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GAS CHROMATOGRAPHIC DETERMINATION OF OXO- AND HYDROXYCARBOXYLIC ACIDS IN SERUM AND URINE OF DIABETIC AND NORMAL SUBJECTS

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SUMMARY

Oxo- and hydroxycarboxylic acids in serum and urine are metabolites of valine, leucine and isoleucine and products of ketogenesis. They are simultaneously determined in the form of their methyl esters and methyl esters–O-methyloximes by gas chromatography, using internal and external standards. Normal values for the urinary excretion of these amino acid metabolites are between 3 ± 2 μmole per 24 h (mean ± standard deviation) for 2-oxoisocaproic acid and 122 ± 58 μmole per 24 h for 3-hydroxyisobutyric acid. In diabetic ketoacidosis the values are increased by a factor of 2–10. In the urine of diabetic patients under fasting conditions all metabolites are elevated and reach a maximum on about the seventh day of fasting. In the serum only 2-oxoisocaproic acid, 3-oxobutyric acid and 3-hydroxybutyric acid reach elevated levels under fasting conditions.

INTRODUCTION

Oxo- and hydroxycarboxylic acids are metabolites of the branched-chain amino acids valine, leucine and isoleucine or products of ketogenesis. In the context of increased amino acid metabolism, their levels in blood serum and urine are elevated during diabetic ketoacidosis. Quantitative data on normal ranges and concentrations in different pathophysiological situations have been incomplete. Enzymatic and especially gas chromatographic (GC)[1–4] and high-performance liquid chromatographic[5,6] procedures have been applied to the determination of some of the metabolites. Oxo- and hydroxycarboxylic acids in diabetes and inborn errors of metabolism have been reviewed[7,8]. In this study, a method has been developed for the simultaneous determination of 2-oxo, 3-oxo, 2-hydroxy- and 3-hydroxycarboxylic acids, representing different pathways of amino acid metabolism and ketogenesis.

EXPERIMENTAL

Samples

Urine samples (24 h) were collected from fifteen healthy individuals (23–63 years old) and fifteen diabetic patients with increased excretion of ketone bodies

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(10–80 years old). From two non-insulin-dependent patients who were fasting for 8 days, 10-ml venous blood samples were drawn on the 2nd, 3rd, 4th, 7th and 8th days. To obtain the serum, the samples were centrifuged at 2000 *g* for 10 min. In addition, 24-h urine samples were taken from four non-insulin-dependent patients, fasting for 8 days, on the 1st, 3rd, 4th, 7th and 8th days. All serum and urine samples were stored at –20°C prior to analysis.

Sample preparation for serum specimens

To 5 ml of serum were added 30 µl of an internal standard solution containing 25 mg of 2-oxocaproic acid, 24 mg of 4-oxobutyric acid, 25 mg of 2-hydroxyvaleric acid and 30 mg of 4-hydroxybutyric acid in 10 ml of distilled water. After mixing the sample with 10 ml of 2-propanol to precipitate the serum proteins, 25 mg of O-methylhydroxylamine hydrochloride were added. The sample was kept at 65°C for 1 h to convert the oxocarboxylic acids into their O-methyloximes. The organic acids were isolated by anion-exchange chromatography on Amberlyst A-26 (Serva, Heidelberg, F.R.G.) and derivatized with diazomethane to form the methyl esters. The methyl esters were prefractionated into four fractions by preparative thin-layer chromatography (TLC), and the fractions were analysed by GC. The details of the method have been described previously[9]. The four fractions were located on the TLC plate by using methyl indolebutyrate as reference substance. With 1.0 as the relative retention value (R_{rel}) for methyl indolebutyrate and 0 for the starting point of the TLC separation, the fractions corresponded to the following zones: fraction 1, R_{rel} = 1.3–1.7; fraction 2, R_{rel} = 1.0–1.3; fraction 3, R_{rel} = 0.7–1.0; and fraction 4, R_{rel} = 0.35–0.7.

Sample preparation for urine specimens

To 30 ml of urine were added 100 µl of the internal standard solution. After mixing the sample with 60 ml of 2-propanol to precipitate any urinary proteins, 150 mg of O-methylhydroxylamine hydrochloride were added and the mixture was processed as described for serum.

Gas chromatographic separation

The GC separations were performed on a Vega 6130 gas chromatograph with a flame ionization detector (Carlo Erba, Hofheim, F.R.G.) under the following conditions: 25 m × 0.25 mm I.D. fused-silica column, coated with OV-1701 (Macherey, Nagel & Co., Düren, F.R.G.); carrier gas, nitrogen at a flow-rate of 4 ml/min; column temperature, 40°C for 10 min, programmed at 2°C/min to 160°C and then at 6°C/min to 230°C, finally held at 230°C for 30 min; injection block temperature, 230°C; sample size, 1 µl at a splitting ratio of 1:10. The gas chromatograph was connected with a CDS-111 integrator (Varian, Darmstadt, F.R.G.).

Identification

The identifications of the oxo- and hydroxycarboxylic acids were based on their methylene units and previous characterizations by GC–mass spectrometry[10]. Mass spectrometric controls were also made when the identification based on methylene units was uncertain. To avoid peak overlapping, samples and hydrocarbon standards (C_5–C_{16}) were analysed separately and in triplicate for the determination of the methylene units.

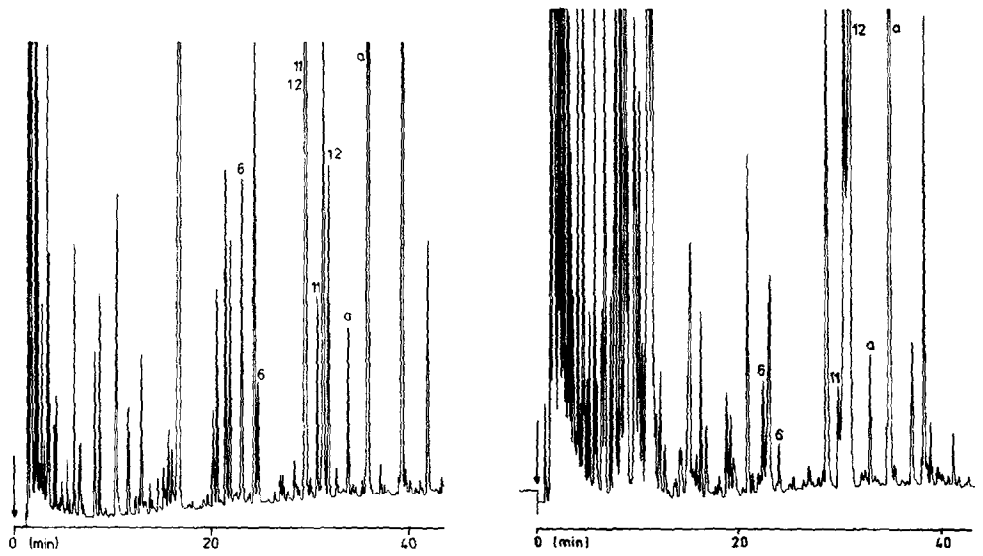

Fig. 1. Gas chromatograms of fraction I (early parts) of the organic acids in the urine of diabetic patient A with ketoacidosis (left) and in the serum of diabetic patient B under fasting conditions, 7th day (right). Peaks: 6 = 2-oxoisovaleric acid; 11 = 2-oxo-3-methylvaleric acid; 12 = 2-oxoisocaproic acid; a = internal standard, 2-oxocaproic acid.

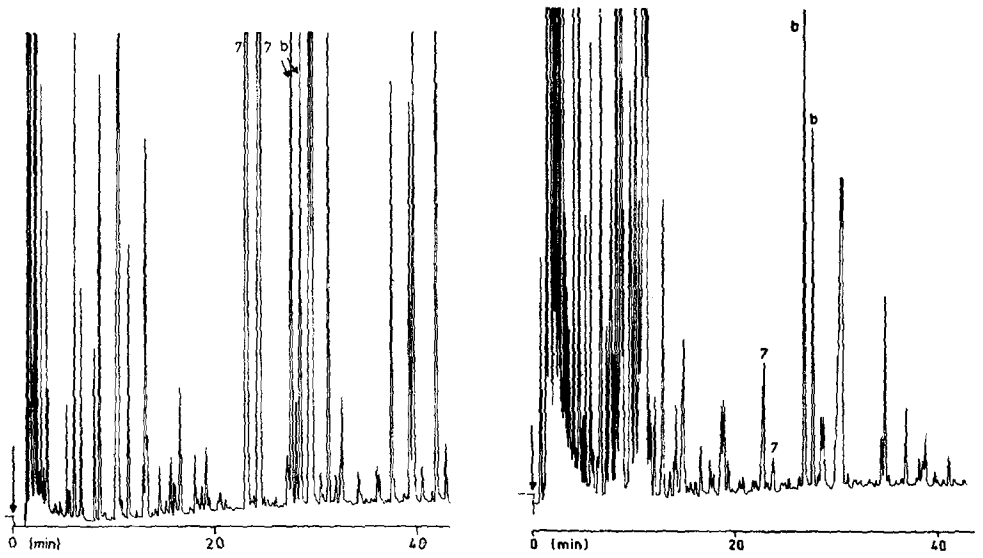

Fig. 2. Gas chromatograms of fraction 2 of the urine sample from patient A (left) and the serum sample from patient B (right). Peaks: 7 = 3-oxobutyric acid; b = internal standard, 4-oxobutyric acid.

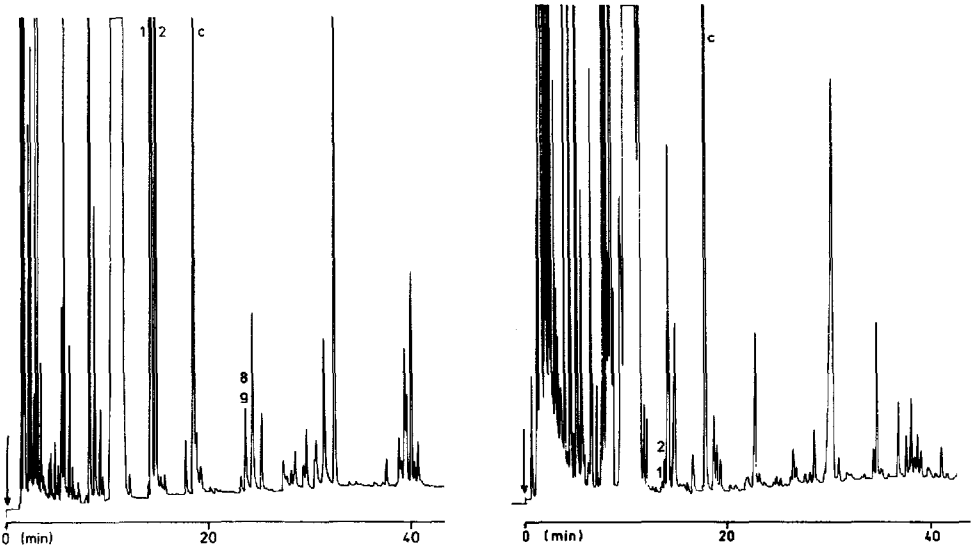

Fig. 3. Gas chromatograms of fraction 3 of the urine sample from patient A (left) and the serum sample from patient B (right). Peaks: 1 = 3-hydroxyisovaleric acid; 2 = 2-hydroxyisovaleric acid; 8 = 2-hydroxy-3-methylvaleric acid; 9 = 2-hydroxyisocaproic acid; c = internal standard, 2-hydroxyvaleric acid.

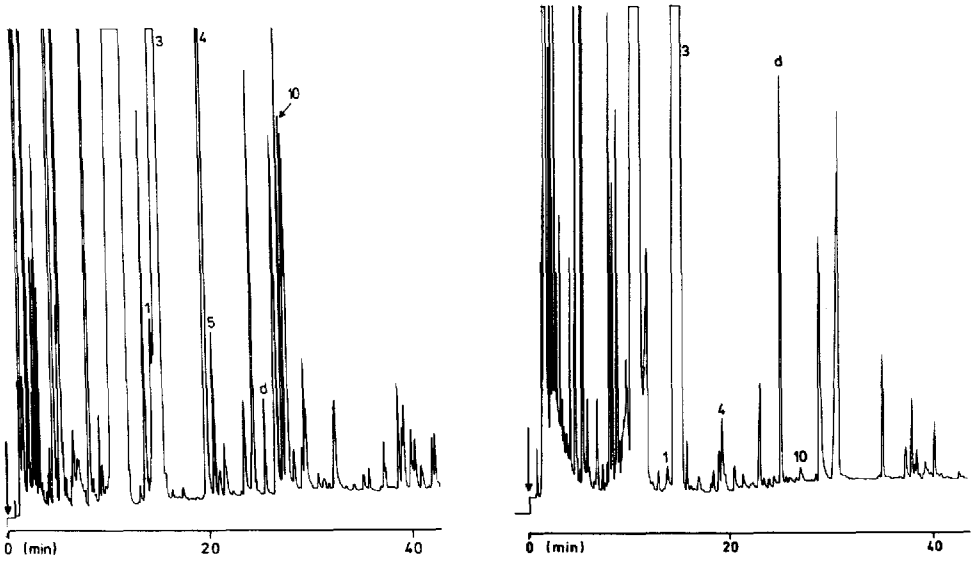

Fig. 4. Gas chromatograms of fraction 4 of the urine sample from patient A (left) and the serum sample from patient B (right). Peaks: 1 = 3-hydroxyisovaleric acid; 3 = 3-hydroxybutyric acid; 4 = 3-hydroxyisobutyric acid; 5 = 3-hydroxy-2-methylbutyric acid; 10 = 3-hydroxy-2-ethylpropionic acid; d = internal standard, 4-hydroxybutyric acid.

Quantitation

Quantitation was based on the ratio of the peak area of the acid to be determined to that of the internal standard and on calibration graphs obtained with four external standard solutions. The standards were prepared by spiking a serum sample with 60, 120, 180 and 240 μg and a urine sample with 120, 240, 360 and 480 μg of each of the reference acids.

RESULTS AND DISCUSSION

Prefractionation

The advantage of prefractionation by TLC, even though it lengthens the sample preparation procedure, is that it helps to avoid interferences in the GC separation and to enrich components present at low concentrations in the biological samples. In the total profile of organic acids, a number of such interferences are observed, which render the quantitation of several low-concentrated oxo- and hydroxycarboxylic acids difficult, especially in pathological samples. For example, after prefractionation, 2-hydroxyisovaleric acid can be measured in samples with large amounts of 3-hydroxybutyric acid and 2-oxoisovaleric acid can be determined in the presence of 3-oxobutyric acid.

With the exception of 3-hydroxyisovaleric acid, which occurs in fractions 3 and 4, all components to be quantitated appear in only one fraction. The distribution of the acids in the four fractions is shown in Figs. 1–4. The oxocarboxylic acids appear in fractions 1 and 2 and the more polar hydroxycarboxylic acids in fractions 3 and 4.

TABLE I

MEAN CONCENTRATIONS OF OXOCARBOXYLIC ACIDS AND HYDROXYCARBOXYLIC ACIDS IN THE URINE OF 15 NORMAL INDIVIDUALS AND 15 DIABETIC PATIENTS WITH KETOACIDOSIS

Compound	*Mean concentration ± S.D. (μmole per 24 h)*	
	Normal individuals	*Diabetics with ketoacidosis*
Valine metabolites:		
2-Oxoisovaleric acid	13 ± 9	35 ± 29
2-Hydroxyisovaleric acid	7 ± 7	252 ± 593
3-Hydroxyisobutyric acid	122 ± 58	691 ± 687
Leucine metabolites:		
2-Oxoisocaproic acid	3 ± 2	16 ± 10
3-Hydroxyisovaleric acid	48 ± 20	101 ± 62
Isoleucine metabolites:		
2-Oxo-3-methylvaleric acid	22 ± 13	48 ± 24
3-Hydroxy-2-methylbutyric acid	13 ± 7	40 ± 30
3-Hydroxy-2-ethylpropionic acid	64 ± 32	258 ± 167
Ketone bodies:		
3-Oxobutyric acid	156 ± 128	6800 ± 5900
3-Hydroxybutyric acid	15 ± 19	2800 ± 2300

The acids to be determined (numbered in Figs. 1–4) are sufficiently well separated from the other organic acids.

Internal standards

The reference substances 2-oxocaproic acid (fraction 1), 4-oxobutyric acid (fraction 2), 2-hydroxyvaleric acid (fraction 3) and 4-hydroxybutyric acid (fraction 4) were chosen as internal standards from among many others tested because they appear in only one fraction, because they are isomers or homologues to the substances to be determined and have similar retention behaviours, because they fit into nearly empty spaces in the complex chromatograms and because they do not occur or occur only in negligible amounts as endogenous compounds, as shown by performing analyses in the absence of the internal standards. This holds true for both the urine and the serum samples (Figs. 1–4).

Separation and quantitation

Except for 2-hydroxy-3-methylvaleric acid and 2-hydroxyisocaproic acid (compounds 8 and 9 in Fig. 3), all hydroxycarboxylic acids are separated. In some instances no baseline separation is achieved. The O-methyloximes of the oxocarboxylic acid methyl esters occur in form of *syn/anti* isomers and are separated into two peaks; for quantitation the peak areas are added. With 2-oxo-3-methylvaleric acid and 2-oxoisocaproic acid, the peaks of the isomeric pairs overlap (Fig. 1). Because it has been found that under the conditions used for sample preparation the ratios of the first and second peaks are relatively constant (2-oxo-3-methylvaleric acid, 10.1 ± 2.2; 2-oxoisocaproic acid, 0.3 ± 0.04), quantitation is based on the two second peaks only.

Working with spiked urine samples, the recoveries for the entire procedure were determined to range between 31% (3-hydroxy-2-methylbutyric acid) and 55% (2-oxoisovaleric acid). On the basis of aqueous solutions with known concentrations (360 and 720 μg/dl) of the reference substances, the accuracy of the method was found to be within ±7–10% (difference from the reference values).

The advantage of the method described over other techniques is that a wide range of metabolites of branched-chain amino acids and the ketone bodies under normal and pathological conditions can be determined simultaneously. The disadvantage is that the procedure is time consuming.

Urinary excretion in normal individuals and diabetic patients with ketoacidosis

For the 2-oxocarboxylic acids, as the primary metabolites of valine, leucine and isoleucine[9], the urinary levels in normal individuals are on average between 3 and 22 μmole per 24 h (Table I). The values for their reduction products are lower by a factor of 2 (*e.g.*, 2-hydroxyisovaleric acid, $\bar{x}$ = 7 μmole per 24 h). The main degradation products are the 3-hydroxy metabolites with mean excretion values of up to 122 μmole per 24 h. During diabetic ketoacidosis, there is not only a large increase in the ketone bodies, 3-oxobutyric acid and 3-hydroxybutyric acid in the urine, but also the urinary excretion of all 2-oxo, 2-hydroxy and 3-hydroxy metabolites of the branched-chain amino acids is elevated 2–5-fold. For 2-hydroxyisovaleric acid, the increase is even higher. The individual variation for this metabolite is very large. Among the 3-hydroxy compounds, 3-hydroxyisobutyric acid reaches the highest level ($\bar{x}$ = 691

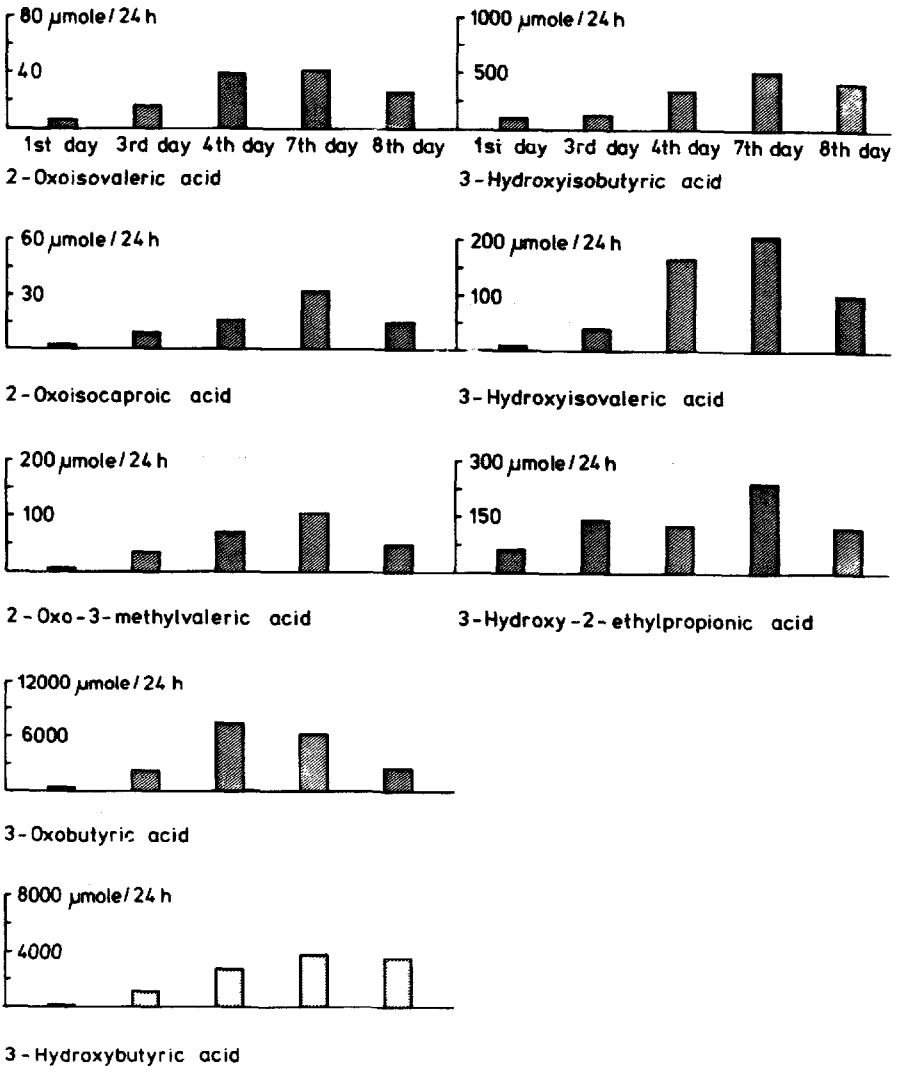

Fig. 5. Oxo- and hydroxycarboxylic acids in the urine of diabetic patients during an 8-day fast period (mean values for four patients).

µmole per 24 h), and as a single substance appears to be the most suitable marker for the amino acid degradation.

Diabetic patients under fasting conditions

In the urine of non-insulin-dependent diabetic patients under fasting conditions, all metabolites of the branched-chain amino acids increase together with ketone bodies (Fig. 5). The maximum is reached on about the 7th day, after which a decrease is generally observed.

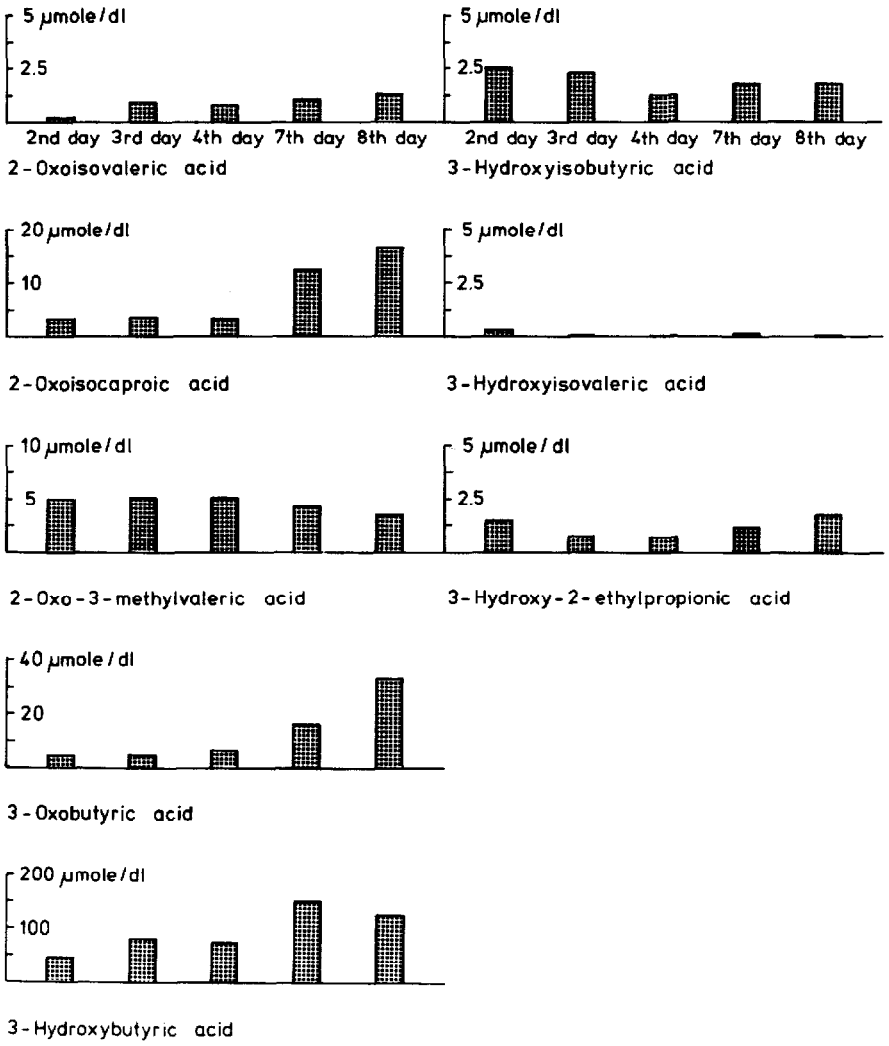

Fig. 6. Oxo- and hydroxycarboxylic acids in the serum of diabetic patients during an 8-day fast period (mean values for two patients).

In the serum an increase is observed only for 2-oxoisocaproic acid and the ketone bodies, 3-oxobutyric acid and 3-hydroxybutyric acid (Fig. 6).

CONCLUSIONS

With the method described, the oxo and hydroxy metabolites of branched-chain amino acids and of the ketone bodies are determined simultaneously and reliably in the normal ranges. The increased amino acid metabolism in conjunction with diabetic ketoacidosis under fasting conditions can be followed in both serum and urine on the basis of several parameters.

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