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RAPID QUANTITATIVE MICROANALYSIS OF KETONES IN URINE BY GAS CHROMATOGRAPHY–MASS FRAGMENTOGRAPHY

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SUMMARY

A quantitative method is described for determination of 4-heptanone and its precursor in urine. The method involves a single-step extraction of urine with cyclohexane and the analysis of 1 μl of the extract by gas chromatography–mass fragmentography. The detection limit is $8 \cdot 10^{-12}$ g, corresponding to 2 μg per 1000 ml of urine. The method is linear in the concentration range of $7.6 \cdot 10^{-9}$–$1.5 \cdot 10^{-5}$ g/ml and the relative standard deviations for the individual values are between 1.2 and 10%. Approximately 40 samples can be analyzed per day or 1 sample in 7–8 min. By varying the experimental conditions, the method is applicable to other ketones.

INTRODUCTION

In the past 5 years a number of workers[1–4] have shown that low-molecular-weight and gas chromatographically volatile metabolites are excreted in urine, especially ketones, alcohols, furans, pyrroles and sulphur compounds. The analyses of the profiles of these substances were qualitative. Under strictly standardized conditions for the sampling procedure and the gas chromatographic or mass fragmentographic analysis, semi-quantitative results were obtained. Under such conditions abnormal and pathological profiles can be recognized.

Detailed results have been described on pathologically increased concentrations of ethanol, *n*-propanol, *n*-butanol, isobutanol and isopentanol in the urinary profiles of patients with diabetes mellitus[5–8]. It has also been established that the concentrations of the ketones, especially 4-heptanone, correlate with metabolic disorders related to diabetes[6,8].

We postulate that, analogous to acetone, the higher ketones are formed from β-keto acids by decarboxylation. We could show that the concentrations of the ketones and of their precursors in the urine of patients with diabetes mellitus depend on several factors. The levels of 4-heptanone and the corresponding precursor are influenced by the blood glucose level, the glucose concentration in urine, the insulin activity and probably by the type of diabetes. 4-Heptanone can be considered to be a very sensitive indicator for metabolic changes.

To test the behaviour of this ketone, to establish the correlations between 4-heptanone and the other clinical–chemical parameters and to investigate its clinical

relevance, we had to develop a precise and rapid quantitative method that can be used as a routine procedure and enables one to analyze a large number of urines in a reasonable time.

The method involves a simple single-step extraction and analysis by gas chromatography–mass fragmentography. In this paper, transformation of the precursor, extraction yield, sensitivity, linearity, precision, interferences and practicability of the method for the determination of 4-heptanone are described. By varying the experimental parameters, the method can be used for other ketones and also other low-molecular-weight constituents in urine.

EXPERIMENTAL

Apparatus

A combination of a Model 2700 gas chromatograph and a CH 5 mass spectrometer (Varian-MAT, Bremen, G.F.R.) was used. The gas chromatograph and the mass spectrometer were directly interfaced over a 30 cm × 0.1 mm I.D. platinum capillary. The total effluent from the gas chromatographic column entered the ion source of the mass spectrometer.

Reagents

The following chemicals were used: GC-grade 2-heptanone (Ega-Chemie 12,336-6; Ega-Chemie, Steinheim, G.F.R.), 3-heptanone (Ega-Chemie H 315-1), 4-heptanone (Ega-Chemie 10,174-5), cyclohexanone (Merck 9664, Merck, Darmstadt, G.F.R.) and cyclohexane (Merck 9666); and Triton X-100 (Serva 37238, Serva Feinbiochemica, Heidelberg, G.F.R.). A Triton solution was prepared containing 0.1% of Triton X-100 in double-distilled water.

Standard solutions

Stock solutions. Three standard stock solutions were prepared from 2-heptanone, 3-heptanone and 4-heptanone, by dissolving 80 mg of the ketone in 100 ml of Triton solution, resulting in a concentration of $8.0 \cdot 10^{-4}$ g/ml.

Internal standard working solution. A 0.1-ml volume of the 3-heptanone stock solution was diluted to 25 ml with Triton solution ($3.2 \cdot 10^{-6}$ g/ml).

Standard working solutions. Fourteen standards were prepared by pipetting equal aliquots of the 2-heptanone and the 4-heptanone stock solutions (0.005–10.0 ml) and 0.1 ml of the 3-heptanone stock solution into Triton solution up to a final volume of 25 ml. All 14 standards had the same concentration of 3-heptanone as the internal standard working solution. When stored at 4°, the solutions were stable for at least 6 weeks.

Extraction procedure

To 4 ml of urine, 0.2 ml of the internal standard working solution was added and this mixture was extracted with 1 ml of cyclohexane by shaking for 1 min. After the extraction, the sample was centrifuged for 5 min at 1500 *g* in order to separate the two phases.

To determine the calibration graphs for 2-heptanone and 4-heptanone, 14

aqueous samples prepared by dissolving 0.2 ml of the standard working solutions in 4.0 ml of double-distilled water (concentrations $7.6 \cdot 10^{-9}$–$1.5 \cdot 10^{-5}$ g/ml) were extracted with cyclohexane in the same manner as the urine samples.

Determination of the extraction yield of 4-heptanone

Two standards containing 4-heptanone in Triton solution ($1.5 \cdot 10^{-7}$ and $1.5 \cdot 10^{-6}$ g/ml) were extracted with cyclohexane using five different extraction ratios: 1, 2, 4, 5 and 10 ml of standard with 1 ml of cyclohexane. To 0.5 ml of each extract, 25 μl of a solution of the internal standard 3-heptanone in cyclohexane ($3.2 \cdot 10^{-6}$ g/ml) were added, resulting in a 3-heptanone concentration of $1.5 \cdot 10^{-7}$ g/ml in the extract.

Each of the standards was extracted a second time using half of the aqueous portion for extraction with 0.5 ml of cyclohexane. To 0.25 ml of the second extracts, 12.5 μl of the internal standard solution were added.

The extraction yields were determined on the basis of a calibration graph obtained by directly injecting several concentrations of 4-heptanone in cyclohexane (internal standard 3-heptanone, $1.5 \cdot 10^{-7}$ g/ml).

Transformation of the precursors into ketones

By heating the urine samples (4 ml) in PTFE-lined screw-capped vials for 60–90 min in a water-bath at 90°, the ketones were formed from their precursors. Approximately 20 urine samples were processed simultaneously.

Gas chromatographic and mass fragmentographic procedure

The gas chromatographic separation was performed on a 100 m × 0.5 mm I.D. stainless-steel column, coated with Emulphor ON-870, at an oven temperature of 80° and an injector temperature of 150°. Helium was used as the carrier gas at a flow-rate of 5 ml/min. The mass spectrometric conditions were as follows: electron energy of the ion source, 70 eV; emission current, 300 μA; accelerating voltage, 3 kV; multiplier voltage, 3.1 kV; ion source temperature, 220°; interface temperature, 200°; resolution, 400; and operating pressure, $4 \cdot 10^{-5}$ torr. For the determination of 4-heptanone and 2-heptanone, the mass spectrometer was focused on the molecular ion *m/e* 114. The sample size was 1 μl of the extract. A 30-cm pre-column was used to protect the separating column from higher boiling substances.

RESULTS AND DISCUSSION

Formation of 4-heptanone from its precursor

4-Heptanone as such is excreted in urine in only small amounts, ranging in normal urines between 10 and 30 μg per 24 h. By heating the urine, its concentration is increased 5- to 15-fold. The same behaviour was observed for 2-heptanone. Figs. 1 and 2 show the formation of the two ketones in a normal urine and a diabetic urine, respectively. The amounts of 4-heptanone in the urines after heat treatment were 184 μg per 24 h in the normal urine and 900 μg per 24 h in the diabetic urine. For 2-heptanone, we determined approximately 27 μg per 24 h in the normal urine and 62 μg per 24 h in the diabetic urine.

We propose that the increase in concentration of the ketones is caused by the

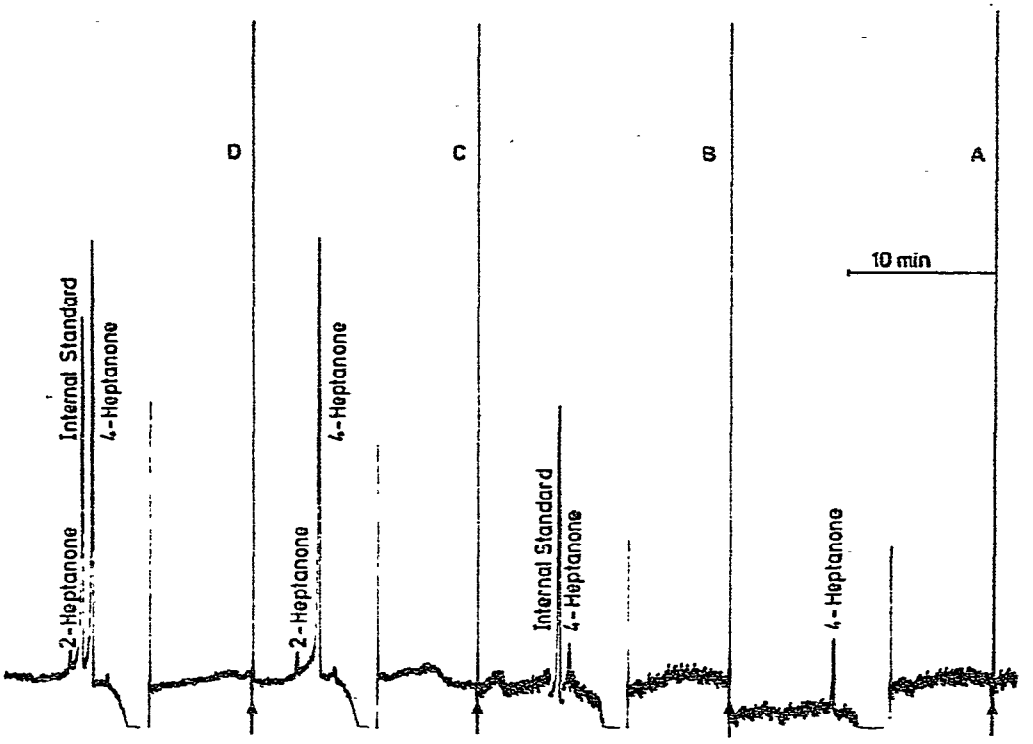

Fig. 1. Mass fragmentograms of a normal urine, *m/e* 114. A, urine not heated, full-scale recorder signal 5 mV; B, urine not heated, addition of internal standard, recorder 5 mV, internal standard with 10 mV; C, urine heated at 90° for 90 min, recorder 10 mV; D, urine heated at 90° for 90 min, addition of internal standard, recorder 10 mV. The arrows below the baseline indicate the starting points of the analyses. The drops in the baseline are caused by the pressure change by the solvent.

transformation of a precursor into the ketone. By analogy with the formation of acetone from acetoacetic acid, β-ketocarboxylic acids can be postulated as precursors of the other ketones. This postulation is supported by the observation that the concentration of 4-heptanone is only slightly increased by heating the sample when the urine is alkalinized with potassium hydroxide prior to heat treatment.

The correlation between concentration of 4-heptanone and reaction time was studied at 70°, 90° and 99° (Fig. 3). We conclude that the precursor can be quantitatively transformed. At 90° and 99°, the transformation was virtually complete after 90 min. By the method described in this paper, the actual amount of 4-heptanone in fresh urine and the total amount of 4-heptanone and its precursor can be determined. In our routine analyses of normal urines and of urines of diabetic patients, we determine the total 4-heptanone after heating the urine sample at 90° for 90 min.

Extraction yield

The non-polar solvent cyclohexane is suitable for the mass fragmentographic determination of the C_7 ketones because of its very low background at mass *m/e* 114. No interfering peaks are observed. The extraction yield was determined for 4-hepta-

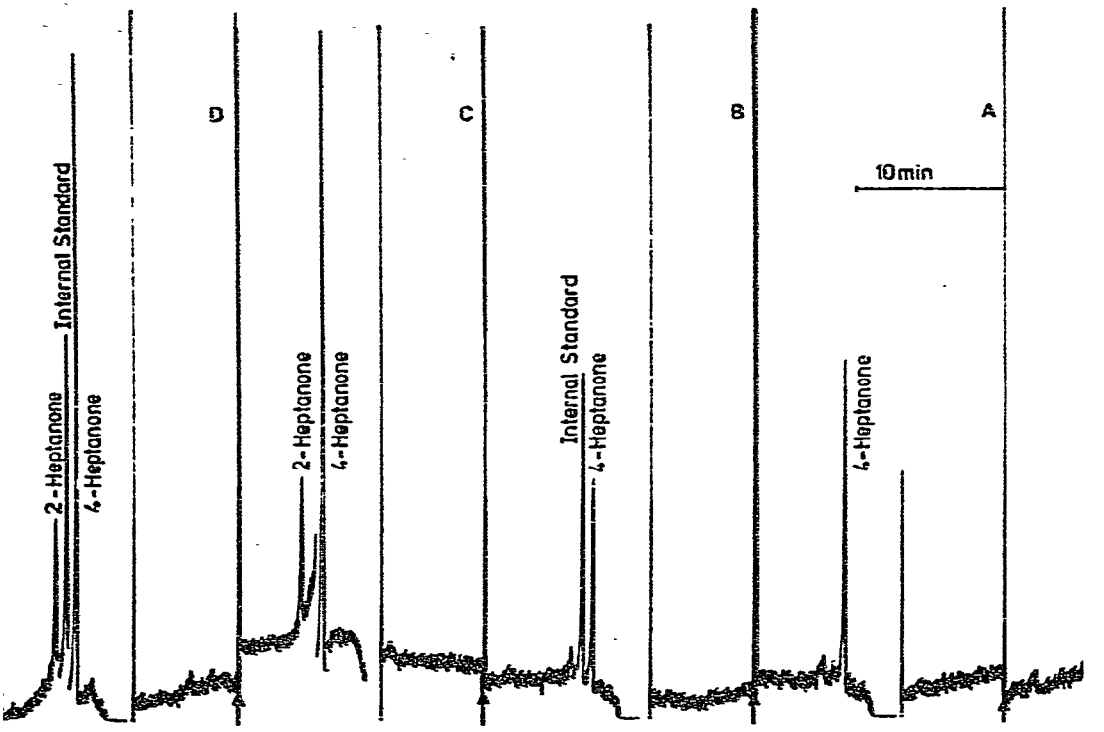

Fig. 2. Mass fragmentograms of urine of a diabetic patient, *m/e* 114. A, urine not heated, recorder 5 mV; B, urine not heated, addition of internal standard, recorder 5 mV, internal standard with 10 mV; C, urine heated at 90° for 90 min, recorder 5 mV, 4-heptanone with 50 mV; D, urine heated at 90° for 90 min, addition of internal standard, recorder 5 mV, 4-heptanone with 50 mV and internal standard with 10 mV. The arrows below the baseline indicate the starting points of the analyses. The drops in the baseline are caused by the pressure change by the solvent.

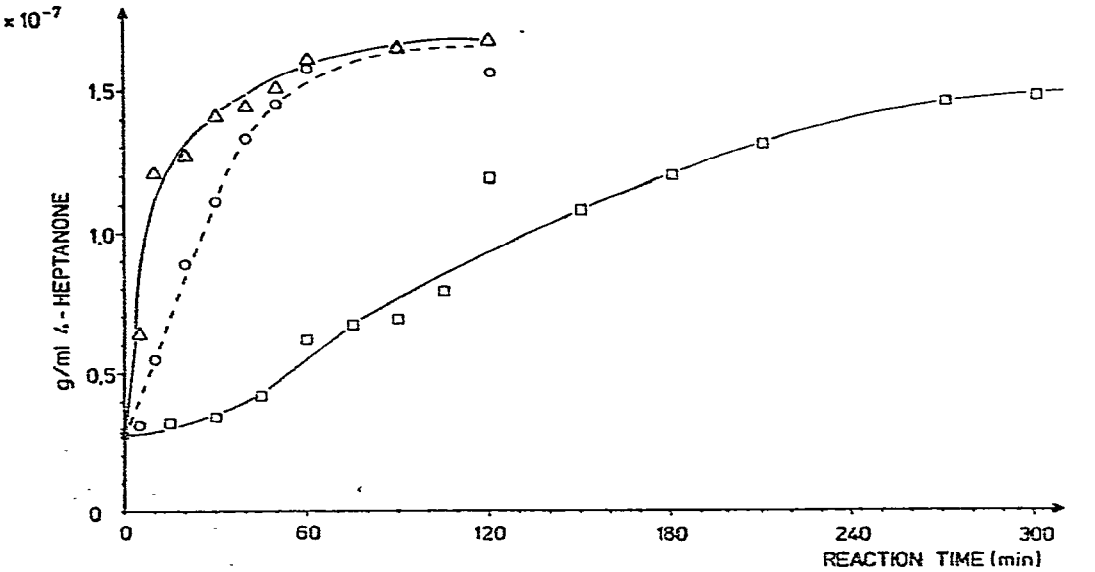

Fig. 3. Concentration of 4-heptanone *versus* reaction time. □, 70°; ○, 90°; △, 99°.

none and found to be satisfactory. At an extraction ratio of 1 ml of urine and 1 ml of cyclohexane, the extraction yield for one extraction step is 97–100% (Fig. 4). To increase the sensitivity of the method, in routine analyses we use an extraction ratio of 4 ml of urine and 1 ml of cyclohexane, resulting in an extraction yield of 95%.

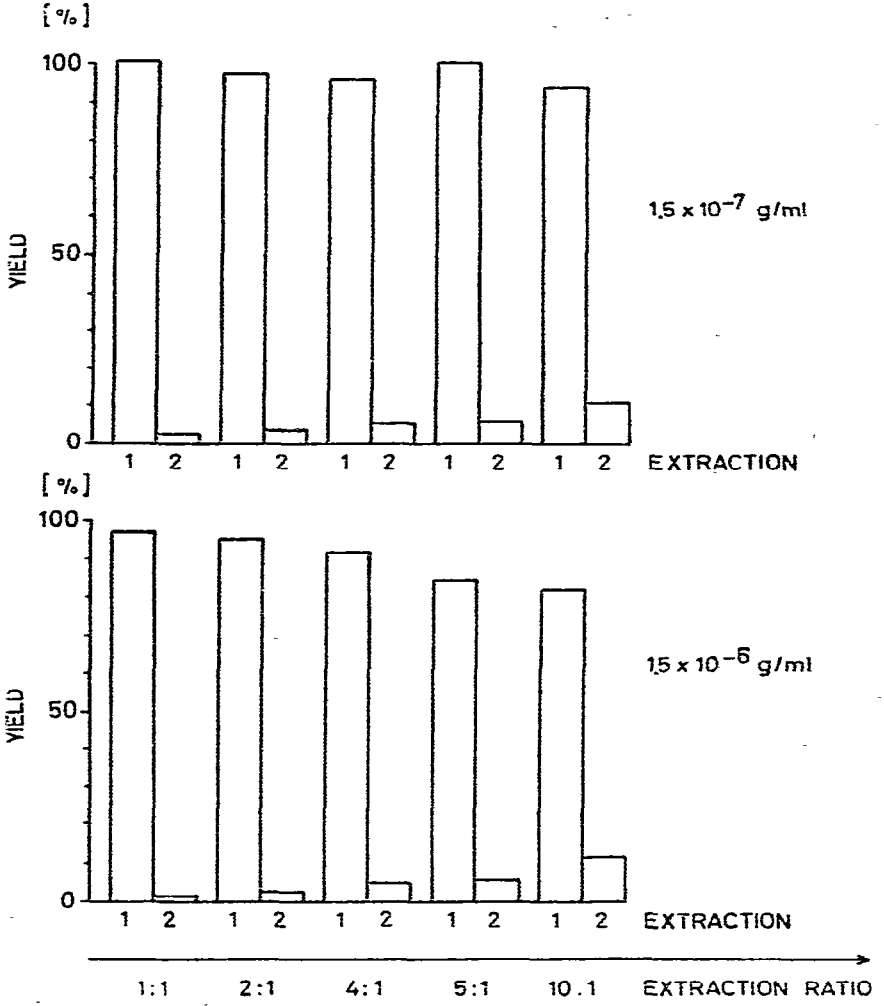

Fig. 4. Extraction yields of 1 and 2 extractions of 4-heptanone *versus* extraction ratios. Extraction ratios, *X* ml urine:*Y* ml cyclohexane: 1:1; 2:1; 4:1; 5:1; and 10:1.

Sensitivity of the method

Because of the high sensitivity of mass fragmentographic detection, the extract can be analyzed directly without any concentration. Therefore, no irreproducible losses, which are difficult to avoid when a sample has to be concentrated, can occur. The detection limit for 4-heptanone under the described conditions was determined to be $8 \cdot 10^{-12}$ g, corresponding to a concentration of 2 μg per 1000 ml of urine. This

sensitivity of the method enables us to use it as a microanalytical procedure, in which only 1 μl of the non-concentrated extract is required.

Linearity of the method and calculation of the concentrations

From 14 standard working solutions in the concentration range $7.6 \cdot 10^{-9}$–$1.5 \cdot 10^{-5}$ g/ml, the calibration graph for 4-heptanone was determined. Fig. 5 shows that with a concentration range of more than three orders of magnitude the method has a very broad range of linearity. The calibration graph for 4-heptanone was plotted on double-logarithmic paper. The same range of linearity was found for 2-heptanone.

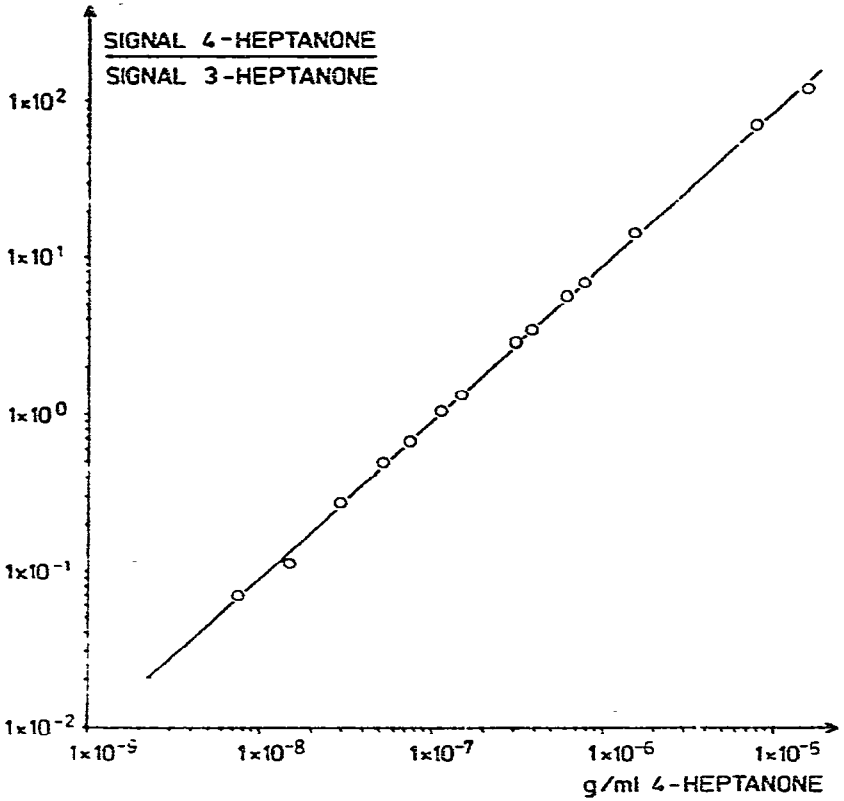

Fig. 5. Calibration graph for determination of 4-heptanone.

The calculation of the 4-heptanone concentration is based on the ratio of the peak heights of 4-heptanone and the internal standard 3-heptanone. By this procedure, the effect of errors during the extraction and uncontrolled variations of the instrumental conditions is eliminated. Controls have shown that a calibration graph can be used for at least 1 month. For routine determinations of 4-heptanone, we use the following equation, which was derived from the calibration graph:

$$X = 1.13 \cdot 10^{-7} Y$$

where X (g/ml) is the concentration of 4-heptanone and Y is the 4-heptanone:3-heptanone signal ratio.

Precision

The values used for plotting the calibration graph for 4-heptanone are mean values of double determinations, three of them being mean values of five-fold determinations. For the five-fold determinations we calculated the absolute standard deviation (S) and the relative standard deviation (S_{rel}) and the results are given in Table I. With S_{rel} values of 1.2 and 1.7% in the normal range of concentration the precision of the method is very good. At the higher concentration, the ratio of 4-heptanone to 3-heptanone is less favourable, resulting in $S_{rel} = 8.3\%$. In this range of concentration of 4-heptanone, S_{rel} can be improved significantly by using a higher concentration of internal standard.

TABLE I

PRECISION OF THE METHOD

Mean value, X (g/ml)	*Absolute standard deviation, S (g/ml)*	*Relative standard deviation, S_{rel} (%)*
$7.6 \cdot 10^{-8}$	$1 \cdot 10^{-9}$	1.2
$3.0 \cdot 10^{-7}$	$1 \cdot 10^{-8}$	1.7
$7.6 \cdot 10^{-7}$	$7 \cdot 10^{-8}$	8.3

The precision discussed here was determined with the standard working solutions, which were not heated. Using urine samples subject to the heating process the precision is somewhat worse (S_{rel} 8–10%), which can be explained by irreproducible losses of ketones when the urine samples are heated. Methodical improvements are possible.

Interferences

No interfering substances were found except in one instance. In urine from a patient with porphyria, 3-heptanone was present and interfered with the internal standard. In all other urines from normal individuals and from patients with diabetes mellitus and other diseases, no interference from 3-heptanone was found.

Practicability of the method

The procedure described for determining the concentration of 4-heptanone is suitable for use in routine analyses. Fig. 6 shows two examples of double determinations of total 4-heptanone after heating the urines. The analysis time was 13 min, but we reduced the time to 7–8 min by using an overlapping injection procedure. In this way we can analyze approximately 40 samples in a normal working day, including the extraction of the samples. In closed vials and at 4°, the cyclohexane extracts are stable for 1 day.

Substances that are extracted from the urine together with the ketones do not interfere in the analysis. After several hundred injections of urine extracts, no increased column bleeding and no deterioration of the column were observed. The

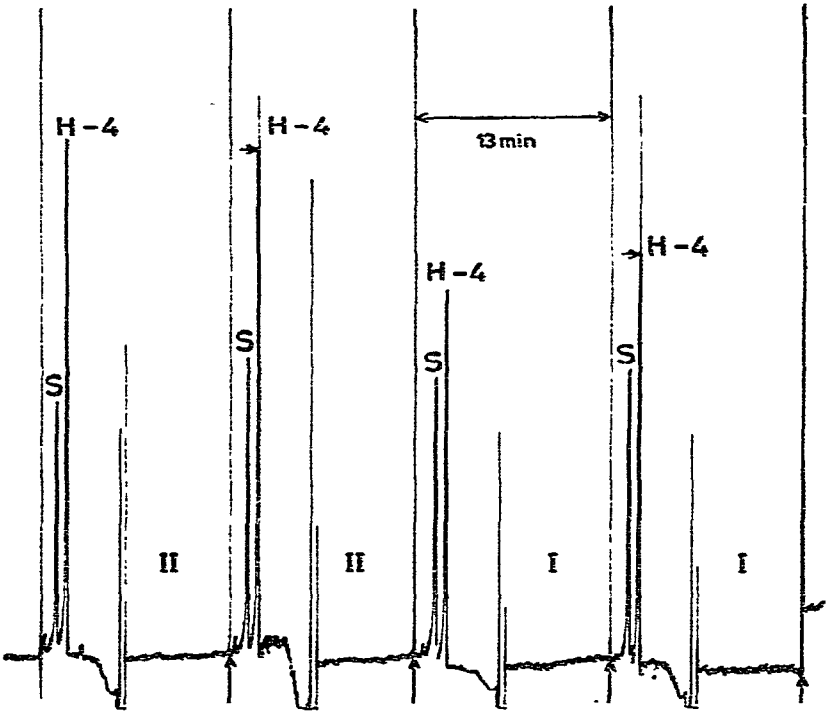

Fig. 6. Double determinations of 4-heptanone in urines I and II. H-4, 4-heptanone; S, internal standard. Urine I, full-scale recorder signal 10 mV, 4-heptanone with 20 mV; urine II, recorder signal 10 mV. The arrows on the peaks indicate sensitivity changes. The arrows below the baseline indicate the starting points of the analyses. The drops in the baseline are caused by the pressure change by the solvent.

glass insert of the injector block is cleaned after every 100 samples and the protecting pre-column is washed after every 200–300 samples.

2-Heptanone can be determined simultaneously with 4-heptanone. By focusing the mass spectrometer on *m/e* 86 or 98, the method can be applied to the determination of 2-pentanone and cyclohexanone, respectively.

Because of the broad range of linearity, samples of pathologically high concentrations of 4-heptanone do not have to be diluted. In our study of normal and diabetic urines we determined concentrations between 7 and 4000 μg per 24 h. The high sample capacity makes the method suitable for correlating the concentration of 4-heptanone and its precursor with other clinical–chemical parameters on a statistical basis.

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