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VOLATILE SUBSTANCES IN BLOOD SERUM: PROFILE ANALYSIS AND QUANTITATIVE DETERMINATION

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SUMMARY

Alcohols, ketones and aldehydes have been identified in the profiles of volatile substances in blood serum. A 5-ml sample is required in order to obtain a complete gas chromatographic profile, selective profiles of alcohols or individual ketones by computer mass fragmentography, and to permit mass spectrometric identification of the compounds.

The quantitative determination of total 4-heptanone using an extraction and gas chromatographic–mass fragmentographic procedure demonstrated concentrations of 10–50 nmole/l in normal serum, whereas in the serum of patients with chronic renal insufficiency the concentrations were 10- to 40-fold higher.

Ethanol and total acetone were quantitatively measured by direct injection of serum. For 10 patients, concentrations between 25 and 85 μmole/l of acetone and 10–170 μmole/l of ethanol were determined.

INTRODUCTION

Volatile metabolites excreted in urine have been extensively studied and normal patterns have been established by gas chromatographic or mass fragmentographic profile analysis, mass spectrometric identification of the substances and, in some instances, by quantitative determination[1–8]. Abnormal excretions of alcohols, ketones and ketone precursors have been demonstrated in cases of metabolic disorders, such as diabetes mellitus or fasting[7–11]. The metabolic pathway and clinical significance of the volatile substances are still under investigation.

Few data are available on the occurrence and concentration of volatile substances in blood. Technical problems such as the availability of sufficiently large samples, the protein content of blood serum and the lower concentration of the substances compared with urine have rendered their qualitative and quantitative determination more difficult. Zlatkis *et al.*[12] adsorbed volatile constituents of pooled serum or plasma on Tenax GC and identified several components by combined gas chromatography–mass spectrometry. Dowty *et al.*[13] used the same adsorbing material to analyse components in plasma of patients with chronic renal failure. They identified approximately 20 substances and found increased concentrations compared with the plasma of normal individuals. Stoner *et al.*[14] extracted volatile substances from plasma

with diethyl ether. The sample size used for these analyses ranged between 5 and 55 ml of plasma.

Zlatkis and coworkers[15,16] introduced micro-scale methods for profile analyses using only 100 μl or even 25 μl of plasma or serum. They applied extraction and column elution methods to transfer volatile and soluble constituents from the biological sample on to a glass-wool trap. The substances were not identified. Stafford *et al.*[17] applied the micro-extraction method to the analysis of 100-μl plasma samples from newborn infants and mothers at the time of delivery, and tentatively identified six compounds.

Quantitative determinations of volatile substances in blood, such as methanol, ethanol and 2-propanol, have been described mainly for toxicological specimens[18–21]. Except for acetone, which has been determined with chemical[22] and gas chromatographic methods[23–25], few quantitative data are available for physiological levels.

This paper describes the analysis of profiles of volatile metabolites in blood serum and the quantitative determination of 4-heptanone by mass fragmentography. It further describes an approach to the quantitative determination of acetone and ethanol by a simple mass fragmentographic method applying direct injection of serum.

EXPERIMENTAL

Apparatus

The gas chromatographic analyses were performed with a Model 900 gas chromatograph with a flame-ionization detector (Bodenseewerk Perkin-Elmer, Überlingen/See, G.F.R.). For the mass fragmentographic determinations a combination of a Model 2700 gas chromatograph and a CH 5 mass spectrometer (Varian-MAT, Bremen, G.F.R.) interfaced over a 30 cm × 0.1 mm I.D. platinum capillary was used. This system, combined with a Spectrosystem 100 MS computer (Varian-MAT), was also used for the mass spectrometric identifications and the selective profiles by computer mass fragmentography.

Reagents

The following chemicals were used: 3-heptanone (H 315-1, Ega-Chemie, Steinheim, G.F.R.), 4-heptanone (Ega-Chemie 10,174-5), cyclohexane (Merck, Darmstadt, G.F.R.), Triton X-100 (Serva Feinbiochemica, Heidelberg, G.F.R.), ammonium sulphate, ethanol and acetone (all from Merck).

Standard solutions

All standard solutions were prepared with doubly distilled water.

Internal standard for the 4-heptanone determination. A solution of $6.4 \cdot 10^{-6}$ g/ml of 3-heptanone in water containing 0.1% of Triton X-100 was used.

4-Heptanone standard solutions. Seven standard solutions with 4-heptanone in water containing 0.1% of Triton X-100 were prepared in the concentration range $1.52 \cdot 10^{-8}$–$1.52 \cdot 10^{-6}$ g/ml. The internal standard and 4-heptanone standard solutions are stable for at least 6 weeks.

Ethanol and acetone standard solutions. For each of the two compounds four aqueous standard solutions were prepared with the following concentrations: $3.95 \cdot 10^{-7}$, $7.9 \cdot 10^{-7}$, $7.9 \cdot 10^{-6}$ and $7.9 \cdot 10^{-5}$ g/ml.

Serum samples

Freshly collected venous blood was centrifuged for 10 min at 1500 *g*. The serum was separated from the cellular material and immediately subjected to the analysis or stored at 4°.

Adsorption technique, gas chromatography and mass spectrometry for the profile analysis

A mixture of 5 ml of serum and 10 g of ammonium sulphate in a 250-ml sample bottle which was kept in a water-bath at 90°, was extracted with helium according to the precedure described for urine[10]. Only one adsorption trap was used. When pooled serum was analysed, larger sample sizes (up to 25 ml of serum and 50 g of ammonium sulphate) were used. After the adsorption, the components were separated gas chromatographically as described elsewhere[10]. The separation was performed on a 100 m × 0.5 mm I.D. stainless-steel column, coated with Emulphor ON-870 (column A), with nitrogen as the carrier gas (or helium in the combination with the mass spectrometer) at a flow-rate of 5 ml/min. The temperature of the injector block for desorption of the substances was 300°, while the column temperature was kept at 60° for 16 min, then programmed to 175° at 2°/min and held at this temperature. All chromatograms were recorded at attenuation 32 with a full-scale recorder deflection of 5 mV. The mass spectrometric conditions are summarized in Table I. The total ion current was recorded with a secondary ion source (total pressure monitoring source). Computer mass fragmentograms from the mass spectrometric data on magnetic tape, using *m/e* 31 for alcohols and *m/e* 114 for 4-heptanone, were recorded on a Complot plotter (Houston Instruments, Bellaire, Texas, U.S.A.).

TABLE I

MASS SPECTROMETRIC CONDITIONS

Parameter	*Value*	*Parameter*	*Value*
Electron energy of ion source	70 eV	Multiplier voltage:	
Electron energy of total pressure monitoring source	20 eV	for profile analyses	2.9 kV
Accelerating voltage	3 kV	for 4-heptanone determination	3.1 kV
Ion source temperature	220°	for acetone and ethanol determination	2.75 kV
Interface temperature	220°	Attenuation of the multiplier signal for mass fragmentography	3
Resolution	500	Scan speed for profile analyses	2.5 sec/decade
Operating pressure	$4 \cdot 10^{-5}$ torr	Repetitive scanning in intervals of	7 sec
Emission current:		Mass range	*m/e* 15–280
for profile analyses	100 μA	Specific ions:	
for 4-heptanone determination	300 μA	for 4-heptanone	*m/e* 114
for acetone and ethanol determination	300 μA	for acetone	*m/e* 58
		for ethanol	*m/e* 31

Determination of 4-heptanone

A mixture of 2 ml of serum and 1 g of ammonium sulphate was heated in glass-stoppered and clamped vials for 90 min in a water-bath at 90° to complete the

decarboxylation of the 4-heptanone precursor. After cooling and addition of 0.1 ml of internal standard, the reaction mixture was extracted with 0.5 ml of cyclohexane. The cyclohexane phase was separated by centrifugation for 5 min at 1500 *g* and separated on column A at an oven temperature of 85° and a helium flow-rate of 5 ml/min. The injector block temperature was 150° and the size of the cyclohexane extract sample was 1 μl. The mass spectrometric conditions are summarized in Table I. The calculations of the concentrations were based on a calibration graph according to the procedure for urine[8].

Determination of acetone and ethanol

A stainless-steel column (100 m × 0.5 mm I.D.) coated with Emulphor ON-870 was prepared and used only for direct injections of aqueous solutions. For determinations of acetone the oven temperature was 50° and for ethanol 55°. A helium flow-rate of 5 ml/min was maintained for all analyses and the injector block was kept at 150°. An amount of 0.5 μl of serum was injected. At the end of each working day, the glass liner of the injection port was cleaned. The mass spectrometric conditions are described in Table I.

To by-pass the water in front of the mass spectrometer, a T-connection and two valves were used between the outlet of the column and the interface. All connections of the column, the interface, the T-connection and the valves were 1/16 in. The valves

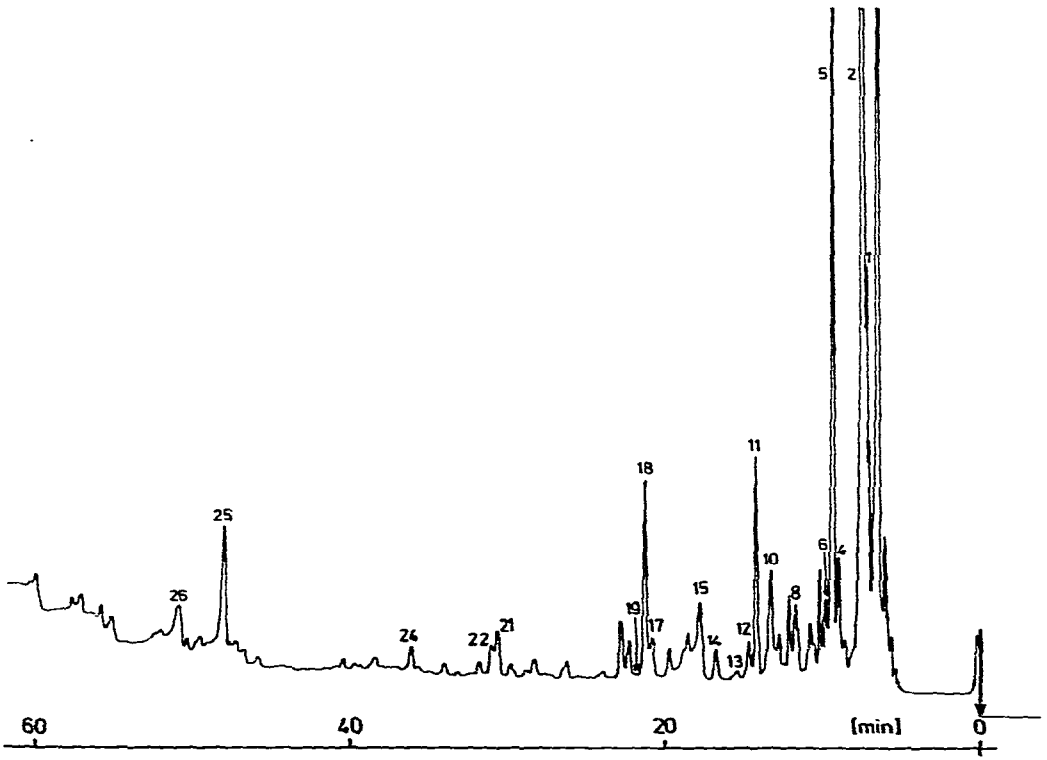

Fig. 1. Gas chromatogram of volatile compounds in serum of a normal individual. Sample size, 5 ml. Conditions as described in the text. Peaks: see Table II.

were mounted in such manner that they were operated from the outside of the gas chromatograph, while all connections were inside the oven. Two normal valves and the T-connection (Kontron-Technik, Eching, G.F.R.) were used instead of a three-way valve because they have a low dead volume and because they were found to perform well with respect to thermal stability and tightness. After the elution of the acetone or ethanol, the effluent of the column was by-passed by turning the valves. The gas flow into the ion source was shut off for 15 min. During this time the elution of the water was completed, and the system became ready for the next analysis.

The concentrations of acetone and ethanol were determined with calibration graphs for each of the substances.

RESULTS AND DISCUSSION

Profile analyses

In order to prevent non-volatile but extractable constituents of serum being transferred into the injection port of the gas chromatograph and thereby possibly causing artifacts by thermal decomposition at the desorption temperature of 240–300°, an adsorption technique analogous to that used for urine was chosen for the serum profiles. It has been shown for urine that by increasing the temperature of the sample during the adsorption process, the yield of the volatile substances in increased and the

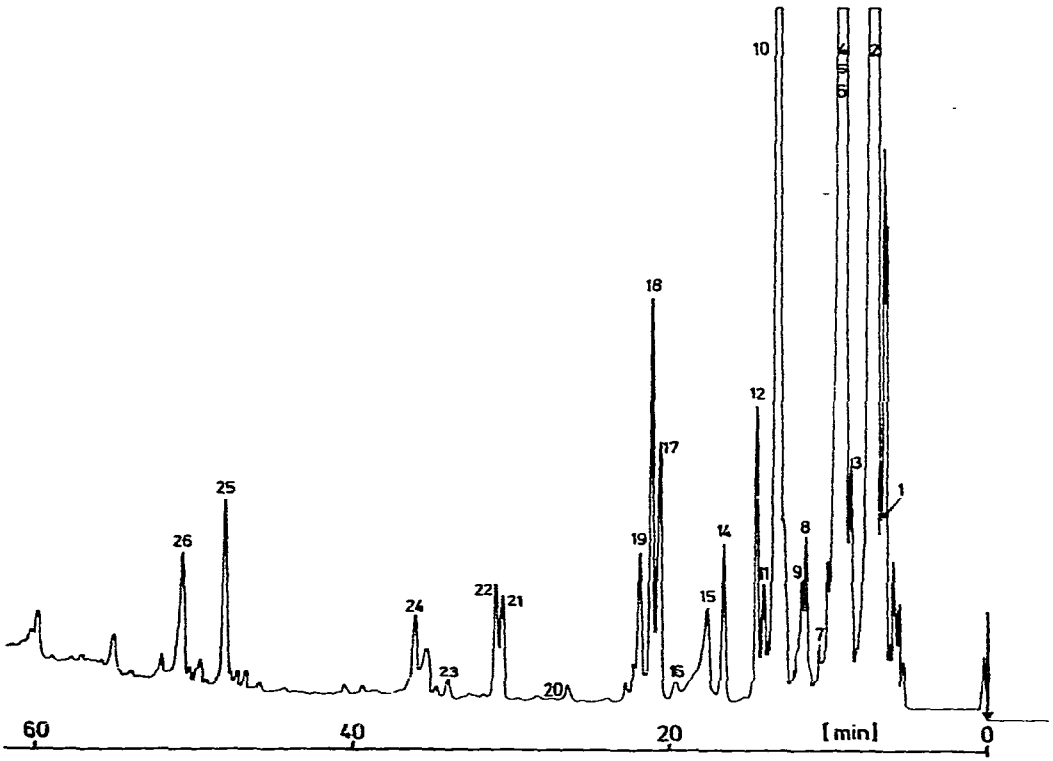

Fig. 2. Gas chromatogram of volatile compounds in pooled serum from patients. Sample size, 25 ml. Conditions as described in the text, except attenuation 64. Peaks: see Table II.

TABLE II

SUBSTANCES INDICATED BY NUMBERS IN FIGS. 1 AND 2

Peak No.	*Compound*	*Peak No.*	*Compound*
—	Acetaldehyde	13	Dimethyl disulphide
1	Propionaldehyde	14	Isobutanol
2	Acetone	15	Hexanal
3	Methanol	16	3-Penten-2-one
4	Butanone	17	*n*-Butanol
5	Ethanol	18	A pentenol (tentative)
6	Isopropanol	19	4-Heptanone
7	2,3-Dimethylfuran	20	Heptanal
8	Pentanone	21	*n*-Pentanol
9	Pentanal	22	Styrene
—	Chloroform	23	Cyclohexanone
10	*n*-Propanol	24	C_9 aromatic
11	Toluene	25	An octenol (tentative)
12	Dioxane	26	Benzaldehyde

adsorption time shortened[10]. For serum the temperature is limited to 55–60°; above this temperature, serum clots and cannot be stirred effectively, resulting in a low yield. By adding glass beads or salt to the serum, continuous stirring is possible at a temperature of 90°. Ammonium sulphate, conditioned at 200° prior to its use, was found to be the most suitable additive. It deproteinates the serum, resulting in a mixture of protein precipitate, excess of ammonium sulphate and deproteinated serum. Only slight contamination is introduced by the ammonium sulphate.

For gas chromatographic profiles, selective profiles by computer mass fragmentography and for mass spectrometric identifications, *ca.* 5 ml of serum are required. The concentrations of most of the compounds in serum are estimated to be generally lower than in urine. This is in agreement with the quantitative determinations of three of the constituents, total 4-heptanone, acetone and ethanol (total 4-heptanone

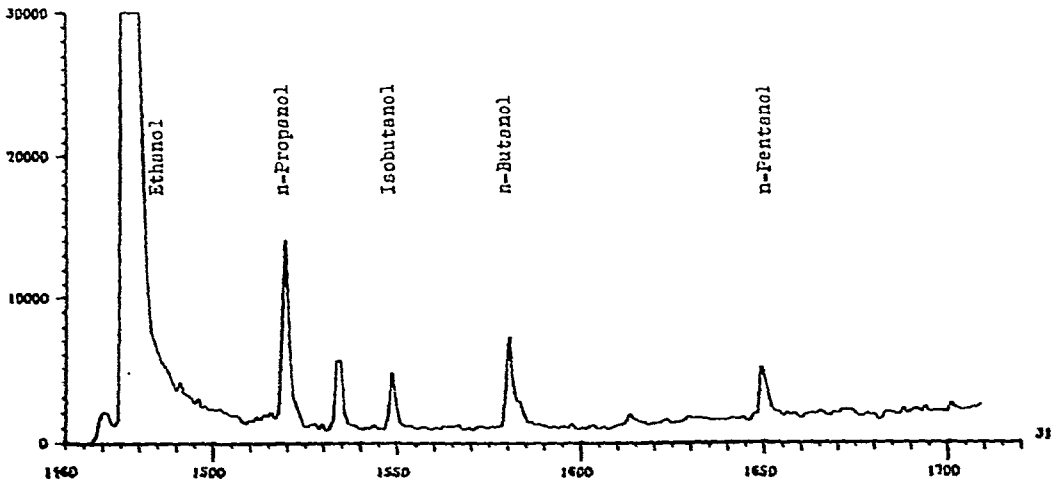

Fig. 3. Computer mass fragmentogram of alcohols in serum, *m/e* 31.

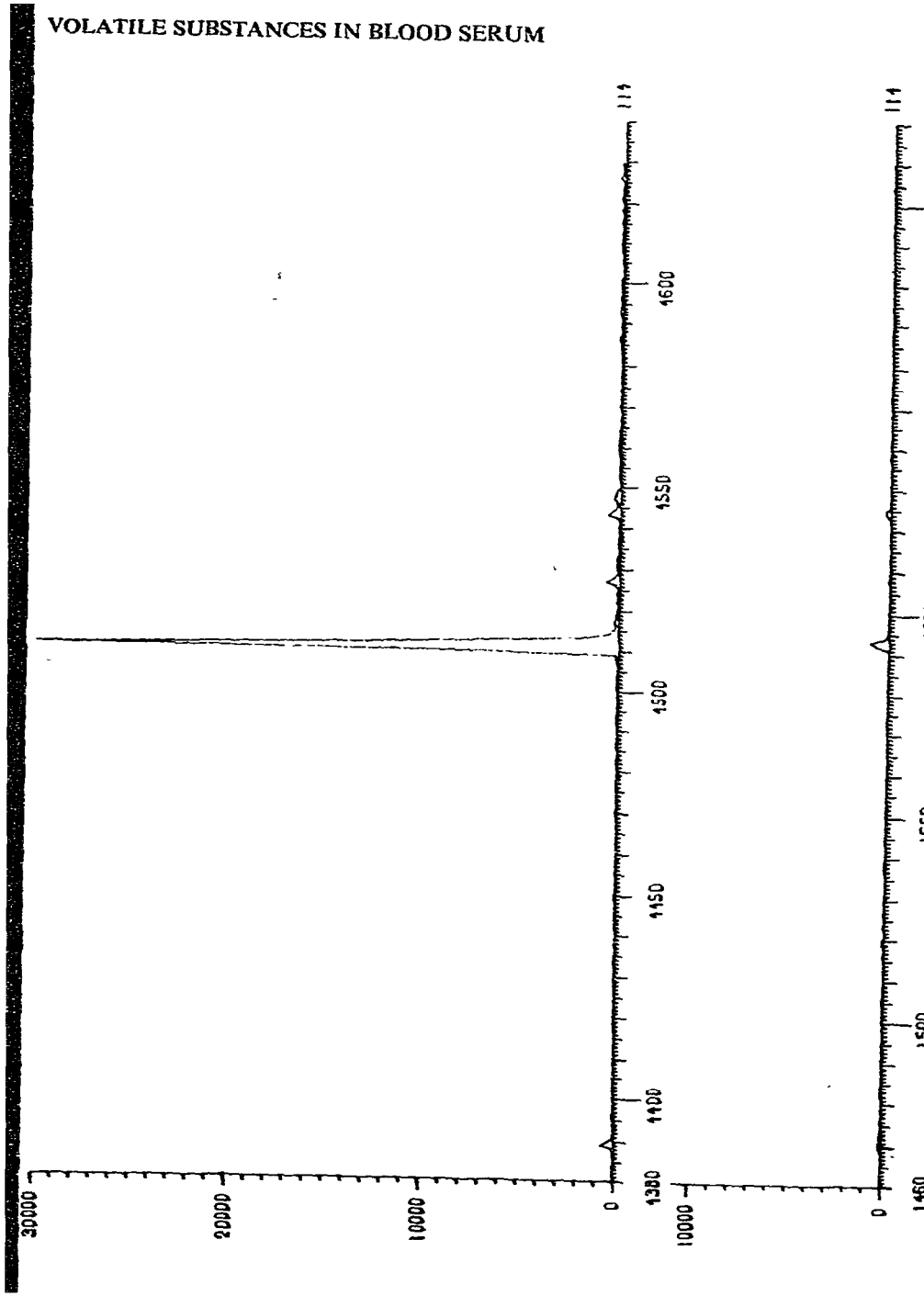

Fig. 4. Computer mass fragmentogram of 4-heptanone, *m/e* 114. Top: patient with chronic renal insufficiency. Bottom: normal subject.

means the sum of free 4-heptanone and the 4-heptanone formed from its precursor by decarboxylation).

As demonstrated in two examples, the profile of serum of a normal individual (Fig. 1) and the profile of a pooled serum of hospital patients (Fig. 2), mainly acetone, ethanol and, in some of the samples, *n*-propanol, occur in higher concentrations. Table II summarizes the constituents that were identified by mass spectrometry. In addition to these compounds, benzene, C_8 aromatics, dichlorobenzene and trace amounts of aliphatic hydrocarbons were found. However, these components were also detected in the blanks.

Compared with the volatile substances in urine, the percentage of alcohols among the total volatile components in serum is higher and the percentage of ketones is lower. This result indicates that alcohols are subject to a higher degree of re-absorption in the kidneys and further metabolization in the body than the ketones and ketone precursors. Fig. 3 shows a typical example of serum alcohols. In all of the serum samples studied *n*-pentanol, which is not normally found in urine, was detected. The concentration of total 4-heptanone in the serum of normal persons is very low (10–50 nmole/l according to the quantitative determination). In the serum of patients with strong renal insufficiency its concentration is increased approximately 10- to 40-fold (Fig. 4), apparently owing to a reduced filtration capacity.

Quantitative determination of 4-heptanone

By analogy with the procedure described for urine[8], total 4-heptanone was determined in serum. Ammonium sulphate was added in order to deproteinate the serum and prevent the sample from clotting. After heat transformation of the precursor into the ketone and addition of the internal standard, the reaction mixture was extracted. By direct mass fragmentographic determination on the unconcentrated

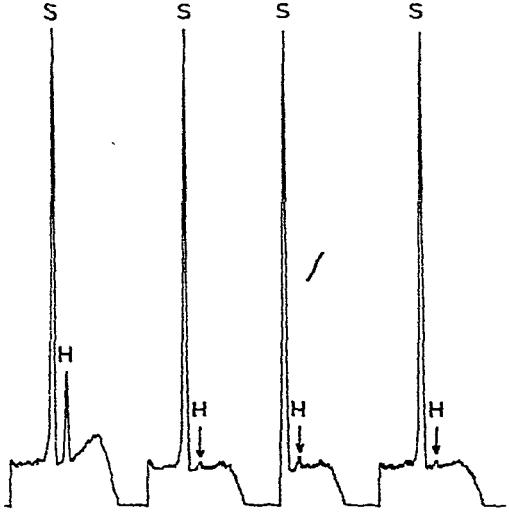

Fig. 5. Mass fragmentograms of four serum samples, *m/e* 114. H = 4-heptanone; S = internal standard. The three samples on the right-hand side are of patients without kidney defects; the sample on the left-hand side is pooled serum from patients with renal insufficiencies. Recorder, 10 mV. Overlapping injections were made.

extract (Fig. 5), the serum levels of total 4-heptanone in normal subjects were estimated to range between 10 and 50 nmole/l, which is lower by a factor of *ca.* 100 than in urine (900–3500 nmole per 24 h). This result indicates a high renal excretion of total 4-heptanone and low re-absorption. In cases of renal insufficiency, the excretion of total 4-heptanone is reduced. In five patients who had to undergo haemodialysis, serum levels between 350 and 1000 nmole/l were determined (Figs. 5 and 6). The concentrations of total 4-heptanone in serum of normal individuals (10–50 nmole/l) are in the range of the detection limit of the procedure. More exact measurements in this range are possible by concentrating the extract and by using lower concentrations of internal standard.

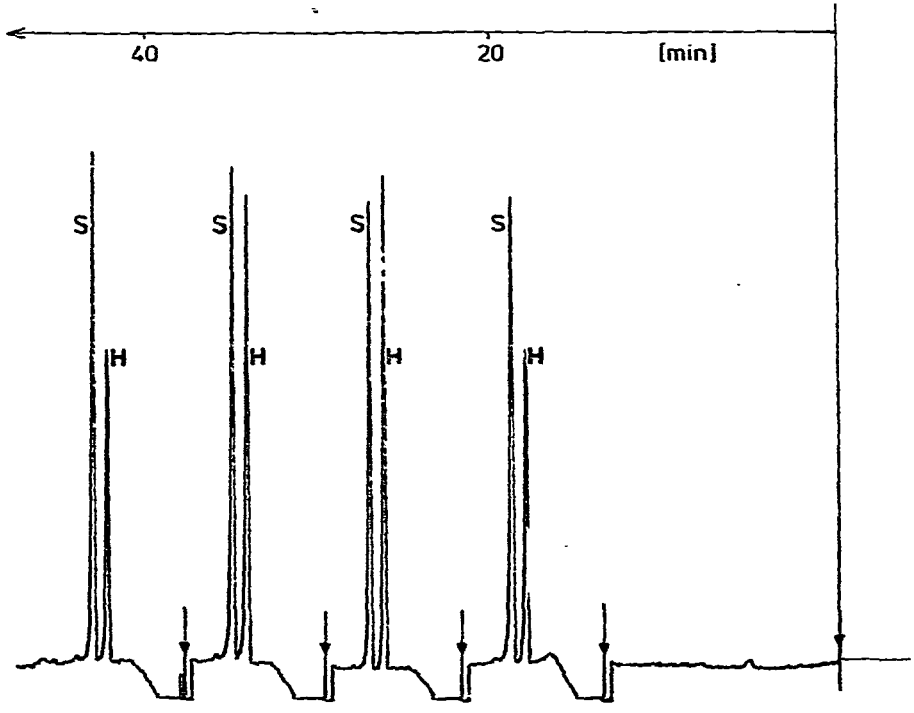

Fig. 6. Mass fragmentograms, *m/e* 114, of serum samples from four patients with chronic renal failure. H = 4-heptanone; S = internal standard. Recorder, 10 mV for H, 20 mV for S. Overlapping injections were made.

Quantitative determination of acetone and ethanol

Because the extraction of hydrophilic substances causes more problems than the extraction of ketones, we attempted a mass fragmentographic determination of such substances by direct injection of serum, using ethanol and acetone as examples. Only a few data are available on physiological concentrations of ethanol in serum, and the published values for normal concentrations of acetone in blood or serum vary considerably. In order to avoid irreproducible sensitivity changes and sensitivity losses in the mass spectrometer system due to water entering the ion source, the water was by-passed using a valve system between outlet of the gas chromatographic column and interface to the ion source. A slow deterioration of the column can be tolerated. The results are shown in Figs. 7 and 8.

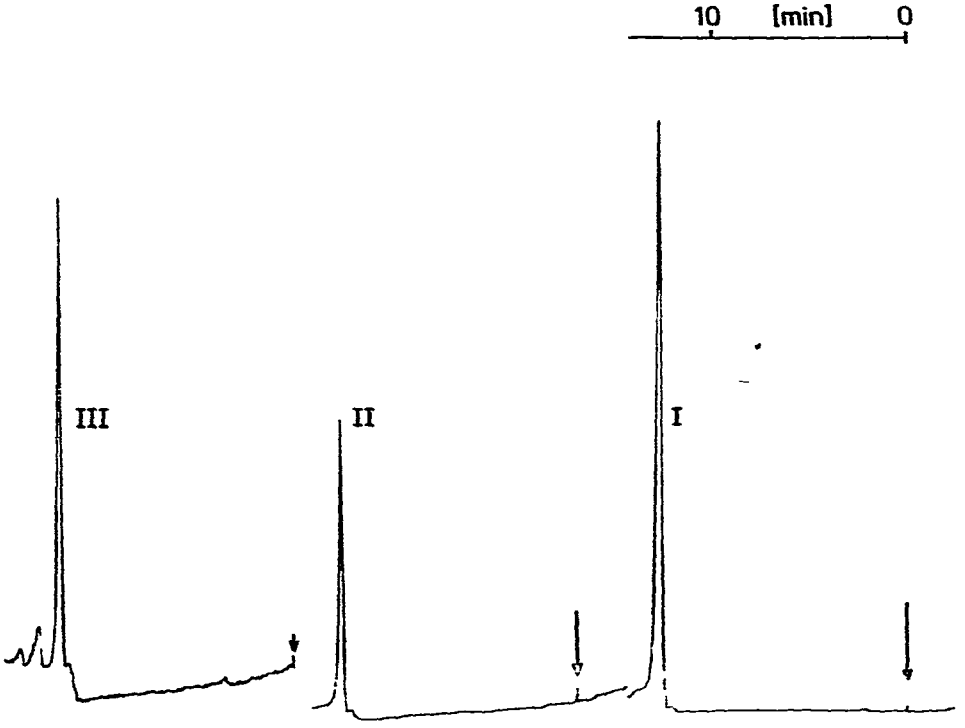

Fig. 7. Mass fragmentograms for acetone determination, *m/e* 58. I, 7.9 µg/ml acetone standard, recorder 50 mV; II, serum sample, recorder 50 mV; III, serum sample, recorder 20 mV.

Depending on the working conditions, different amounts of acetoacetic acid are decarboxylated to acetone[23,24]. Only with headspace analysis does it seem possible to determine the momentarily present acetone[25]. Under the conditions of this study, total acetone (which means free acetone and acetoacetic acid which decarboxylates in the injector block) is determined. Complete decarboxylation under the conditions described has been demonstrated for urine. Heating the urine at 90° for 40 min in tightly closed vials prior to analysis did not increase the concentration of acetone. By using this procedure, complete decarboxylation occurs[25].

Using the ion at *m/e* 58 for the mass fragmentographic determination of acetone, no interfering substances were observed in serum, except propionaldehyde, which is present in low concentration. The gas chromatographic column must be tested prior to its use, if it separates this pair of compounds. Isopropanol may interfere in the determination of ethanol with the fragment ion at *m/e* 31. While for ethanol the relative intensity of *m/e* 31 is 100%, it is only 5% for isopropanol. Therefore, interferences can be neglected provided that the concentration of isopropanol is low. If the separation of isopropanol from ethanol is not sufficient, a correction is possible by measuring the isopropanol level using the ion at *m/e* 60.

The calculations of the concentrations of acetone and ethanol are based on calibration graphs obtained with aqueous standards. For each series a new calibration graph is determined. The procedure could be improved be using internal standards. The determination of 10 randomly selected serum samples from adult patients

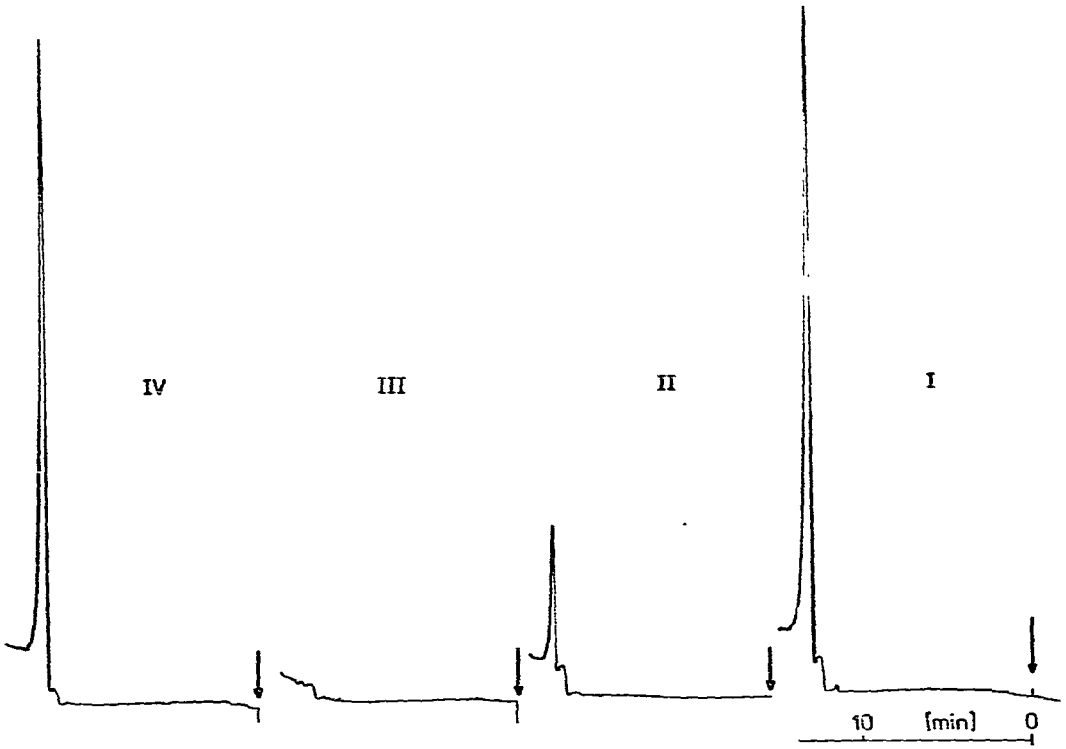

Fig. 8. Mass fragmentograms for ethanol determination, *m/e* 31. I, Pooled serum sample; II, serum sample; III, water blank; IV, 7.9 μg/ml ethanol standard. For I–IV the recorder was set at 50 mV for full-scale deflection.

without obvious metabolic abnormalities gave acetone levels between 25 and 85 μmole/l and ethanol levels between 10 and 170 μmole/l. These total acetone levels are in agreement with gas chromatographically determined acetone[23,24] and enzymatically determined acetoacetic acid[26].

The method of direct injection of serum or urine in quantitative mass fragmentographic determinations of volatile substances should be considered for all substances that are not readily extractable.

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