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GAS CHROMATOGRAPHIC—MASS SPECTROMETRIC DETERMINATION OF TOTAL 4-HEPTANONE, A NEW MARKER IN DIABETES MELLITUS

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SUMMARY

Total 4-heptanone is the sum of a β-oxocarboxylic acid, 2-ethyl-3-oxohexanoic acid, and its decarboxylation product, 4-heptanone.

The β-oxocarboxylic acid is found in serum and in urine, and is detected by gas chromatography—mass spectrometry in the form of its methyl ester or its O-methyloximated acid methyl ester. The ketone is detected within the profile of volatile metabolites in serum and in urine. However, the analytical procedure includes some ketone coming from in vitro decarboxylation.

Total 4-heptanone is measured by gas chromatography—mass fragmentography. The method includes a quantitative transformation of the β-oxocarboxylic acid into the ketone. A comparative study with 270 patients with diabetes mellitus, 28 healthy individuals and 143 non-diabetic hospitalized patients showed that the urinary excretion of total 4-heptanone is increased in diabetes mellitus. The mean values are 1073 μg per 24 h for diabetics, compared with 207 μg per 24 h and 246 μg per 24 h for healthy individuals and non-diabetic patients, respectively. Diabetic ketoacidosis and fasting conditions decrease the total 4-heptanone.

INTRODUCTION

Ketone bodies are, besides glucose and pH value, the classical parameters to indicate in a diabetic patient a decompensation in the sense of hyperglycemia and insulin deficiency. The ketone bodies, which are formed by ketogenesis after increased lipolysis and fatty acid oxidation, are normalized when the patient is restabilized by therapy. In addition to the regular ketone bodies, acetoacetic acid, acetone and β-hydroxybutyric acid, higher-molecular-weight ketone bodies are found in blood and urine, especially the ketones themselves which are analytically best detectable [1—4]. We could show that the levels of the higher-molecular-weight ketone bodies, 2-pentanone, 2-penten-3-one and 2-heptanone, as well as the β-oxocarboxylic acid which acts as precursor for 2-pentanone, also rise during increased ketogenesis and normalize when the diabetic patient is under good therapeutical control.

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On the other hand, we observed that two other ketonic substances, 4-heptanone and its precursing β-oxocarboxylic acid, 2-ethyl-3-oxohexanoic acid, behave differently. They are not normal when the glucose levels of diabetic patients are well controlled, and therefore reflect an abnormality in the metabolism of diabetics even if by therapy the patients are normalized. This paper describes mainly the results of a broad study on the urinary total 4-heptanone (ketone plus its precursor) in diabetic patients in comparison with healthy individuals and non-diabetic hospital patients.

EXPERIMENTAL

Apparatus

The analyses were performed on a Model 900 gas chromatograph (Bodenseewerk Perkin-Elmer, Überlingen, G.F.R.), a Model 3700 gas chromatograph (Varian, Darmstadt, G.F.R.) and a combination of a Model 2700 gas chromatograph, CH 5 mass spectrometer and Spectrosystem 100 MS computer (Varian-MAT, Bremen, G.F.R.). The gas chromatograph and the mass spectrometer were interfaced by a 30 cm × 0.1 mm I.D. platinum capillary.

Detection of 2-ethyl-3-oxohexanoic acid

2-Ethyl-3-oxohexanoic acid was detected in a fraction of the organic acids in serum and in urine in the form of its methyl ester or in the form of its O-methyloximated acid methyl ester. The method for extraction, derivatization and pre-fractionation leading to the O-methyloximated acid methyl ester derivative was described previously [5]. To obtain the methyl ester with the free carbonyl group left, the method was modified such that the reaction of the serum or urine sample with O-methyl hydroxylamine hydrochloride was omitted.

Fraction 2 of the pre-fractionated sample contained the 2-ethyl-3-oxohexanoic acid derivative and was analyzed by gas chromatography—mass spectrometry (GC—MS) under the following conditions: 25 m glass capillary column coated with OV-17 (Bodenseewerk Perkin-Elmer); carrier gas, helium at 4 ml/min; column temperature, 40°C for 10 min, then programmed at 2°C/min; injector block temperature, 250°C; sample size, 1 μl; ionization by electron impact; ionization energy, 70 eV; accelerating voltage, 3 kV; multiplier voltage, 2.5 kV; emission current, 300 μA; ion source temperature, 220°C; interface temperature 220°C; resolution 700; recording mode, automatic repetitive scanning, the mass spectra were recorded over the mass range *m/e* 15—380 and stored on magnetic tape.

Detection of total 4-heptanone

4-Heptanone was detected in the profile of volatile substances. The gas phase extraction and adsorption technique used in the work-up of the sample, and the GC procedure were described for urine [2] and serum [3].

Quantitative determination of total 4-heptanone in urine

Total 4-heptanone was determined by gas chromatography—mass fragmentography (GC—MF). A 6.4 mg/l solution of 3-heptanone in water containing 0.1%

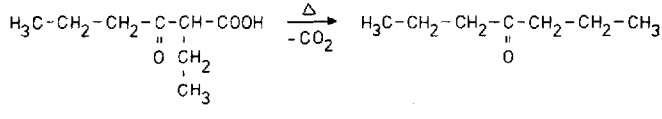
of Triton X-100 was used as the internal standard.

Urine samples (4-ml; usually in series of 20 samples) were heated in glass-stoppered and clamped vials for 90 min in a water-bath at 90°C to complete the decarboxylation of 2-ethyl-3-oxohexanoic acid to form 4-heptanone. After cooling, 0.2 ml of the internal standard solution was added to each of the samples resulting in internal standard concentrations of 305 μg/l. The mixtures were extracted with 1 ml of cyclohexane by shaking for 1 min. The extracts were separated from the aqueous phases, and 1-μl aliquots of the extracts were subjected to GC—MF analysis under the following operating conditions. Two different columns were used with equal suitability: column A, 100 m × 0.5 mm I.D. stainless-steel column, coated with Emulphor ON-870, at an oven temperature of 80°C; column B, 25 m × 0.2 mm I.D. fused silica column, coated with OV-1701 (Scientific Glass Engineering, Weiterstadt, G.F.R.), at an oven temperature of 40°C, injector block temperature 150°C. Mass spectrometric conditions were as described above, except for resolution (400) and recording mode (the mass spectrometer was focused on the molecular ion *m/e* 114).

The calculation of the concentrations of total 4-heptanone was based on the ratios of the peak heights of 4-heptanone and internal standard, and on calibration graphs obtained from eight aqueous standard solutions with 4-heptanone concentrations between 15 and 3200 μg/l. The amount of total 4-heptanone excreted in 24 h was calculated by multiplying the concentration with the 24-h urine volume.

RESULTS AND DISCUSSION

We define total 4-heptanone as the sum of 2-ethyl-3-oxohexanoic acid and 4-heptanone. The ketone is formed from the labile β-oxocarboxylic acid by decarboxylation according to the reaction



It must be anticipated that this decarboxylation partly occurs in vivo, analogous to the decarboxylation of acetoacetic acid to form acetone. In vitro, heat enhances this reaction.

The β-oxocarboxylic acid is found in urine and in serum. It can be detected in fraction 2 of the organic acids either in the form of its methyl ester with the carbonyl group left underivatized, or in the form of the methyl ester with the carbonyl group being derivatized to form the O-methyloxime. By computer—MF, especially the methyl ester is detected with good sensivity and specificity. Its detection is based on the intense ion *m/e* 71, supported by the molecular ion *m/e* 172 and the fragment ions *m/e* 129 and *m/e* 141 (Fig. 1). The method is suitable for the qualitative detection and identification of 2-ethyl-3-oxohexanoic acid. To a certain degree, semiquantitative information can be derived. A quantitative determination of the acid is restricted by its lability. The extent of decarboxylation during the work-up procedure is

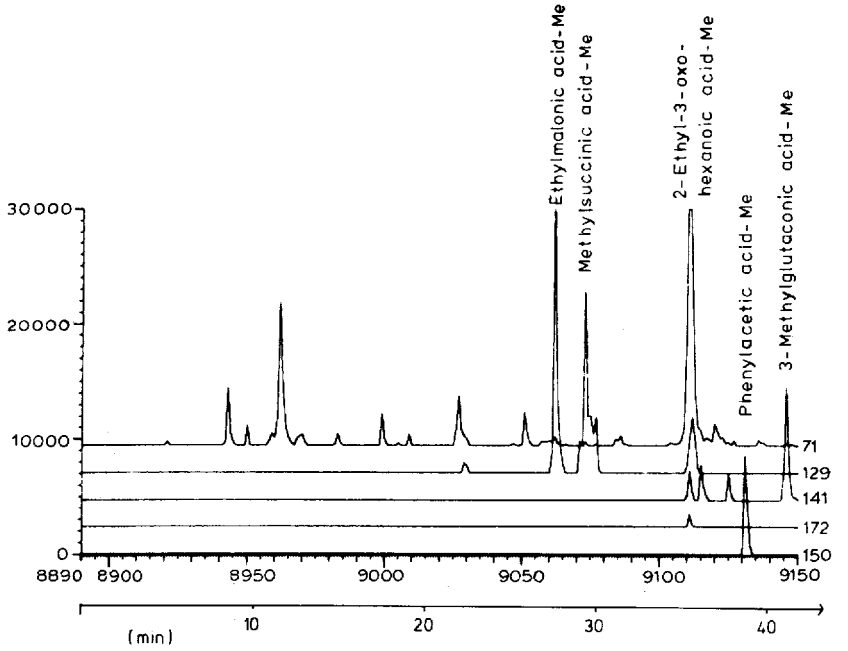


Fig. 1. Computer mass fragmentogram of fraction 2 of the organic acids in urine of a patient with diabetes mellitus, for the detection of the methyl ester of 2-ethyl-3-oxohexanoic acid (traces of the ions *m/e* 71, 129, 141, 172 and 150).

difficult to control. Even with the O-methyloximated derivative a correct quantification appears doubtful.

The amount of ketone which occurs in vivo in urine and in serum is difficult to detect, since by decarboxylation of the β-oxocarboxylic acid in vitro, the amount of the ketone will rise. The 4-heptanone which is detected within the profile of volatile metabolites in urine (Fig. 2) and serum (Fig. 3), corresponds only to some extent to the original ketone. The greater part is produced during the analytical procedure by decarboxylation of the β-oxocarboxylic acid. Nevertheless, when the analytical procedure is strictly standardized, the profiles of volatile metabolites allow the reproducible detection and recognition even of small variations and shifts in the concentration of 4-heptanone.

However, since there is no indication that the β-oxocarboxylic acid and the ketone have different physiological significance, it is justified to quantify the total 4-heptanone. By heating the sample, 2-ethyl-3-oxohexanoic acid is quantitatively decarboxylated. The reaction kinetics (Fig. 4) demonstrate that after 90 min at 90°C the decarboxylation process is completed. Fig. 5 shows the recording of the GC—MF determination of total 4-heptanone in three different samples. The extracts are injected every 8 min. Interfering substances are not observed.

We determined the total 4-heptanone in the urine of 270 patients with diabetes mellitus. The control groups consisted of 28 healthy individuals and 143 hospitalized patients with no obvious metabolic abnormalities. For each of the patients and the healthy individuals, the measurements were made in

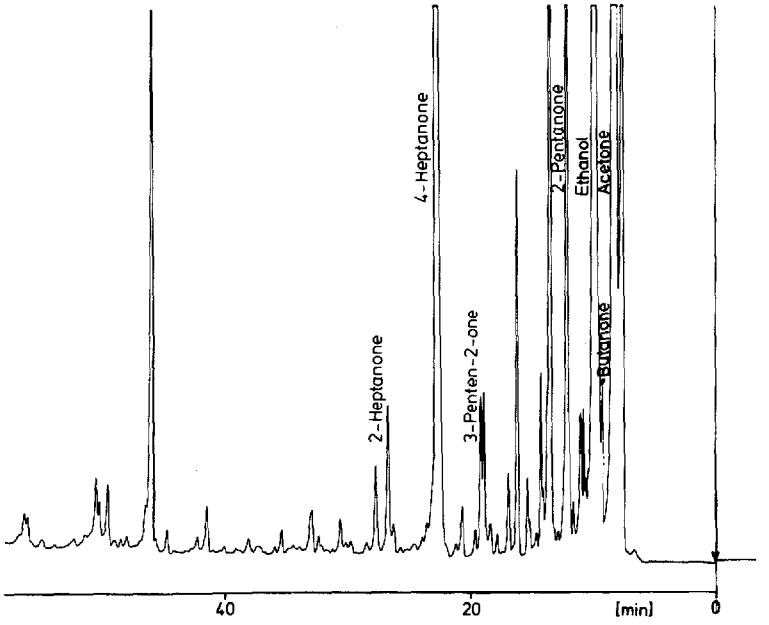


Fig. 2. Profile of the volatile metabolites in urine of a patient with diabetes mellitus, for the detection of 4-heptanone.

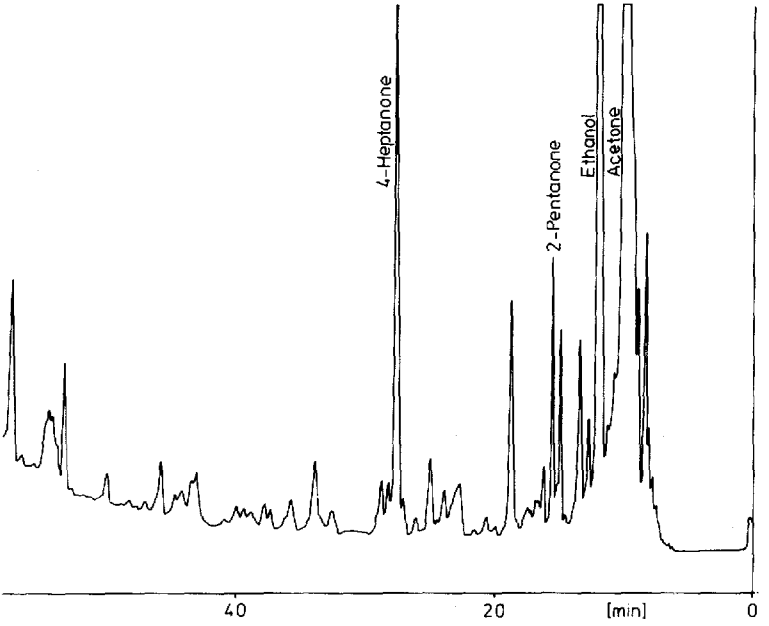


Fig. 3. Profile of the volatile metabolites in serum of a patient with diabetes mellitus, for the detection of 4-heptanone.

three consecutive 24-h urines. Of the diabetic cases 234 were classified as diabetes mellitus type I (insulin-dependent) and diabetes mellitus type II (not insulin-dependent).

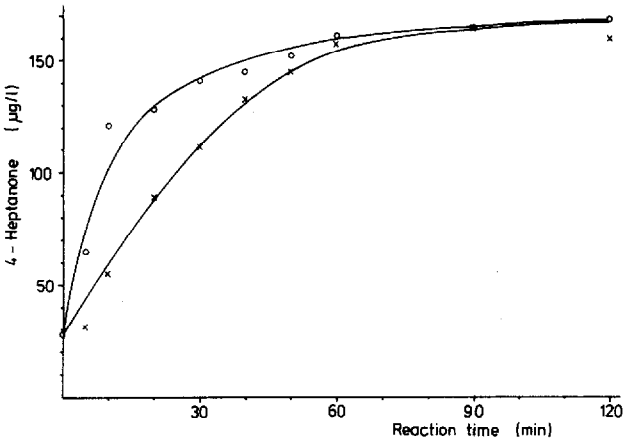


Fig. 4. Kinetics of the formation of 4-heptanone by heat decarboxylation of 2-ethyl-3-oxo-hexanoic acid in urine. (×) 90°C; (○) 99°C.

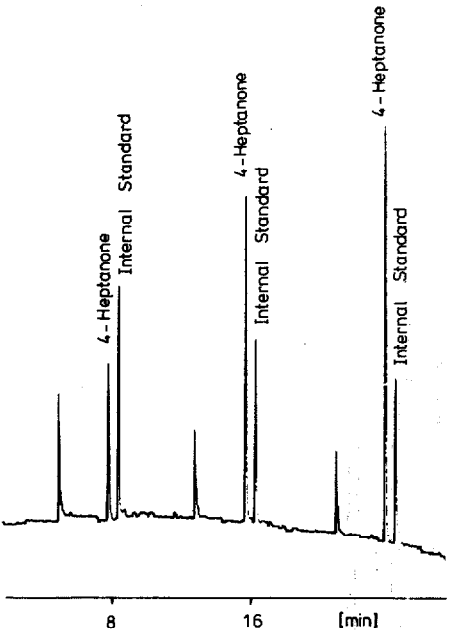


Fig. 5. GC—MF trace of the determination of total 4-heptanone in three urine samples, *m/e* 114.

The results are summarized in Table I. Whereas the mean values of urinary total 4-heptanone in healthy individuals and non-diabetic hospitalized patients are very similar (207 µg per 24 h and 246 µg per 24 h, respectively), the mean value is increased by a factor of 4 to 5 in diabetics (1073 µg per 24 h). Comparing diabetes mellitus type I and type II, we find that, on average, type I diabetics excrete more total 4-heptanone than type II diabetics. Elevated total 4-heptanone values are also found in patients with steroid-induced diabetes. Patients without overt diabetes mellitus, but abnormal glucose tolerance, already show increased total 4-heptanone (Table I).

TABLE I

TOTAL 4-HEPTANONE IN URINE

Group	Mean excretion (μg per 24 h)	Range of excretion (μg per 24 h)
Healthy individuals (n = 28)	207	60—470
Non-diabetic patients (n = 143)	246	40—810
Diabetic patients (n = 270)	1073	15—5590
Type I diabetes (n = 109)	1215	19—5590
Type II diabetes (n = 125)	978	15—4640
Steroid-induced diabetes (n = 10)	1020	610—2000
Abnormal glucose tolerance (n = 22)	448	50—1500
Fasting, non-diabetics (n = 14)	35	5—75
Fasting, diabetics (n = 8)	86	25—170

Characteristic of the excretion of total 4-heptanone in the group of the diabetic patients is the fact that the range is very broad. As well as in Table I, the distribution is illustrated in Fig. 6. From a detailed analysis of the clinical situation of the diabetic patients we could draw some conclusions.

During ketoacidotic periods total 4-heptanone decreases significantly and turns to its high level after the ketoacidosis is overcome. Total 4-heptanone also decreases when obese diabetic patients are put on fasting conditions (Table I). The same observation is made when obese but otherwise healthy individuals are put on fasting conditions (Table I). Not only complete fasting conditions result in a decrease of total 4-heptanone. A reduced diet already leads to a drop in the urinary total 4-heptanone. In conjunction with a study in which diabetic and non-diabetic patients were fed exclusively by infusion of glucose and fructose for 48 h, receiving only 25 kcal/kg body weight in 24 h, the total 4-heptanone decreased on average to half of the starting value in the control group (Fig. 7) and to much less than half in the diabetic group (Fig. 8). We con-

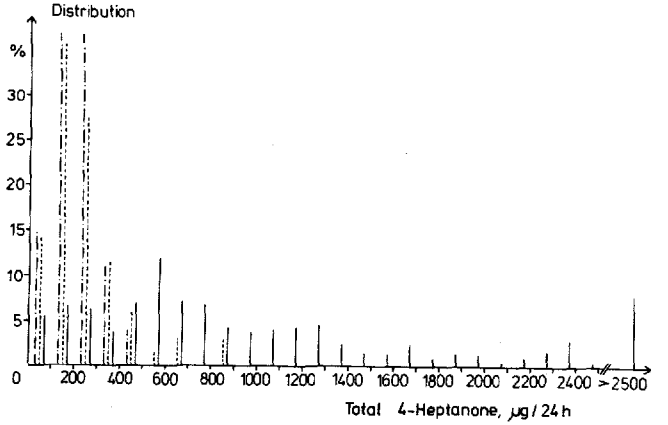


Fig. 6. Distribution of the urinary excretion of total 4-heptanone. (• — •) Healthy individuals, n = 28. (— — —) Patients without diabetes mellitus, n = 143. (———) Diabetic patients, n = 270.

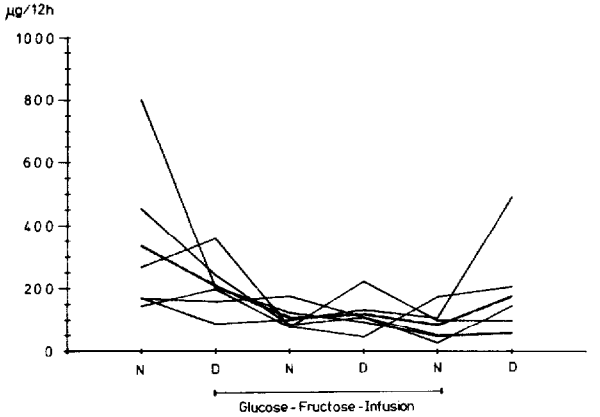


Fig. 7. Total 4-heptanone in urine of a control group being fed by infusion of glucose and fructose, 25 kcal/kg body weight in 24 h. N = night, D = day.

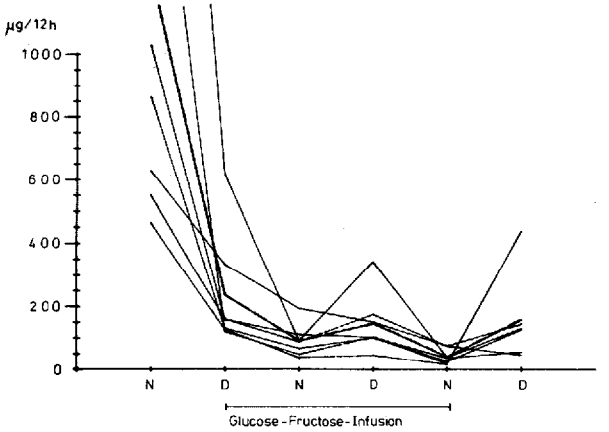


Fig. 8. Total 4-heptanone in urine of diabetic patients under the same conditions as the control group in Fig. 7.

clude from these observations that total 4-heptanone decreases in all those physiological and pathophysiological situations in which lipolysis and fatty acid oxidation is increased and fatty acid synthesis is decreased.

This behaviour of total 4-heptanone has to be considered in the comparison of diabetic patients with control persons. Consistent results are obtained when for the determination of total 4-heptanone the patient is on a balanced diet and not in a hyperglycemic and ketoacidotic period. Most of the 4-heptanone values of the diabetic patients falling in the normal range must be understood as pseudonormal since, at the time when the urine was collected, the patients were either ketoacidotic or under fasting conditions or on a reduced diet.

In diabetic patients who are on a balanced diet and are not ketoacidotic, the extent of increase of the total 4-heptanone covers a broad range. The distribution curve shows a maximum for values between 500 and 600 µg per 24 h (Fig. 6). However, many values are higher and reach several thousand µg per 24 h. It is observed that patients with very high total 4-heptanone are in many cases patients who are therapeutically difficult to control and whose blood

glucose levels are very unstable.

On the basis of the large number of diabetic patients and control persons included in the described study, we conclude that an increased urinary excretion of total 4-heptanone is inherently connected with diabetes mellitus and is an additional marker for the disease. On the other hand, total 4-heptanone is a sensitive indicator for the interplay between fatty acid oxidation and fatty acid synthesis.

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