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PROFILING OF URINARY MEDIUM-SIZED PEPTIDES IN NORMAL AND UREMIC URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

This report describes the profiling of medium-sized peptides in both normal and uremic urine by ion-pair reversed-phase high-performance liquid chromatography using an acetonitrile—heptafluorobutyric acid solvent system as eluent. Several medium-sized peptide peaks could be detected in both normal and uremic urine at low picomole level by using post-column fluorescence derivatization with fluorescamine. Contrary to expectation, uremic urine contained slightly larger amounts of medium-sized peptides compared with normal urine.

INTRODUCTION

In recent years, the so-called "middle molecules" (MMs) in the molecular weight range of 300—5000 as postulated in the middle molecule hypothesis [1], which would normally be removed by the kidneys, have been considered to play a major role in uremic toxicity [2]. Many authors have reported that MMs are peptidic substances [2—5].

High-performance liquid chromatography (HPLC) is an emerging new technology that is of value in the analysis and separation of peptides [6—8]. The excellent resolving power of HPLC is especially advantageous for the analysis and separation of peptides existing in urine that are present in trace amounts in complex mixtures.

It seems highly probable that some of the MMs are excreted in the urine. We have therefore attempted to profile the medium-sized peptides existing in both normal and uremic urine by HPLC.

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EXPERIMENTAL

Urine samples

The normal urine samples were obtained from three healthy subjects. The uremic urine samples were obtained from three dialysis patients who excreted about 400—900 ml of urine per day. Freshly voided urine was collected and stored frozen at −60°C until use.

Apparatus and chemicals

A Shimadzu Model LC-3A HPLC system (Shimadzu, Kyoto, Japan) was used, which included a Model SIL-1A injector, a Model SGR-1A step gradient former, a Model GRE-2B linear gradient former, a Model CRD-5A chemical reaction detector, a Model SPD-2A variable-wavelength UV detector equipped with an 8-μl flow-cell, and a Model RF-500LC spectrofluorometer equipped with a 12-μl flow-cell. Sources of polyamines and standard peptides used have been given previously [9,10]. All other reagents were obtained from Nakarai (Kyoto, Japan) and were of analytical or HPLC grade. All glassware used was siliconized.

Sample treatment

The normal urine samples were filtered through a 0.45-μm Millipore filter (Millipore, Bedford, MA, U.S.A.) and the uremic urine samples were filtered through a Centriflo CF-50A (Amicon, Lexington, MA, U.S.A.) which has a nominal molecular weight cut-off of about 50,000. The peptide condensation, desalting and the separation of peptides from amino acids were performed by the method of Bohlen et al. [11] with some modifications. The filtrates of urine were pumped through a LiChroprep RP-18 (Merck, Darmstadt, G.F.R.) column, 10 × 0.8 cm, at a flow-rate of 2.0 ml/min. The column was washed with trifluoroacetic acid (TFA)-water (1:99, v/v), after which the column was eluted with *n*-propanol—TFA-water (60:1:39) at a flow-rate of 2.0 ml/min. The column effluent was monitored by UV spectrophotometry at 210 nm. The eluted fraction from the column was collected and lyophilized (cross-hatched area, Fig. 1).

Ion-pair reversed-phase HPLC

The lyophilized materials (see above) were redissolved in acetonitrile—heptafluorobutyric acid (HFBA)—water (10:0.1:89.9) and injected onto a LiChrosorb RP-18 (5-μm, Merck) column, 25 × 0.46 cm. The elution was carried out with acetonitrile-HFBA—water (10:0.1:89.9) isocratically for 30 min followed by a linear acetonitrile gradient of 0.4%/min at a flow-rate of 1.5 ml/min. The column effluent was monitored by UV spectrophotometry at 210 nm or by post-column fluorescence derivatization with fluorescamine [12]. At full-scale sensitivity, about 5—10 pmol of peptides could be detected by the fluorescamine method. All chromatograms were run at room temperature.

Molecular weight distribution

The molecular weight distribution was estimated by high-performance gel

chromatography reported earlier [9,10] with the use of a TSK-GEL 2000SW column (60 × 0.75 cm; Toyo Soda, Tokyo, Japan). The elution was done with 0.05 *M* sodium phosphate buffer, pH 7.2, containing 0.3% (w/v) sodium dodecyl sulfate at a flow-rate of 0.3 ml/min. The column effluent was monitored by UV spectrophotometry at 210 nm.

RESULTS

A typical chromatogram of normal urine samples on a LiChroprep RP-18 column is shown in Fig. 1. The fraction of the cross-hatched area was collected and subjected to ion-pair reversed-phase HPLC.

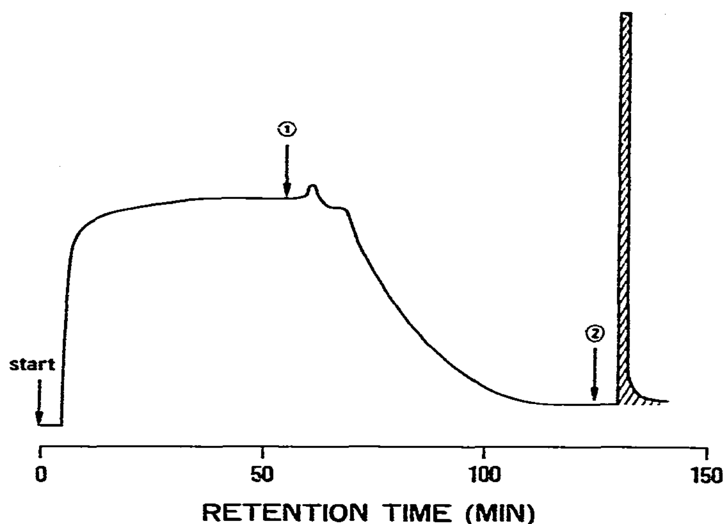

Fig. 1. Peptide condensation, desalting and amino acid removal. Arrows 1 and 2 indicate the beginning of the elution with TFA—water (1:99) and with *n*-propanol—TFA—water (60:1:39), respectively. Detection: 1.28 a.u.f.s. at 210 nm.

The retention times of standard samples chromatographed on a LiChrosorb RP-18 column are tabulated in Table I. The reproducibility of the results was better than ± 2.0% (relative standard deviation). The sample volume of injection, up to 1000 μl, had no significant effect on the result. The medium-sized peptides with molecular weights above 500 showed suitable retention times and were well separated from polyamines. The elution order of peptides seems to follow the molecular weight and hydrophobicity.

Typical elution profiles of normal urine samples, representing original urine volumes of 10 and 50 ml, are shown in Figs. 2 and 3, respectively. Fig. 2 shows that many fluorescamine-positive peaks could be well separated. The UV absorbance profile showed a similar result to that of the fluorescamine method.

The molecular weight distribution of fractions A and B (see Fig. 3) was estimated by high-performance gel chromatography. The results revealed that both fractions contained a large amount of medium-sized substances (Fig. 4).

TABLE I

RETENTION TIME AND MOLECULAR WEIGHT OF STANDARD SAMPLES CHROMATOGRAPHED ON A LICHROSORB RP-18 COLUMN

Sample	Retention time (min)	Molecular weight
Spermidine	54	145
Spermine	50	202
Leu-enkephalin	81	556
Met-enkephalin	73	574
Vasopressin	73	1084
Angiotensin I	112	1297
Angiotensin II	100	1046
Dynorphin	118	1724
Glucagon	119	3485

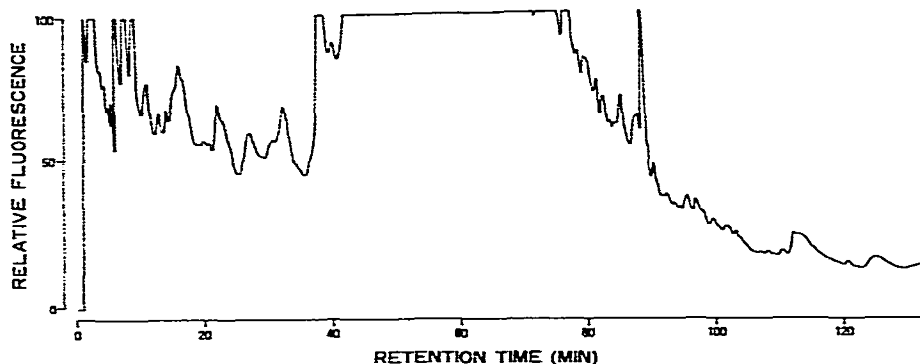

Fig. 2. Typical elution profile of normal sample, representing an original urine volume of 10 ml, chromatographed on a LiChrosorb RP-18 column. The sensitivity setting of the fluorometer was an eight-fold attenuation of the full-scale sensitivity.

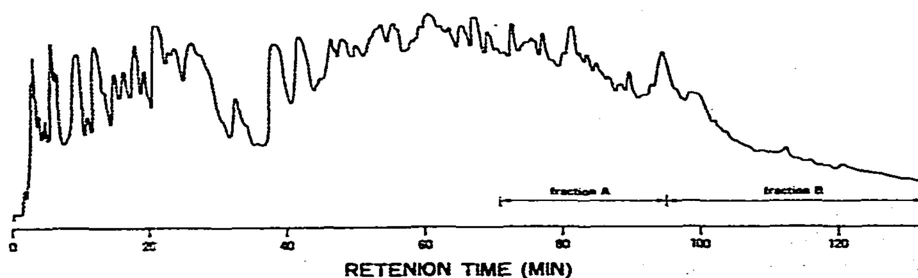

Fig. 3. Typical UV-absorbance profile of a normal sample, representing an original urine volume of 50 ml, chromatographed on a LiChrosorb RP-18 column. Detection: 1.28 a.u.f.s. at 210 nm.

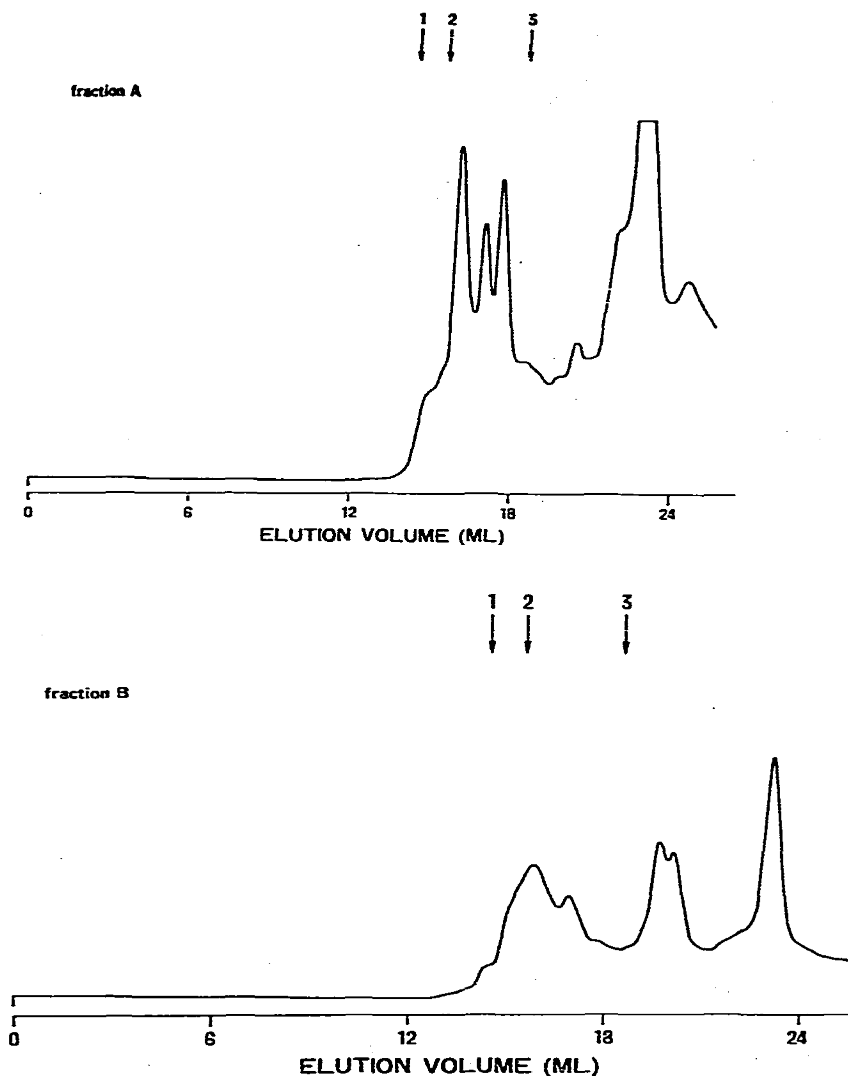

Fig. 4. The estimation of molecular weight distribution by high-performance gel chromatography. Fractions A and B (Fig. 3), representing an original urine volume of 20 ml, were used for samples. Arrows 1, 2 and 3 indicate the elution volumes of insulin, oxytocin and Leu-enkephalin, respectively. Detection: 0.64 a.u.f.s. at 210 nm.

A typical elution profile of uremic samples is shown in Fig. 5. The concentrations of medium-sized substances having a retention time over 80 min were slightly higher than those of the normal samples. However, the sample obtained from a nephrotic uremic patient showed a different elution profile (Fig. 6). No peptide peak unique to uremic or normal urines could be detected.

DISCUSSION

Until now, the separation of peptides from amino acids has been a difficult

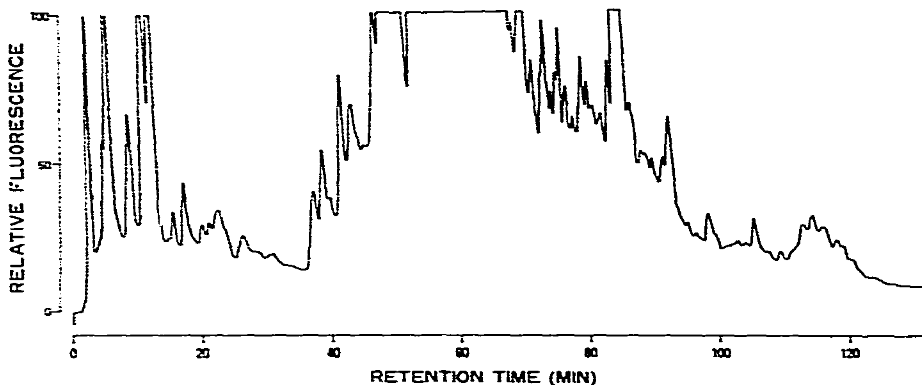

Fig. 5. Typical elution profile of a uremic sample, representing an original urine volume of 10 ml. The conditions were the same as in Fig. 2.

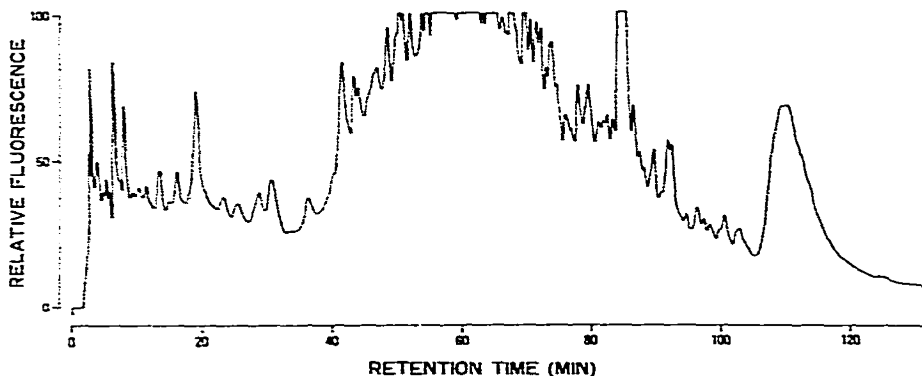

Fig. 6. Elution profile of a uremic sample, representing an original urine volume of 10 ml, obtained from a nephrotic uremic patient. The conditions were the same as in Fig. 2.

task despite a number of specific techniques that have been introduced (for example, see ref. 13). The technique of batch adsorption of peptides in a large amount of body fluids to octadecasilyl-silica particles is a very efficient first step in the concentration, desalting and separation from amino acids [11, 14, 15]. The acetonitrile—HFBA solvent system is excellent for the elution of peptides from a reversed-phase column [16, 17]. This system is volatile and allows detection of peptides at wavelengths in the range 200—220 nm.

Fluorescamine is a selective reagent for substances containing primary amino groups such as proteins, peptides and amino acids [18]. In addition, as little as 10 pmol of peptides can be easily detected.

The retention time, fluorescamine reactivity, UV absorbance characteristics and molecular weight distribution strongly indicated that most peaks existing in fractions A and B were peptidic substances. It is likely that a large number of urine samples will reveal many more medium-sized peptide peaks.

Contrary to expectation [9], the urine samples obtained from dialyzed patients whose creatinine clearance was less than 3 ml/min contained slightly

larger amounts of peptides compared with normal samples. Many peptides and low molecular weight proteins such as lysozyme, β_2-microglobulin and various peptide hormones are freely filtered through the glomeruli and removed from the luminal fluid by proximal endocytosis or luminal hydrolyzation and subsequent reabsorption [19, 20]. Therefore, it seems highly probable that the peptides in uremic urines are due to tubular dysfunction in a diseased kidney. That is to say, the peptides filtered through the glomeruli are very scarce in uremia, but most of these peptides are excreted in the urine without reabsorption and degradation. These results are consistent with the important role of residual renal function in the elimination of MMs [21]. The urine sample obtained from a nephrotic uremic patient contained a large medium-sized peptide peak, but the significance of this peak could not be elucidated in this study. This requires further study.

Further characterization, especially as to toxicity, of medium-sized peptides existing in normal and uremic urine is now in progress in our laboratory.

REFERENCES

1 A.L. Babb, M.J. Strand, D.A. Uvelli, J. Milutinovic and B.H. Schribner, Trans. Amer. Soc. Artif. Intern. Organs, 18 (1972) 98.
2 P. Furst, L. Zimmerman and J. Bergstrom, Clin. Nephrol., 5 (1976) 178.
3 A. Klein, M. Sarnecka-Keller and Z. Hanicki, Clin. Chim. Acta, 90 (1978) 7.
4 L. Zimmerman, A. Baldesten, J. Bergstrom and P. Furst, Clin. Nephrol., 13 (1980) 183.
5 J. Menyhart and J. Grof, Clin. Chem., 27 (1981) 1712.
6 M.T.W. Hearn and W.S. Hancock, Trends Biochem. Sci., 4 (1979) N58.
7 M. Rubinstein, Anal. Biochem., 98 (1979) 1.
8 F.E. Regnier and K.M. Gooding, Anal. Biochem., 103 (1980) 1.
9 H. Mabuchi and H. Nakahashi, J. Chromatogr., 224 (1981) 322.
10 H. Mabuchi and H. Nakahashi, J. Chromatogr., 213 (1981) 275.
11 P. Bohlen, F. Castillo, N. Ling and R. Guillemin, Int. J. Pept. Protein Res., 16 (1980) 306.
12 R.W. Frei, L. Michel and W. Santi, J. Chromatogr., 126 (1976) 665.
13 E. Rothenbuhler, R. Waibel and J. Solms, Anal. Biochem., 97 (1979) 367.
14 H.P.J. Bennett, A.M. Hudson, C. McMartin and G.E. Purdon, Biochem. J., 168 (1977) 9.
15 J.A. Koehn and R.E. Canfield, Anal. Biochem., 116 (1981) 349.
16 A.N. Starratt and M.E. Stevens, J. Chromatogr., 194 (1980) 421.
17 H.P.J. Bennett, C.A. Browne and S. Solomon, J. Liquid Chromatogr., 3 (1980) 1353.
18 S. Udenfriend, S. Stein, P. Bohlen, W. Dairman, W. Leimgruber and W. Weigele, Science, 178 (1972) 871.
19 T. Maak, Amer. J. Med., 58 (1975) 57.
20 F.A. Carone and D.R. Peterson, Amer. J. Physiol., 238 (1980) F151.
21 J. Milutinovic, R.E. Cutler, P. Hoover, B. Meijsen and B.H. Scribner, Kidney Int., 8 (1975) 185.