Journal of Chromatography, 217 (1981) 255–262
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,971

ANALYSIS OF THE OXOCARBOXYLIC ACID FRACTION IN SERUM AND URINE AS O-METHYLOXIMES BY THERMIONIC SPECIFIC DETECTION

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SUMMARY

After O-methyloximation of the carbonyl functions, the organic acids in serum and urine are extracted by anion-exchange chromatography, transformed into the methyl esters and pre-fractionated by thin-layer chromatography. In one of the four fractions and on the basis of the nitrogen in the O-methyloxime esters, the profiles of the oxocarboxylic acids are analyzed by gas chromatography with thermionic specific detection. The method has good specificity for the oxocarboxylic acids and is suitable for comparative studies.

During diabetic or fasting ketoacidosis the serum concentrations of 3-oxobutyric acid and of the amino acid metabolites 2-oxobutyric, 2-oxoisovaleric and especially 2-oxo-3-methylvaleric and 2-oxoisocaproic acid are increased. In urine mainly 3-oxobutyric acid and only small amounts of the 2-oxocarboxylic acids are excreted.

INTRODUCTION

Aliphatic oxocarboxylic acids can be used as markers for acidotic conditions caused by decompensated fatty acid and amino acid metabolism. Whereas 3-oxobutyric acid is formed by ketogenesis in conjunction with increased fatty acid oxidation, the 2-oxocarboxylic acids originate from transamination of amino acids. It has been shown that the methyl-branched amino acids (*i.e.*, valine, leucine and isoleucine) as well as 2-aminobutyric acid are elevated in case of diabetes mellitus[1,2] especially during ketoacidosis[3,4].

A number of derivatives has been chosen by different authors for the gas chromatographic (GC) analysis of oxocarboxylic acids. Using synthetic compounds, the GC behaviour of oxime trimethylsilyl esters and of O-methyl-, O-ethyl- and O-benzyloxime trimethylsilyl esters was compared[5]. For the analysis of the acids in serum and urine of patients with maple sirup urine disease, the oxime trimethylsilyl ester derivatives were used[6,7]. In the investigation of the same metabolic defect, as well as other disorders, O-trimethylsilylquinoxalinols were introduced as derivatives[8].

In this study the oxocarboxylic acids in serum and urine of patients with ketoacidosis were detected on the basis of the nitrogen in the O-methyloxime methyl ester derivatives by thermionic specific detection. The method was devised as part of a comprehensive procedure for the different classes of organic acids in serum and urine.

0021-9673/81/0000–0000/$02.50

EXPERIMENTAL

Samples

Serum and urine samples were collected from normals, from patients with controlled diabetes mellitus, and patients with diabetes under ketoacidosis, from individuals under fasting conditions and from uremic patients who had to undergo hemodialysis. Serum was obtained from venous blood by centrifugation. Urine was collected either for 12 h or for 24 h.

Deproteinization and O-methyloximation

To 10 ml of serum or urine, 20 ml of isopropanol were added, thoroughly shaken for 2 min and centrifuged at 1650 *g* for 15 min. The supernatant was transferred by means of a pipette to 50 mg of O-methyl hydroxylamine hydrochloride. The mixture was kept at 65°C for 1 h and centrifuged to obtain a clear solution.

Extraction of the organic acids

The organic acids were extracted by anion-exchange chromatography using Amberlyst A-26 (Serva, Heidelberg, G.F.R.) in 25-cm long columns with a bed volume of 30 ml. The columns were regenerated by applying 100 ml of 1 *M* hydrochloric acid, washed until neutral with distilled water, the resin transformed into the OH^- form with 100 ml of 1 *M* sodium hydroxide solution, again washed until neutral with distilled water, and equilibrated with 100 ml of isopropanol–water (2:1). The deproteinized and O-methyloximated solution was passed through the anion-exchange column rate of 2 ml/min. Then the column was washed with 100 ml of distilled water, 70 ml of methanol and 70 ml of diethyl ether. The organic acids were eluted with 100 ml of formic acid–methanol (4:96) and 100 ml of formic acid–diethyl ether (4:96). The volume of the eluate was reduced to 0.2 ml on a rotary evaporator at 40°C.

Formation of the methyl esters

The concentrated eluate was diluted with 3 ml of methanol. Then a solution of diazomethane in diethyl ether was added dropwise until no further formation of foam and bubbles was observed. The solution was kept at room temperature for 12 h before it was evaporated to dryness under a stream of nitrogen. The residue was redissolved in chloroform–methanol (2:1).

Thin-layer chromatography

The mixture of the methyl esters was separated into four fractions using 20 × 20 cm thin-layer plates (Kieselgel 60 F_{254}; Merck, Darmstadt, G.F.R.). The sample was applied to the left side of the plate as a band 13 cm in length. On the right side, a mixture of the reference substances (*i.e.*, methyl stearate, methyl phenylacetate, methyl indolbutyrate and dimethyl tartrate) was applied. The plate was developed with two solvent mixtures in succession. The first solvent mixture (*n*-heptane–diethyl ether–chloroform, 40:30:30) was allowed to run to the upper edge of the plate. After marking the position of methyl indolbutyrate under a UV lamp (254 nm) the plate was developed with the second solvent mixture (cyclohexane–chloroform–methanol,

40:55:5) up to the marked position of methyl indolbutyrate. While the left side of the plate was covered, the right side was treated with a 2,7-dichlorofluorescein spray in order to make the reference substances visible at 366 nm.

With the aid of the reference substances, the left side of the thin-layer plate was divided into four zones: fraction 1, zone between methyl stearate and methyl phenylacetate; fraction 2, zone between methyl phenylacetate and methyl indolbutyrate; fraction 3, zone between methyl indolbutyrate and dimethyl tartrate; and fraction 4, zone between dimethyl tartrate and starting line. The four fractions were scraped off separately and extracted from the silica gel with dichloromethane continuously over 12 h in a Soxhlet extractor. The dichloromethane was evaporated under a stream of nitrogen, the residue redissolved in ethyl acetate and concentrated to a volume of 50 μl.

Gas chromatographic analysis

Fraction 2 was used for the analysis of the oxocarboxylic acids. The separation was performed on a Model 3700 gas chromatograph with a thermionic specific detector (Varian, Darmstadt, G.F.R.) under the following conditions: glass capillary column coated with UCON 75 H 90000, 25 m (WGA, Griesheim, G.F.R.); carrier gas, nitrogen at 5 ml/min; column temperature, 40°C for 10 min, then programmed at 2°C/min; injector block temperature, 250°C; sample size, 1 μl at a splitting ratio of 1:10.

RESULTS AND DISCUSSION

Gas chromatographic separation

Depending on the steric conditions the O-methyloximes of the oxocarboxylic acid methyl esters may form *syn–anti* isomers. Whereas the derivatives of the unbranched 2-oxocarboxylic acids (pyruvic and 2-oxobutyric acids) show single peaks in the gas chromatogram (peaks 1 and 3 in Fig. 1), the branched acids (*i.e.*, 2-oxoisovaleric, 2-oxo-3-methylvaleric and 2-oxoisocaproic acids) give rise to pairs of peaks (peaks 2, 5 and 6 in Fig. 1). *Syn–anti* pairs are also observed in case of 3-oxobutyric acid and 4-oxovaleric acid (peaks 4 and 7 in Fig. 1) with the carbonyl groups not adjacent to the carboxyl groups.

Single peaks and peak doublets are satisfactorily separated on the UCON phase, except that the second peak of the 3-oxobutyric acid derivative and the first peak of the 2-oxo-3-methylvaleric acid derivative coincide. However, the presence and the ratio of these two acids can be estimated from the peaks of the other two pairs.

Profiles of oxocarboxylic acids

Figs. 2–9 give representative examples of the patterns of oxocarboxylic acids in serum and in urine of normal individuals and of patients with different rates of fatty acid and amino acid metabolisms.

The profiles of oxocarboxylic acids in serum of normal individuals are characterized mainly by pyruvic acid and 2-oxoisocaproic acid (Fig. 2). The concentrations of the other oxocarboxylic acids are low. In urine only pyruvic acid is excreted in considerable but varying amounts (Fig. 3).

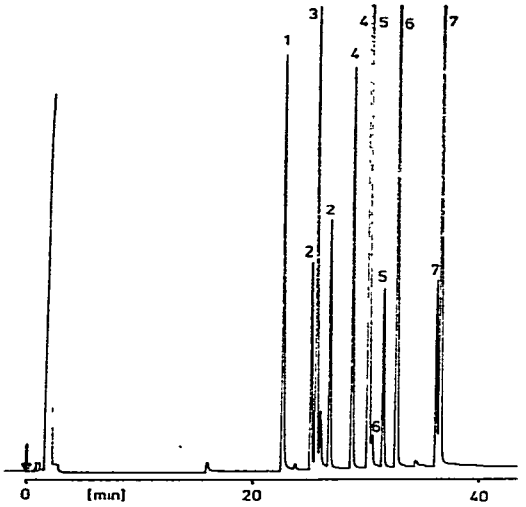

Fig. 1. Gas chromatography–thermionic specific detection of the O-methyloxime methyl esters of a reference mixture of oxocarboxylic acids. Peaks: 1 = pyruvic acid; 2 = oxoisovaleric acid; 3 = 2-oxobutyric acid; 4 = 3-oxobutyric acid; 5 = 2-oxo-3-methylvaleric acid; 6 = 2-oxoisocaproic acid; 7 = 4-oxovaleric acid.

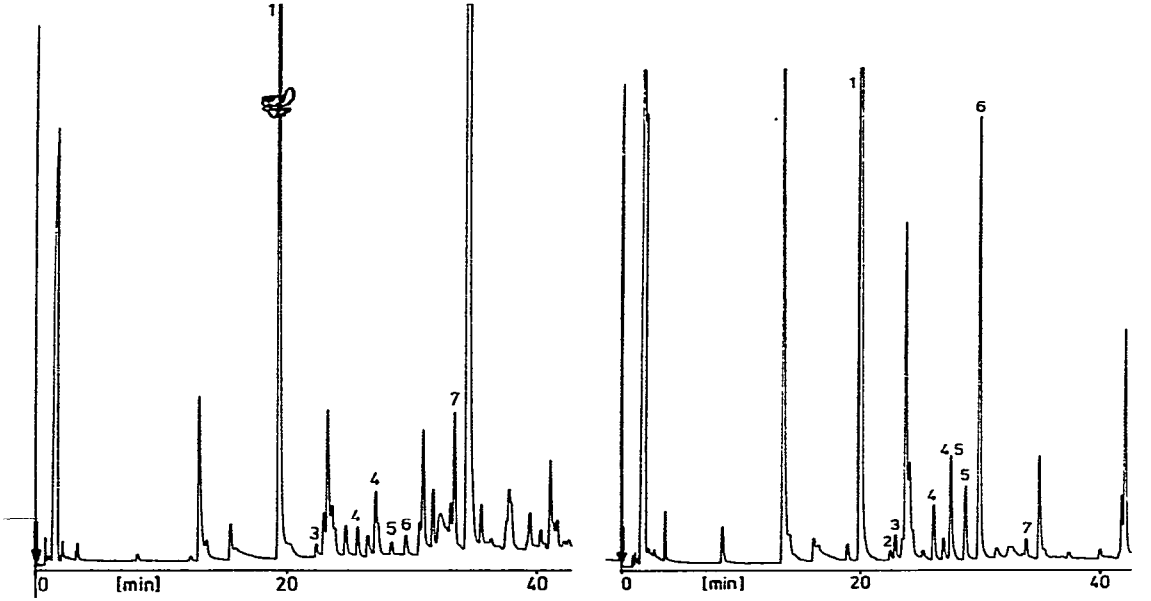

Fig. 2. Gas chromatography–thermionic specific detection of the oxocarboxylic acid derivatives from serum of a normal individual. Peak numbers as in Fig. 1.

Fig. 3. Gas chromatography–thermionic specific detection of the oxocarboxylic acid derivatives from urine of the same patient as in Fig. 2 on the same day. Peak numbers as in Fig. 1.

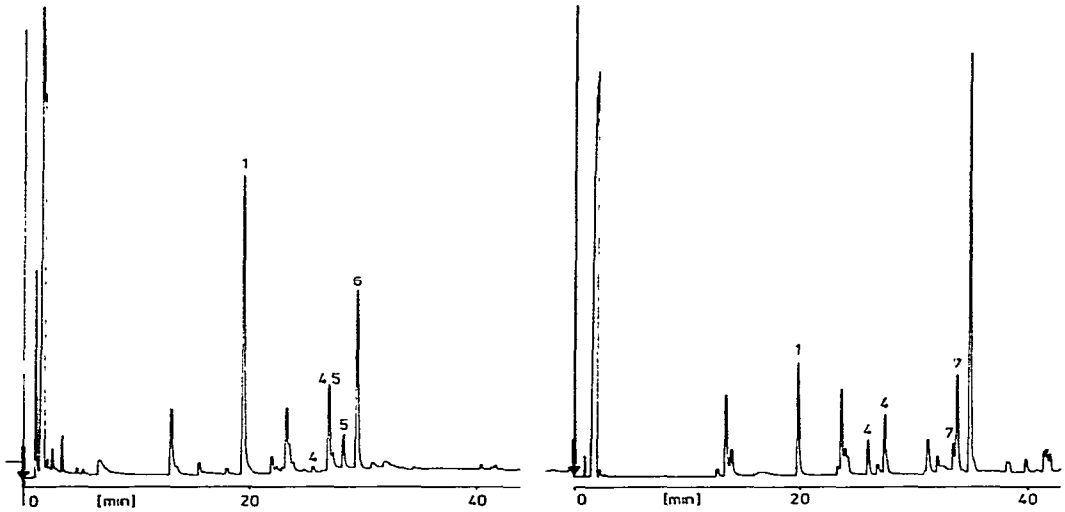

Fig. 4. Gas chromatography–thermionic specific detection of the oxocarboxylic acid derivatives from serum of a diabetic patient under good therapeutical control. Peak numbers as in Fig. 1.

Fig. 5. Gas chromatography–thermionic specific detection of the oxocarboxylic acid derivatives from urine of the same patient as in Fig. 4 on the same day. Peak numbers as in Fig. 1.

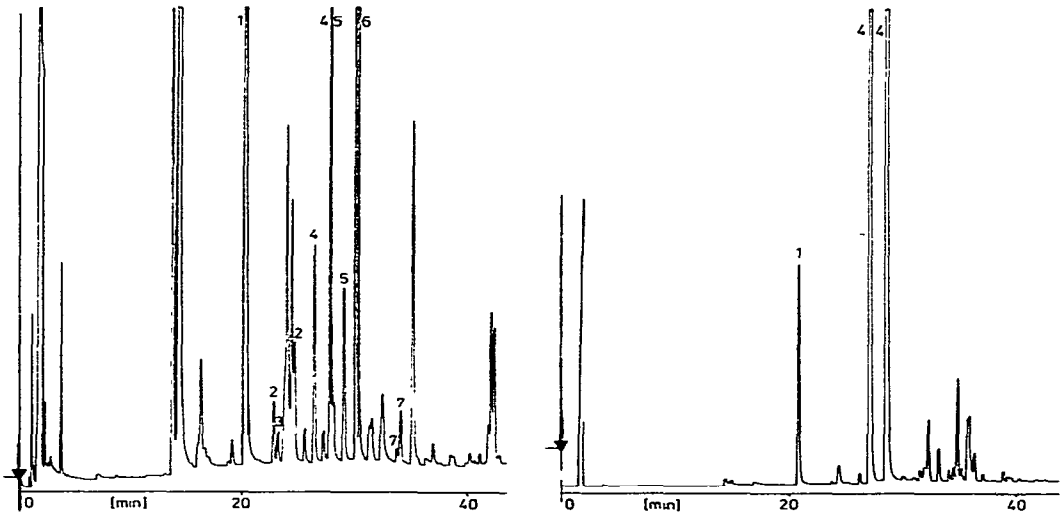

Fig. 6. Gas chromatography–thermionic specific detection of the oxocarboxylic acid derivatives from serum of a patient with diabetic ketoacidosis. Peak numbers as in Fig. 1.

Fig. 7. Gas chromatography–thermionic specific detection of the oxocarboxylic acid derivatives from urine of the same patient as in Fig. 6 on the same day. Peak numbers as in Fig. 1.

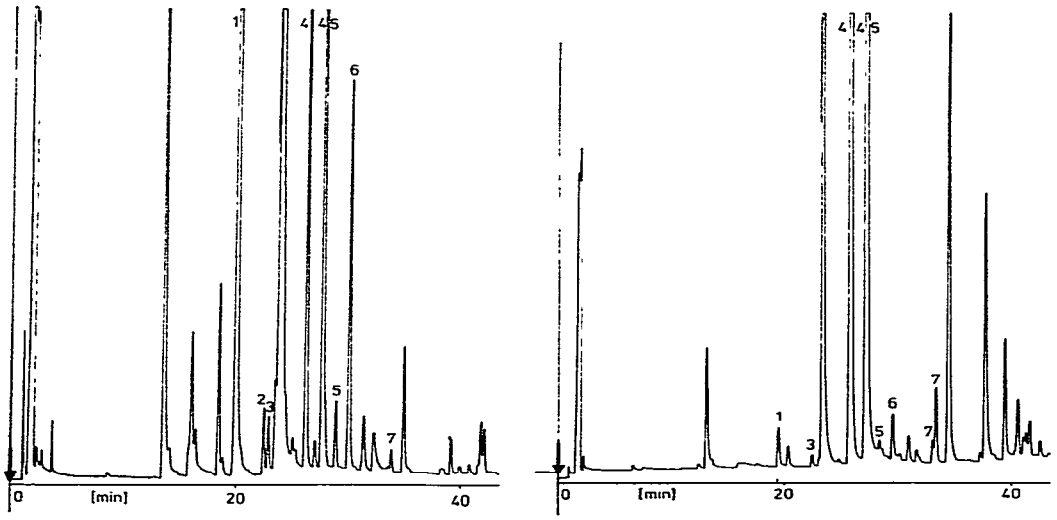

Fig. 8. Gas chromatography–thermionic specific detection of the oxocarboxylic acid derivatives from serum of an individual under fasting conditions. Peak numbers as in Fig. 1.

Fig. 9. Gas chromatography–thermionic specific detection of the oxocarboxylic acid derivatives from urine of the same individual as in Fig. 8 on the same day. Peak numbers as in Fig. 1.

As demonstrated in Figs. 4 and 5 which show serum and urine of the same patient on the same day, diabetic patients under good therapeutical control have low concentrations of 3-oxobutyric acid and the amino acid metabolites in serum (Fig. 4). In urine only some 3-oxobutyric acid is excreted (Fig. 5). Pyruvic acid appears in varying concentrations in serum and is excreted in urine normally in small amounts.

In different forms of ketoacidosis there is an increase in the concentration of 3-oxobutyric acid and the amino acid metabolites. Figs. 6 and 7 are examples for serum and urine from a diabetic ketoacidosis, and Figs. 8 and 9 are examples for serum and urine from a fasting individual on the fifth day. Among the amino acid metabolites 2-oxoisocaproic acid, originating from leucine, appears in the highest concentration. It is followed by 2-oxo-3-methylvaleric acid coming from isoleucine. The metabolites of valine and aminobutyric acid (*i.e.*, 2-oxoisovaleric and 2-oxobutyric acids) are present in small concentrations. The concentration of pyruvic acid is generally high. In urine large amounts of 3-oxobutyric acid are excreted, but hardly any amino acid metabolites. This shows that during ketoacidosis the body loses ketone moieties, and indirectly fatty acids, but only small amounts of 2-oxocarboxylic acids and therefore amino acids.

A considerable increase in the concentration of 3-oxobutyric acid and the 2-oxocarboxylic acids (especially 2-oxoisocaproic acid in serum) was also observed in case of uremia (Fig. 10).

Practicability of the method

The characteristic feature of the described method is that the oxocarboxylic acids are analyzed in one of four fractions of the entire group of organic acids in

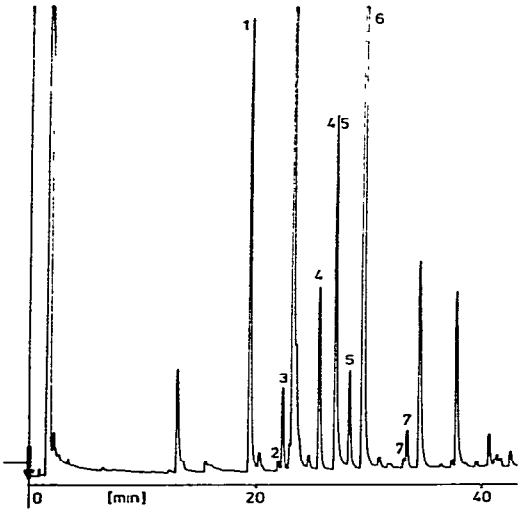

Fig. 10. Gas chromatography–thermionic specific detection of the oxocarboxylic acid derivatives from serum of a uremic patient. Peak numbers as in Fig. 1.

serum and in urine. Fraction 1 contains mainly the free fatty acids; fraction 2 is characterized by the oxocarboxylic acids, by dicarboxylic acids and aromatic acids; fraction 3 consists of hydroxycarboxylic acids, aromatic acids and acid conjugates; and fraction 4 contains acid conjugates. For each fraction a profile analysis is performed. The method is equally suitable for serum and urine.

The described clean-up procedure is somewhat time-consuming and should be applied to the oxocarboxylic acids especially when additional classes of acids (*e.g.*, as in the case of an acidosis when the free fatty acids, the dicarboxylic acids or the hydroxycarboxylic acids are also investigated) must be studied.

Gas chromatographic profile analysis of the four fractions and mass spectrometric (MS) analysis and confirmation of the substances require *ca.* 10 ml of sample. For the GC profiles of the four fractions only 3–5 ml of sample will suffice.

The method is suitable for comparative profile analyses. It provides qualitative information on the composition of each acid fraction and on variations and abnormalities between profiles of different individuals (semi-quantitative information). For the oxocarboxylic acids an exact quantification on the basis of internal standards is complicated by the occurrence of *syn–anti* isomers the ratios of which sometimes differ. The recovery of the oxocarboxylic acids is rather low. For 2-oxoisocaproic acid the rate of recovery was determined to be 40%.

Based on the detection of nitrogen in the O-methyloxime methyl esters, the oxocarboxylic acids can be very specifically measured in fraction 2. Some additional nitrogen-containing substances, which are probably introduced during the clean-up procedure, are separated from the acids under investigation. The identity and GC purity of the peaks of the acid derivatives were controlled by GC–MS and by using blanks. When the total, unprefractionated derivatized extract of the acids (instead of fraction 2) is used for the analysis of the oxocarboxylic acids by thermionic specific detection, several interferences may occur, resulting from *e.g.*, phosphoric acid and

the extremely large amounts of 3-hydroxybutyric acid in samples from ketoacidotic patients.

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