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GAS CHROMATOGRAPHIC–MASS SPECTROMETRIC STUDY OF VOLATILE ORGANIC METABOLITES IN URINES OF PATIENTS WITH *DIABETES MELLITUS*

H. M. LIEBICH and O. AL-BABBILI
Medizinische Universitätsklinik, Abteilung IV, Otfried-Müller-Strasse, 74 Tübingen (G.F.R.)

SUMMARY

Abnormally increased concentrations of the aliphatic alcohols ethanol, *n*-propanol, isobutanol, *n*-butanol and isopentanol and the ketones 4-heptanone and cyclohexanone in human urine reflect metabolic disorders related to *diabetes mellitus*. For the determination of these low-molecular-weight metabolites, the components are trapped on an adsorbent, separated by gas chromatography and identified by mass spectrometry. After standardization of the adsorption and desorption techniques, the procedure is applicable for comparative studies and for screening.

INTRODUCTION

Disorders in the metabolism of carbohydrates, lipids and proteins that are related to *diabetes mellitus* can be recognized in abnormalities of a variety of biochemical parameters, including elevated blood glucose level, inadequate glucose tolerance, reduced insulin activity in blood, glucose in urine, acetone in urine and, during the stage of acute decompensation, diabetic ketoacidosis or lactic acidosis.

Profile studies of volatile metabolites in urine using gas chromatography (GC), mass spectrometry (MS) and a sampling technique for the low-molecular-weight and GC volatile substances have been recently introduced[1–5]. It was expected that in addition to the established clinical-chemical parameters, substances of the group of volatile metabolites are excreted in urine that might be characteristic of *diabetes mellitus* and could be used for diagnosis[2]. Profiles of these low-molecular-weight metabolites have been studied in normal subjects showing a wide variety of constituents, part of which were identified by MS. The concentrations of the different substances, mainly ketones, aldehydes, alcohols, sulphur compounds, furan derivatives and pyrroles, were estimated to range between 10 ng and 200 μg per 24-h urine. In normal urines, the concentrations are subject to physiological variations with relatively narrow normal ranges for compounds such as 2-pentanone, 4-heptanone, 2-heptanone and pyrrole and very broad normal ranges for other constituents, *e.g.*, allyl isothiocyanate and carvone, which in some normal urines are absent. No obvious differences were found in the patterns of males and females.

This paper deals with abnormalities in the profile of low-molecular-weight

components in urines of patients with *diabetes mellitus*. An attempt is made to standardize the procedure and make it useful for screening investigations of a large number of patients and samples over an extended period of time.

EXPERIMENTAL

Adsorption technique

The volatile urinary constituents were concentrated in the following manner. To 5% of a 24-h urine sample an aliquot of 20 g of ammonium sulphate per 100 ml of urine was added in order to increase the volatility of the low-molecular-weight organic compounds. The mixture was placed in a 500-ml sample bottle in a water-bath at 90° and stirred with a magnetic stirrer while helium was passed over the urine at the flow-rate of 20 ml/min for 1 h. The volatile components were carried with the helium flow through a water condenser of 10 cm length into two parallel glass tubes (11 cm × 10 mm O.D.) containing 2 ml of the adsorbing material, Tenax GC, which is a porous polymer of 2,6-diphenyl-*p*-phenylene oxide with a particle size of 35–60 mesh (Applied Science Labs., State College, Pa., U.S.A.). Cooling water at 12 ± 1° was supplied over a thermostat.

Gas chromatographic separation

A Model 900 gas chromatograph with a flame ionization detector (Bodenseewerk Perkin-Elmer, Überlingen/See, G.F.R.) was used for separation. By inserting the adsorbent trap into the injector block, the dimensions of which were designed to accept the trap, the components were desorbed at 300° within 10 min. The compounds were re-condensed in a 1 m × 0.75 mm I.D. pre-column, cooled with liquid air, using a flow-rate of 15 ml/min. After re-condensation, the pre-column and separating column (column A, 100 m × 0.5 mm I.D.; column B, 200 m × 0.5 mm I.D.; both stainless steel) were connected. Unless indicated otherwise in the figure legends, column A was used. The pre-column and separating columns were coated with Emulphor ON-870 (Supelco, Bellefonte, Pa., U.S.A.). The GC separations were performed with nitrogen as the carrier gas at the flow-rate of 5 ml/min and a column temperature of 60° for 16 min, then programmed to 175° at 2°/min and held at that temperature (attenuation 256). Identical GC conditions were used in the combination with MS. The integration of peak areas was effected with a Varian Aerograph Model 480 integrator.

Mass spectrometric identification

A combination of a Model 2700 gas chromatograph, a CH5 mass spectrometer and Spectrosystem 100 MS instrument (Varian-MAT, Bremen, G.F.R.) was used. The gas chromatograph and mass spectrometer were directly connected over a 30 cm × 0.1 mm I.D. platinum capillary interface. The ion source of the mass spectrometer was equipped with a high-efficiency oil diffusion pump (600 l/sec), permitting the total effluent from the GC column to enter the ion source. Mass spectra were recorded exponentially in the mass range *m/e* 15–280 at a scan rate of 2.5 sec/decade applying the mode of automatic repetitive scanning. With a programmed delay of 4 sec after each scan, one spectrum was recorded approximately every 7 sec. The experimental conditions are summarized in Table I. The total ion current was recorded with a

TABLE I

EXPERIMENTAL CONDITIONS FOR MASS SPECTROMETRY

Parameter	*Value*
Electron energy of ion source	70 eV
Electron energy of total pressure monitoring source	20 eV
Emission current	100 μA
Accelerating voltage	3 kV
Multiplier voltage	2 kV
Ion source temperature	220°
Interface temperature	220°
Resolution	750
Operating pressure	$5 \cdot 10^{-5}$ torr

second ion source (total pressure monitoring source). All mass spectra were stored on magnetic tape. The substances were identified from their mass spectra and the identifications were confirmed by comparison with the spectra of reference compounds.

RESULTS AND DISCUSSION

The methodology used in this study is qualitative with some semi-quantitative character. It involves an adsorption technique with limited quantitative application to the entire group of urinary volatiles. Using standards, quantitation can be achieved for selected components. However, reproducible results over a long period of comparisons are obtained for all constituents of the profile when the technique is strictly standardized. In this way, it is possible to screen normal individuals and diabetic patients, to run comparative studies and to recognize pathological patterns of compounds.

Standardization of the method

Urine must be carefully collected by the patient over a period of 24 h. In this study, no polyuria with more than 4000 ml and no oligouria with less than 300 ml of urine were studied. Aliquots of the total urine volume, aliquots of ammonium sulphate, sampling temperature, helium flow-rate and adsorption time must be carefully controlled.

Effect of the temperature of the cooling water

The temperature of the cooling water for the condenser affects the yield of volatile compounds and must be kept constant to within ±1°. The effect of variations in the water temperature on the adsorption efficiency is shown in Figs. 1 and 2. The numbers of the peaks in Figs. 1 and 2 (and also in Figs. 6–11) refer to the substances listed in Table II. At 5°, the greatest yield of very low-boiling constituents is observed, while higher boiling compounds are reduced in concentration. At 12°, the loss at the low end of the profile is tolerable while the concentration at the high end is improved. A temperature of 12° is considered to be optimal. At 16°, a distinct loss is observed for the low-boiling materials, and higher temperatures are not useful. At 30°, the trapping efficiency is low for both the low-boiling and high-boiling portions of the profile.

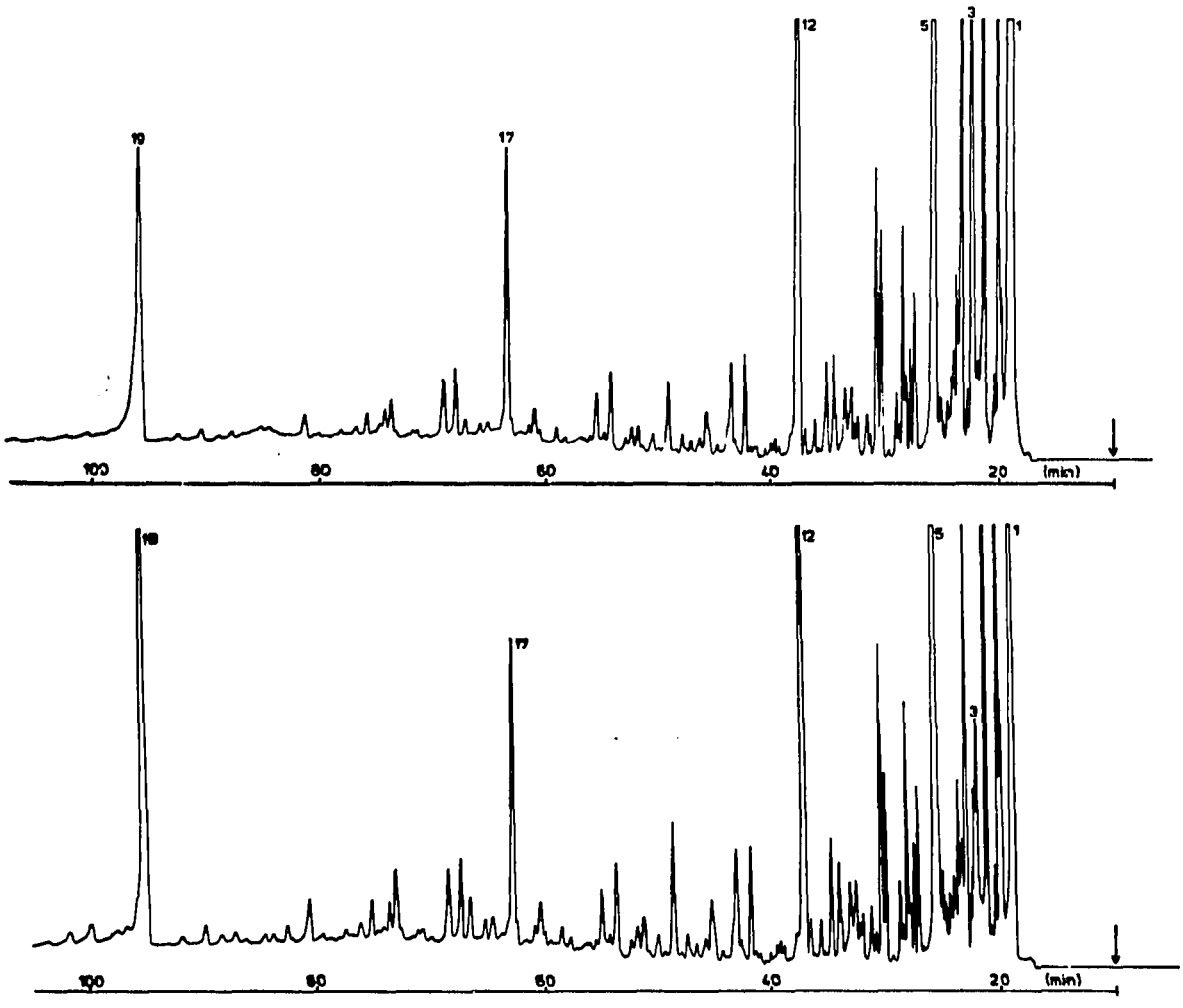

Fig. 1. Gas chromatogram of volatile compounds in a normal urine (column B). Top: cooling water temperature 5°. Bottom: cooling water temperature, 12°.

Effect of the desorption temperature

A constant desorption temperature is critical for comparison purposes. A standard solution of 150 ng of each of the compounds 2-pentanone, toluene, *n*-butanol, 4-heptanone, 2-heptanone and cyclohexanone in 0.5 μl of acetone was injected on Tenax GC and then processed in the same manner as the urine samples. The chromatograms in Figs. 3–5 show that low desorption temperatures result in significant losses for the ketones in the standard. A temperature of 300° is recommended as the desorption temperature.

Sampling temperature and sampling time

In order to accelerate and complete the collection of the volatile compounds on the adsorbent, the urine is heated at 90° in a water-bath. Lower water-bath tem-

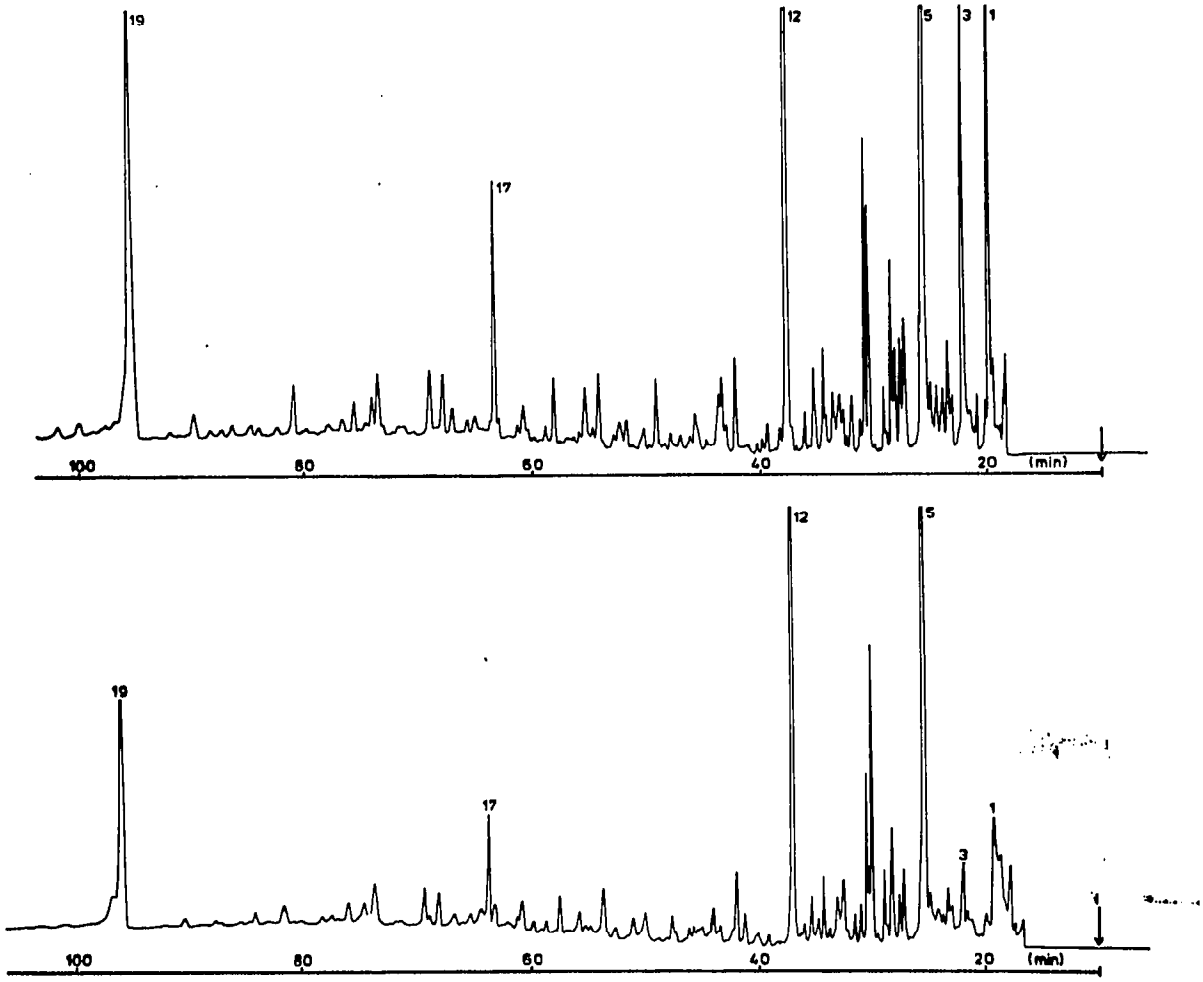

Fig. 2. Gas chromatogram of volatile compounds in a normal urine (same urine as in Fig. 1, column B). Top: cooling water temperature 16°. Bottom: cooling water temperature, 30°.

TABLE II

SUBSTANCES INDICATED BY NUMBERS IN FIGS. 1, 2 AND 6–11

Peak	*Component*	*Peak*	*Component*
1	Acetone	12	4-Heptanone
2	2-Butanone	13	Isopentanol
3	Ethanol	14	2-Heptanone
4	2,3-Butanedione	15	Cyclohexanone
5	2-Pentanone	16	Allyl isothiocyanate
6	*n*-Propanol	17	Pyrrole
7	Dimethyl disulphide	17a	Butenyl isothiocyanate
9	3-Penten-2-one	18	Benzaldehyde
10	N-Methylpyrrole	19	Carvone
11	*n*-Butanol		

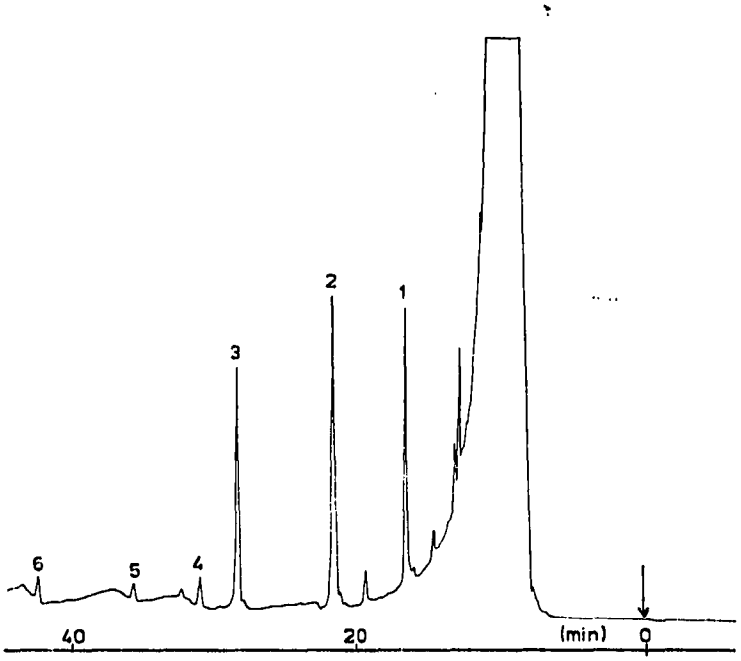

Fig. 3. Gas chromatogram of standard mixture. Peaks: 1 = 2-pentanone; 2 = toluene; 3 = *n*-butanol; 4 = 4-heptanone; 5 = 2-heptanone; 6 = cyclohexanone. Desorption temperature, 220°.

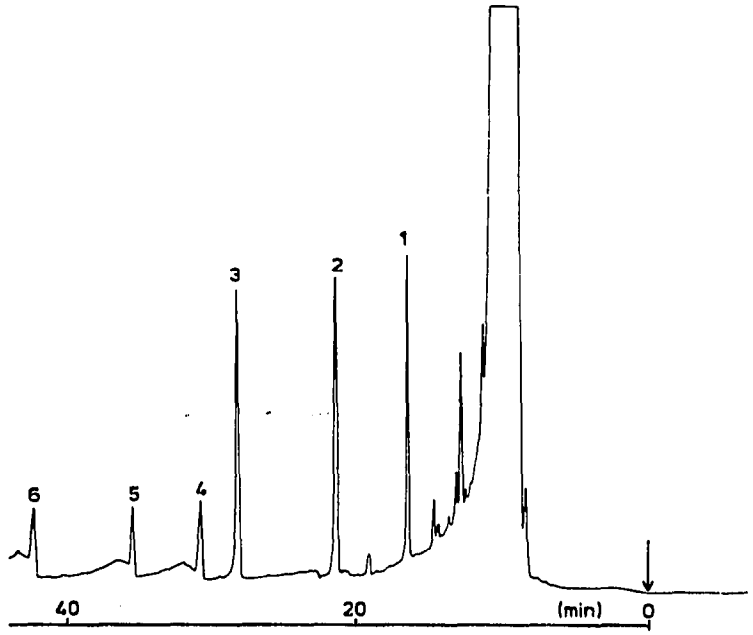

Fig. 4. Gas chromatogram of standard mixture. Peaks as in Fig. 3. Desorption temperature, 270°.

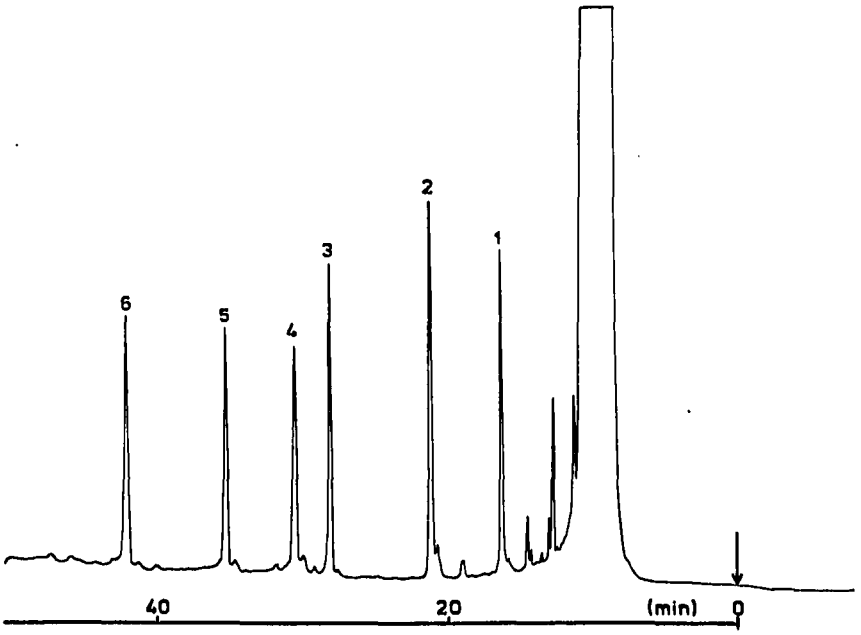

Fig. 5. Gas chromatogram of standard mixture. Peaks as in Fig. 3. Desorption temperature, 300°.

peratures produce lower yields of volatile compounds, which can be partially compensated for by using a longer collection time. Fig. 6 demonstrates the comparison of sampling for 1 h at 90° with sampling of the same urine for 15 h at 30°. Apart from the longer analysis time, a disadvantage of low-temperature sampling is that stringent precautions are necessary in order to reduce the amount of impurities emanating from the helium and the laboratory atmosphere. In addition, the very low-boiling material is not trapped effectively on the adsorbent over the long sampling period.

The use of a urine temperature of 90° involves a risk of generating artifacts from thermally labile urinary constituents. The comparison of the two chromatograms in Fig. 6 shows that this is not an important factor with respect to the compounds studied here. In addition, the urinary constituents described here were also identified by an extraction procedure that involves milder conditions[1].

Assay of urines of patients with diabetes mellitus

A selection of 54 samples of 18 hospitalized patients with overt *diabetes mellitus* was studied according to the standardized procedure. The patients were receiving different therapies: control by diet, control by oral antidiabetic medication (sulphonylurea, biguanide) and control by insulin. At least one sample of each patient was analyzed by combined GC–MS in order to identify and confirm the compounds that are considered to be characteristic parameters among the low-molecular-weight metabolites in urine. Fig. 7 gives a typical example of the analysis of a diabetic urine by GC–MS. Compared with the chromatograms from the flame ionization detector, the monitoring

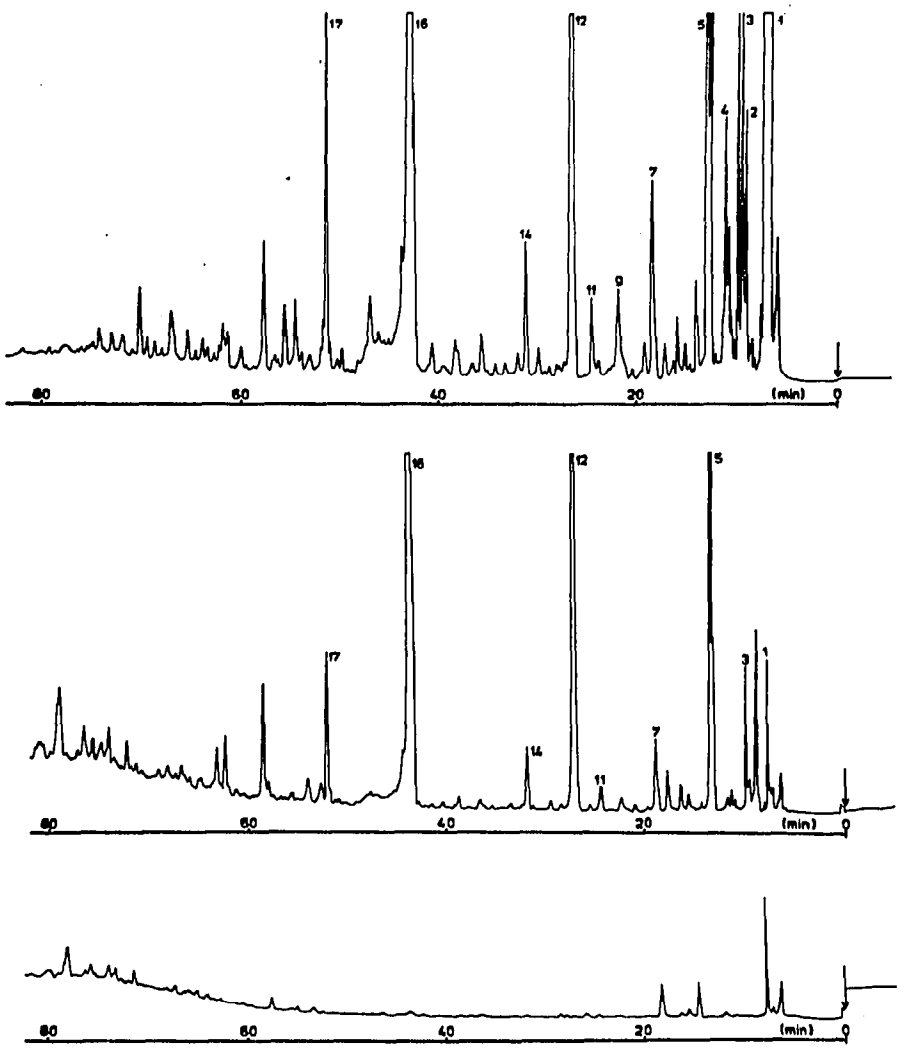

Fig. 6. Gas chromatogram of volatile compounds in a normal urine. Top: urine temperature 90°, collection time 1 h. Middle: urine temperature 30°, collection time 15 h. Bottom: blank for 30° and collection time 15 h.

of the total ion current includes a large peak for water, which is also trapped on the adsorbent.

As etiology, first occurrence, symptoms, clinical–chemical values, severeness, therapy, duration, complications and control of diabetes vary widely, it is to be expected that the constituents under investigation do not follow a uniform pattern. The selection of chromatograms shown in Figs. 9–11 demonstrates this fact very clearly. Fig. 8 depicts a normal control. However, a number of components excreted in the urines of diabetic patients can be considered to be very typical metabolic parameters, the increase or decrease in concentration of which characterizes disorders connected with *diabetes mellitus*. These parameters are the primary aliphatic alcohols

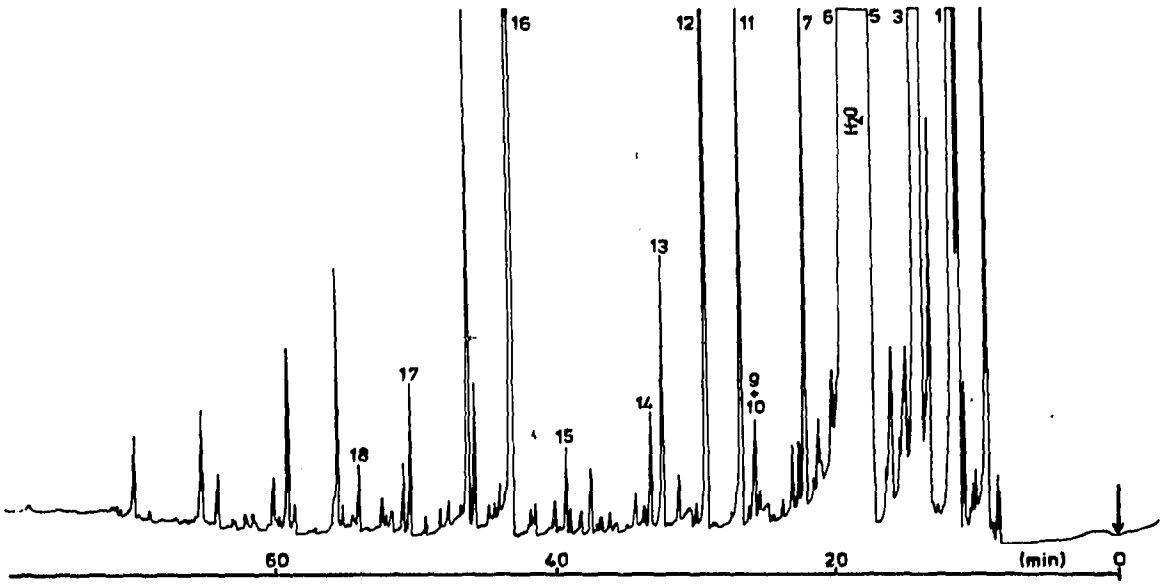

Fig. 7. Volatile compounds in urine of patient A (see Table III). Recorded total ion current from total pressure monitoring source.

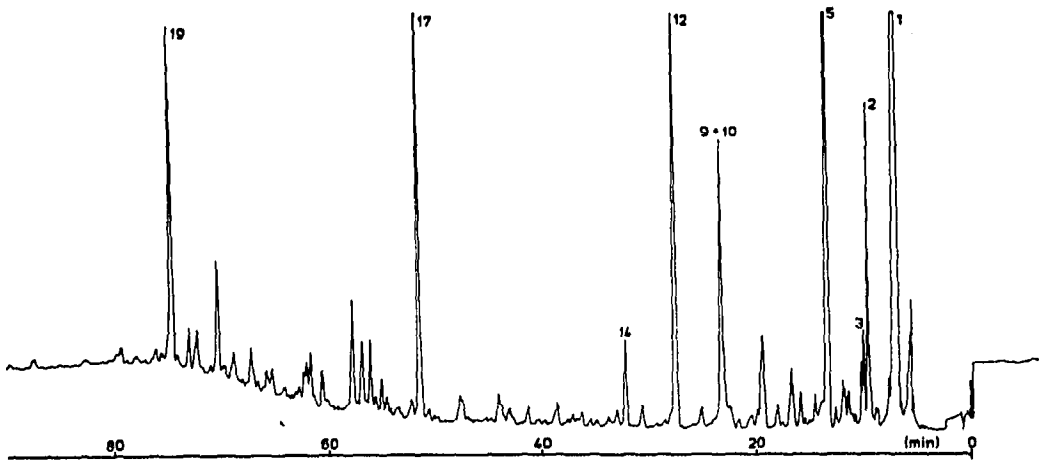

Fig. 8. Gas chromatogram of volatile compounds in urine of a 28-year-old normal male.

ethanol, *n*-propanol, isobutanol, *n*-butanol and isopentanol and the ketones 4-heptanone and cyclohexanone. The concentrations of the substances were compared on the basis of peak areas. "Normal ranges" were derived from the peak areas in the chromatograms of normal urines.

Ethanol, *n*-propanol, isobutanol and *n*-butanol are excreted in normal urines, but only ethanol is detectable in larger concentration. Isopentanol is not found in normal subjects. In most urines of patients with diabetes, some or all five of the aliphatic alcohols are increased in concentration. During the study, normal subjects and diabetic

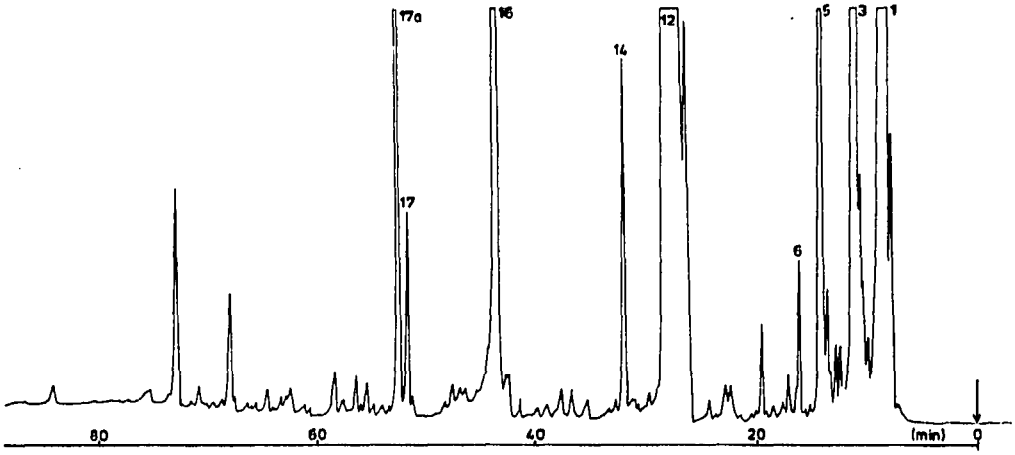

Fig. 9. Gas chromatogram of volatile compounds in urine of patient B (see Table III).

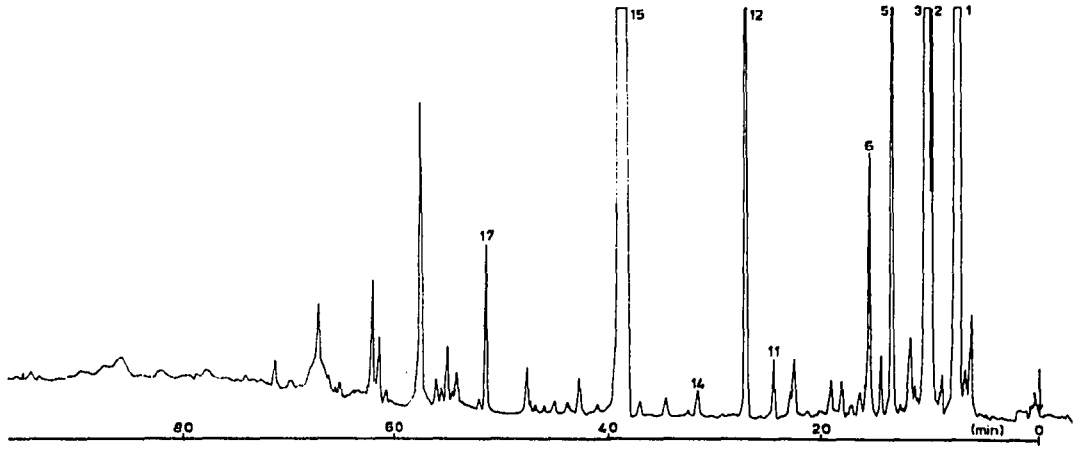

Fig. 10. Gas chromatogram of volatile compounds in urine of patient C (see Table III).

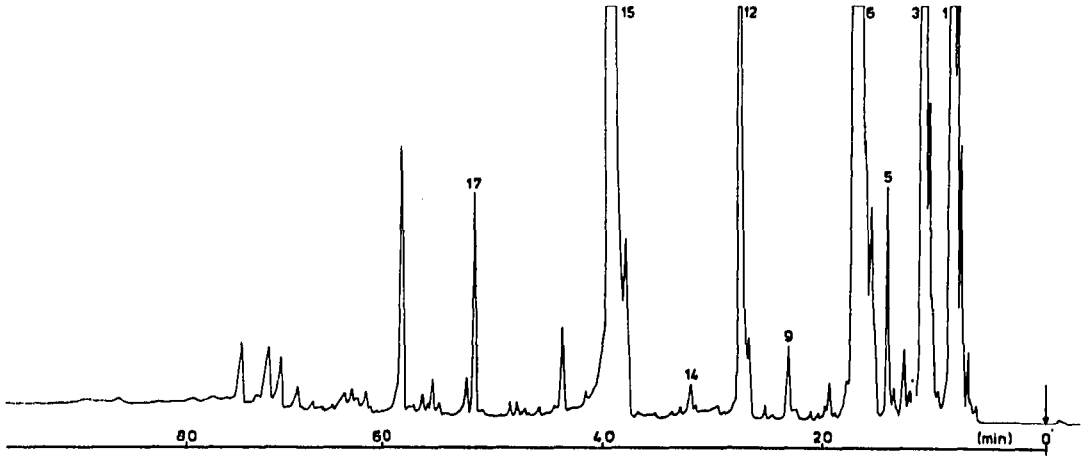

Fig. 11. Gas chromatogram of volatile compounds in urine of patient D (see Table III).

patients did not drink any alcoholic beverages because the consumption of ethanol increases the concentration of ethanol in urine. In the 54 samples of 18 patients, elevated concentrations of the alcohols were observed as follows: ethanol in 48 samples (89%), *n*-propanol in 47 samples (87%), isobutanol in 37 samples (69%), *n*-butanol in 30 samples (56%) and isopentanol in 15 samples (28%).

The C_7-ketone 4-heptanone is consistently found in normal urines among the compounds of higher concentration (estimated mean value for 4-heptanone = 50 μg per 24-h urine), whereas cyclohexanone occurs only in trace amounts. In diabetic urines, elevated 4-heptanone levels were found in 18 samples (33%) and cyclohexanone in 29 samples (54%).

Figs. 7 and 9–11 show examples of cases with extreme elevations of one or several parameters. The concentrations of ethanol and *n*-propanol were increased in all four examples, with an extreme value for *n*-propanol in the urine of a 75-year-old patient whose diabetes was diagnosed at the age of 58 (patient D). Isobutanol and *n*-butanol levels were elevated in the case of a 55-year-old patient with diabetes diagnosed at the age of 37 (patient A) and in the case of a 66-year-old patient with diabetes diagnosed at the age of 50 (patient C). In both cases the isobutanol level was slightly increased. Isopentanol was found only in patient A. Pathologically high concentrations of cyclohexanone were observed in the urines of patients A, C and D with extreme values for C and D. Patient B, aged 20, with *diabetes mellitus* since 3 years of age, is an example of an extremely high 4-heptanone level. On the day the urine was collected, this patient developed hypoglycaemic shock with a blood glucose concentration of 17 mg per 100 ml. The 4-heptanone level decreased on the following day

TABLE III

CLINICAL AND CLINICAL-CHEMICAL DATA ON PATIENTS

Patient	*Sex*	*Age*	*Diabetes since age*	*Therapy*	*Blood glucose*		*Urine glucose**	*Urine acetone**	*Increased values*
					Time	*mg per 100 ml*			
A	Female	55	37	Nadisan®	8:00	145	—	—	Ethanol, *n*-propanol, isobutanol, *n*-butanol, isopentanol, cyclohexanone
					11:00	185			
					16:00	186			
B	Male	20	3	28 + 16 U insulin	8:00	17	+++	—	Ethanol, *n*-propanol, 4-heptanone
					11:00	181			
					16:00	62			
C	Female	64	50	60 U insulin	8:00	400	++++	—	Ethanol, *n*-propanol, isobutanol, *n*-butanol, cyclohexanone
					11:00	305			
					16:00	376			
D	Female	75	58	Diet	8:00	79	—	—	Ethanol, *n*-propanol, cyclohexanone
					11:00	152			
					16:00	171			

* —, negative; +, ++, +++ and ++++, positive on a relative scale.

when the blood glucose level was controlled. However, during the entire control period, 4-heptanone never decreased to the normal level. Clinical and clinical-chemical data on the patients are summarized in Table III.

The results of our study of 54 selected urines of patients with *diabetes mellitus* indicate that the aliphatic alcohols ethanol, *n*-propanol, isobutanol, *n*-butanol and isopentanol and the ketones 4-heptanone and cyclohexanone are substances whose abnormal concentrations in urine are typically related to metabolic disorders in patients with diabetes. Depending on form, stage and severeness of the disease, one or several of these parameters are changed in concentration. With increased concentrations in 89% and 87% of the samples studied, ethanol and *n*-propanol seem most indicative of *diabetes mellitus*. Isopentanol, cyclohexanone and 4-heptanone are more specific for certain forms, stages and situations of the disease. Further studies are necessary in order to find detailed correlations between these metabolites and the clinical aspects of *diabetes mellitus*.

ACKNOWLEDGEMENT

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