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Scaling up in isolation of medium-size uraemic toxins

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ABSTRACT

Plasmatic accumulation of uraemic toxins in the middle molecular mass range has been reported to be associated with several pathologies observed in uraemic patients. The very low concentration of these toxins in uraemic body fluids makes classical chromatography techniques inadequate in isolating sufficient amounts of these endogenous substances, thus precluding their identification. A scaling up of gel permeation and ion-exchange chromatographies was therefore developed. This considerably increased the amount of uraemic toxins isolated, thus allowing the study of their chemical nature and facilitating understanding of their biological activities.

INTRODUCTION

Uraemic haemodialysed patients classically develop several associated disorders such as uraemic neuropathy and disturbances of ionic metabolism (Na^+, Ca^{2+}, etc.). These pathologies are commonly attributed to plasmatic accumulation of endogenous substances in the molecular mass range 200–2000, the so-called uraemic middle molecules (UMM) [1].

In previous papers [2,3] we demonstrated that UMM, isolated by successive gel permeation and anion-exchange chromatography, exhibit some biological effects correlated with several pathologies such as neuropathy and Na^+ metabolism disorders which are associated with end-stage renal failure treated by haemodialysis.

However the concentrations of these toxins in uraemic body fluids are very low. Hence the standard chromatographic technique described previously [4] is inadequate for isolating sufficient amounts of these active molecules, thus precluding their identification and a better understanding of their biological activities. We therefore scaled up the standard chromatographic process to increase the amounts of UMM isolated. In this paper we report the UMM separations obtained using scale-up and standard chromatographic conditions simultaneously for comparison.

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EXPERIMENTAL

Biological samples

Ultrafiltrates of plasma from uraemic patients (UF) were obtained at the beginning of dialysis by applying a negative pressure (400 mmHg) in the dialyser compartment before the dialysis fluid was run through the dialyser. Samples were stored at −20°C until use.

Gel permeation chromatography (GPC)

Preparative scale. A 100-ml volume of UF was separated on a glass column (900 × 50 mm I.D.) packed with Sephadex G-15. The column was eluted with Tris–HCl buffer (10^{-2} *M*, pH 8.6) at a flow-rate of 296 ml h^{-1} (linear velocity 15.07 cm h^{-1}). The absorbance was monitored at 254 nm and the eluate was collected in 9-ml aliquots.

Standard conditions. A 20-ml volume of UF was chromatographed on a 900 × 26 mm I.D. column of Sephadex G-15 eluted with Tris buffer at a flow-rate of 80 ml h^{-1}.

Ion-exchange chromatography (IEC)

Preparative scale. A UMM sample (corresponding to 400 ml of UF) was injected onto a glass column (230 × 26 mm I.D.) packed with DEAE-Sephadex A-25 and equilibrated with Tris–HCl buffer (10^{-2} *M*, pH 8.6) at a flow-rate of 275 ml h^{-1}.

The column was eluted for 60 min with the same buffer, followed by a 12-h exponential gradient of NaCl at the same flow-rate. At any time the NaCl concentration was

$$C_t = C_f[1 - \exp(-Qt/V)]$$

where C_t represents the NaCl concentration at time t, C_f the final NaCl concentration (0.13 *M*), Q the flow-rate and V the volume of the gradient mixing chamber (1000 ml). The absorbance was monitored continuously at 254 nm and aliquots (13 ml) were collected.

Standard IEC. A UMM sample from 40 ml of UF was separated using a 23 cm × 8 mm I.D. column (DEAE-Sephadex A-25) equilibrated with Tris–HCl buffer at a flow-rate of 26 ml h^{-1}. Elution was carried out with an exponential gradient of NaCl (0–0.15 *M*) using a 125-ml mixing volume chamber.

RESULTS

Fig. 1 shows typical elution profiles of 100 ml and 20 ml of UF separated by GPC (Sephadex G-15) according to preparative (top) and standard (bottom) conditions, respectively. The elution profiles obtained with the preparative procedure and standard chromatographic method(s) were identical. Thus, GPC provided 8–10 fractions. Peak 2 is observed in uraemic but not in normal plasma ultrafiltrates [4]. The apparent molecular mass of the peak is about 1000 dalton, hence this compound falls in the molecular mass range of the so-called "uraemic middle molecules" [5], which have been suggested to be connected with various uraemic disorders [1,6,7]. Thus peak

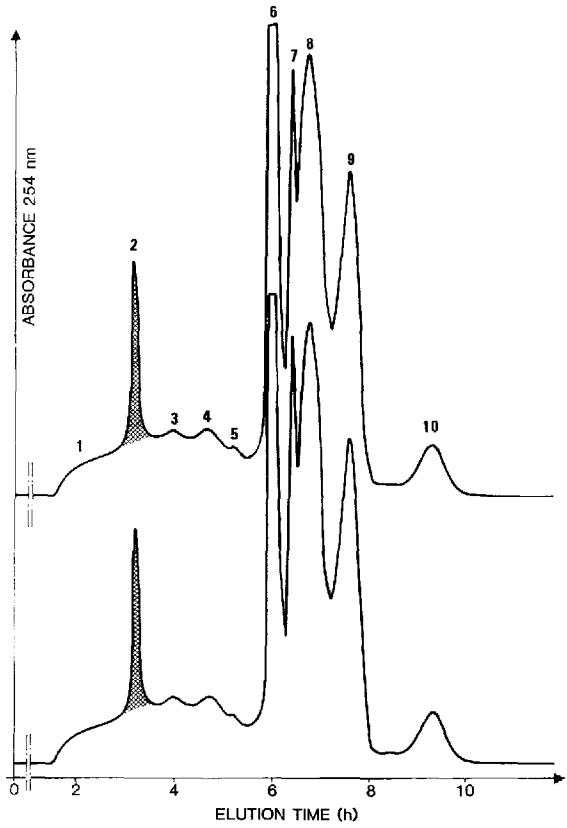

Fig. 1. Typical GPC of uraemic plasmatic ultrafiltrates: scale up (top) and standard procedures (bottom). The hatched fraction represents the elution of UMM (peak 2). For peaks, see Results section.

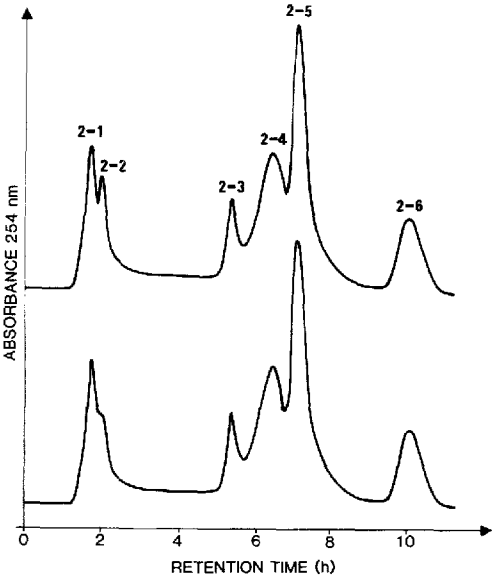

Fig. 2. Typical IEC elution profiles of crude UMM (peak 2): scale up (top) and standard procedures (bottom).

2 was called "crude UMM". It contains compounds which have been reported to be toxic [2,3]. IEC separation was therefore performed on this peak.

Fig. 2 represents IEC separations of crude UMM (peak 2). As in GPC, the chromatograms obtained were identical. IEC allows the separation of peak 2 into six fractions numbered in order of elution 2-1, ..., 2-6. The method of peak labelling is based on the decimal system, where 1, 2, 3, etc., refer to the primary peaks obtained with GPC. Next, sub-peaks obtained in the subsequent separation of peak 2 by IEC are designed in order of elution: 2-1, 2-2, 2-3, etc. This numbering system follows previously established nomenclature [8].

DISCUSSION

Separation of UMM by the standard technique using two successive chromatographic steps is an efficient method but it does not allow the isolation of sufficient amounts of compounds. Therefore, in order to obtain a greater amount of these substances, we scaled up the GPC and IEC procedures.

For GPC an increase in productivity was achieved by increasing the column diameter (from 26 mm to 50 mm), which increased the stationary phase volume (from 478 to 1767 ml) and allowed the separation of an increased sample volume (from 20 to 100 ml). In order to preserve the chromatographic resolution and the separation time, the flow-rate was increased (from 80 to 296 ml h^{-1}) to obtain a constant linear velocity of eluent (15.07 cm h^{-1}).

The comparative results obtained (Fig. 1) show that scaling up in GPC allows a five-fold increase in sample volume treated per chromatographic run.

For the scaling up in IEC, the column size was adapted to the sample volume treated. To select an appropriate column, we used the equation

$$S.F. = \frac{(I.D._2)^2 \times L_2}{(I.D._1)^2 \times L_1}$$

where $S.F.$ is the scale factor for separation, $I.D._2$ is the I.D. of the preparative column and L_2 is its length, $I.D._1$ is the I.D. of the standard column and L_1 is its length.

To preserve the resolution we maintained the column length ($L_1 = L_2 = 23$ cm). Because the sample volume to be processed per chromatographic run increases from 40 to 400 ml, the $S.F.$ value was 10. Under these conditions, as $I.D._1$ was 8 mm, the calculated $I.D._2$ was about 26 mm. Therefore, the preparative column selected for IEC was 230 × 26 mm I.D. (see Experimental). In addition, to preserve the separation time, we adjusted the flow-rate to 275 ml h^{-1} to maintain a constant linear velocity of 51.8 cm h^{-1}.

As elution was achieved using an increasing exponential NaCl gradient (see Experimental), modification of the flow-rate modified the slope of the gradient. Therefore, in order to preserve a similar gradient curve, we recalculated the other gradient parameters taking into account the new flow-rate. The mixing gradient chamber volume was therefore increased from 125 to 1000 ml and the final NaCl concentration was 0.13 M.

Under these optimized conditions, the separation of UMM (peak 2) by scaling up in IEC is comparable to the separation carried out under the standard conditions

(Fig. 2). Moreover, scaling up makes it possible to process ten times more UMM sample volume per run.

Consequently, this scaling up in the isolation steps of UMM considerably increases the amount of compounds isolated, thus making it possible to study their chemical nature and biological activity.

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