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# First Evidence for Accumulation of Protein-Bound and Protein-Free Pyrraline in Human Uremic Plasma by Mass Spectrometry

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Glucose-derived advanced glycation end products (AGEs) cross-link proteins and cause various biological tissue damage. One of them, pyrraline [ε-2-(formyl-5-hydroxymethyl-pyrrol-1-yl)-L-norleucine], has been demonstrated by utilizing antibody to accumulate in plasma and sclerosed matrix of diabetic individuals, suggesting responsibility for diabetic complications. To elucidate the involvement of pyrraline in uremia, we examined the pyrraline levels in patients with chronic renal failure by a mass spectrometric approach. Here we show that protein-free pyrraline as well as pyrraline with binding protein are significantly increased in non-diabetic uremic plasma compared to healthy subjects. Our results suggest that circulating pyrraline could be a substance contributing to complications in uremia. © 1996 Academic Press, Inc.

Reducing sugars react non-enzymatically with protein amino groups to produce a diverse group of protein-bound moieties called advanced glycation end products (AGEs) (1-3). These reactions occur slowly on proteins with long half-lives, such as collagen, or under conditions of high sugar concentrations. So far, AGEs have been postulated to contribute to the development of pathologies associated with aging and diabetes mellitus such as cataract, nephropathy and vascular diseases because of their highly cross-linked nature (2, 4, 5).

Pyrraline is one of the major AGEs whose chemical structures are established (Fig. 1). The availability of antibodies to pyrraline revealed the elevation of this AGE levels in the plasma (6) and its preferential staining in sclerosed arterial and extracellular matrix (7) of diabetic individuals, providing the possibility of its pathogenetic role. However, pyrraline formation in proteins has been questioned by immunologic technique (8), since accurate quantification is difficult due to its susceptibility to destruction. Recently, Portero-Otin et al. (9) detected pyrraline-like materials in human plasma by high-performance liquid chromatography (HPLC). In the present study, to investigate the involvement of pyrraline in complications accompanied by uremia, we tested the plasma pyrraline levels by a mass spectrometric approach for the rapid identification. To our knowledge, this is the first direct evidence of the accumulation of protein-bound and protein-free pyrraline in plasma of patients with non-diabetic chronic renal failure.

## MATERIALS AND METHODS

*Patients.* Plasma samples were obtained from 30 living subjects; 10 patients with chronic renal failure (CRF) requiring hemodialysis (mean age, 63 ± 17 years), 10 patients who presented end stage renal failure (creatinemia ranging from 9 to 13 mg/dl, 60 ± 15 years) and were studied before institution of hemodialysis, and 10 normal

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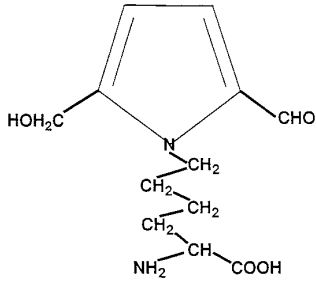


**FIG. 1.** Chemical structure of pyrraline (ε-2-(formyl-5-hydroxymethyl-pyrrol-1-yl)-L-norleucine). The molecular weight is 254 Da.

subjects (51 ± 23 years). CRF was attributed to chronic glomerulonephritis, and diabetic patients were excluded. All hemodialysis patients had been on maintenance hemodialysis 3 times a week for more than 12 months.

*Standard chemicals.* Pyrraline was synthesized according to the method of Miller and Olsson (10) with some modification. The synthesized product was confirmed to be a pure pyrraline using $^1$H-NMR spectrum, mass spectrometry, and elemental analysis.

*Sample preparation.* To quantificate protein-bound pyrraline in the plasma, the enzyme digested samples were prepared as previously described (9). Briefly, to 1 ml plasma from each patient, 1 ml cold 10% trichloroacetic acid was added and mixed. Precipitated proteins were pelleted by centrifugation, and then 2.0 ml diethyl ether was added to the pellet and mixed. Ether layer was separated by centrifugation and the resultant pellet was dried for lyophilization. The weight of lyophilized protein powder was measured by electronic balance. Ten mg of lyophilized protein from each sample was incubated with different peptidases at three times: 2% peptidase (Sigma, USA, from porcine intestine), 2% peptidase (Sigma, USA, from streptomycex griseus), 2% pronase E (Sigma, USA). After digestion, samples were separated by centrifugation, and the supernatant obtained was filtered through a 0.22 μm filter (Millipore, USA). Six hundreds pmol of synthesized pyrraline was added into 100 μl of each filtered sample prior to mass spectrometric analysis.

To quantificate plasma protein-free pyrraline, 1 ml of each plasma was sterilezed through a 0.22 μm filter, and was filtrated through MPS-1 (micropartition system, Amicon, Div. Inc., USA) for separation of free from protein-bound microsolute. Just before analysis, 600 pmol of synthesized pyrraline was added to 100 μl of each filtrated sample. In both measurement of protein-bound and -free pyrraline, the recovery of additive pyrraline was more than 99% because it was added just before assay by mass spectrometry.

*Mass spectrometry (MS).* A TSQ 7000 triple stage quadropole mass spectrometer (Finnigan MAT, Instruments Inc., USA) was used. For electrospray ionization (ESI)-MS, an ionizing energy, a current of spray, and a voltage of spray were 72 eV, 1.5 mA, and 4.5 kV, respectively. Each 5 μl of the samples was introduced into the ion sources by high-performance liquid chromatography (Hewlett Packard, USA) using a solvent of 29/69/2 methanol/$H_2O$/acetic acid (v/v/v) at a flow rate of 0.6 ml/min. The structure of pyrraline was assigned by MS/MS spectra according to the pyrrol compounds. For pyrraline quantification, selected ion monitoring (SIM) method was used. The standard curve of synthesized pyrraline was expressed in m/z=255 ion peak (the protonated molecular ion of pyrraline), and peak areas were linearly correlated with concentration over a range from 5 pmol to 5000 pmol (r=0.990). The contents of each sample were determined as follows: 600 pmol × (an area in a sample without standard pyrraline) / {(an area in a sample with standard pyrraline) − (an area in a sample without standard pyrraline)}

area: m/z=255 ion peak area

Protein-bound pyrraline values were expressed as pmol per one mg total protein in plasma, and protein-free pyrraline concentration was shown as pmol per one ml of plasma.

*Statistical analysis.* The results were expressed as means ± SD. p values for comparisons were determined by unpaired Student's t-test. All p values of less than 0.05 were considered to indicate statistical significance.

## RESULTS AND DISCUSSION

*Plasma protein-bound pyrraline levels in normal subjects and uremic patients.* To determine the pyrraline contents of the peptide fraction of plasma, we used mass spectrometry (ESI/MS)

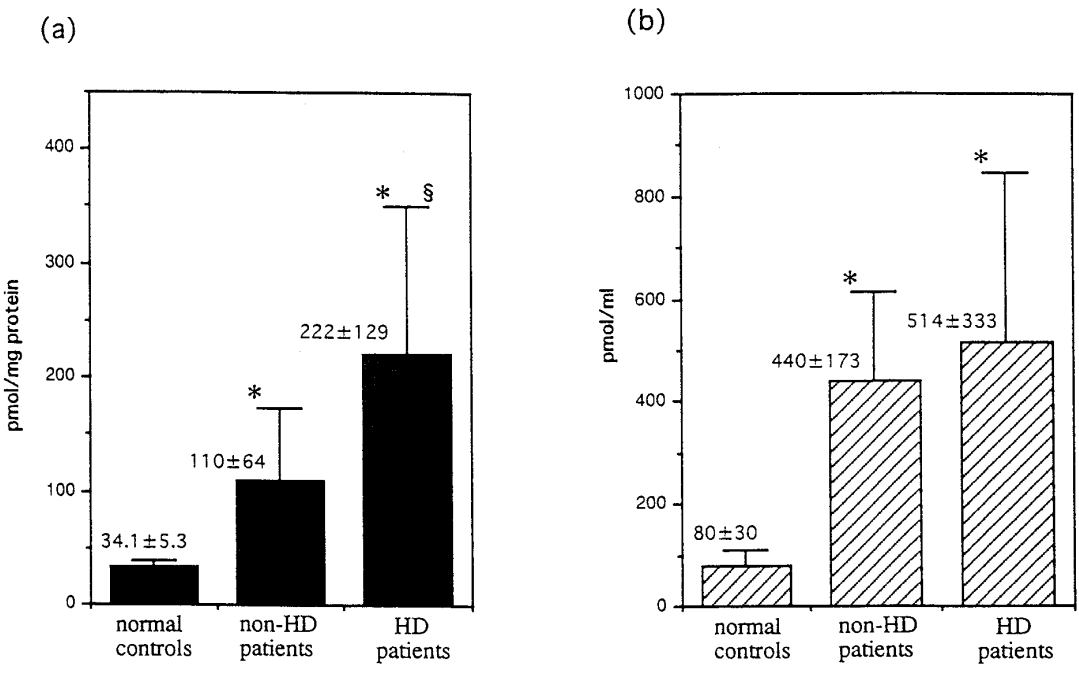


**FIG. 2.** Plasma levels of the protein-bound pyrraline (a) and the protein-free pyrraline (b) in hemodialysis (HD) patients, uremic non-dialyzed (non-HD) patients and normal subjects. Data represent means ± SD of each 10 patients. * denotes $p<0.001$ for the comparison with normal subjects; § denotes $p<0.005$ for the comparison with non-HD patients.

with high sensitivity and accuracy. As shown in Fig. 2a, both the plasma pyrraline levels in uremic non-dialyzed (non-HD) patients and those in hemodialysis (HD) patients were much higher than in normal controls ($p<0.001$). When comparing the levels in both uremic plasma, plasma of HD patients showed a significant increase of protein-bound pyrraline ($p<0.005$). These results reflected a dramatic accumulation of protein-bound pyrraline in uremic plasma, indicating increased production or decreased removal of this circulating AGE in renal failure. Moreover, hemolysis in uremia may affect the plasma pyrraline contents since Odetti et al. (11) reported significantly elevated levels of pentosidine, one of the AGEs, in uremic erythrocytes.

*Plasma protein-free pyrraline levels in normal subjects and uremic patients.* The high sensitivity of ESI/MS urged us to investigate the possible existence of a free form of pyrraline with no binding protein in human plasma. Unexpectedly, the ESI/MS method served to identify free pyrraline in the plasma of all subjects (Fig. 3). Consistent with protein-bound pyrraline, the levels of free pyrraline in uremic plasma were significantly higher than in controls ($p<0.001$, Fig. 2b). The accumulation of free pyrraline may be mainly due to the diminution of glomerular filtration, considering that the molecular weight of pyrraline (254 Da) is low enough to be filtered through the glomerulus. Interestingly, there was no significant difference in the free pyrraline levels between non-HD and HD patients in contrast with the levels of protein-bound form. The discrepancy between protein-bound and free pyrraline might be explained by the easy removal of free pyrraline like creatinine through hemodialysis.

Since pyrraline is believed to be always produced on proteins in vivo, the issue of the origin of the free pyrraline must be addressed. It does not seem reasonable that free pyrraline would

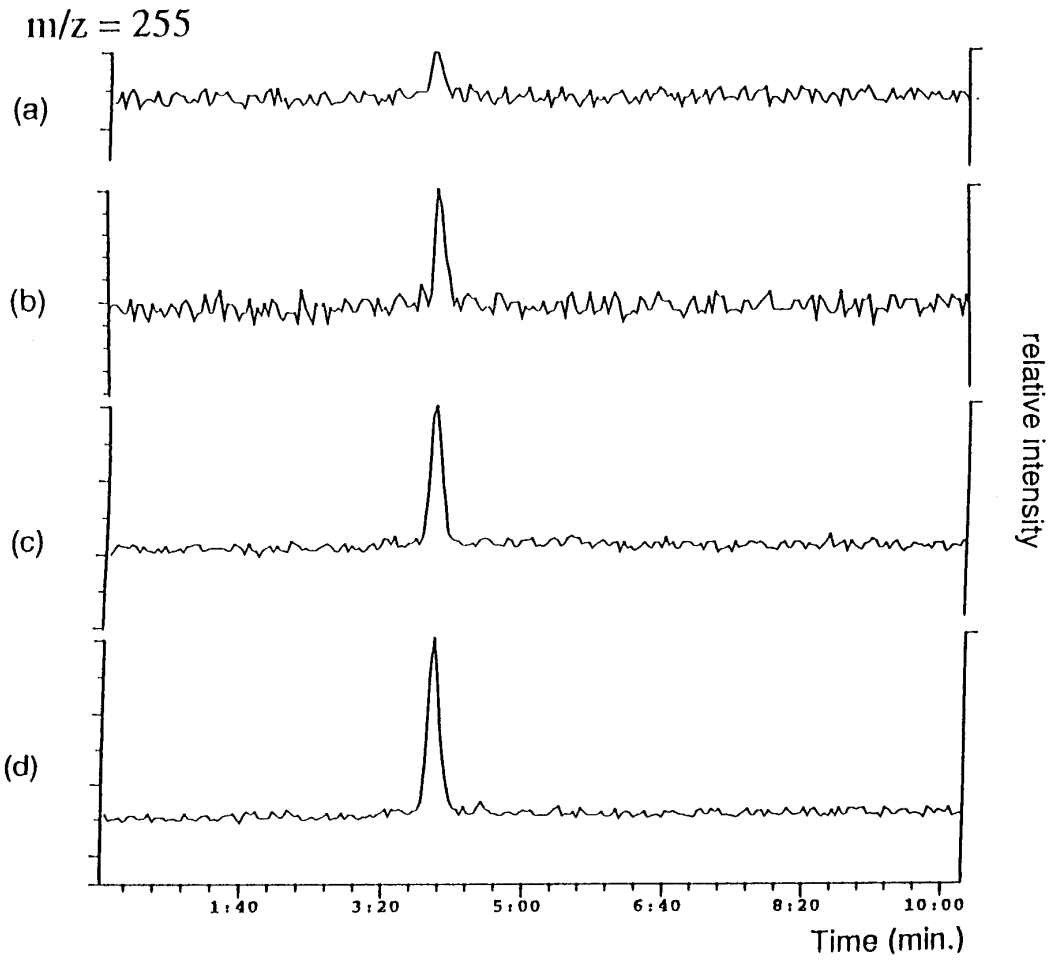


**FIG. 3.** Representative selected ion monitoring (SIM) mass chromatograms of m/z=255 ion of protein-free pyrraline obtained from normal subjects (a), non-HD patients (b), HD patients (c), and normal subjects with 600 pmol of standard pyrraline (d).

be formed in the plasma, because the extremely slow reactivity of aldoses in pyrraline formation is coupled with the relatively fast turnover of lysine. Free pyrraline might be released from long-lived pyrraline-modified proteins in the degradation process. Alternatively, free pyrraline appearing in circulation may be due to absorption from diets, since various foodstufs contain proteins modified with AGEs (12, 13).

Several investigators have suggested the pathological role of compounds in uremic plasma that are not eliminated by hemodialysis (14-16). These so-called uremic toxins may have a critical role in the development of complications accompanied by chronic renal failure such as microangiopathy, peripheral neuropathy and arthropathy (14-18). In our previous work, we observed that $\beta_2$-microglobulin ($\beta_2$M) of amyloid deposits is modified with AGEs in dialysis-related amyloidosis exhibiting arthropathy (19). More recently, we found a significant amount of pentosidine in amyloid-fibril $\beta_2$M (20). Aside from whether or not pyrraline or pentosidine belong to the uremic toxins, the circulating AGEs may contribute to further tissue damage by deposition.

In conclusion, we demonstrated the accumulation of both protein-bound and protein-free pyrraline in plasma of patients with non-diabetic CRF, especially dialysis patients. Further investigations will be pursued to elucidate the role of pyrraline in the pathogenesis of complications associated with uremia.

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