethylsilylated 2-hydroxy-2-methyllevulinic acid (upper spectrum) and of a peak (lower spectrum) in the gas chromatogram of the oxime-trimethylsilylated ethyl acetate extract from diabetic ketotic urine.

ing suggests that the occurrence of the compound is specific not to "diabetic" ketoacidosis but to the ketotic state itself.

DISCUSSION

2-Hydroxy-2-methyllevulinic acid has not been previously reported to be present in physiological fluids. This compound was first detected in the urine and serum of diabetic patients with ketoacidosis. The occurrence of the compound seems to be correlated only to ketosis and not to diabetes, since it was detectable in the urine of a child with non-diabetic ketosis.

The source of 2-hydroxy-2-methyllevulinic acid is not known at present. The compound possibly derives from the condensation of pyruvate and acetone, which is an abnormal metabolite during ketosis. The result presented in this report directs attention to the occurrence in ketosis of a previously unnoticed metabolite, viz., 2-hydroxy-2-methyllevulinic acid. Further studies are in progress to elucidate the metabolic pathways involved in the formation of this compound.

The profile analysis of the organic acids in urine and serum of patients with a metabolic disease using high-resolution GC—MS is very useful to detect and identify new metabolites for clinical examination and investigation of the metabolism.

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