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3-HYDROXYHEXANOIC ACID: AN ABNORMAL METABOLITE IN URINE AND SERUM OF DIABETIC KETOACIDOTIC PATIENTS

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SUMMARY

A new organic acid, 3-hydroxyhexanoic acid, was identified in the urine or serum of five diabetic patients with ketoacidosis. The compound was not detected in the urine and serum of healthy subjects or diabetic patients without ketosis. The compound was also detected in the urine of a non-diabetic ketotic patient with dicarboxylic aciduria, suggesting that the occurrence of the compound is more related to the ketotic state than to "diabetic" ketosis.

INTRODUCTION

Ketoacidosis is a clinical condition caused by the accumulation of increased amounts of ketone bodies, namely 3-hydroxybutyrate, acetoacetate and acetone, in the body fluids. The other acids have been found in large amounts in the urine of ketoacidotic patients. The increased urinary excretion of adipic acid and suberic acid was found in diabetic patients with ketosis [1, 2]. These

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aliphatic dicarboxylic acids are formed through ω-oxidation of free fatty acids followed by β-oxidation. The increased urinary excretion of 3-hydroxyisovaleric acid [3], 3-hydroxyisobutyric acid and 2-methyl-3-hydroxybutyric acid [4] was found in ketoacidosis. The acids are known to be the intermediates of the metabolism of the branched-chain amino acids, leucine, valine and isoleucine, respectively. The increased urinary excretion of these acids is due to the enhanced catabolism of protein in ketoacidosis and to the inhibition of their further metabolic breakdown by the accumulated 3-hydroxybutyric acid and acetoacetic acid [5]. The urinary excretion of 2-hydroxybutyric acid [6, 7] and 2-hydroxyisovaleric acid [8] was increased in patients with lactic acidosis and ketoacidosis. Recently, we found abnormal metabolites, 5-hydroxyhexanoic acid, 3-hydroxyvaleric acid and 2-hydroxy-2-methyllevulinic acid, in the urine of diabetic ketoacidotic patients [9, 10]. In the present study, 3-hydroxyhexanoic acid was demonstrated in the urine and serum of diabetic ketoacidotic patients. To the authors' knowledge this metabolite has never been reported in human physiological fluids to date.

METHOD

Chemicals

3-Hydroxyhexanoic acid was synthesized according to the method of Cornforth et al. [11].

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

Samples

Urine samples were obtained from five patients with diabetic ketoacidosis, five diabetic patients without ketosis, and five healthy subjects. Serum samples were obtained from five patients with diabetic ketoacidosis, five diabetic patients without ketosis and five healthy subjects.

Case 1 with diabetic ketoacidosis showed hyperglycaemia (398 mg/dl), acidosis (pH 7.26, P_{CO_2} 23 mmHg, P_{O_2} 160 mmHg, base excess −16 mequiv./l under O_2 aspiration), lethargy, glycosuria, ketonuria, and dehydration. Case 2 showed hyperglycaemia (650 mg/dl), acidosis (pH 7.12, P_{CO_2} 43 mmHg, P_{O_2} 181 mmHg, base excess −16 mequiv./l under O_2 aspiration), lethargy, glycosuria, ketonuria, and dehydration. Case 3 showed hyperglycaemia (524 mg/dl), excitation, glycosuria and ketonuria. Gas analysis was not performed before insulin treatment. Case 4 showed hyperglycaemia (556 mg/dl), acidosis (pH 7.21, P_{CO_2} 14 mmHg, P_{O_2} 151 mmHg, base excess −19 mequiv./l under O_2 aspiration), lethargy, glycosuria, ketonuria, and dehydration. Case 5 showed hyperglycaemia (1418 mg/dl), acidosis (pH 7.21, P_{CO_2} 25 mmHg, P_{O_2} 80 mmHg, base excess −16 mequiv./l), coma, glycosuria, ketonuria and dehydration.

Urine was obtained from a 55-year-old, non-diabetic patient with ketosis and normoglycaemia. The patient complained of vomiting, vertigo, paraesthesia, and incontinence.

Sample preparation

Serum was filtered through CF-25 cone membrane filter (Amicon, Lexington, MA, U.S.A.). A 1-ml volume of serum ultrafiltrate or urine was acidified to pH 1 by addition of 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 extract was dehydrated over anhydrous sodium sulphate and evaporated with a stream of nitrogen. Methoxylamine hydrochloride (1 mg) in 50 μl of ethyl acetate was added to the extract and allowed to react at 60°C for 30 min. The extract was dried with a nitrogen stream and trimethylsilylated with 20 μl or 40 μl of N,O-bis(trimethylsilyl)trifluoroacetamide for serum samples or for urine samples, respectively. A 3-μl volume of each sample was used for gas chromatography—mass spectrometry (GC—MS).

Gas chromatography—mass spectrometry

A Hewlett-Packard 5710A gas chromatograph combined with a JMS D-300 mass spectrometer (JEOL) was used. The data were processed by a JMA 2000 computer from 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 ionization (EI) mass spectra were recorded at an ionizing energy of 22 eV, an ionization current of 300 μA, and an accelerating voltage of 3 kV.

RESULTS

Fig. 1A shows the gas chromatogram of organic acids in the urine of the diabetic patient with ketoacidosis (case 4). Each component of the gas chromatogram was identified by EI and chemical ionization (CI) mass spectra. The EI mass spectrum of peak 19 is shown in Fig. 2B. The CI mass spectrum showed that the molecular ion of the compound was 276. The ion at *m/z* 261 is due to $[M-CH_3]^+$. The ion at *m/z* 233 is due to $[M-CH_3CH_2CH_2]^+$. The ion at *m/z* 219, $[M-57]^+$, is derived from the loss of CH_3 and CH_2CO, suggesting the structure of 3-hydroxycarboxylic acid [12]. The ion at *m/z* 204 is due to $[M-CH_3CH_2CH_2-CHO]^+$. The intense ion at *m/z* 145 is derived from the loss of CH_2COO-TMS, also suggesting the structure of 3-hydroxycarboxylic acid. Since the TMS (trimethylsilyl) derivative of synthesized 3-hydroxyhexanoic acid and peak 19 in Fig. 1 showed identical retention times on the gas chromatograms (Fig. 1) and identical EI mass spectra (Fig. 2), peak 19 was identified as 3-hydroxyhexanoic acid. 3-Hydroxyhexanoic acid was detected in two urine samples out of five diabetic ketoacidotic patients. The acid was not detected in the urine of five healthy subjects nor in the urine of five diabetic patients without ketosis. In diabetic ketoacidotic patients, the urinary excretion of lactic acid, acetoacetic acid, 2-hydroxybutyric acid, 3-hydroxybutyric acid, 2-hydroxyisovaleric acid, 2-methyl-3-hydroxybutyric acid, 3-hydroxyisovaleric acid, 3-hydroxyvaleric acid, 5-hydroxyhexanoic acid, 2-hydroxy-2-methyllevulinic acid and adipic acid was found to be increased.

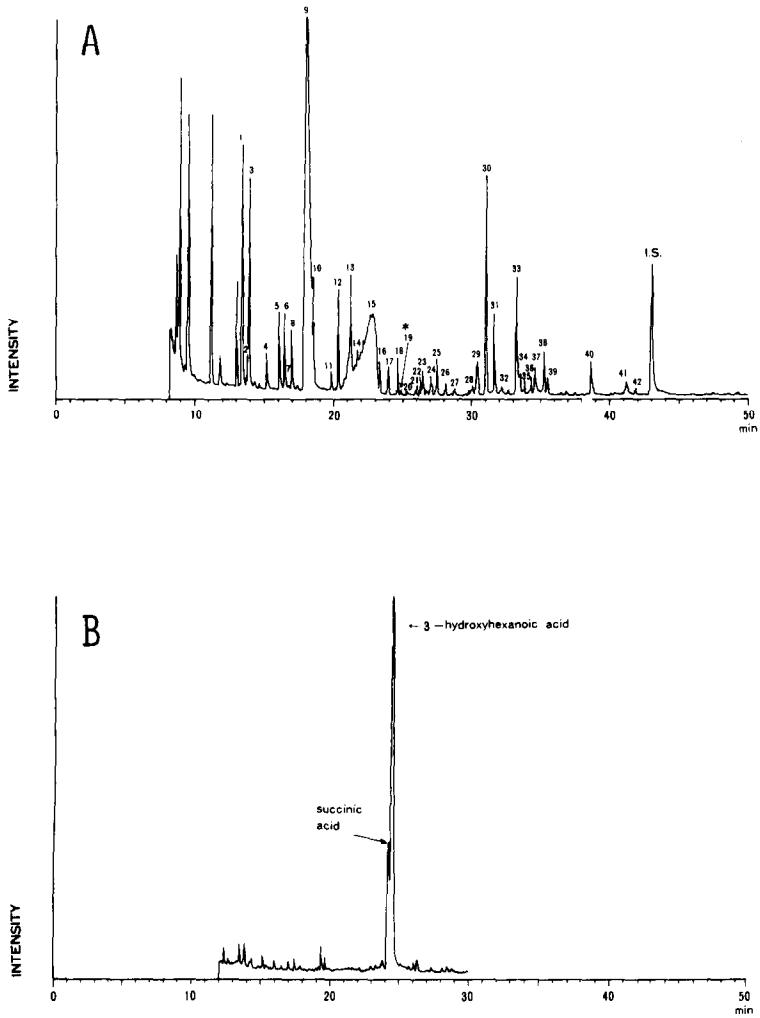

Fig. 1. Gas chromatograms of the methoxime-trimethylsilylated ethyl acetate extract from urine of a diabetic ketoacidotic patient (A) and of trimethylsilylated 3-hydroxyhexanoic acid (B). Peak identification: 1, lactic acid; 2, 2-hydroxyisobutyric acid; 3, glycolic acid; 4, 5, acetoacetic acid; 6, 2-hydroxybutyric 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; 19, 3-hydroxyhexanoic acid; 22, glyceric acid; 23, fumaric acid; 24, 5-hydroxyhexanoic acid; 25, 4-deoxythreonic acid; 28, 3-deoxytetronic acid; 29, 2-hydroxy-2-methyllevulinic acid; 30, 2-deoxytetronic acid; 33, adipic acid; 37, 3-methyladipic acid; 38, 2,3-dideoxypentonic acid; 40, 4-hydroxyphenylacetic acid; 42, isosaccharinolactone.

Fig. 3 shows the gas chromatogram of organic acids in the serum of the diabetic patient with ketoacidosis (case 4). 3-Hydroxyhexanoic acid was also detected in the diabetic ketoacidotic serum. 3-Hydroxyhexanoic acid was detected in four out of five diabetic ketoacidotic patients' sera. The acid was not detected in the sera of five healthy subjects nor in the sera of five diabetic patients with no ketosis. In the diabetic ketoacidotic patients the serum

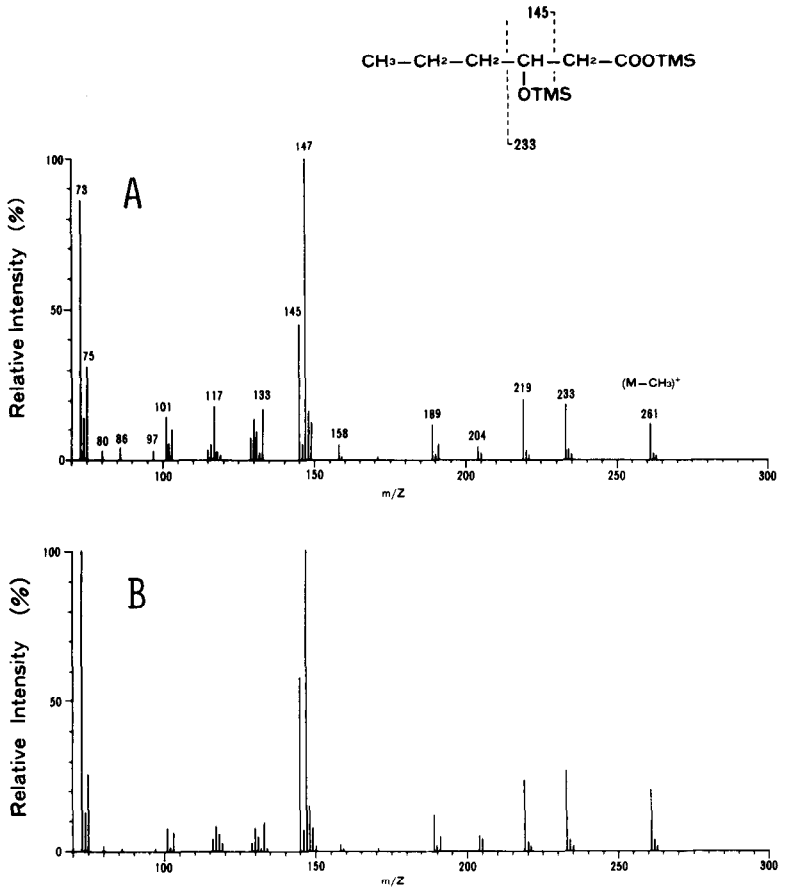

Fig. 2. EI mass spectra of trimethylsilylated 3-hydroxyhexanoic acid (A) and of peak 19 (B) in Fig. 1.

concentrations of lactic acid, 2-hydroxybutyric acid, 3-hydroxybutyric acid, 2-methyl-3-hydroxybutyric acid, 3-hydroxyisovaleric acid, 3-hydroxyvaleric acid, and 2-hydroxy-2-methyllevulinic acid were elevated.

Table I shows the concentrations of 3-hydroxyhexanoic acid in the urine and serum of diabetic ketoacidotic patients. In urine of two diabetic ketoacidotic

TABLE I

CONCENTRATION OF 3-HYDROXYHEXANOIC ACID IN URINE AND SERUM OF DIABETIC KETOACIDOTIC PATIENTS

	Diabetic ketoacidosis					Non-ketotic diabetes (n = 5)	Healthy subjects (n = 5)
	1	2	3	4	5		
Urine (μg/mg creatinine)	0.43	ND*	ND	1.81	ND	ND	ND
Serum (μg/ml)	ND	0.25	1.42	0.95	0.25	ND	ND

*Not detected.

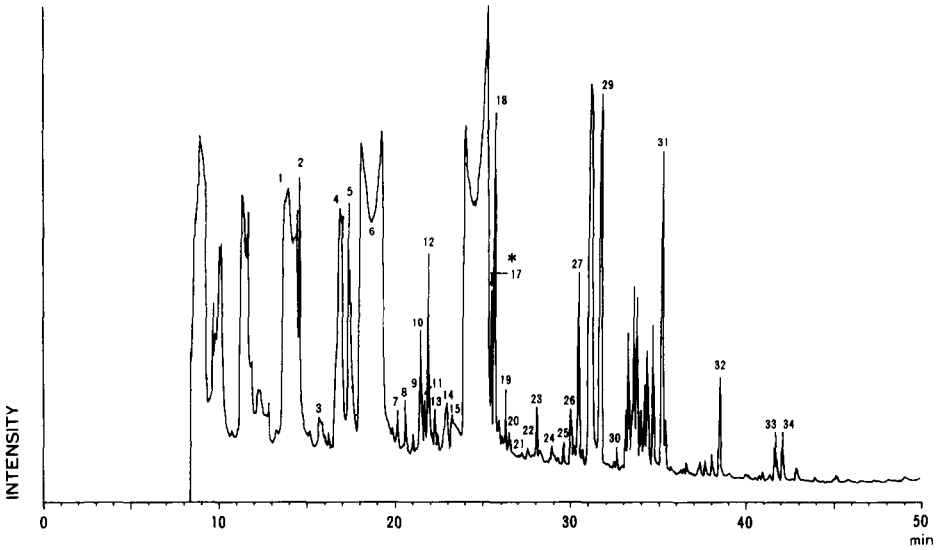

Fig. 3. Gas chromatogram of methoxime-trimethylsilylated organic acids in serum of a diabetic ketoacidotic patient. The peaks were identified as follows. 1, lactic acid; 2, glycolic acid; 4, 2-hydroxybutyric acid; 5, 3-hydroxypropionic acid; 6, 3-hydroxybutyric acid; 7, 2-methyl-3-hydroxybutyric acid; 8, 3-hydroxyisovaleric acid; 9, 2-ethylhydracrylic acid; 11, 3-hydroxyvaleric acid; 14, urea; 17, 3-hydroxyhexanoic acid; 20, glyceric acid; 21, 4-deoxyerythronic acid; 22, 4-deoxythreonic acid; 26, 3-deoxytetronic acid; 27, 2-hydroxy-2-methyllevulinic acid; 28, 2-deoxytetronic acid; 31, 2,3-dideoxypentonic acid; 33, isosaccharinolactone.

patients, 3-hydroxyhexanoic acid was detected at concentrations of 0.43 and 1.81 μg/mg creatinine. In sera of four diabetic patients, 3-hydroxyhexanoic acid was detected at concentrations of 0.25, 1.42, 0.95 and 0.25 μg/ml.

3-Hydroxyhexanoic acid was detected in the urine of a non-diabetic ketotic patient with dicarboxylic aciduria. The compound became undetectable in the patient's urine when ketosis and subjective complaints disappeared.

DISCUSSION

3-Hydroxyhexanoic acid has not been reported to be present in physiological fluids so far. This acid was first detected in the urine and serum of diabetic patients with ketoacidosis. The occurrence of the acid seemed to be correlated to ketosis, since the acid was detected in the urine of a non-diabetic patient with dicarboxylic aciduria and ketosis.

The formation of the compound seems to be closely related to the enhanced β-oxidation of free fatty acids. A possible source of 3-hydroxyhexanoic acid is an intermediate of the enhanced β-oxidation in mitochondria due to the impaired sequence of β-oxidation pathway, which is caused by the relative deficiency of coenzyme A and NAD^+ in mitochondria. Another possible source of the acid is an intermediate of β-oxidation in peroxisome due to the overwhelming supply of free fatty acids for the capacity of β-oxidation in mitochondria, or due to the impaired transport of acyl-coenzyme A into mitochondria, which may be caused by the relative deficiency of carnitine.

Although the occurrence of some higher-molecular-weight 3-hydroxycarboxylic acids (C_8, C_9, C_{10} and C_{12}) in plasma was reported by Pfordt and Spiteller [13], we could not identify these acids except the possible detection of 3-hydroxyoctanoic acid by monitoring its characteristic fragment ions at *m/z* 289 $[M-CH_3]^+$, 247 $[M-CH_3-CH_2CO]^+$, 233 $[M-C_5H_{11}]^+$, and 173 $[M-CH_2COO\text{-}TMS]^+$. A peak suggestive of 3-hydroxyoctanoic acid was detected in four diabetic ketoacidotic sera (cases 2, 3, 4, and 5). It is possible that both acids have a common pathway of formation.

Further study is needed to clarify the metabolic pathway involved in the formation of the compounds and the clinical significance of the metabolites' appearance in body fluids.

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