Improved Separation and Quantification of the "Middle Molecule" b4-2 in Uremia

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The plasma of uremic patients usually contains high concentrations of the so-called middle molecules (molecular mass, 300 to 1500 Da), which exert various toxic effects. Among these numerous substances, only one, named peak b4-2, has been correlated with uremic neuropathy. We describe our improvement of a two-stage chromatographic method, gel permeation followed by anion-exchange chromatography (J. Chromatogr. 148: 55-65, 1978), for separation and quantification of b4-2 in body fluids. In analyzing more than 300 samples from 43 uremic patients and 12 healthy subjects, we found a linear correlation between peak area at 254 nm and b4-2 concentration in the range 0.8 to 32 mg/L. The coefficient of variation, including data acquired during seven changes of columns, was 5%. Analysis time (80 min) was shorter than required with other methods. Our results confirm previous data for urinary b4-2 excretion by healthy subjects and for b4-2 removal rate in uremic patients undergoing hemodialysis or hemofiltration. Patients treated with continuous ambulatory peritoneal dialysis have a higher b4-2 excretion than do healthy subjects, suggesting a higher production of this solute in uremic patients.

Additional Keyphrases: chromatography, gel-permeation, chromatography, anion-exchange, hemodialysis, urine, kidney disease

Patients with chronic renal failure have many metabolic abnormalities. Their clinical state can be improved by various techniques in which artificial systems remove toxic products of metabolism that would normally be excreted by the kidney. This improvement suggests that the uremic symptoms are attributable to the presence of toxins in the body fluids at concentrations greater than those encountered in healthy subjects.

Among the numerous attempts made to correlate the concentration of specific metabolites in plasma (or in other body fluids) with changes in uremic symptoms, the "middle molecule hypothesis" proposed by Babb et al. (1) has stimulated much investigation during the last decade. According to this hypothesis, "middle molecules" are solutes in the 300-1500 Da range that accumulate in body fluids of uremic patients and that can be more or less removed by hemofiltration, peritoneal dialysis, and hemodialysis, to name but a few techniques for treating chronic renal failure. Analytical techniques, metabolic toxicity, and clinical aspects of "middle molecules" in uremia and other diseases were recently reported in a symposium in Avignon, France (2).

A great number of toxic actions found in uremic sera, hemofiltrate, hemodialysate, or urine have been related to "middle molecules" (3). These actions were shown mainly in vitro experiments such as inhibition of induced lymphocyte proliferation, inhibition of platelet release, inhibition of the growth of cultured mouse 3T3 fibroblasts, inhibition of the frog sural nerve action potential, and inhibition of phagocytosis (4-8).

Polyneuritis is a principal symptom of middle molecule retention in uremic patients. Two of us treated six patients with a dialysis strategy involving a high removal rate for middle molecules, by using a high-permeability membrane (AN-69; Rhône-Poulenc, Vitry, France) (9). The neurological status of all these patients improved after three months of adequate dialysis (10); however, this improvement was found to be correlated only with a decrease in the concentration in plasma of a middle molecule named peak b4-2 (11), the in vitro neurotoxicity of which has been proved by the frog sural nerve test (12). Cuelle described a two-stage chromatographic procedure for measuring b4-2 in body fluids (13).

We describe a modification of Cuelle's method. Our procedure allows better recovery of peak b4-2, and substantial reduction of the coefficient of variation for peak surface area, thus improving reproducibility. Analysis time is shorter than required by Fürst et al. (14) and by Chapman et al. (15) for quantifying other uremic middle molecules (peak 7c) by gel-permeation chromatography followed by ion-exchange chromatography. This improved method allowed us to evaluate the peritoneal and renal excretion of the toxin b4-2 in uremic patients treated with continuous ambulatory peritoneal dialysis, as compared with urinary excretion in healthy subjects.

Materials and Methods

Apparatus

Figure 1 shows the chromatographic system we used. Samples were injected with a microliter glass syringe (style no. 3; Hamilton, 7402 Bonaduz, Switzerland) into the flow through a septum injector (Chromatam; Touzart et Matignon, 94400 Vitry, France) on the top of the steric exclusion column (borosilicated glass, 75 cm × 6.35 mm, i.d.; Technicon, 95330 Domont, France). We used a positive-displacement pump (Model 29633; Dosaprop Milton Roy, 27360 Pont Saint Pierre, France) to pump 2.5 mmol/L Na2SO4 (pH 6.6) at a flow rate of 36 ml/h. The eluent, monitored with an ultraviolet detector (0.32 A full-scale) at 254 nm vs an air-filled reference cell (Varian, Palo Alto, CA 94303), could be either discarded to waste or collected in a 5-mL syringe by turning a three-way valve. The peak containing middle molecules was collected and pumped (flow rate 30 ml/h) by a peristaltic pump (Minipuls 2; Gilson, 95400 Villiers-le-Bel, France) through a four-way valve toward the septum injector (Chromatam) of the ion-exchange column (borosilicated glass, 7 cm × 6.35 mm, i.d.; Technicon). Switching the four-way valve allowed elution of the ion-exchange column with a gradient (Mixograd; Gilson) from 10 to 500 mmol/L Na2SO4 (pH 6.6) at a flow rate of 30 ml/h. Absorbance of the
eluent from the second column was monitored at 254 nm, under the same conditions as for the steric exclusion column, but at 0.02 A full-scale. Both detector signals were recorded on a two-channel stripchart recorder (2 mV, 2.5 mm/min; Servotrace, Model PED VX 100; Sefram, 75015 Paris, France). Tubings were made either of Teflon (3 mm o.d.) from the buffer to the septum injector of the first column and from the buffers to the gradient former of the ion-exchange column, or of polyethylene (2 mm o.d.). The pump tubes were made of polyvinylchloride (Technicon). To maintain constant daily flow rates and reproducible chromatographic profiles, the pumping tubes were changed every six weeks. Freshly made eluent solvent was injected, followed by the injection of the standard solute to verify elution volumes and b4-2 peak area.

Reagents

De-ionized water was used throughout. Na₂SO₄ (Merck, Darmstadt, F.R.G.) buffers, 2.5, 10, and 500 mmol/L, were adjusted to pH 6.6 with diluted NaOH. The steric exclusion column was filled with Sephadex G 15 (Pharmacia) previously swollen for 3 h at 20 °C in 2.5 mmol/L Na₂SO₄. The ion-exchange column was filled with DEAE Sephadex A-25 (Pharmacia), previously swollen for 30 min at 20 °C in 500 mmol/L Na₂SO₄. Both slurries were degassed during 20 min before filling the columns, as were the elution liquids. No antimicrobial agent was added, either to the reagents or to the samples.

Procedures

Chromatography. All the analyses were run at room temperature.

For the steric-exclusion column (Sephadex G 15), pressure was continuously checked and kept within the range 25 to 100 kPa. Injected volumes varied from 25 to 500 μL. With a flow rate of 36 mL/h, nine peaks are distinguishable on the chromatograms (Figure 2). The first eluted peak (peak a, molecular masses >1500 Da) had an elution volume (Vₑ) of 8.4 mL, as determined with ovalbumin (relative molecular mass = 45 000; Carlo Erba, 20159 Milano, Italy). Peak b, corresponding to the middle molecule fraction (elution volume = 1.11 Vₑ), was routinely collected between 7.2 and 11.6 mL eluted, and this 4.4 mL was injected onto the ion-exchange column. In the case of a very large peak b, the original sample volume injected onto the steric-exclusion column was reduced, to collect all of the eluent corresponding to peak b in 4.4 mL.

Use of the ion-exchange column (DEAE Sephadex A-25) further separated peak b into ultraviolet-absorbing or non-absorbing components (16). Peaks were eluted with an ion gradient of from 10 to 500 mmol/L in a 1-h interval (Figure 3). Six peaks that absorb at 254 nm were detected (b1 to b6, b1 being first eluted). As previously described (17), b4 was the peak of interest in uremia. This peak contains two subpeaks: one detected at 206 nm (b4-1, not shown), the other detected at 254 nm (b4-2). Only the latter apparently correlates with neuropathy in uremic patients (10, 11).

Sample preparation. Blood samples were collected in 10-mL plastic tubes containing lithium heparin anticoagulant (Labo Express Service, 91170 Viry-Châtillon, France). After centrifugation (20 min, 3000 rpm) plasma was deproteinized by ultrafiltration through a polycrylonitrile membrane (AN-69 from an RP 6 hemodialyzer; Rhône-Poulenc, 75008 Paris, France). Ultrafiltration was performed with 400-kPa air pressure and stopped when the ultrafiltrate reached 50% of the initial measured plasma volume. The retentate was discarded and the middle molecules in the ultrafiltrate were measured.

Urine samples were filtered through white paper (no. 08322-71; Prolabo, 75011 Paris, France) to remove particulate matter before analysis.

If necessary, hemodialysate samples were concentrated five- to 10-fold under reduced pressure at 37 °C (Rotovap; Heidolph, OSI, 75015 Paris, France). As with urine, paper filtration was used to remove precipitates.

Hemofiltrate samples generally required neither concentration nor deproteinization before analysis, and thus were injected without pretreatment.

Peritoneal dialysate samples were handled in the same way as plasma, with ultrafiltration through polycrylonitrile membrane to remove small proteins that had filtered
from the urine of healthy subjects (final concentration, 21 mg/L) to determine the concentration in the samples.

A linear standard curve (\( y = 0.257 + 0.366x \)) was obtained by plotting peak area (cm²) against the concentration of standard b4-2 over the range of 2 to 21 mg/L (\( r = 0.9996, n = 5 \), data not shown).

**Patients**

**Healthy subjects.** We assayed plasma and 24-h urines of healthy subjects on a free diet.

**Hemodialysis.** Plasma and hemodialysate came from uremic patients submitted to regular hemodialysis treatment three times a week with a closed batch-dialysate delivery system (Cuprophan hemodialyzer, 1.8 m² effective surface).

**Hemofiltration.** Hemofiltration (three times a week) was performed with a Filtral hemodialyzer, and b4-2 was measured in the pooled hemofiltrate from one session.

**Peritoneal dialysis.** Seven patients treated with continuous ambulatory peritoneal dialysis according to the technique of Oreopoulos et al. (20) for 120–575 days were admitted for mass balance study. The patients, in stable clinical condition, had creatinine clearances ranging from 1.1 to 7.4 mL/min per 1.73 square meters of body surface. Blood samples were withdrawn at the beginning and at the end of the 24-h study period. Bags of fresh and used dialysate were carefully weighed for mass balance. Peritoneal dialysate flow rates were in the range 4.1–6.9 mL/min. Used dialysates were pooled for each patient, well mixed, and sampled for b4-2 determination; 24-h urines were collected for the same purpose.

**Results**

**Analysis time.** A whole run was completed in less than 80 min.

**Precision.** The reproducibility of the method was checked in two ways. First, the within-day CV for retention volume of b4-2 was 1.9% (n = 11). Secondly, reproducibility among runs was followed from September 1980 to August 1981. Repeated injections of the same amount of purified b4-2 corresponded to a mean peak area of 74 (SD 3.1) cm²/mL injected (n = 23, CV = 4.2%). For the same period the surface area given by one specific sample (100 μL of fivefold diluted urine from a healthy subject) was 8.0 (SD 0.7) cm²/mL (n = 15, CV = 9.0%). Retention volumes were 12.14 (SD 0.70) mL (n = 23) and 12.26 (SD 0.62) mL (n = 15), respectively, for purified b4-2 and for the urine sample. This one-year period included seven changes of columns and more frequent changes of buffers.

**Detection limit.** Taking into account the baseline perturbations occurring at the sensitivity scale we used, no accurate quantification could be made at concentrations less than 0.8 mg/L, owing the nonlinearity of the calibration curve in this concentration range (not shown). Practically, this corresponded to a minimal amount of 0.4 μg of b4-2 (in 500 μL) that could be applied to the top of the column.

**Accuracy.** Urine from a healthy subject was diluted two-to 40-fold with water. Measured concentrations of the 10 diluted samples (over the 40-fold concentration range) were plotted against theoretical dilution values, giving a linear correlation (\( y = 0.168 + 31.12x, r = 0.998, n = 11 \)) in the range of 0.8 to 32 mg/L (not shown). Furthermore, after correction for dilution, mean ± SD was 32.0 ± 1.3 mg/L, for a CV of less than 4%. Analyses were in fact run in the range 2–21 mg/L.

**Potential interferences.** Peak b4-2 being correlated with neuropathy, we focused our attention on artifacts that could impede its accurate quantification, especially the peaks immediately preceding and following it (b4-1 and b5).
than 300 samples were analyzed (from 43 patients and 12 healthy subjects) and checked for the presence of a high peak b4-1 or b5 (i.e., > peak b4-2). Apparently such interference could be related either to the preparation of the samples or to the drugs the patients were receiving. For example, acetylsalicylic acid, ingested orally, produced a high peak b5 in the urine of healthy subjects and in the dialysate of uremic patients. Neither the use of sodium azide (NaN₃) as preservative in hemodialysate, urine, or hemofiltrate, nor the use of heparin as anticoagulant caused artifactual peaks. Blood samples collected into tubes containing fluoride + oxalate, however, gave a high peak b5. In peritoneal dialysate that was not deproteinized by ultrafiltration on AN-69, a higher peak b3 was observed. By not allowing accurate estimation of the baseline, this impeded calculation of the area of peak b4-2. Hemolysate, which is known to contain middle molecules (15, 21), was found to give a very high peak b4-1. Erythrocyte middle molecules had an elution volume close to that of b4-2. When whole blood was analyzed after complete hemolysis, it was not possible to measure peak b4-2 accurately because the erythrocytes absorbed strongly at 254 nm. However, erythrocytes do not seem to contain a significant amount of peak b4-2.

**Evaluation of b4-2 excretion.** Table 1 shows b4-2 concentration in plasma and the excretion rate obtained for healthy subjects and for uremic patients treated with hemodialysis or hemofiltration (predialysis values). In the plasma of healthy subjects, the b4-2 concentration remained below the detection limit of the method (<0.8 mg/L). Expressed in terms of standard body surface area, the daily urinary excretion in healthy subjects was 36 ± 2 mg/1.73 m².

In plasma of seven patients undergoing continuous ambulatory peritoneal dialysis, b4-2 concentration was 4.5 (SEM 0.8) mg/L. Daily excretion in dialysate (mean ± SEM) was 32 ± 8 mg/1.73 m² and daily excretion in urine was 23 ± 5 mg/1.73 m². The total daily excretion (54 ± 7 mg/1.73 m²) is thus greater than in healthy subjects (p < 0.05).

**Discussion**

Furst et al. (14) described a two-stage chromatographic method for the analysis of middle molecules in body fluids. By increasing buffer flow rates, Chapman et al. (15, 22) devised a method that enables more rapid completion of analyses (3.25 vs 8 h). Cueille (13) described another two-stage chromatographic method for much faster separation of middle molecules. Our modification of this last method allows completion of six analyses per 8-h working day. If not investigating the peaks eluted after b4-2, one can shorten the gradient to one run in about 60 min.

The resistance of middle molecule b4-2 to metabolic modifications should be noted, there being no need to add antimicrobial agent. The reproducibility of the purified standard and of a reference urine sample shows good stability of b4-2 over a one-year period when kept frozen at −20 °C. Moreover, analysis can be done at room temperature and does not require any cooling system.

The CV over a one-year period (9%) is less than the 16% formerly obtained (13) and is about the same as that obtained by Chapman et al. (8%) in the quantification of the middle molecules of their peak 7 (15). This improvement could be explained by the fact that the peak b is collected separately (see Figure 1) at atmospheric pressure and then re-injected on the ion-exchange column at the same flow rate as the gradient is eluted. This allows accurate collection and adequate re-injection of peak b.

The detection limit does not enable precise quantification in healthy subjects of plasma b4-2 concentrations less than 0.8 mg/L, so that preliminary concentration of plasma ultrafiltrate is required. However, this sensitivity is quite sufficient for quantifying b4-2 in uremic plasma, hemofiltrate, urine (healthy and uremic subjects), and peritoneal dialysate. Furthermore, hemodialysates, which are sometimes very dilute, can be readily concentrated under reduced pressure, thus enhancing the detection limit.

In our method, preventing artifactual peaks requires some simple precautions in collecting samples. We use lithium heparinate as the anticoagulant, carefully deproteinize plasma and peritoneal dialysate by ultrafiltration through polyacrylonitrile membrane, and avoid hemolysis.

Having checked the reliability of our improved method for separation and quantification of b4-2, we measured the concentration of this solute in healthy subjects and in uremic patients treated with various dialysis strategies. The b4-2 concentration in plasma and the removal rate in uremic patients treated by hemodialysis or hemofiltration compare quite well with our previous results (10). However, the urinary excretion from healthy subjects exceeds the previously reported values. This could be explained by (a) the healthy subjects' receiving an uncontrolled free diet, (b) a more adequate collection of peak b, and/or (c) our re-analysis, after dilution, of those samples having too high a middle molecule concentration, which gave too great a peak b.

The b4-2 concentration in plasma of healthy subjects and uremic patients is shown in Table 1. It is clearly lower in uremic patients than in healthy subjects.

**Table 1. b4-2 Concentration in Plasma and Removal Rate in Healthy Subjects and Uremic Patients**

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<thead>
<tr>
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<th>b4-2 concn. mg/L</th>
<th>Excretion</th>
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<tbody>
<tr>
<td>Healthy subjects (n = 9)</td>
<td>&lt;1</td>
<td>38 ± 3 mg/24h</td>
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<tr>
<td>Uremic patients</td>
<td></td>
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<tr>
<td>Hemodialysis (n = 6)</td>
<td>4.0 ± 0.7</td>
<td>57 ± 9 mg/session</td>
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<td>(Cuprophan membrane)</td>
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<tr>
<td>Hemofiltration (n = 7)</td>
<td>3.5 ± 0.6</td>
<td>61 ± 12 mg/session</td>
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<td>(AN-69 membrane)</td>
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*Mean ± SEM.

In conclusion, our modified method for the measurement of b4-2 in body fluids improves the reproducibility and reliability of the quantification. Our data confirm the previous results (10) obtained for uremic patients treated with hemodialysis or hemofiltration. Moreover, the removal rate study in patients treated with continuous ambulatory peritoneal dialysis suggests that our method can be used routinely for assessment of dialysis adequacy.

**References**