Characterization of Uremic "Middle Molecular" Fractions by Gas Chromatography, Mass Spectrometry, Isotachophoresis, and Liquid Chromatography

Ad C. Schoots,1 Frans E. P. Mikkers,3 Henk A. Claessens,1 Rita De Smet,2 Nadine Van Landschoot,2 and Severin M. G. Ringol2

Uremic ultrafiltrates (and normal serum, for comparison) were fractionated by means of gel filtration. The collected fractions were further investigated by combined analytical techniques: "high-performance" liquid chromatography, gas chromatography, mass spectrometry, and isotachophoresis. Ultrafiltrate fractions in the so-called middle molecular mass region \( M_r 500-2000 \) contained a considerable amount of substances of low molecular mass, such as carbohydrates, organic acids, amino acids, and ultraviolet absorbing solutes. Ultraviolet absorbance in the "middle molecular mass region" of the gel chromatogram is mainly due to the presence of these rather low-molecular-mass solutes. Therefore this signal is not a quantitative measure of molecules with a "middle" molecular mass.

Additional Keyphrases: peritoneal dialysis · uremia · kidney disease

Numerous organic substances are known to accumulate in the serum during chronic renal failure, ranging from very simple molecules such as urea to complex molecules such as \( \beta_2 \)-microglobulin.

Many of these retained components can act as cell toxins and enzyme inhibitors or may cause abnormal membrane transport in tissue and cells (1). Patients with renal failure have an increased susceptibility to bacterial infections, the cause of 15% of all deaths in hospital dialysis treatment in Europe in 1975 (2).

Many authors have investigated the role of lower-molecular-mass substances, such as guanidines, guanidino acids, amino acids, amines, phenolic acids, and polylols (3-6). Although in many cases appreciably increased concentrations were found in the body fluids, attempts to relate these to uremic symptoms generally failed. Most of these studies, however, were directed at specific analytes or a specific class of analytes. Scribner (7) suggested that with more-permeable dialysis membranes certain accumulating substances could be more efficiently removed. His suggestion stemmed from the clinical finding that patients on long-term peritoneal dialysis were doing well, despite relatively high concentrations of creatinine, uric acid, and urea in serum—i.e., the peritoneum is such a membrane. However, not only is permeability important for dialysis efficiency, but also membrane surface area and the number of hours of dialysis per week. Babb et al. (8) proposed a "square meter–hour" hypothesis, relating these factors; however, their original suggestion postulated the presence of unknown, but pathophysiologically important, molecules with a relative molecular mass \( M_r \) intermediate to that of rather small molecules (i.e., \( M_r < 500 \)) and that of large solutes such as lipoproteins, polypeptides, and proteins. In 1972 the "square meter–hour" hypothesis was changed to the "middle molecule" hypothesis (9). Since the proposal of the "middle molecule" hypothesis, dialyzers with large surface area have been increasingly used to shorten dialysis time, and Babb and Scribner (10) have developed the concept of the "dialysis index" to facilitate quantitation and prescription of dialysis therapy.

Though there is some clinical evidence for the importance of "middle molecules," their role in uremic toxicity remains controversial. Nowadays the middle molecule hypothesis refers to serum solutes in the relative molecular mass range of 500 to 2000. Many investigators have emphasized the importance of "middle" molecules, but there has been no convincing analytical demonstration of the presence of these solutes; as such, the middle molecule hypothesis remains to be proven.

Most investigators trying to analyze, identify, and isolate "middle" molecules have used gel filtration (11-17) for separation, with ultraviolet (UV) detection.4 Generally, the results of gel filtration are taken as a quantitative measure for accumulation and elimination of "middle molecules," i.e., the gel-chromatographic peaks were "identified" as "middle" molecules.

Only a few investigators have re-analyzed the gel filtration fractions and claim to have isolated or identified solutes of the required molecular mass (18-20). Moreover "middle molecule" fractions from gel filtration showed in vitro toxicity effects, such as inhibition of glucose utilization, inhibition of phagocytic activity, and inhibition of the activity of several enzymes. In some cases these toxic effects were directly attributed to the "identified" middle molecules (21-25).

Because of the complexity of uremic body fluids and their gel-filtration fractions, various analytical techniques have to be used to screen different classes of solutes. In this study we have used the following high-resolution separation techniques to characterize "middle molecular" fractions from uremic ultrafiltrates: gas chromatography (GC), mass spectrometry (MS), isotachophoresis (ITP), and "high-performance" liquid chromatography (HPLC). The application of these techniques to the screening of whole or processed uremic body fluids has been previously described (26-35). In our experiments we fractionated uremic ultrafiltrate (and, for comparison, normal

1 Laboratory of Instrumental Analysis, Department of Chemical Engineering, Eindhoven University of Technology, P.O. Box 513, 5600 MB Eindhoven, The Netherlands.

2 Division of Nephrology, Department of Medicine, University of Ghent, Belgium.


Received May 29, 1981; accepted Oct. 5, 1981.

4 Nonstandard abbreviations: UV, ultraviolet; GC, gas chromatography; MS, mass spectrometry; ITP, isotachophoresis; and HPLC, "high-performance" liquid chromatography.
serum) by gel filtration, and analyzed the resulting fractions by the above-mentioned techniques. To relate toxicity and chemical composition of gel-filtration fractions, we assessed these fractions for inhibition of phagocytic activity (25).

Materials and Methods

**Gel filtration.** We used a 60 × 1.6 cm (i.d.) column packed with Sephadex G-15 gel (Pharmacia, Uppsala, Sweden) (25, 26). The sample volume applied was 5 mL for uremic ultrafiltrates and 2 mL for normal serum. The eluent was an aqueous ammonium acetate buffer (30 mmol/L, pH 6.9) at a flow rate of 15 mL/h. After measuring UV absorbance at 206 and 280 nm, we collected 5-mL fractions.

**Gas chromatography** was performed as described (30, 31). After pretreatment (evaporation of sample and chemical derivatization), the fractions were analyzed on a nonpolar coated-glass capillary column.

**Mass spectrometry** was used on-line with gas chromatography for identification under the conditions described earlier (30, 31). For identification or determination of the molecular mass of material represented by liquid-chromatographic peaks, we used a thermal-desorption direct-insertion probe operated under electron impact and chemical ionization conditions. The electric current through the platinum wire of the probe, on which the samples were deposited, was programmed from 0 to 2 A within a 2-min period (wire diameter was 0.15 mm). For chemical ionization we used a 10/90 by vol mixture of ammonia and methane as the reagent gas. The mass spectrometer was of the quadrupole type (Model 4000 GC/MS; Finnigan-MAT, Sunnyvale, CA 94086).

**Isotachophoresis.** Anionic solutes at low pH were separated as earlier described (26, 27).

**ITP profiles** were obtained by recording UV absorption at 254 nm and conductometric detector signals.

**“High-performance” liquid chromatography.** We used a 25 cm × 4.6 mm (i.d.) column packed with nonpolar octadecyl modified silica (reversed phase; Lichrosorb RP-18, particle diameter 5 μm; Merck AG, Darmstadt, F.R.G.). To pack the column, we used a reciprocating piston pump (Model DSTV-122; Haskel Engineering and Supply Co., Burbank, CA 91502), at a pressure of 70 MPa. The eluent was a linear gradient from 100% aqueous ammonium formate buffer (0.05 mol/L, pH 4) to 100% highly purified methanol (Rathburn Chemicals Ltd., Walkerburn, Scotland) over a 60-min period. Real analysis time was generally 35 min. The HPLC apparatus involved two Waters 6000A pumps, and a Waters M650 solvent programmer (Waters Associates, Inc., Milford, MA 01757), and an external sample loop valve sample-injector (Valco Inc., Houston, TX 77024). Absorbance at 254 nm was measured with a variable-wavelength UV detector (Model LC-3; Pye Unicam Ltd., Cambridge, England). Samples of diluted gel fractions were injected directly.

**Test of inhibition of phagocytic activity.** To initiate phagocytosis we added polystyrene latex beads, inulin, or zymosan to a normal blood sample incubated with [14C]glucose. Phagocytosis includes the utilization of glucose to form CO2 via the hexose monophosphate shunt. After addition of the ultrafiltrate fraction we measured 14CO2 production by liquid scintillation counting.

Uremic ultrafiltrates were obtained during a sequential ultrafiltration and diffusion procedure on an uremic patient with use of a polycyrlonitrile membrane (in vivo). Uremic ultrafiltrates and normal serum were fractionated by gel filtration yielding 40 5-mL fractions. These fractions were tested for phagocytosis inhibition. The remaining aliquots of these fractions were lyophilized and stored at −18°C until further use. For the screening by combined analytical techniques, we redissolved the lyophilized samples in 500 μL of doubly-distilled water. From this, 200 μL was used for preparing the GC samples, 100 μL was injected on the liquid chromatograph, and 1−5 μL was needed for isotachophoresis.

This entire procedure was carried out three times on samples of ultrafiltrate from three different patients.

Results

Figure 1 shows the gel-filtration profile for uremic ultrafiltrate. Below the curves, phagocytosis inhibition is plotted in the form of a histogram. Table 1 includes a list of the solutes found and identified in the GC and ITP profiles of the gel filtration fractions. The listing does not account for the substances found in the HPLC profiles, the identity of which is still being investigated by mass spectrometry and Fourier infrared spectroscopy. Preliminary results, however, with thermal-desorption mass spectrometry indicate that mainly they are solutes of low molecular mass (M, between 100 and 300), possibly originating from purine and pyrimidine me-
Table 1. Solutes (and Their Molecular Masses) Found In Adjacent Gel-Filtration Fractions *

<table>
<thead>
<tr>
<th>Solute</th>
<th>Mr</th>
<th>13</th>
<th>14</th>
<th>15</th>
<th>16</th>
<th>17</th>
<th>18</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium (chloride)</td>
<td>58</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>0</td>
<td>O</td>
<td>0</td>
</tr>
<tr>
<td>Creatinine</td>
<td>113</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>0</td>
<td>O</td>
<td>0</td>
</tr>
<tr>
<td>Urea</td>
<td>60</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
</tr>
<tr>
<td>Acetate</td>
<td>60</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
</tr>
<tr>
<td>Lactate</td>
<td>90</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
</tr>
<tr>
<td>Gluconate</td>
<td>196</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>133</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
</tr>
<tr>
<td>Phosphate</td>
<td>98</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
</tr>
<tr>
<td>Serine</td>
<td>105</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
</tr>
<tr>
<td>Threonine</td>
<td>119</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
</tr>
<tr>
<td>Glucose</td>
<td>180</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
</tr>
<tr>
<td>Fructose</td>
<td>180</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
</tr>
<tr>
<td>Glucitil</td>
<td>182</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
</tr>
<tr>
<td>Arabinitol</td>
<td>152</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
</tr>
<tr>
<td>Erythritol</td>
<td>122</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
</tr>
<tr>
<td>Myo-inositol</td>
<td>180</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
</tr>
</tbody>
</table>

* Open circles indicate the presence, black dots symbolize the fraction with maximum concentration, of the indicated solute in the fraction.

Tabolism as well as from protein catabolism.

Fractions 12 to 15 should contain the presumed “middle molecules.” This middle molecular mass region was determined on the basis of the elution volume of injected markers of a well-known molecular mass (Figure 1). From the list of solutes in Table 1 it should be concluded that a considerable amount of the low-molecular-mass solutes elutes in the middle molecular mass region. Fractions 20 to 40 did not contain any appreciable amount of low-molecular-mass solutes as far as they could be detected by the techniques we used. The liquid-chromatographic (Figure 2) and isotachopheretic (Figure 3) profiles of middle-molecular-mass fractions 13, 14, and 15 showed a considerable amount of UV-absorbing solutes and anionic substances. The UV detection wavelength for liquid chromatography was 254 nm, whereas that for gel filtration was 206 nm (and 280 nm). Some fractions were also monitored at 206 nm in liquid chromatography. The only difference from the 254-nm trace was that at 206 nm the overall peak height was smaller while the pattern remained unchanged. Therefore, we confined the experiments to detection at 254 nm in liquid chromatography. As an illustration of the large amount of UV-absorbing solutes, Figure 2 shows the HPLC profiles of ultrafiltration fractions 12, 14, 15, and 16. Moreover, it can be seen that most carbohydrate-like and anionic substances (Table 1, Figure 3) elute in fractions 13, 14, and 15.

As stated before, the UV-absorbing solutes are not yet positively identified; however, analysis by electron-impact and chemical-ionization thermal-desorption mass spectrometry indicates that these are solutes of low molecular mass (Mr between 100 and 300).

Figure 3 shows the GC, ITP, and HPLC profiles of some of the above-mentioned fractions. Because the ITP profile of fraction 14 showed a very large zone for acetate, the more informative profile of fraction 13 is illustrated. The UV-detection trace in this profile shows absorptions between the zones of phosphate, lactate, and acetate. An estimation of ionic mobilities in this isotachopheretic system at pH 3.5 of the leading electrolyte (27) leads to the conclusion that it must consist of low-molecular-mass solutes (Mr up to 400). A comparison of the HPLC profiles of fraction 14 from uremic ultrafiltrate and from normal serum (Figure 3) reveals that there are marked differences in concentrations for almost all the metabolites detected.

Because phagocytosis inhibition is maximal in fraction 14, it may be related to the high amount of low-molecular-mass solutes or to high osmolality in this and neighboring fractions. Extremely high concentrations of sodium and acetate were found here. The measured osmolalities of fractions 13, 14, and 15 were between 100 and 800 mosmol/kg.

Discussion

Gel filtration fails as a size-discriminating analytical technique for separation of “middle” molecules from other molecules in complex biological fluids. Obviously, in gel filtration of such fluids, several retention mechanisms (43) are active rather than size exclusion alone. Charge of functional groups in the gel matrix and charge, concentration, and chemical structure of sample components will all play an important role (44, 45). Therefore, in gel filtration of complex heterogeneous mixtures, no single mechanism is responsible for retention, but rather a combination of several (e.g., size exclusion, ion inclusion, ion exclusion, and absorption) (43). Therefore the elution volume of the gel chromatographic peaks is not related to the molecular mass. Different authors use the UV peak area...
in the "middle molecular mass region" of the gel chromatogram as a quantitative measure for the so-called middle molecules (13, 14, 24). Our results show that the middle molecule fractions contain a large amount of solutes of low molecular mass, of which a part must be held responsible for the UV-signal in the gel chromatogram. Though a slight improvement in the gel filtration procedure may be possible, this will not lead to a major change in the elution pattern. Even in somewhat more efficient procedures where gel filtration is combined with gradient-elution ion-exchange chromatography, "middle molecular" subpeaks appeared to contain UV-absorbing solutes of lower molecular mass (20, 37, 41, 42).

Therefore the quantification of "middle molecules" by UV peak area is incorrect even in these cases (39, 40). Evidently, gel filtration can not be used as an analytical technique for determination of "middle molecules". Furthermore, the use of gel filtration as a preparative method for obtaining middle molecules is doubtful. No doubt, middle molecules do exist, although at a far lower concentration than generally suggested.

As the general attention to "middle molecules" is largely based upon results of gel-filtration studies, it seems relevant to reconsider the importance of solutes of lower molecular mass in uremic intoxication of chronically dialyzed patients.

References


