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Note

# Analysis of small peptides in uremic serum by high-performance liquid chromatography

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In recent years, middle molecular weight uremic solutes in the molecular weight range of 300—5000 as postulated in the middle molecule hypothesis [1] have been considered to play a major role in uremic toxicity such as uremic neuropathy, but proof remains elusive. Several investigators have reported that serum peptide concentrations are increased in uremic patients and that these peptides have toxicity [2—8]. Several peptides have been isolated from uremic body fluids and totally or partially characterized [9—12]. Recently, we have reported that small peptides having molecular weights below 1000 are increased in uremic serum compared with normal serum [13]. We report here the systematic analysis of small peptides in both normal and uremic serum at the picomole level by high-performance liquid chromatography (HPLC) developed in our laboratory [13, 14].

## EXPERIMENTAL

### *Sample preparation*

Serum samples were obtained from six patients undergoing maintenance hemodialysis and from four healthy subjects. All serum samples were stored frozen at —30°C until analyzed. Serum samples were ultrafiltrated through a Centriflo CF-25 filter (Amicon, Lexington, MA, U.S.A.) which has a nominal molecular weight cut-off of about 25,000. A 10-ml volume of ultrafiltrate was lyophilized and the residue was redissolved in 1 ml of 0.05 *M* sodium phosphate buffer (pH 7.2) containing 0.3% sodium dodecyl sulfate (SDS), and was filtered through a 0.45-$\mu$m Millipore filter (Millipore, Bedford, MA, U.S.A.).

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*Apparatus and chemicals*

A Shimadzu Model LC-3A HPLC system (Shimadzu, Kyoto, Japan) was used, which included a Model SIL injecter equipped with a 1000-$\mu$l sample loop, a Model SGR-1A step gradient former, a Model CRD-5A chemical reaction detector, a Model SPD-2A variable-wavelength UV detector equipped with an 8-$\mu$l flow cell and a Model RF-500LC spectrofluorometer equipped with a flow interrupter (Gilson, Middleton, WI, U.S.A.). All glassware used was siliconized. All reagents used were of analytical or HPLC grade and were obtained from Nakarai (Kyoto, Japan). The solvents used were filtered through a 0.45-$\mu$m Millipore filter and de-gassed before use. Sources of standard samples have been given previously [13, 14].

*High-performance gel chromatography*

The high-performance gel chromatography was performed by a method described previously [13]. Briefly, a 1000-$\mu$l sample was injected onto a TSK-GEL 2000SW column (60 × 0.75 cm; Toyo Soda, Tokyo, Japan) and elution was carried out with 0.05 *M* sodium phosphate buffer (pH 7.2) containing 0.3% SDS at a flow-rate of 0.3 ml/min. Detection was by UV absorbance at 210 nm and eluate fractions were collected at 4-min intervals. Then a 1000-$\mu$l volume of each of fractions 14—16 was submitted to ion-pair reversed-phase HPLC.

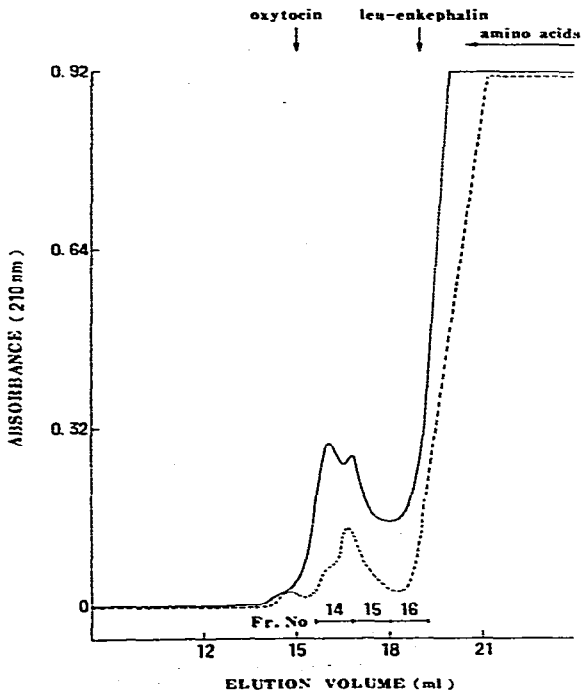


Fig. 1. Typical elution profiles of normal (——) and uremic (——) samples chromatographed on a TSK-GEL 2000SW column. Eluate fractions were collected at 4-min intervals.

*Ion-pair reversed-phase HPLC*

The analysis was performed by a method reported previously [14] with some modifications. A 1000-$\mu$l sample (see above) was injected onto an octadecylsilane column (Cosmosil $5C_{18}$, particle size 5 $\mu$m, 15 × 0.45 cm; Nakarai, Kyoto, Japan). A stepwise gradient elution at a flow-rate of 1.5 ml/min was used. The program of mobile phases was as follows: (1) acetonitrile—water (40:60, v/v) containing 15 m*M* SDS and 10 m*M* phosphoric acid, 10 min; (2) acetonitrile—water (50:50) containing 15 m*M* SDS and 10 m*M* phosphoric acid, 10 min; (3) acetonitrile—water (65:35) containing 15 m*M* SDS and 10 m*M* phosphoric acid, 10 min; (4) acetonitrile—water (75:25) containing 15 m*M* SDS and 10 m*M* phosphoric acid, 10 min. The detection system used was post-column fluorescence derivatization with fluorescamine. The peaks with heights at least three times greater than the baseline elevation were adopted as significant peaks. The minimum detectable level of standard peptides was about 50 pmoles.

## RESULTS

The elution profiles of high-performance gel chromatography were compatible with our previous report [13] (Fig. 1), that is, peptides having

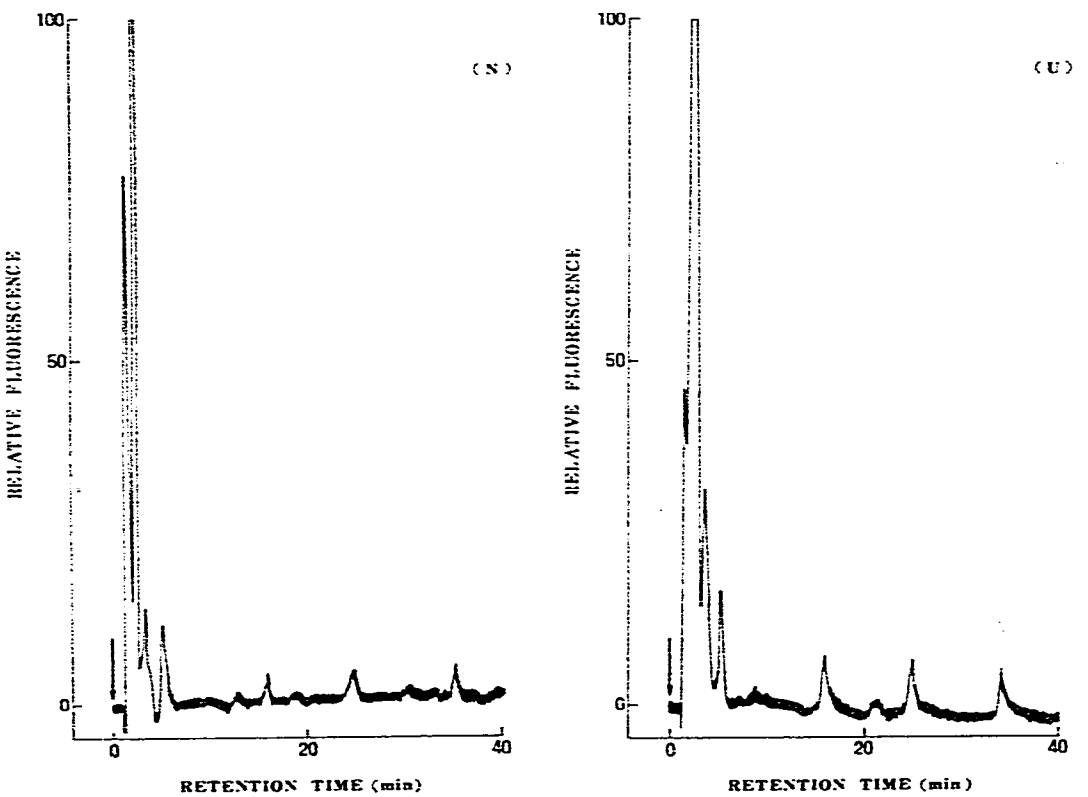


Fig. 2. Typical elution profiles of fraction 14 of normal (N) and uremic (U) samples obtained from a TSK-GEL 2000SW column by ion-pair reversed-phase HPLC.

molecular weights below 1000 are increased in uremic serum compared with normal serum, but those with molecular weights above 1000 are not increased. Typical chromatograms of ion-pair reversed-phase HPLC of fractions 14—16 are shown in Figs. 2—4, respectively. In our previous report [13], the accumulation of peptides having molecular weights below 1000 was observed in the region of fractions 14—16 using post-column fluorescence derivatization with fluorescamine. The molecular weight range is about 500—1000 for fraction 14, about 500 for fraction 15, and below 500 for fraction 16. It is seen that several peptide peaks can be separated. However, when the peaks with heights at least three times greater than the baseline elevation were adopted as significant peaks, no peak unique to uremia could be detected.

Peptide concentrations are increased in uremic serum compared with normal serum, and these peptides are toxic [2—8]. Also, several investigators have reported that the blood level of peptide hormones is elevated in patients with uremia (see ref. 15, for example), and some investigators have reported that the metabolic degradation of peptide hormones is impaired in the diseased kidney (for example, see ref. 16). Abiko and co-workers [9—11] have isolated the tripeptide (His-Gly-Lys), the heptapeptide (His-Pro-Ala-Glu-Asn-Gly-Lys) and the pentapeptide (Asp-Leu-Trp-Gln-Lys) from uremic body fluids. Recently, we have reported the accumulation of small peptides in uremic body fluids using

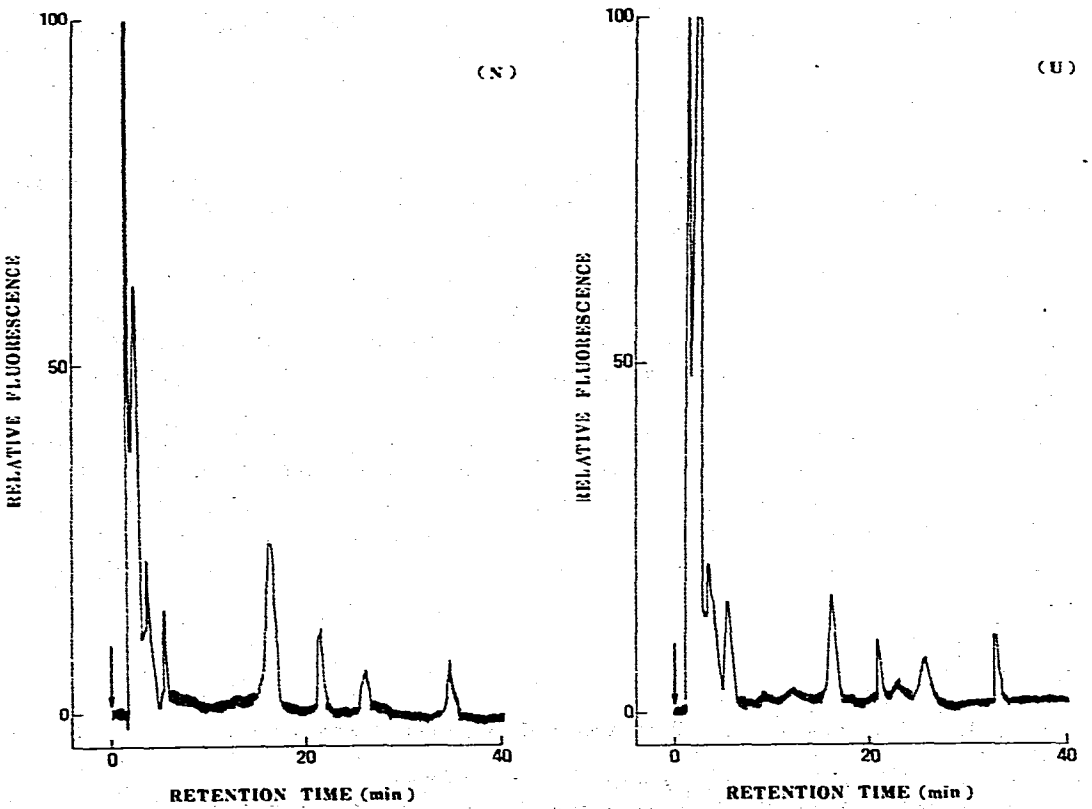


Fig. 3. Typical elution profiles of fraction 15 of normal (N) and uremic (U) samples obtained from a TSK-GEL 2000SW column by ion-pair reversed-phase HPLC.

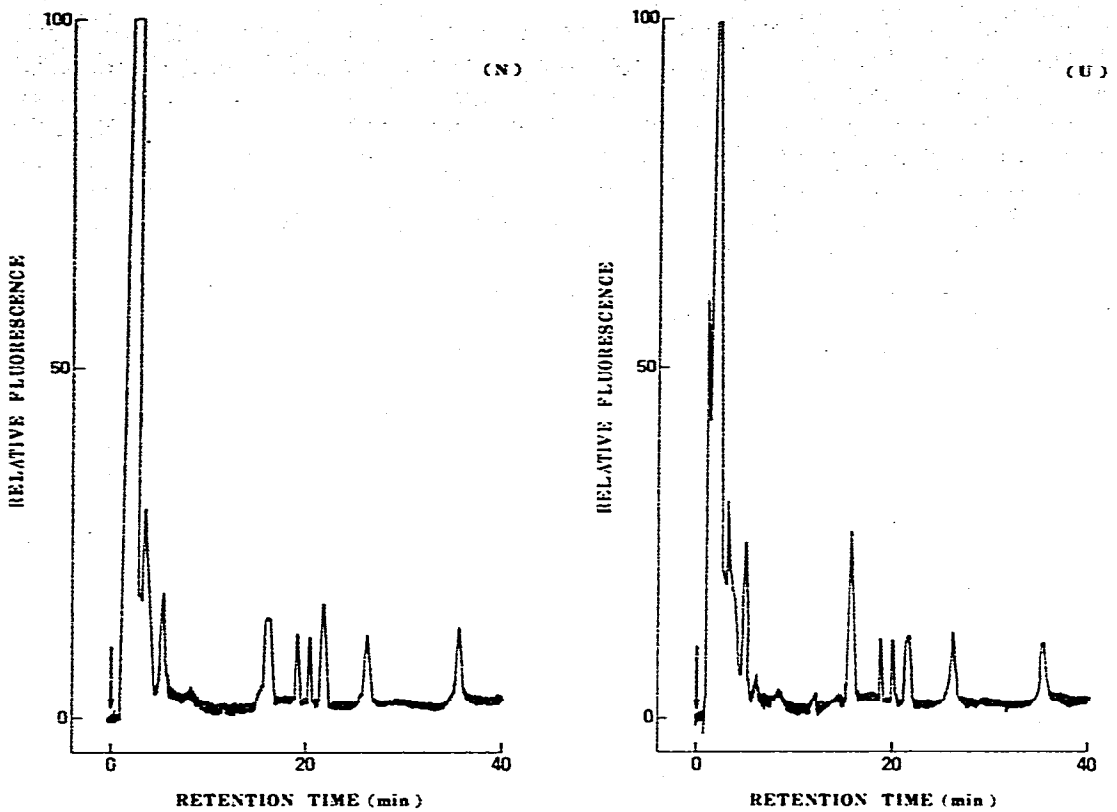


Fig. 4. Typical elution profiles of fraction 16 of normal (N) and uremic (U) samples obtained from a TSK-GEL 2000SW column by ion-pair reversed-phase HPLC.

high-performance gel chromatography combined with post-column fluorescence derivatization with fluorescamine [13]. However, we could not detect the small peptides with molecular weights below 1000, which are unique to uremia at a level of about 5 pmoles/ml serum, by ion-pair reversed-phase HPLC.

In general, biologically active peptides are present in trace amounts in serum, and these are usually detectable only by radioimmunoassay or specific bioassay. Therefore, a large amount of sample and/or a more sensitive detection system are necessary to determine the small peptides unique to uremia; this work is now under progress in our laboratory.

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