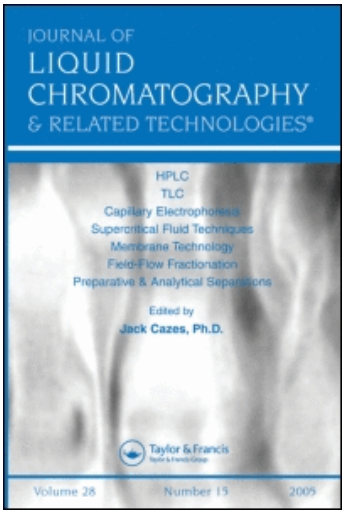

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An Improved Semi-Preparative Liquid Chromatography Technique to Purify A Uremic Toxin Fraction Inhibiting Na , K -Atpase

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AN IMPROVED SEMI-PREPARATIVE LIQUID CHROMATOGRAPHY TECHNIQUE TO PURIFY A UREMIC TOXIN FRACTION INHIBITING Na^+, K^+-ATPase

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ABSTRACT

We reported previously inhibition of Na^+,K^+-ATPase by a fraction (fraction 2-3) of medium size uremic toxins. Due to considerable amount of sulfates in this fraction, a semi preparative high performance liquid chromatography turned out to be inadequate to isolate the active compound. Therefore we developed a novel chromatographic method using ion exchanger Sephadex QAE A25 in acidic medium. This method allows the elimination of sulfates and the partial purification of the active component.

INTRODUCTION

Among uremic toxins the so-called "uremic middle molecules" (UMM) with a molecular mass around 1000 Da are well known to accumulate in uremic plasma. These substances are eliminated in the urine of healthy subjects and seem to be associated with various uremic disorders (1).

Since Welt (2) observed decreased red cell Na^+,K^+-ATPase activity in some uremic patients there has been overwhelming evidence that the Na^+,K^+-pump is depressed in uremia (3). Studies showing inhibition of the pump in normal erythrocytes incubated with uremic plasma (4,5) and the corrective effect of hemodialysis on the Na^+ transport alterations (6-8) suggest that a dialysable circulating factor may cause Na^+,K^+-pump suppression in uremia.

Using ^{23}Na-Nuclear Magnetic Resonance (^{23}Na-NMR) we confirmed in our own previous work some uremic patients exhibit indeed high levels of intracellular sodium (9). We also isolated next, using gel permeation followed by ion exchange chromatographies, from uremic plasma ultrafiltrates and normal urines, a fraction of UMM (fraction 2-3) (10) and demonstrated its capacity to inhibit Na^+,K^+-ATPase, in vitro, with doses corresponding to those found in plasma of uremic patients (11). Next, using ^{23}Na-NMR technique we demonstrated this fraction 2-3 causes impairment of the Na^+,K^+-pump in intact living erythrocytes (12).

But fraction 2-3 studied hitherto, had not been isolated in pure form, as demonstrated by HPLC analysis, which resolved it into many UV-absorbing solutes (13). In addition, fraction 2-3 contains about 90% sodium sulfate, as we later found

on chemical analysis (unpublished results). Because of the presence of high amounts of diluting substance, the HPLC technique failed in the case of semi preparative separation of the active component.

Therefore in the present study we have developed a new liquid chromatography method in order to eliminate sulfates and increase the purification of the Na^+,K^+-ATPase inhibitor present in the fraction 2-3 of UMM.

MATERIALS AND METHODS

Biological Samples

Plasma ultrafiltrates (UF) from four uremic patients treated by hemodialysis and urine from three healthy subjects were processed.

UF samples were obtained at the beginning of dialysis by applying a negative pressure (400mm Hg) in the dialyzer compartment after priming the dialyzer and stopping the dialyzate flow.

Urines samples were centrifugated for 60 min. at 3000g.

All samples were stored at -20°C until use.

Isolation of Fraction 2-3

Fraction 2-3 from UMM was isolated according to a preparative liquid chromatographic method recently developed (14). Let us recall briefly the two steps of this technique:

UF or normal urine is separated by gel permeation chromatography on Sephadex G15, eluted with Tris-HCl buffer pH=8.6into 8-10 fractions numbered in order of elution.

Fraction 2 is identified to UMM because its chromatographic behaviour corresponded to compounds with molecular mass of about 1000 Da.

Then, UMM (fraction 2) are fractionated by ion exchange chromatography on Sephadex DEAE A25 using an increasing exponential gradient of NaCl in Tris-HCl buffer pH=8.6 as eluent. The 6 fractions obtained were labeled 2-1 through 2-6 in order of elution.

Finally fraction 2-3 is desalted, analysed for its sulfates content and stored in lyophilized form.

Purification of Fraction 2-3

6 ml of fraction 2-3 in water solution (1 mg/ml) are injected onto a glass column (230 x 8 mm I.D.) packed with Sephadex QAE A25 and equilibrated with dilute HCl solution (pH=2.4) at a flow-rate of 26 $mlxh^{-1}$.

The column is eluted for 180 min. with the same HCl solution, followed by a 960 min.increasing exponential gradient of NaCl at the same flow-rate.

Three different NaCl final concentrations (0.15 M, 0.05 M and 0.025 M) are applied. At any time the NaCl concentration was:

$$C_t = C_f \left(1 - e^{-\frac{Qt}{V}} \right)$$

where C_t represents the NaCl concentration at time t; C_f the final NaCl concentration used (0.15 M, 0.05 M or 0.025 M); Q, flow-rate; V, volume gradient mixing chamber (125 ml).

The absorbance is monitored at 254 nm and the eluate is collected in 10 ml aliquots.

The sulfate elution is checked manually by classical precipitation with $BaCl_2$ solution (10%) in acidic medium.

Depending on the elution profile, the eluate is divided into six fractions: 2-3-1 through 2-3-6.

Finally the fractions are collected, lyophilized, reconstituted in 0.2 ml of Tris-HCl buffer 0.1 M pH=7.4 and tested for their inhibitory effect on Na^+,K^+-ATPase at a final concentration of 6 times the original fraction 2-3 water solution.

Inhibitory effect is estimated at 37°C by the rate of inorganic phosphorus (P_i) release from 3 mM Na_2ATP (Vanadate free) pH=7.4 in a 1 ml reaction mixture containing 100 mM NaCl, 20 mM KCl, 1 mM EGTA, 20 mM $MgCl_2,6H_2O$, 100 mM Tris-HCl buffer and 0.1 unit of purified Na^+,K^+-ATPase from dog kidney (Sigma) according to previous report (10).

After incubation for 5 min., reaction is stopped by addition of cold perchloric acid (0.75 M final concentration). P_i released is measured according to Hurst's method (15).

Enzymatic activities obtained with chromatographic fractions are compared with those determined with enzyme alone and results are expressed as inhibition percentage.

RESULTS AND DISCUSSION

Fraction 2-3 from UF (4 patients) and normal urine (3 subjects) are prepared according to isolation steps involving gel permeation and ion exchange chromatographies at pH=8.6.

Under these conditions sulfates, issued from biological samples, coelute into fraction 2-3 as determined by chemical analysis. Consequently sulfate content of fraction 2-3 (about 90%) interferes with its purification and biological study.

Therefore , to eliminate these cumbersome ions, fraction 2-3 is processed by using a chromatographic technique involving anion exchange on Sephadex QAE A25 in acidic medium (pH=2.4) and an increasing exponential gradient of NaCl as eluent.

First, fraction 2-3 sample is chromatographed using a 0.15 M final concentration of NaCl for gradient elution. These experimental conditions allow an efficient separation of sulfate ions from the UV-absorbing solutes as shown in Figure 1A. In addition we note a separation beginning of fraction 2-3 into several components. But under these experimental conditions, the resolution is unsufficient.

Therefore, to improve the separation, the exponential gradient conditions are modified by decreasing the final concentration of NaCl.Using 0.05 M and 0.025 M NaCl typical elution profiles shown in Figure 1B and 1C are obtained respectively.

These gradient conditions allowed an improvement in the separation of the UV-absorbing solutes. At the same time sulfate retention increases considerably (Figure 1B) and with 0.025 M NaCl gradient concentration, these ions are not eluted (Figure 1C). Therefore we choose the latter as standard chromatographic condition.

The UV-absorbance profile show that chromatography on Sephadex QAE A25 column allows separation of fraction 2-3 into several peaks. The ones poorly adsorbed by ion exchanger are eluted isocratically with HCl solution (pH=2.4) and constitute fraction 2-3-1, the others eluted under gradient of 0.025 M NaCl yield fraction 2-3-2 through 2-3-6 as shown in Figure 1C.

Figure 2 compares typical elution profiles of fraction 2-3 from UF (top) and normal urine (bottom). In spite of variations in peak intensities, the chromatograms are qualitatively similar. This is in good accordance with data

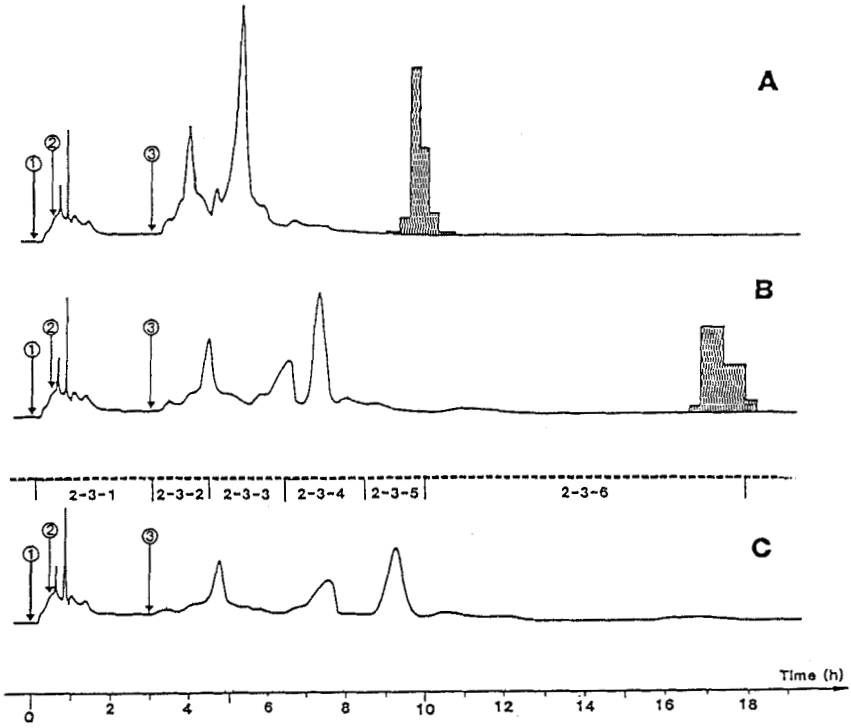

FIGURE 1: Typical UV-absorbance profiles of fraction 2-3, chromatographed on a Sephadex QAE A25. Arrows 1, 2 and 3 indicate the sample injection, the beginning of the elution with HCl solution (pH=2.4) and the start of gradient elution with NaCl final concentration 0.15 M (A), 0.05 M (B) and 0.025 M (C) respectively. Detection 0.2 a.u.f.s. at 254 nm . Hatched fractions point out the sulfates characterization in eluate. Collection mode is indicated on the hatched line above part C of the figure.

showing that UMM accumulated in plasma of uremic patients are eliminated in urine of healthy subjects (1,10).

These results show that our new chromatographic step in purification of fraction 2-3 from UF or normal urine eliminates sulfates and yields 6 fractions: 2-3-1 through 2-3-6.

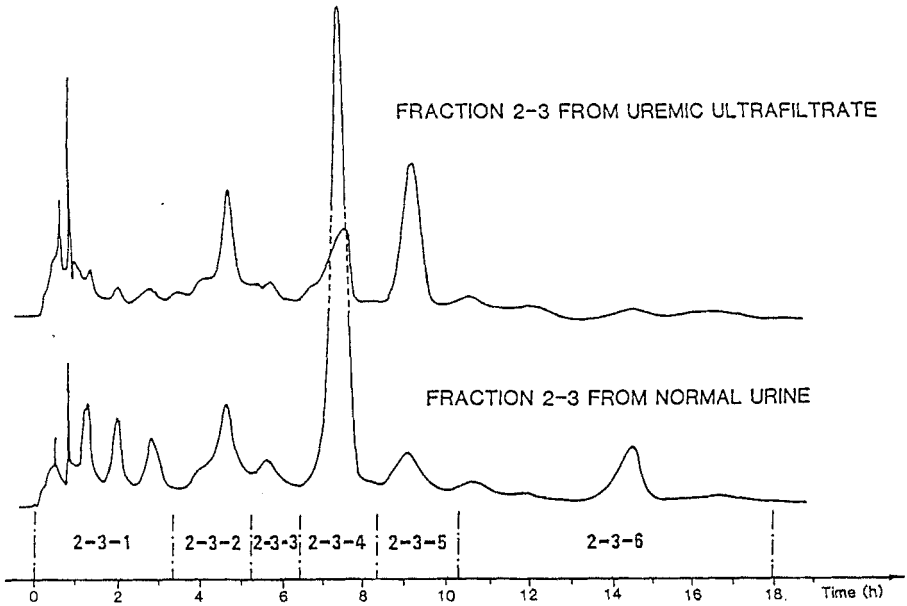

FIGURE 2: Typical elution profiles of the fraction 2-3 from UF (top) and normal urine (bottom). Chromatographic conditions were as described in the text using a final NaCl concentration of 0.025 M for gradient elution. Detection 0.2 a.u.f.s. at 254 nm. Hatched lines indicate the collection mode of fractions.

Since fraction 2-3 contains as we showed, a Na^+,K^+-ATPase inhibitor (11,12), fractions 2-3-1 through 2-3-6 isolated from four UF and three normal urines (Figure 2) were tested in order to determine what of them contains the Na^+,K^+-ATPase inhibitor.

Results in Table 1 show that fractions, isolated from 6 mg of fraction 2-3, have no effect except for fraction 2-3-1 which exerts a significant inhibition.

Simultaneous presence of the Na^+,K^+-ATPase inhibitor into the fraction 2-3-1 from UF or normal urine leads to the idea

TABLE 1

Inhibitory Effect on Na^+,K^+-ATPase (%) of Fractions isolated by Sephadex QAE A25 Chromatography from 6ml Fraction 2-3 (1 mg/ml) of UF and Normal Urine.

Sample	Na^+,K^+-ATPase Inhibition (%)[a]	
	UF (n=4)	Normal Urine (n=3)
2-3-1	94±5	96±4
2-3-2	8±3	10±3
2-3-3	2±1	4±2
2-3-4	2±1	6±2
2-3-5	4±1	10±3
2-3-6	2±1	9±2

a Results are given as the mean value ± SD

that its toxicity in uremic patients may be due to an excessive accumulation, since in healthy subjects it is normally eliminated in urine.

To conclude, chromatography on Sephadex QAE A25 in acidic medium appears to be an efficient and reproducible step in the partial purification of the Na^+,K^+-ATPase inhibitor present in fraction 2-3 of UMM. Further study is needed, however, to isolate and to establish the chemical nature and the clinical significance of this inhibitor which accumulates in uremic plasma and is eliminated in normal urine.

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REFERENCES

1 Bergström, J. and Fürst, P., Replacement of Renal Function by Dialysis, Drukker., W., Parson., F.M. and Maher., J.M., eds., Martinus Nujohff, Boston, 1983, p. 354.

2 Welt, L.G., Sachs, J.R. and Mc Manus, T.J., An ion transport defect in erythrocytes from uremic patients, Trans. Assoc. Am. Phys., 77, 169, 1964.

3 Deepak, K. and Kahn, T., Na^+-K^+ pump in chronic renal failure, Am. J. Physiol., 252, F785, 1987.

4 Cole, C.H., Balfe, J.W. and Welt, L.G., Induction of a ouabain sensitive ATPase defect by uremic plasma, Trans. Assoc. Am. Phys., 81, 213, 1968.

5 Kramer, H.J., Gospodinov, D. and Kruck, F., Functional and metabolic studies on red blood cell sodium transport in uremia, Nephron, 16, 344, 1976.

6 Izumo, H., Izumo, S., DeLuise, M. and Flier, J.S., Erythrocyte Na,K pump in uremia. Acute correction of a transport ion defect by hemodialysis, J. Clin. Invest., 74, 581, 1984.

7 Quarello, F., Boero, R., Guarena, C., Rosali, C., Giraudo, G., Giacchino, F. and Piccoli, G., Acute effects of hemodialysis on erythrocyte sodium fluxes in uremic patients, Nephron, 41, 22, 1985.

8 Zannad, F., Kessler, M., Royer, R.J. and Robert, J., Effect of hemodialysis on red blood cell Na^+-K^+-ATPase activity in terminal renal failure, Nephron, 40, 127, 1985.

9 Monti, J.P., Gallice, P., Crevat, A., El Mehdi, M., Durand, C. and Murisasco, A., Intra-erythrocytic sodium in uremic patients, as determined by high resolution ^{23}Na nuclear magnetic resonance, Clin. Chem., 32, 104, 1986.

10 Gallice, P., Fournier, N., Crevat, A., Briot, M., Frayssinet, R. and Murisasco, A., Separation of one uremic middle molecules fractions by high performance liquid chromatography, Kidney Int., 23, 764, 1983.

11 Gallice, P., Monti, J.P., Braguer, D., Durand, C., Manchon, H., Murisasco, A. and Crevat, A., Separation from uremic body fluids and normal urine of Na^+,K^+-ATPase inhibitor, Adv. Exp. Med. Biol., 223, 215, 1987.

12 Gallice, P., Monti, J.P., Baz, M., Murisasco, A. and Crevat, A., ^{23}Na nuclear magnetic resonance study of Na^+-K^+ pump inhibition by a fraction from uremic toxins, Clin. Chem., 34, 2044, 1988.

13 Gallice, P., Monti, J.P., Legall, J.M., Crevat, A. and Murisasco, A., Ion-pair high-performance liquid chromatography profiling of a uremic toxin fraction, J. Chromatogr., 422, 263, 1987.

14 Gallice, P., Crevat, A. and Berland, Y., Scaling up in isolation of medium size uremic toxins, J. Chromatogr., in press.

15 Hurst, R.O., The determination of nucleotide phosphorus with a stannous chloride-hydrazine sulphate reagent, Can. J. Biochem. 42, 287, 1964.