Evaluation of Dialysis Treatment in Uremic Patients by Gel Filtration of Serum
Jesus Oseca,1 Teodosio Gee,2 Carmen Sanz,1 Isabel Millan,3 and Julio Botella4

A group of substances of molecular masses between 300 and 1500 Da have been found to be toxic metabolites in patients with uremia. We determined the concentration in serum of these molecules in the following groups of patients: two hemodialyzed groups (one with cuprophane and the other with polyacrylonitrile dialyzers), one group treated with continuous ambulatory peritoneal dialysis, one group of nondialyzed azotemic patients, and one control group of healthy persons. Ultrafiltrates of the subjects' sera were fractionated on Sephadex G-15 followed by ion-exchange chromatography. Eluates were monitored by absorbance at 254 and 206 nm. Partially characterized peaks P1, P2, and P3 were obtained by gel filtration, correlated with the concentration of creatinine in serum; their concentrations were significantly P <0.01) larger in hemodialyzed groups than in peritoneal dialyzed or in nondialyzed azotemic patients. After ion-exchange chromatography, two peaks (P5 and P6) correlated with serum creatinine and also were larger in hemodialyzed patients than in the other groups. Apparently, adequate discrimination is obtained by gel-filtration analysis and further analysis by ion-exchange chromatography does not provide additional information in most of the affected patients.

Additional Keyphrases: hemodialysis · peritoneal dialysis · chromatography, ion-exchange · creatinine · azotemia

Materials and Methods

Patients and Sera

Pre-dialysis blood samples were collected from five groups: Group A, six patients hemodialyzed with a cuprophane dialyzer; Group B, six patients hemodialyzed with polyacrylonitrile; Group C, six patients dialyzed with chronic ambulatory peritoneal dialysis (CAPD); Group D, six nondialyzed azotemic patients (NDAP), whose conditions did not require any dialysis; and Group E, healthy individuals with normal kidney function. The procedures followed were in agreement with the Helsinki Declaration.

The blood samples were taken and serum was obtained by centrifugation, within 60 min after collection. In every case, samples were analyzed in duplicate runs.

We diluted 2 mL of each serum sample with 2 mL of distilled water, then passed this through a Centrifu CF-50 membrane (Amicon, Danvers, MA) for 20 min at 900 × g in a Beckman refrigerated centrifuge (Beckman Instruments, Brea, CA). The nonfiltered residue was resuspended in 2 mL of distilled water and the ultrafiltration step was repeated. Both ultrafiltrates were pooled and lyophilized. Before assay, we dissolved the lyophilized material in 2 mL of distilled water.

Methods

Each subject was monitored for serum creatinine and creatinine clearance. Serum creatinine was estimated according to the Jaffé kinetic colorimetric method, with an Astra-4 analyzer (Beckman Instruments Inc.). Creatinine clearance was determined as described by Henry et al. (11).

Analytical chromatography by gel filtration. Gel filtration was performed according to the method of Fürst et al. (12), with use of a 75 × 0.5 cm column packed with Sephadex G-15 (Pharmacia, Uppsala, Sweden). After equilibrating the column with 0.01 mol/L Tris·HCl buffer, pH 8.6, we applied 0.5 mL of the ultrafiltrate. The column was eluted with the same buffer at a flow rate of 10 mL/h. The eluates were monitored at 254 nm and 206 nm with a dual-channel ultraviolet spectrometer (Uvicord III; LKB, Bromma, Sweden). At both wavelengths the range of absorbance was 0 to 1 A. Peak separation was complete in 2 h. Effluents were collected with a fraction collector (7000 Ultrarac; LKB). Fractions with molecular masses between 1000 and 1500 Da (determined by comparison with the oxytocin elution peak) were collected and lyophilized. The dry powder was resuspended in 0.4 mL of 0.01 mol/L Tris·HCl buffer, pH 8.6.

Analytical ion-exchange chromatography. Two 35 × 0.5 cm columns were used, one as a comparison column (to control background) and the other as a sample column. Both were packed with diethylaminoethyl-Sephadex A-25 (Pharmacia), equilibrated with 0.01 mol/L Tris·HCl buffer, pH 8.6, and eluted with a linear gradient of NaCl (0–1 mol/L), performed with an LKB 11900 Ultrogard Gradient mixer. The flow rate was 10 mL/h. Detection at 206 nm was monitored in the range of 0–1 A; however, at 254 nm we used a 0.2–0.5 A range. Separation was complete.
in 3 h (12). Concentrations were expressed as millimeter of peak height per milliliter of serum.

Statistical analysis. The Shapiro-Wilk test was applied to establish the behavior of distributions. Whenever the Shapiro-Wilk test rejected the hypothesis of normal distribution, or when the Bartlett test for homogeneity of variances was significantly different, we calculated the overall significance of differences with the Kruskal-Wallis (one-way analysis of variance) test. If the differences were significant, we then tested the differences between the groups pair-wise, using the Mann-Whitney U-test (13). Differences were considered nonsignificant when P > 0.05.

Association between variables was assessed by Spearman’s rank-order correlation coefficient (r_s) (14). Precision studies were carried out by duplicate analysis of samples in different days and results were expressed as CV (%).

Results

Figures 1 and 2 show the chromatographic profiles obtained by gel filtration and ion-exchange chromatography, respectively. Gel filtration of uremic serum yielded five or six peaks at 254 nm. In contrast, no peak appeared in healthy controls (data not shown). At 206 nm, the chromatogram showed peaks in both groups. Peaks P_1 and P_2, with retention times of 39 and 50 min, respectively, were collected and further processed by ion-exchange chromatography. The profile of uremic serum at 254 nm showed 11 peaks by ion-exchange chromatography, whereas no peaks could be detected at this wavelength in sera from healthy subjects. The reproducibility (run-to-run CV) of retention times for the peaks was 6% and 8% in gel filtration and ion-exchange chromatography, respectively.

Analytical precision (CV) was <1%. To calculate this CV, we used results of duplicate analyses on different days, 31 pairs of data for each peak. The means and ranges for each peak (mm/mL) were as follows: P_1 at 254 nm, 6.8 (0–20); P_1 at 206 nm, 28 (0–67); P_2 at 254 nm, 3.4 (0–10); P_2 at 206 nm, 30 (5.5–56); P'_a at 206 nm, 9.5 (0–23); and P'_a at 254 nm, 5.3 (0–18.5).

Table 1 shows the values for peaks 1 and 2 obtained by gel filtration, as monitored at 254 nm. The highest values were observed in the hemodialyzed patients (cuprophane and polyacrylonitrile), with no statistical variation between the values obtained by these two procedures.

![Fig. 1. Chromatographic profile obtained by gel filtration from a uremic serum](image)

We applied 0.5 mL of ultrafilterate to Sephadex G-15 and eluted it with 0.01 mol/L Tris-HCl buffer, pH 8.6, at a flow rate of 10 mL/h. The eluates were monitored at 254 and 206 nm. Fractions between 1000 and 1200 Da (according to the oxytocin elution peak) were collected.

![Fig. 2. Chromatographic profile obtained by ion-exchange chromatography of a uremic serum](image)

We used diethylamine- Sephadex A-25, equilibrated with 0.01 mol/L Tris-HCl buffer, pH 8.6, and eluted with the same buffer but with a continuous ionic strength gradient to NaCl, 1 mol/L. Flow rate was 10 mL/h.

Table 1. Concentrations of Peaks Obtained by Gel Filtration Monitored at 254 nm

<table>
<thead>
<tr>
<th>Patient group</th>
<th>n</th>
<th>Mean (and range), mm/mL of serum</th>
<th>Peak P_1</th>
<th>Peak P_2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cuprophane</td>
<td>6</td>
<td>15 (5–20)*&lt;sub&gt;b&lt;/sub&gt;&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7 (4–10)*&lt;sub&gt;a&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Polyacrylonitrile</td>
<td>6</td>
<td>12 (6–17)*&lt;sub&gt;abc&lt;/sub&gt;</td>
<td>6 (4–6)*&lt;sub&gt;abc&lt;/sub&gt;</td>
<td></td>
</tr>
<tr>
<td>CAPD</td>
<td>6</td>
<td>4 (1–9)*&lt;sub&gt;de&lt;/sub&gt;</td>
<td>3 (1–4)*&lt;sub&gt;de&lt;/sub&gt;</td>
<td></td>
</tr>
<tr>
<td>NDAP</td>
<td>6</td>
<td>3 (1–5)*&lt;sub&gt;de&lt;/sub&gt;</td>
<td>1 (0.7–2.5)*&lt;sub&gt;de&lt;/sub&gt;</td>
<td></td>
</tr>
<tr>
<td>Healthy controls</td>
<td>7</td>
<td>0 (0–0)</td>
<td>0 (0–0)</td>
<td></td>
</tr>
</tbody>
</table>

* P < 0.01 vs healthy controls; * P < 0.01 vs NDAP; * P < 0.01 vs CAPD; * P < 0.01 vs polyacrylonitrile; and * P < 0.01 vs cuprophane dialysis (Mann-Whitney's U-test to compare the different groups pair-wise).

Figures and Table 2 show the values obtained by ion-exchange chromatography for peaks P'_a (206 nm) and P'_a (254 nm). These are the only two peaks that are significantly associated with serum creatinine or creatinine clearance. The concentration of middle molecules in these two peaks is higher in hemodialyzed patients (without any difference between type of dialyzer) than in peritoneal dialysis and in nondialyzed azotemic patients. This is particularly evident for P'_a, which is absent in healthy subjects (Table 2) but shows the same concentration in peritoneal-dialysis patients as in nondialyzed azotemic patients. Therefore, P'_a does not discriminate between these groups (Table 2). However, the concentration of P'_a discriminates well between hemodialyzed patients and the other groups studied. P'_a is highly variable in CAPD patients (Figure 4) and does not discriminate between this group and polyacrylonitrile-hemodialyzed patients nor between CAPD and nondialyzed azotemic patients. The values for CAPD also overlap with those obtained in healthy subjects.

Figure 5 shows the serum concentrations of creatinine in...
molecules with peripheral neuropathy and uremic anemia (16). In vitro studies have shown that middle molecules have a depressive effect on lymphoblast proliferation and on mixed lymphocyte reaction (17). All these toxic actions of middle molecules point out the need of monitoring concentrations of middle molecules to assess therapy in uremic diseases. Achieving this general use will require a simplified procedure.

In this work, we have used the separation method of Fürst et al. (12), modified by increasing the flow rate to 10 mL/h, which yields a different profile from the one they reported. In addition, material for peak 7c was no longer available to be cochromatographed in our conditions, which obliged us to rename our peaks. Dr. Cueille kindly sent us material that produces the b4.2 peak but, when cochromatographed this did not coincide with any of our observed peaks, a discrepancy referred to by Dr. Cueille (18).

The reproducibility of the procedure adapted from Fürst’s method is similar to that of other studies (19), which allows us to study middle molecules with reasonable confidence in various stages of renal failure. From the results (Tables 1 and 2 and Figures 3 and 4), we can conclude that CAPD is the best dialysis procedure for eliminating these compounds; a similar result was obtained by Bergström et al. (20). The effectiveness of this procedure could be due to steady-state conditions in this type of dialysis, or to the continued lavage of the peritoneum with hypertonic solu-

**Table 2. Concentrations of Peak P’_o Obtained by Ion-Exchange Chromatography**

<table>
<thead>
<tr>
<th>Patient group</th>
<th>Mean</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cuprophane</td>
<td>10.3</td>
<td>5-18.5abc</td>
</tr>
<tr>
<td>Polycrylonitrile</td>
<td>11.6</td>
<td>8.5-16abc</td>
</tr>
<tr>
<td>CAPD</td>
<td>2.4</td>
<td>0-6de</td>
</tr>
<tr>
<td>NDAP</td>
<td>2.2</td>
<td>0-7de</td>
</tr>
<tr>
<td>Healthy controls</td>
<td>0</td>
<td>0-0</td>
</tr>
</tbody>
</table>

Footnotes as in Table 1.

the different groups. Table 3 shows significant Spearman’s coefficient of rank correlation values between serum creatinine or creatinine clearance and P_1, P_2, P_3, and P_4. All of these peaks are positively correlated with the concentration of serum creatinine and negatively with creatinine clearance.

**Discussion**

Plasma middle molecules appear more associated with complications of uremia such as edema, pericarditis, and intercurrent infections than is any other serum analyte (15). Other clinical studies have also associated middle
tions, which would result in increased porosity of the membrane (21), or to a minor metabolic alteration (20). For whatever reason, CAPD appears to remove neurotoxic middle molecules more efficiently than any other type of dialysis (3, 7, 21–24).

The different types of hemodialysis systems studied showed no differences with respect to the handling of middle molecules, as was also found by Asaba (15). Our results contradict previous reports regarding the behavior of polyacrylonitrile membrane derived from in vitro studies with B12 as an example of a middle molecule. The similarity of results in two different populations regarding middle molecule behavior in different dialysis procedures underscores the validity of this method for control of the concentrations of these metabolites in sera of azotemic patients.

Nondialyzed azotemic patients show the lowest values of middle molecules in uremic patients, which makes sense when one considers that this group has the highest mean for creatinine clearance (8 mL/min), compared with the CAPD group (3 mL/min), the polyacrylonitrile-dialyzed group (1 mL/min) or the cuprophane-dialyzed group (0.8 mL/min), and that the concentration of middle molecules considered as a whole (as determined by gel filtration) is inversely associated with creatinine clearance (Table 3). Middle molecules apparently are closely related to the remaining renal function, as reported by Asaba et al. (25) and by our group previously (26). Thus the concentration of middle molecules has been found to be associated with the severity of symptoms in nondialyzed azotemic patients (27). Perhaps the glomerulus filters these substances as easily as low-molecular-mass solutes.

When we consider the values in all studied groups of peaks P1 and P2 obtained by ion-exchange chromatography, in comparison with the values of peak P3 (obtained by gel filtration), all exhibit the same behavior (Tables 1 and 2 and Figures 3 and 5). Therefore, for an initial estimate of the concentration of the middle molecules, gel-filtration chromatography would be adequate, despite its being a non-specific analytical technique, given that the ultrafiltrate contains also carbohydrates, organic acids, amino acids, and other ultraviolet-absorbing solutes (28, 29). Although biochemically such an analysis would be unsatisfactory, especially given that peak 7c is composed of several different middle molecules (30, 31), clinically this approach may be acceptable. Uremic neuropathy has a multifactorial etiology, and, as previously reported by our group, motor conduction velocity is associated with middle molecules as a whole (26). In practical terms, this abbreviated technique for analyzing middle molecules is quite applicable in the clinical setting because of the simplification as well as lower cost and acceptable information. This will provide clinical practitioners a useful method for monitoring concentrations of middle molecules in patients with uremia.

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References

Table 3. Significant (P <0.01) Rank-Order Correlation Coefficients

<table>
<thead>
<tr>
<th></th>
<th>Creatinine</th>
<th>254 nm</th>
<th>206 nm</th>
<th>P1</th>
<th>254 nm</th>
<th>206 nm</th>
<th>P2</th>
<th>206 nm</th>
<th>P3p</th>
<th>206 nm</th>
<th>P3p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creatinine</td>
<td>—</td>
<td>0.71</td>
<td>0.63</td>
<td>0.82</td>
<td>0.57</td>
<td>0.76</td>
<td>0.81</td>
<td>—</td>
<td></td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Creatinine clearance</td>
<td>−0.74</td>
<td>−0.85</td>
<td>−0.85</td>
<td>−0.75</td>
<td>−0.7</td>
<td>−0.76</td>
<td>−0.77</td>
<td>−0.77</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>