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Note

Ion-pair high-performance liquid chromatography profiling of a uremic toxin fraction

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Some uremic patients exhibit above-normal sodium concentrations in their erythrocytes [1–3]. We recently studied this by ^{23}Na nuclear magnetic resonance (NMR) spectroscopy and were led to postulate the presence of a Na^+, K^+-ATPase inhibitor in uremic body fluids [4]. To verify this hypothesis, we studied in vitro the inhibitory effect of uremic toxins, so-called uremic middle molecules, and demonstrated the existence of such an inhibitor in one fraction (2-3 fraction) of these uremic toxins [5].

However, the 2-3 fraction used in the latter study was not isolated in pure form since we later found that it could be partially separated by gel permeation into two parts with quite different UV absorptions. In a first step we studied the high UV-absorbing part of this fraction and the present report concerns its high-performance liquid chromatographic (HPLC) profiling study.

EXPERIMENTAL

Sample preparation

Five 2-3 fractions of uremic toxins were isolated from four different uremic plasmatic ultrafiltrates and from a pool of normal urines according to a semipreparative procedure previously described [6]. Briefly, the first step was gel permeation (Sephadex G15) followed by anion-exchange chromatography (Sephadex DEAE A25). The last step was gel permeation (Sephadex G15). In

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each step the eluate UV detection was carried out at 254 nm. Finally the fractions were stored in lyophilised form. Prior to HPLC analysis, the fractions were solubilized with bidistilled water, and the solutions were filtered through a 0.45-μm membrane (Millipore, Paris, France).

Reagents

Tetrabutylammonium chloride (TBA-Cl) was obtained from Sigma (St. Louis, MO, U.S.A.) and ammonium acetate from Prolabo (Paris, France). Deionized water was bidistilled using a BI 18 bidistillation apparatus from Heraeus (Orsay, France). Uvasol-grade methanol was used as purchased from Merck (Paris, France).

High-performance liquid chromatography

The liquid chromatography separations were performed using a fully automated HPLC system consisting of a Vista 5040 liquid chromatograph and a Vista 401 data system from Varian (Orsay, France) equipped with a Model Valco manual loop injector (10-μl loop). A Kratos Model Spectroflow 783 UV–visible detector was operated at 240 nm and 0.05 a.u.f.s. The analytical column, 250 × 4.9 mm I.D., was packed with Spherisorb C_6, particle size 5 μm (Soparès, Gentilly, France).

The chromatographic system was operated at room temperature. Separation of the 2-3b sample (140–700 μg) was achieved by gradient elution. The mobile phase consisted of solvent A, which was bidistilled water–0.005 *M* TBA-Cl–0.05 *M* ammonium acetate, and solvent B, which was methanol–0.005 *M* TBA-Cl–0.05 *M* ammonium acetate. Before use the solvents A and B were filtered through a 0.45-μm membrane filter.

The initial conditions for the first 10 min of the analysis were 80% A and 20% B. Then the solvent composition was changed linearly over a period of 20 min to 55% A and 45% B. At 45 min, the solvent composition was changed over a period of 1 min back to initial conditions of 80% A and 20% B and was equilibrated for 30 min. The mobile phase flow-rate was 1 ml/min.

RESULTS AND DISCUSSION

From uremic body fluids and normal urines the first gel permeation step yields seven fractions. Fraction 2, corresponding to crude uremic toxins, is separated into six fractions (2-1–2-6) by an anion-exchange step. The last gel permeation step allowed a desalting of the 2-3 fraction and separated it into two parts 2-3a and 2-3b (Fig. 1). The latter exhibited a high UV absorption at 240 nm.

As shown in Fig. 2, reversed-phase HPLC with the ion-pair complex formation gave an efficient separation of the 2-3b fraction. Many UV-absorbing solutes were present in this fraction. More than 30 peaks were resolved.

Qualitatively, we observed the same peaks regardless of the origin of the sample (uremic patients or normal urine). Therefore, the possibility of artifacts due to the presence of drugs co-eluted with uremic toxins was discarded [7]. Moreover, the similarity of the normal uremic elution profiles is indicative of the physiological character of the separated substances.

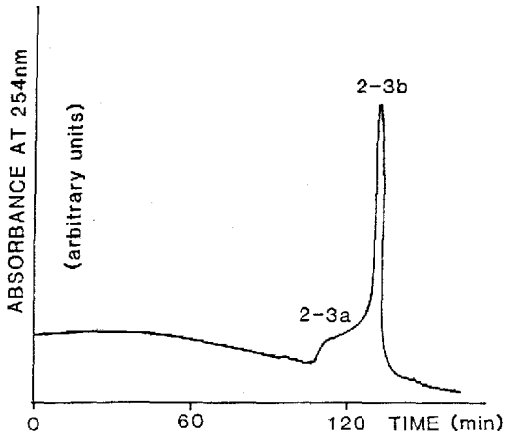

Fig. 1. Typical gel permeation chromatographic profile of the 2-3 fraction. Chromatography was carried out with a 100×2.5 cm I.D. glass column packed with Sephadex G15 using bidistilled water as eluent. The eluate was monitored at 254 nm at a flow-rate of 80 ml/h.

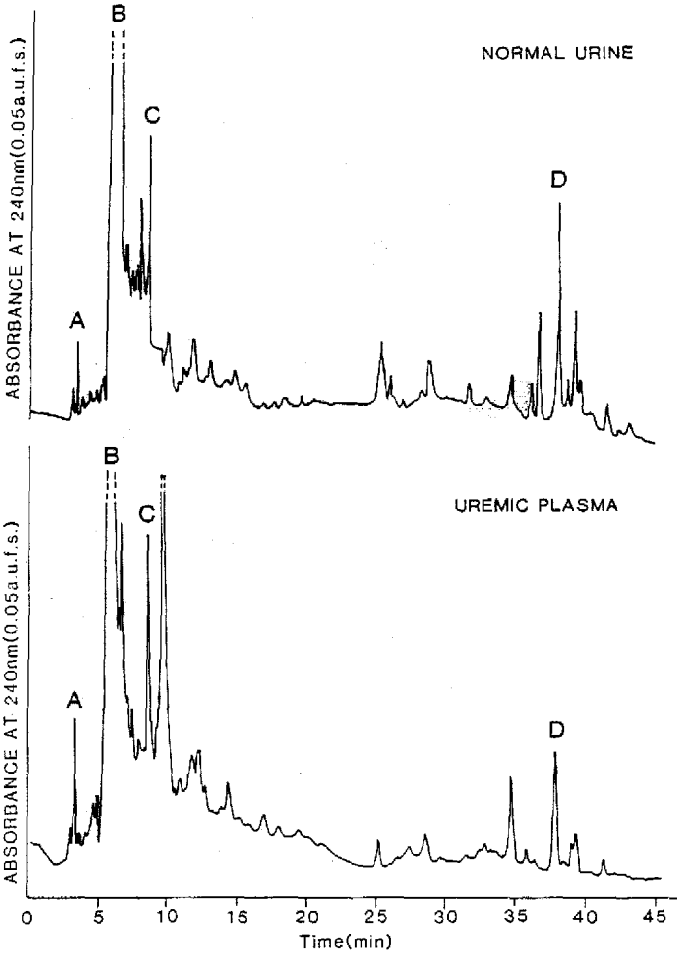

Fig. 2. Typical HPLC elution profiles of the 2-3b fraction from normal urine (top) and from uremic plasmatic ultrafiltrate (bottom). Chromatographic conditions are described in Experimental.

In contrast, the chromatograms obtained were quantitatively different from one sample to another. These differences suggest variations in the accumulation or metabolism of these substances from one patient to another.

To verify the proportionality between the peak intensities and the amount of sample analysed, amounts of 2-3b fraction varying from 140 to 700 μg were analysed, and the integration of some peaks (A, B, C and D in Fig. 2) was determined. A linear response is obtained from these peaks: A, $y=1468x-428$, $r=0.998$; B, $y=51\,758x+3889$, $r=0.997$; C, $y=2539x+62$, $r=0.997$; D, $y=7706x+766$, $r=0.998$.

In order to determine the reproducibility of the entire chromatographic procedure, several analyses were carried out with the same sample. For each peak the coefficient of variation (C.V.) of retention times was always lower than 1%, and the average C.V. was 0.8%.

To conclude, this HPLC method allows the separation of a uremic toxin fraction into its various components, thus permitting the identification of these substances and the determination of their activity on Na^+, K^+-ATPase.

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