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VOLATILE METABOLITES IN SERA OF NORMAL AND DIABETIC PATIENTS

ALBERT ZLATKIS*, COLIN F. POOLE and ROSWITHA BRAZELI

Department of Chemistry, University of Houston, Houston, Texas 77004 (U.S.A.)

DONALD A. BAFUS

Northrup Services, NASA, Houston, Texas 77058 (U.S.A.)

and

PETER S. SPENCER

Institute of Neurotoxicology, Albert Einstein College of Medicine, Bronx, N.Y. 10461 (U.S.A.)

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SUMMARY

The profiles of volatile metabolites in serum samples from normal individuals and from individuals with diabetes mellitus with varying degrees of polyneuropathy have been studied. The transevaporator procedure was used to obtain sample extracts which were chromatographed on a highly efficient glass column coated with Silar 10C (106 m × 0.25 mm I.D.). Differences in profiles between normal subjects and diabetic subjects on no drug therapy were noticed. However, correlations between the severity of the neuropathy and the concentrations of certain ketones could not be established. Compounds present both in diabetic and normal sera have been identified by mass spectrometry.

INTRODUCTION

The pattern of polyneuropathy found in cases of prolonged exposure to 2-hexanone is broadly similar to the symmetrical distal polyneuropathy found in many patients with long-standing diabetes mellitus [1]. The toxicity of 2-hexanone is attributed to its oxidative metabolite, 2,5-hexanedione [2, 3]. Longer-chain γ-diketones also produce experimental neuropathy [4]. To test the hypothesis [5] that the diabetic state produces endogenous neurotoxic diketones which could account for the sensory loss affecting many patients with diabetes mellitus, profiling experiments have been undertaken.

*To whom correspondence should be addressed.

Earlier studies on the volatile metabolites in urines of diabetic patients revealed some abnormally high levels of ketones, as well as alcohols and other compounds [6—10]. We have studied the profiles of volatile metabolites in sera from individuals with diabetes with varying degrees of sensory neuropathy to determine quantitative variations of the ketones, especially those that can undergo ω-1 oxidation to form γ-diketones. The overall profiles have also been compared to those obtained from normal individuals to examine other possible qualitative and quantitative changes.

EXPERIMENTAL

Sample preparation

Samples received from Albert Einstein College of Medicine were shipped frozen in dry ice. Upon arrival, the samples were thawed and transferred to 2-ml glass bottles with PTFE-lined screw caps, placing 0.5-ml quantities per bottle. This transfer process was to eliminate any problems associated with the refreezing and rethawing of the samples as they were used. Samples were then stored at $-10°$ until ready for analysis.

Extracts of 50-μl samples were prepared using the transevaporator procedure as described by Zlatkis et al. [11]. The technique was adapted for this work without change, except for a slight modification on the transevaporator which allowed the connections of the glass bead tubes to be made to the body of the transevaporator with a 10/30 ground glass joint (Fig. 1). Headspace samples were not collected because of the low-level profiles that small sample quantities of serum yield [12]. 2-Chloropropane, purified by distillation from phosphorus pentoxide was used as the solvent and 0.4 ml was the volume used for each extract.

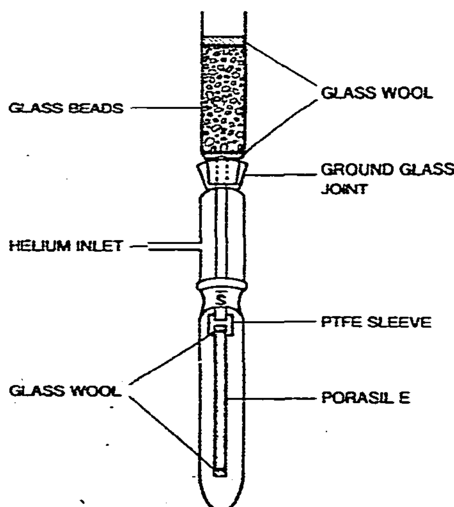

Fig. 1. Schematic diagram of transevaporator sampling apparatus.

Gas chromatography

A Varian 3700 gas chromatograph equipped with flame ionization detector, capillary direct injector insert, and glass-lined detector insert was used in this work. Separations were carried out on a 106 m × 0.25 mm I.D. glass column coated with Silar 10C (Quadrex, New Haven, Conn., U.S.A.). Helium was used as the carrier gas at a flow-rate of 1.5 ml/min and a head pressure of 40 p.s.i. Helium make-up gas was also added at the end of the column to provide a final flow-rate of 30 ml/min through the detector.

A dual trapping system was used in transferring the sample to the analytical column to avoid any losses in efficiency. The volatiles were first stripped from the glass bead tubes which held the concentrates of the extracted samples, and trapped on a precolumn (15 cm × 0.5 mm I.D.) of uncoated stainless steel, as previously described [11]. The precolumn was transferred to the chromatograph where it was connected to the carrier gas inlet via a Swagelok fitting and to a second precolumn (15 cm × 0.25 mm I.D.) of uncoated stainless steel via heat shrinkable PTFE tubing. The second precolumn, which was connected to the analytical column with heat shrinkable tubing, was immersed in liquid nitrogen and the sample transferred to it by heating the first precolumn to 200° with a small current. When the second liquid nitrogen Dewar was removed, the analysis was started. The column was operated isothermally at 40° for 6 min, then programmed at 2°/min to 180° where it was held for an additional 30 min. Peak areas were integrated on a Spectra Physics Autolab System I computing integrator which was tied to the gas chromatograph.

Mass spectrometry

Gas chromatographic—mass spectrometric analyses were made on a Model CH5 mass spectrometer connected to a Spectrosystem 100 data system and coupled to a Varian 1740 gas chromatograph (all from Varian-Mat, Bremen, G.F.R.). Spectra were recorded at 70 eV at an exponential scan mode for a mass range of 21 to 210 every 3 sec. Temperatures were 220° for the analytical ion source and 225° for the separator and transfer lines. Chromatographic conditions and sample transfer conditions were identical to those on the Varian 3700. Sample sizes however were increased to 180 μl of serum.

RESULTS AND DISCUSSION

The transevaporator sampling procedure provides an efficient means of obtaining an extraction profile of small sample quantities and allows for semi-quantitative determinations of a variety of compounds that differ widely in terms of polarity. The reproducibility of the method for a standard mixture of alcohols, ketones, and aldehydes has been previously reported [12].

Fig. 2 shows the chromatogram obtained from a sample of a normal subject. The variations between different normal individuals is small and only slight quantitative changes have been observed. A consistent pattern was obtained even for samples collected in different laboratories or hospitals and presumably therefore under different sample collection procedures. This consistency in the normal profiles facilitates standardization and should help to distinguish any quantitative or qualitative variations found to occur between normal and

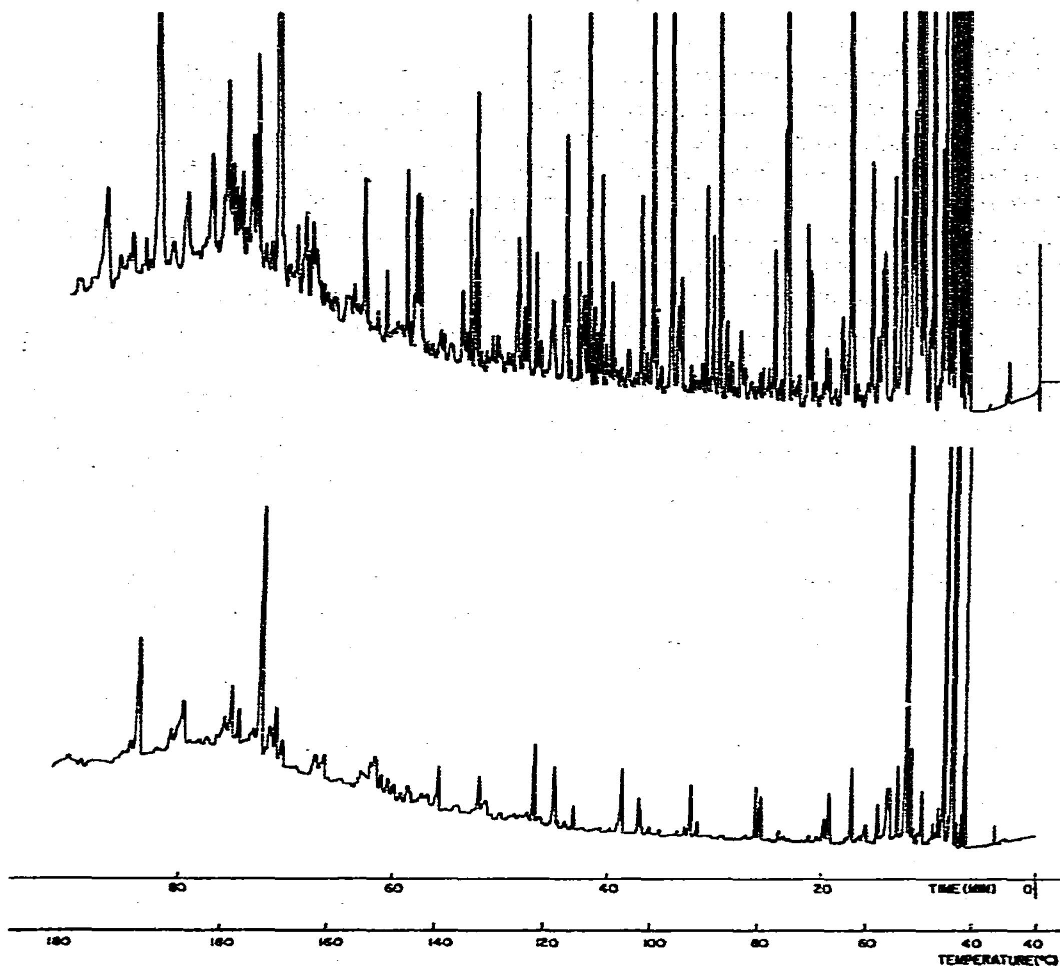

Fig. 2. Chromatograms obtained from normal blood serum (top) and corresponding blank (bottom). Attenuation, 64.

TABLE I

STANDARD DEVIATION OF RETENTION TIME AND PEAK AREA

Five replicate analyses were carried out.

Sample	Retention time			Peak area		
	Mean value (sec)	Standard deviation	Relative standard deviation (%)	Mean value (counts)	Standard deviation	Relative standard deviation (%)
2-Hexanone	1701	11.2	0.66	18046	4118	22.8
4-Heptanone	1860	6.1	0.33	1920	497	25.9
3-Heptanone	1977	9.1	0.46	5258	1064	20.2
2-Heptanone	2089	8.9	0.43	15672	5436	34.7

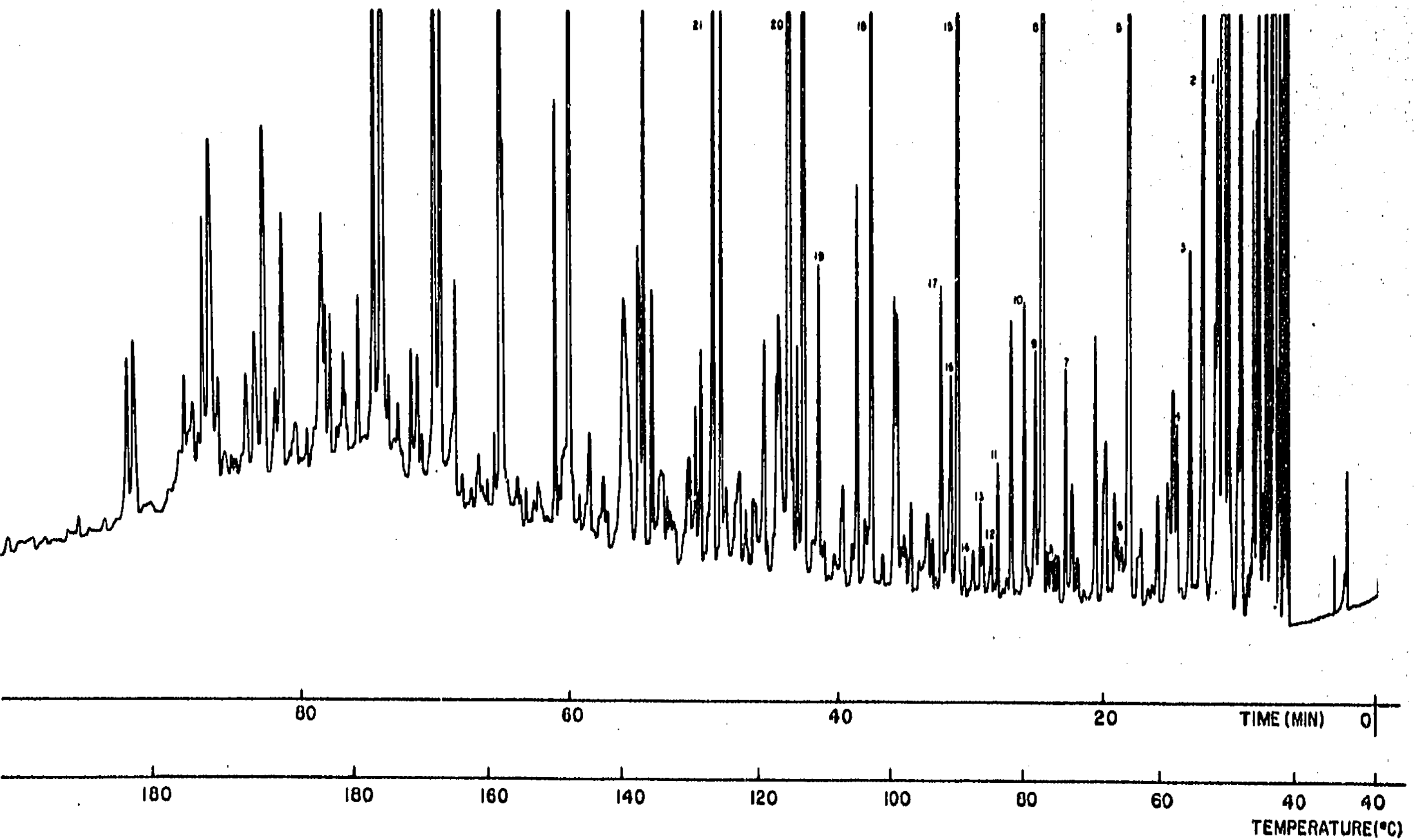

Fig. 3. Chromatogram obtained from long-standing diabetic on no drug therapy. Numbers correspond to compounds listed in Table II. Attenuation, 64.

pathological samples. The standard deviations for some aliphatic ketones identified in a normal control sample, for five replicate runs for both retention time and peak area counts are listed in Table I. The values are close to the average values previously reported for variations for individual peaks in replicate runs of normal serum [11].

Samples from diabetic patients with varying degrees of neuropathy ranging from minimal to severe plus autonomic were investigated. Some of the patients were not on any type of drug therapy while others were receiving insulin or orinase. Significant quantitative variations were found between normal subjects and subjects with long-standing diabetes on no therapy, especially at the high-temperature end of the chromatograms. Qualitative differences were not determined. Fig. 3 shows the chromatogram obtained from a diabetic sample where the patient has a mild case of neuropathy and is not taking medication.

In the analyses of urinary volatile metabolites from insulin-treated diabetics,

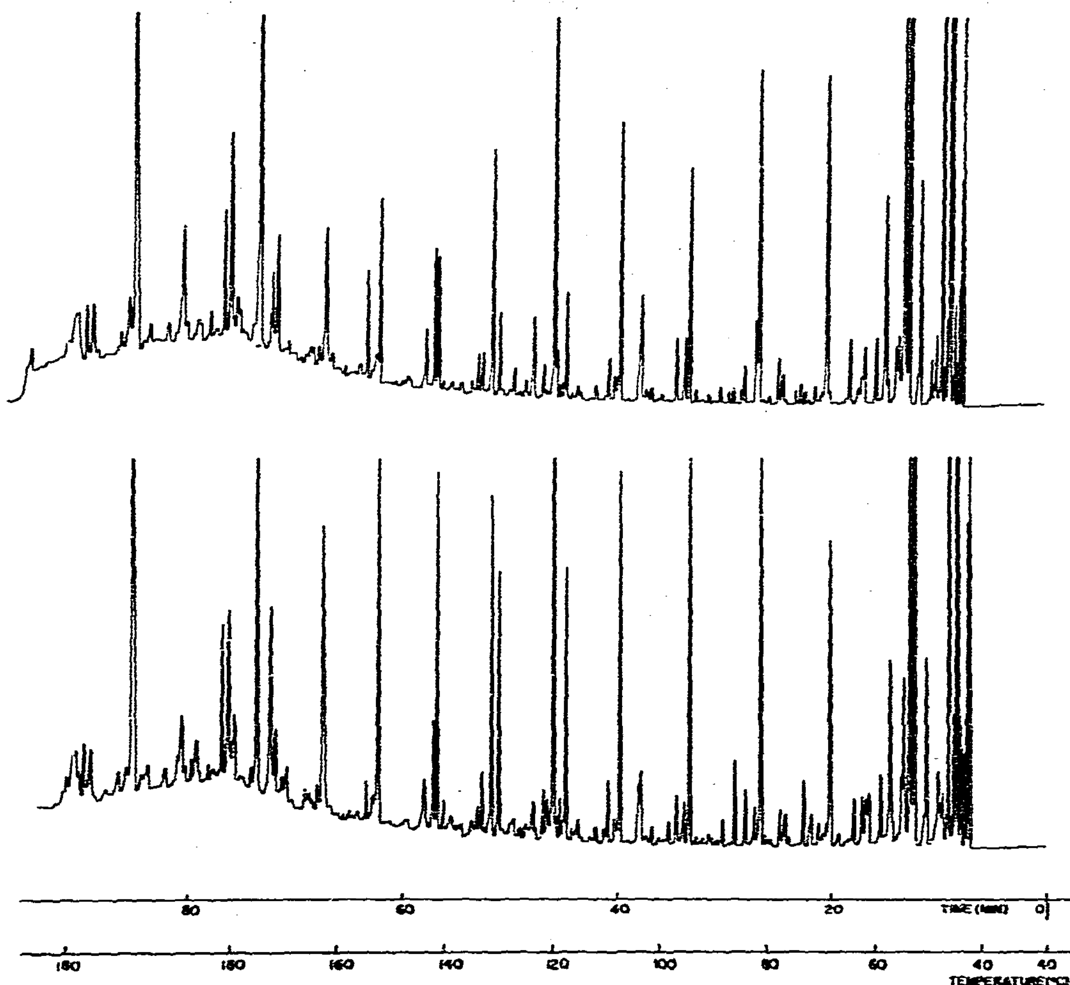

Fig. 4. Chromatograms obtained from diabetic on insulin (top) and from an individual with chemical diabetes (bottom). Attenuation, 160.

characteristic profiles were obtained which differed substantially from normal samples [6]. This distinction was not found in serum samples. Serum profiles from diabetics on insulin closely resembled the profiles obtained from normal individuals. Fig. 4 shows the chromatogram obtained from a diabetic with a moderate case of neuropathy on insulin (top) and from a subject with chemical diabetes (bottom). Individuals with the latter are generally asymptomatic but have abnormal glucose tolerance under standard conditions which is controlled by diet.

Compounds identified by mass spectrometry in diabetic serums were also found in normal serums and are listed in Table II. Sixteen of these compounds had been previously identified [13—15]. Clearly, many of the peaks that are present in large quantities are normal aldehydes. The identity of the aliphatic ketones indicated by mass spectra and listed in Table I were confirmed by comparing their chromatographic retention times with those of the authentic compounds. 2-Hexanone and 3-heptanone were the only ketones found which could undergo ω-1 oxidation to form the γ-diketones. Intermediate metabolites of these ketones which could also lead to the formation of the diketones were not detected nor were the γ-diketones themselves.

TABLE II

VOLATILE COMPOUNDS IDENTIFIED IN HUMAN SERUM

Peak number	Compound	Peak number	Compound
	Formaldehyde	9	*n*-Butanol
	Acetaldehyde	10	2-Hexanone
	Furan	11	*n*-Nona-2,4-dienal
	Propanol	12	4-Heptanone
	Propenal	13	*trans*-2-Methyl-2-butenal
	2-Methylpropenal	14	3-Heptanone
1	2-Propanone	15	*n*-Heptanal
	2-Methyltetrahydrofuran	16	*n*-Pentanol
2	*n*-Butanal	17	2-Heptanone
	Ethanol		Toluene
	2-Propanol	18	*n*-Octanal
3	Benzene		2-Octanone
4	2-Butanone	19	*n*-Pentylbenzene
5	*n*-Pentanal	20	*n*-Nonanal
6	2-Pentanone		Benzaldehyde
7	2-Butenal	21	2-Nonenal
8	*n*-Hexanal		*n*-Decanal
			n-Undecanal

Fig. 5 shows the partial calibration curves for the ketones listed in Table I. They were obtained by injecting known amounts of a standard onto glass bead tubes which were then treated in a manner identical to an actual sample. The curves are linear over the peak area range which the samples contained, and the recovery for the entire sampling procedure was found to average at 70% for each of the ketones.

When individual samples were compared no correlation between the severity of the neuropathy and the concentrations of 2-hexanone or 3-heptanone could be found. Also because of the limited number of control samples we were not

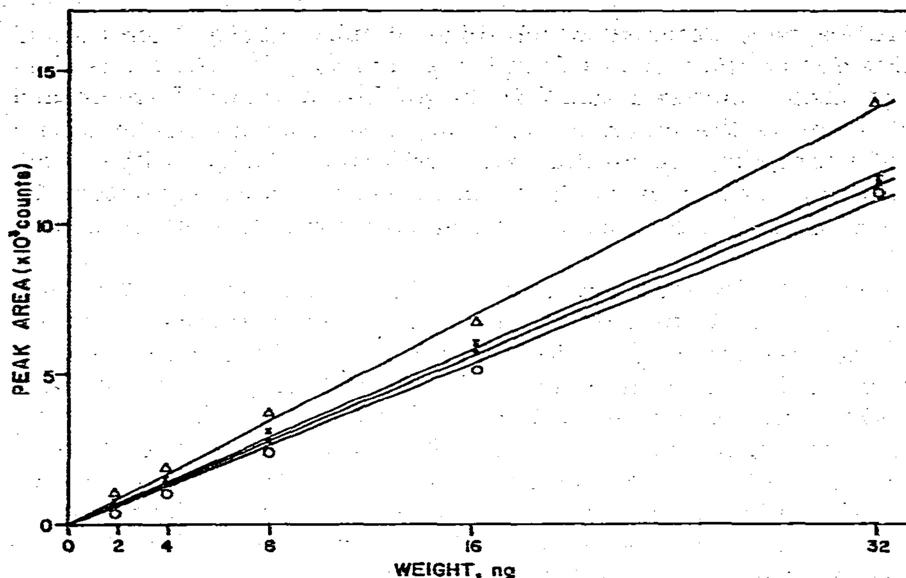

Fig. 5. Calibration curves for ketones identified in serum: 3-heptanone (△); 4-heptanone (X); 2-heptanone (•); 2-hexanone (○).

able to determine if there was any significant concentration differences in 4-heptanone and 2-heptanone in normal versus diabetic samples. It has been established that the concentrations of these two ketones in urine correlate with metabolic disorders related to diabetes [10]. The type of treatment seemed to make no difference. It is possible that the concentrations need to be correlated with blood glucose levels and insulin activity before significant changes in concentration become apparent. In all of the samples analyzed the ketone concentrations were found to be in the low nanogram range and 2-hexanone and 2-heptanone were found to be in the highest concentration while 3-heptanone and 4-heptanone were found to be in the lowest. It should be noted that 2-butanone was not found in greater quantities in the diabetic sera. This is the ketone which is not neurotoxic, but which is able to potentiate the neuropathic property of 2-hexanone [16].

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