Identification of Some Abnormal Metabolites in Plasma from Uremic Subjects

Frederick W. Bultitude and Simon J. Newham

We describe a method for comparing plasma samples from healthy subjects and from chronic uremic patients before and after dialysis. It was used to determine the nature of those metabolites that appear to characterize the uremic state. Preliminary fractionation of the metabolites by gel chromatography was followed by removal of the aqueous effluent by lyophilization and preparation of volatile trimethylsilyl derivatives, which were then examined by gas–liquid chromatography. Gas–liquid chromatography/mass spectrometry was used to characterize and identify individual metabolites. Gas–liquid chromatographic patterns of plasma from healthy and uremic subjects differ markedly, more so than do individual plasma samples within the same class of subjects. Concentrations of many metabolites are increased in uremia, but after dialysis of the patient’s blood, the concentrations become about the same as those in healthy plasma. We have observed some 150–200 metabolites in each category of plasma. We have tentatively identified about a tenth of the compounds that appear to be specific to or increased in uremia, including lactic acid, glycerol, erythritol, erythronic acid, 2-deoxy erythro pentonic acid, arabinol, arabinonic acid, inositol, and lactose. Some of these are present in concentrations >20 mg/liter and have not been previously reported as occurring in the uremic state.

Additional Keyphrases: gas–liquid chromatography/mass spectrometry • gel chromatography

The work described here originated from a program submitted in 1967 by the Chemistry Division, Atomic Weapons Research Establishment to the Department of Health and Social Security Working Party on Intermittent Dialysis. The first account of this work in the scientific literature was published in 1970 (1).


Ed. note: This paper was not one of those presented at the Symposium at the Oak Ridge National Laboratory. Nevertheless, it is included here because it is more appropriate for this issue than for the usual one.

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The clinical state of patients suffering from renal failure can be improved by dialysis of the blood to remove toxic products of metabolism that would normally be excreted by the kidneys. The efficacy of the treatment suggests that the uremic disorder is directly attributable to the presence of toxins in the body fluids at concentrations above those normally attained in healthy persons.

Several workers have attempted to correlate the concentrations of specific metabolites in blood or other body fluids with changes in the uremic state of the patient caused by dietary control or dialysis (2–9). Collectively, the results show that the concentrations of many metabolites in the body fluids are altered from those of healthy subjects and that some of them are not present in healthy plasma. However, no research group has so far systematically studied the differences that exist over the entire spectrum of organic metabolites between plasma from healthy and uremic subjects or between blood from uremic patients before and after clinical dialysis treatment. We considered that a study along these lines, if it proved practicable, would be most informative, and the course adopted in this laboratory was to examine all those organic metabolites that are amenable to gas–liquid chromatography. A recent report indicates that Furst et al. (10) are also undertaking experiments of this nature.

Many components can now be separated from urine and other body fluids by high-resolution liquid chromatography (11, 12) and in addition GLC/MS1 is now being used extensively in the identification of these components (13–17). In the case of the chronic uremic patient, samples that can be examined are limited to blood, because many of the patients are anuric. Spent dialysis fluid would contain metabolites at concentrations much lower than those in

1 Nonstandard abbreviations used: DHSS, Department of Health and Social Security; GLC, gas–liquid chromatography (-ic); MS, mass spectrometry (-ic); TMS, trimethylsilyl; AW-DMCS, acid washed and dimethylchlorosilane-treated.
blood but with a concentration of electrolytes equivalent to that of blood, hampering the investigation. Analysis of blood plasma presents problems additional to those of urine analysis, because the protein that is present may interfere with the chromatography, the size of plasma samples is generally more limited than in the case of urine, and concentrations of metabolites in plasma are frequently lower than those in urine.

The methods we used were developed on the basis that only 5-ml plasma samples would be available from the (characteristically anemic) uremic patients and that those metabolites removed by hemodialysis should be detected and characterized if they were present in a concentration of 1 mg/liter or more in plasma. The blood cells were removed from blood samples by centrifuging; gel chromatography was chosen to remove the plasma protein and to give an initial fractionation of the metabolites of interest. After lyophilization, derivatization was followed by gas–liquid chromatography. The method is thus limited to the study of volatile compounds or those that can be made volatile by derivative formation. Silylation was chosen as the best derivatization procedure, because several trimethylsilylation reagents will react with a wide range of compounds such as urinary acids (18, 19), amines (20), carbohydrates (21), and steroids (22), forming derivatives that are ideally suited to GLC. Very volatile metabolites (16) may not be detected by this system if they are eluted in the solvent peak, and at this stage it is not possible to put an upper limit on the molecular weight of compounds that can be detected, because there will be a great variation in volatilities. These points are for later investigation.

Experimental Methods

Blood Samples

Blood samples were obtained from healthy men and women who were not subjected to dietary control and had not received any medication during the previous 48 h. Uremic patients were generally on a low-protein diet and drug therapy before and after they were included on the dialysis program. Heparin was administered during dialysis, which was performed with a Kiil dialyser with Cuprophan membranes and a single-pass dialysate system.

Samples of whole blood were collected in heparinized tubes and centrifuged, and the plasma was stored at -15 °C before use.

Analytical Procedures

Gel chromatography. The flow system used in the gel-chromatographic stage (Figure 1) incorporated two long columns preceded by a short column. The purpose of the small column is discussed below. The columns were packed with Sephadex cross-linked dextran gel G-10 (40–120 μm mesh size; Pharmacia Labs Inc., Piscataway, N. J. 08854), which was swollen in water and packed by gravity into Pharmacia K16 (1.6 × 20 cm) and K15 (1.5 × 90 cm) columns. The elution rate was 16–18 ml/h, as fixed by use of two Perpx peristaltic pumps (LKB Instruments Inc., Rockville, Md. 20852).

Column effluent was monitored by ultraviolet absorbance at 281 nm with an LKB Uvicord II initially at position 1 (Figure 1), to indicate the point of column switching (see below), and then at position 2 to indicate points for fraction splitting. Round 3-mm quartz flowcells, were included in the flow lines at positions 1 and 2 and the monitor was moved when necessary. The absorbance was recorded vs. time and fraction number on an LKB galvanometric recorder. Fractions of about 4 ml were collected in tubes on an LKB Radirac fraction collector. By determining peak widths and retention times of standard compounds such as β-phenylalanine and tryptophan (1) and by detecting the protein, glucose, urea, and amino acid content of the collected fractions with reagent test strips (Ames Co., Elkhart, Ind. 46514) and thin-layer chromatography followed by development with ninhydrin, the small fractions were bulked so that no one compound would appear in more than two fractions.

Because sorption effects can be superimposed on the normal separation process when compounds are eluted from Sephadex G-10 (24, 25), compounds can be very highly retarded. The elution behavior of a compound on the gel can be described in terms of its $K_d$ value (23), which is the fraction of the inner volume of the gel available to it for diffusion. $K_d$ is a constant for the gel but is independent of the column. For totally excluded molecules $K_d = 0$, for mol-
ecules having access to the total inner volume of the gel $K_d = 1$, and for adsorbed molecules $K_d$ can be greater than unity. GLC examination of the gel chromatography effluent demonstrated that compounds were eluted to a $K_d$ value of 60 when water was used as eluent. As a consequence, very large elution volumes were observed. This effect could be diminished by adding a buffer to the eluent, but this proved unsatisfactory because it gave rise to problems similar to those found in ion-exchange chromatography, namely, difficulties of detection and characterization of metabolites in buffer solutions. Solvent extraction of the metabolites from residual buffer materials after lyophilization was found to be limited by the incomplete extractability of polar compounds, although solvent extraction was moderately successful for group separations of acids, phenols, and neutral compounds from plasma filtered through PM-10 ultrafiltration membranes (Amicon Corp., Lexington, Mass. 02173) with apparent pore sizes of 200 nm (nominal cut-off, 10,000 molecular weight). A better method of reducing the effluent volume was found by introducing a small column into the system (col. 1 in Figure 1). The strongly adsorbed materials were initially retained on this column and, beginning with those having a $K_d$ value of 2, were eluted independently, by-passing columns 2 and 3 by appropriate switching of the flow lines to be collected with a fraction collector at position 1. Three fractions were collected, the first of 120 ml and two of about 800 ml each. Usually no metabolites were observed in the last fraction. Protein was satisfactorily resolved from the bulk of low-molecular-weight species by passage through the three columns, the effluent collected to a $K_d$ of 3 and combined into 10 fractions, which varied in volume from 16 ml for the first to 400 ml for the tenth, as the peak width of the eluted compounds increased with elution volume. Usually no metabolic species were observed in the last two or three fractions, but these were routinely checked. Proteins were present in fractions two and three, and after filtration through a PM-10 membrane numerous low-molecular-weight species were demonstrated by GLC but have not yet been examined in detail.

**Freeze drying.** The metabolites were isolated by removing the water by lyophilization on a Model EF-1 8-port manifold freeze drier (Edwards High Vacuum, Inc., Grand Island, N. Y. 10472) cooled by a slurry of solid CO$_2$/alcohol and coupled to a two-stage Edwards ED75 rotary vacuum pump. The operating pressure measured by a Pirani gauge was <13.3 Pa (<0.1 Torr). Samples were removed within 1 h after they appeared to be dry, to minimize loss of volatile components.

**Trimethylsilyl derivative preparation.** TMS derivatives were prepared by reacting the residue from freeze drying with a minimum of $N,O$-bis-TMS-acetamide or $N,O$-bis-TMS-trifluoroacetamide (Pierce Chemical Co., Rockford, Ill. 61105; freshly opened ampuls) in pyridine (British Drug Houses; AR, distilled and stored over KOH) in a ratio of 1:1 to 3:1, at 60 °C for 4 h.

**Gas–liquid chromatography.** A Model 900 chromatograph (Perkin-Elmer Corp., Norwalk, Conn. 06856) fitted with twin 4 m × 1.75 mm i.d. glass columns packed with 2½% of either OV-1 or OV-17 on 80–100 mesh AW-DMCS treated Chromosorb G (Applied Science Laboratories, Inc., State College, Pa. 16801) and coupled to twin flame-ionization detectors, was operated in the compensated mode. Helium carrier-gas flow rate was 20 ml/min at 70 °C. Injection temperature was 200–250 °C and that of detectors 230–270 °C. The oven temperature program was: 70 °C for 2 min, 0.5 or 2 °C/min to 270 °C, and hold for 65 min. Chromatograms were recorded on a two-pen strip-chart recorder at two sensitivities.

“Methylene units” have been used to express retention data for samples and standards. This method was preferred to quoting relative retention times against a single reference peak because of the wide range in retention times of the various metabolites.

**Gas–liquid chromatography/mass spectrometry.** Mass spectral data were obtained by coupling a Pye Model 104 gas chromatograph, via a Becker-Ryhage double-jet separator, to the source of an AEI MS902 double-focusing mass spectrometer. The chromatograph was fitted with a 3 m × 2 mm i.d. glass column packed with 2½% OV-1 on 80–100 mesh AW-DMCS treated Chromosorb G. Helium flow rate was 30 ml/min at 80 °C. Injection temperature was 200–250 °C. The oven temperature program was 80 °C for 2 min, 1–1.5 °C/min to 270 °C, and hold for 30 min. The temperatures of the connecting lines, separator, and source were maintained at 220 °C above ambient. The pressure in stage 1 of the separator was maintained at 26.6 Pa (0.2 Torr) and stage 2 at 665 mPa (0.005 Torr), allowing the source to operate at 1.33 mPa (<1 × 10$^{-5}$ Torr) (uncorrected).

Low-resolution spectra (static resolving power, 1000; 10% valley definition) were obtained at 70 eV, trap current 500 μA, and accelerating voltage 8 kV, by scanning from the top of the GLC peak, as indicated by the total ion-current monitor coupled to a strip-chart recorder, and recorded on ultraviolet-sensitive paper at three sensitivities over a range of 1000 to 28 atomic mass units.

We examined several different methods of matching spectra. These included, first, the extraction of the eight most intense ions in order of decreasing intensity and, second, extraction of the eight most intense ions in any order. Successful matching by both these methods was limited by variations in spectra caused by continuously varying sample pressure in the ion source while a GLC peak was being scanned. This was overcome to a certain extent by scanning from the peak maximum down the trailing side of the peak, but this assumed perfect GLC resolution from other components. The third method involved comparing the two or three most intense peaks from blocks of 14 atomic mass units, and it is that used by
the National Institutes of Health system (26). This overcame the bias caused by changing sample pressure, and is probably the method of choice if adequate computer facilities are available. In a fourth method, a Modular One computer is used to record mass spectra on disc, and to display them on a television monitor for visual comparison. With only 1500 spectra of trimethylsilyl compounds in our file, this visual comparison method appears to be adequate, if a little time-consuming. Mass spectra from each GLC peak scanned were compared with spectra from GLC peaks obtained from other gel-chromatographic fractions and other plasma samples when the GLC peaks had retention times within ±10% of each other and also with reference spectra in the library compiled from the literature and from standard compounds. A tentative match was considered to have been achieved when all major ions were the same in each spectrum and their intensities were essentially similar, allowing for slight discrepancies caused by changing sample pressures on scanning GLC peaks and differences in instrumentation.

High-resolution, accurate mass measurements were obtained “on the fly” by the peak-matching technique with a static resolution of 10 000, 10% valley definition. Perfluoredecalin and perfluorotributylamine, were used as reference compounds by bleeding into the mass spectrometer source from the cold inlet system via a glass “leak.” Accurate masses were calculated by interpolation between reference and unknown and the data analyzed by a Modular One 16-bit 1.11 processor with 16K of fast store and 1M words of backing store on a 1.53 fixed-disk system. A list of possible elemental formulas for each measurement was printed out through an ASR 33 teletype.

High-resolution data for individual ions in each spectrum are now being obtained. This is a slow process, as only one ion can be measured on each GLC peak, assuming 1-min peak separation, and the number of times this can be done is often limited by the amount of sample available. Measurements are confidently within ±10 ppm and the computer printout of elemental formulas is programmed to include all compounds containing C, H, N, O, and Si, with unlimited C and H, but zero or up to a maximum of 6, 8, and 10 N, O, and Si atoms, respectively. Elemental formulas matching the measured mass exactly are

Fig. 2. Gas/liquid chromatograms on 2% OV-1 of fraction 4 after gel chromatography of plasma from: 2a, uremic male (patient 1), before starting the hemodialysis program; 2b, uremic female (patient 2), six weeks before starting the hemodialysis program; 2c, uremic female (patient 2), immediately before the first hemodialysis treatment; 2d, uremic female (patient 2), at end of the first hemodialysis treatment; 2e, healthy female
not more likely to be correct than those varying by 10 ppm, so that investigation of the structural possibilities is required.

Results and Discussion

Blood samples from seven healthy subjects, 17 uremic patients maintained by dietary control, and a uremic patient before and after the first and subsequent routine dialysis have been examined by the procedures outlined above.

The nonprotein-containing fractions gave gas chromatograms with three to more than 80 peaks per fraction of metabolites at concentrations equivalent to 1 mg/liter or greater in the original plasma, giving a total of 150 to 200 compounds in both uremic and healthy plasma. Figure 2 shows typical low-sensitivity GLC traces of fraction four of plasma from uremic subjects, before and after dialysis, and from a healthy subject.

The gas chromatograms of plasma from different uremic patients before they commenced the hemodialysis program showed only minor differences (examples are shown in Figures 2a, 2b, and 2c); i.e., we obtained gas chromatograms typical of the uremic state. The gas chromatograms from healthy subjects were also very similar to each other (a representative one is shown in Figure 2e) but they differed markedly from those of pre-dialysis plasma from a uremic subject. The heights of most of the peaks obtained from the uremic subject’s plasma were much higher than those in the healthy subject, indeed, about 100 compounds in uremic plasma were at concentrations exceeding 5 mg/liter, but in contrast only about a dozen of these compounds appeared in control plasma above this concentration. One or two peaks obtained on GLC of plasma from healthy persons were sometimes higher than the equivalent peaks in samples from uremic subjects.

Gas chromatograms of plasma fractions from healthy subjects and a post-dialysis uremic patient are very similar (see, for example, Figures 2d and 2e). Concentrations of metabolites in the plasma of dialyzed uremic patient were in some cases decreased to concentrations below those in the healthy subject, which could indicate a diminution of essential components on dialysis. If it were found to be beneficial to do so, such compounds could be replaced, orally or intravenously. The samples of plasma were taken near the end of hemodialysis and the low concentrations might reflect the greater depletion of constituents in plasma than in the water in tissue. Samples taken some hours after the end of hemodialysis would demonstrate any “rebound” effect resulting from diffusion from the cellular (33).

To date, good low-resolution mass spectra have been obtained for most of the metabolites that occur in plasma in concentrations of 5 mg/liter or greater. These number about 100 in plasma from a pre-dialysis uremic patient but only about 20 of them have so far been tentatively identified. Table 1 lists those appearing regularly at concentrations much exceeding those in plasma from healthy subjects, along with GLC retention data as methylene units on OV-1 and the sources of the reference mass spectra. Several of these are polyhydroxy compounds with, in two cases, the alcohol and the corresponding acid both appearing, often at concentrations greater than 20 mg/liter, compared with concentrations that may be lower than the detection limit (1 mg/liter) in healthy plasma. This finding has not been previously reported in uremia, although abnormalities of carbohydrate metabolism in uremia have been known for many years (31, 32) and disturbances in carbohydrate metabolism are common in liver diseases and diabetes.

We conclude that the method described here is suitable for separating a wide range of metabolites in body fluids. Although the time required for a complete metabolic profile (two to three weeks) of course prohibits any routine application of the procedure described here, simpler techniques could be devised to serve the more limited requirements of recognizing particular conditions, not only uremia, once the specific metabolites in question have been identified. The long-term aim would be a direct and early diagnosis before the appearance of recognizable clinical symptoms or before identification by currently used diagnostic techniques. The development of improved treatments entailing less metabolic disturbance might similarly be investigated.

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Mr. J. R. Morris of the AWRE Chemistry Division participated in the development of gel-separation procedures, and his contribu-

Table 1. Tentative Identification of Compounds that Occur at High Concentrations in Uremia

<table>
<thead>
<tr>
<th>Compound</th>
<th>Methylene units (2% OV-1)</th>
<th>Mol wt TMS compound</th>
<th>Matched with low-resolution spectrum in ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactic acid</td>
<td>10.58</td>
<td>234</td>
<td>27</td>
</tr>
<tr>
<td>Urea</td>
<td>12.77</td>
<td>204</td>
<td>27</td>
</tr>
<tr>
<td>Phosphate</td>
<td>12.78</td>
<td>314</td>
<td>28</td>
</tr>
<tr>
<td>Glycerol</td>
<td>12.88</td>
<td>308</td>
<td>29</td>
</tr>
<tr>
<td>Erythritol</td>
<td>15.34</td>
<td>410</td>
<td>29</td>
</tr>
<tr>
<td>Erythronic acid</td>
<td>15.71</td>
<td>424</td>
<td>30</td>
</tr>
<tr>
<td>2-Deoxy erythro-pentonic acid</td>
<td>16.85</td>
<td>438</td>
<td>30</td>
</tr>
<tr>
<td>Arabinitol</td>
<td>17.62</td>
<td>512</td>
<td>29</td>
</tr>
<tr>
<td>Arabinonic acid</td>
<td>18.20</td>
<td>526</td>
<td>30</td>
</tr>
<tr>
<td>Inositol</td>
<td>21.36</td>
<td>612</td>
<td>27</td>
</tr>
<tr>
<td>Lactose</td>
<td>25.71</td>
<td>918</td>
<td>27</td>
</tr>
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tion to the program is gratefully acknowledged. Thanks for technical assistance are due to Mr. C. Young of the AWRE Chemistry Division and Messers. M. Nicholson, G. Wilson, D. Wright, and A. McLachlan of the Physical Measurements Unit, AWRE.

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