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# Mass spectrometric study on the protein chemical modification of uremic patients in advanced Maillard reaction

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**Abstract**

The Maillard reaction, initiated by the nonenzymatic reaction of reducing sugar with protein, is proposed to play a significant role in protein aging and the complications of aging and diabetes. In this study, we detected and quantified some advanced glycation endproducts (AGEs) in human serum proteins of control and uremic patients by a highly selective and specific assay, electrospray ionization liquid chromatography–mass spectrometry–mass spectrometry (ESI-LC–MS–MS). From our results, levels of each AGEs in serum of uremic patients were significantly elevated, compared to age-matched controls. These results provide the evidence for increased modifications of proteins by Maillard reaction in uremia. 

*Keywords:* Maillard reaction; Proteins

## 1. Introduction

Advanced glycation endproducts (AGEs) are formed over months by a nonenzymatic reaction between aldoses and proteins, known as the Maillard reaction [1,2]. The reaction is initiated with the reversible formation of a Schiff base between a reducing sugar and the amino group of a protein. The relatively unstable Schiff base undergoes rearrangement to form a more stable Amadori product, which, over a period of several months to years, undergoes a series of further reactions through α-dicarbonyl intermediates to form AGEs. In general, in diabetes, AGE accumulation is accelerated and linked to atherosclerosis [3–6], nephropathy, neuropathy, retinopathy and cataracts [7–9]. During the last decade, over a dozen such AGEs as biomarkers have been identified in vivo [10–12] and can now be measured by spectrophotometric, chromatographic [13–15] or immunochemical techniques [16–18]. In this study, we describe a more specific and selective electrospray ionization mass spectrometry–mass spectrometry (ESI-MS–MS) procedure for simultaneous measurement of some AGEs, ε-2-(formyl-5-hydyroxymethyl-pyrrol-1-yl)-L-nor-leucine (pyrraline), $N^{\varepsilon}$-(carboxymethyl) L-lysine (CML), pentosidine, glyoxallysine dimer (GOLD), and methylglyoxallysine dimer (MOLD), in human serum proteins of control and uremic patients. We report here that levels of all these AGEs were significantly elevated in serum of uremic patients, compared to age-matched controls. These results propose that increased chemical modification of serum proteins by Maillard glycation or glycoxida-

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tion reaction may be important in the pathogenesis of vascular disease in uremia.

## 2. Experimental

### 2.1. Materials

Acetonitrile, acetic acid, diethyl ether, trifluoroacetic acid (TFA) and trichloroacetic acid (TCA) were analytical reagent grade and purchased from Wako (Osaka, Japan). AGEs that we measured in this study were ε-2-(formyl-5-hydroxymethyl-pyrrol-1-yl)-L-norleucine (Pyrraline), $N^{\varepsilon}$-(carboxymethyl) L-lysine (CML), pentosidine, glyoxallysine dimer (GOLD), and methylglyoxallysine dimer (MOLD). Pyrraline was synthesized according to the method of Miller and Olsson [19] with some modification. Pentosidine was kindly supplied by Dr. Toshio Miyata, and CML, GOLD, MOLD, and $D_4$-CML, $^{15}$N-GOLD, $^{15}$N-MOLD as internal standards were kindly supplied by Dr. Baynes.

### 2.2. Patients

Serum samples were obtained from 30 living subjects; 15 chronic renal failure (CRF) patients (seven males and eight females), all with nondiabetic nephropathy (mean age, 58±4 years), and 15 normal controls (eight males, seven females; mean age, 55±5 years). The serum levels of creatinine in uremic patients and healthy subjects were 11.3±0.5 (mean±SD, $n=15$) mg dl$^{-1}$ and 0.5±0.3 (mean±SD, $n=15$) mg dl$^{-1}$, respectively.

### 2.3. Sample preparation

To quantify protein-bound AGEs in human serum, the enzyme digested samples were prepared as previously described [15]. Briefly, to 1 ml serum from each patient, 1 ml of cold 10% PCA was added and mixed. Precipitated proteins were pelleted by centrifugation, and 2 ml diethyl ether was then added to the pellet and mixed. The ether layer was separated by centrifugation and the resultant pellet was dried for lyophilization. The concentration of the serum protein was determined, prior to TCA precipitation, using the method described by Lowry et al. [20]. 100 mg of lyophilized protein from each sample was incubated with different peptidases on three occasions [13]. After digestion, samples were separated by centrifugation, and the supernatant obtained was filtered through a 0.22 μm filter (Millipore, USA).

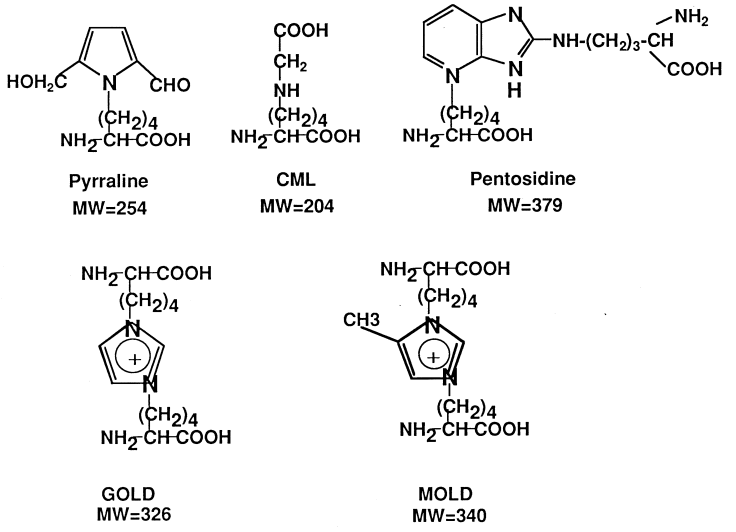


Fig. 1. Chemical structures of AGEs. M.W.=molecular mass.

### 2.4. ESI-LC–MS–MS conditions

All AGEs were resolved by reversed-phase high-performance liquid chromatography (RP-HPLC) using a Model 1050 pump (Hewlett-Packard, Palo Alto, CA, USA) and analyzed by ESI-MS using a TSQ 7000 triple stage quadrupole mass spectrometer (Thermoquest, San Jose, CA, USA) and a DEC 3000 (Digital Equipment, Bedford, MA, USA). Each of the 5 μl of the samples was introduced into the ion sources using a HPLC system (Hewlett-Packard, USA), equipped with a Monitor C-18M column (5 μm, 150×4.6 mm ID, Column Engineering, USA) equilibrated with solvent A (0.03% TFA in $H_2O$), and elution with a linear gradient to 50% solvent B (40% acetonitrile, 60% $H_2O$, 0.02% TFA) for the first 30 min, to 50% in the next 20 min, to 100% in the next 5 min, and the column was then reequilibrated with 100% solvent A for the last 20 min at a flow-rate of 0.3 ml $min^{-1}$. To identify them in serum protein, the RP-HPLC-ESI–MS–MS method and selected reaction monitoring (SRM) method were

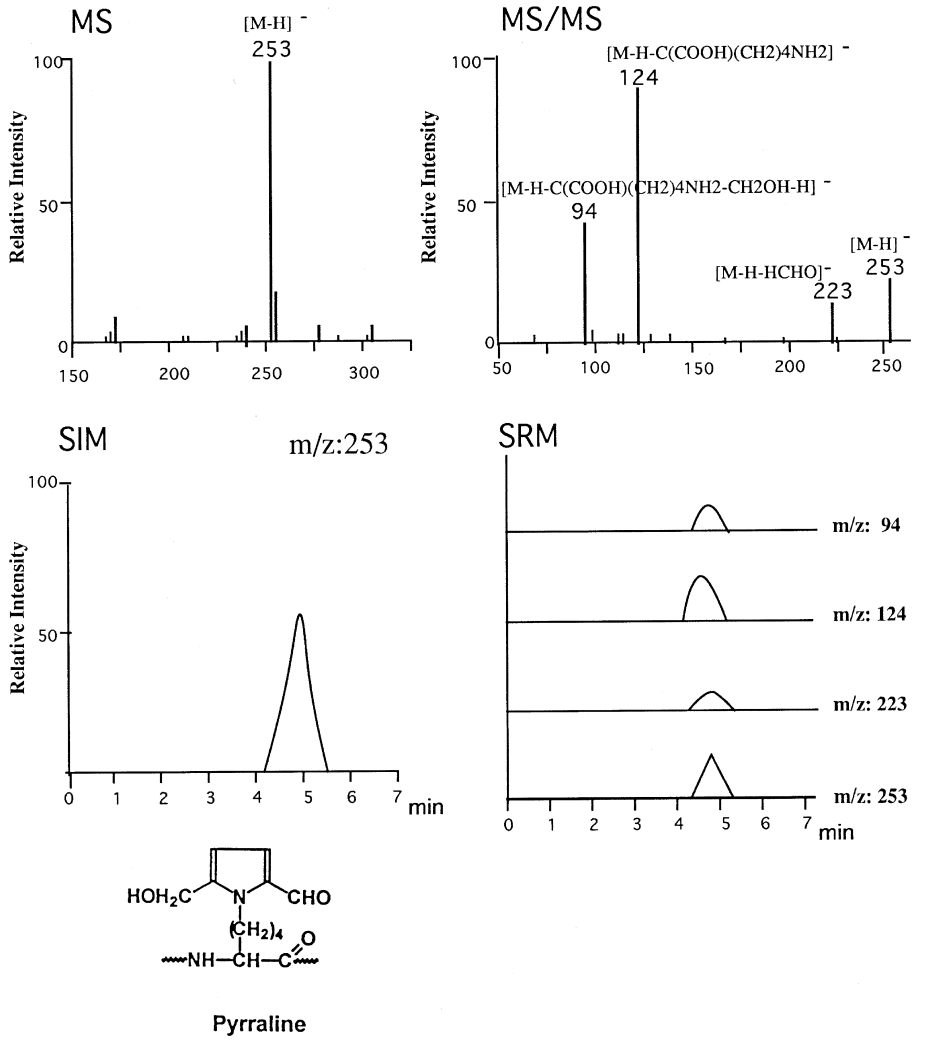


Fig. 2. ESI-LC–MS, MS–MS, SIM and SRM spectra of standard pyrraline.

used. The selected ion monitoring (SIM) method was used for quantitation of these five AGEs. For ESI-MS, the ionizing energy, current and voltage of spray were 72 eV, 1.5 mA, and 4.5 kV, respectively. For ES-MS–MS analysis, collision-induced dissociation was performed using argon as the collision gas at a pressure of 2.0 mTorr. The collision offset was −25 V (positive mode) or 22.5 V (negative mode).

## 3. Results

The chemical structures of AGEs measured in this study are shown in Fig. 1. ESI-LC–MS, SIM, ESI-MS–MS, and SRM spectra of each AGE are shown in Figs. 2–6. Pyrraline gave a deprotonated molecular ion peak $[M-H]^-$, $m/z=253$; and three daughter ion peaks: $[M-H-HCHO]^-$, $m/z=223$; [M–H–

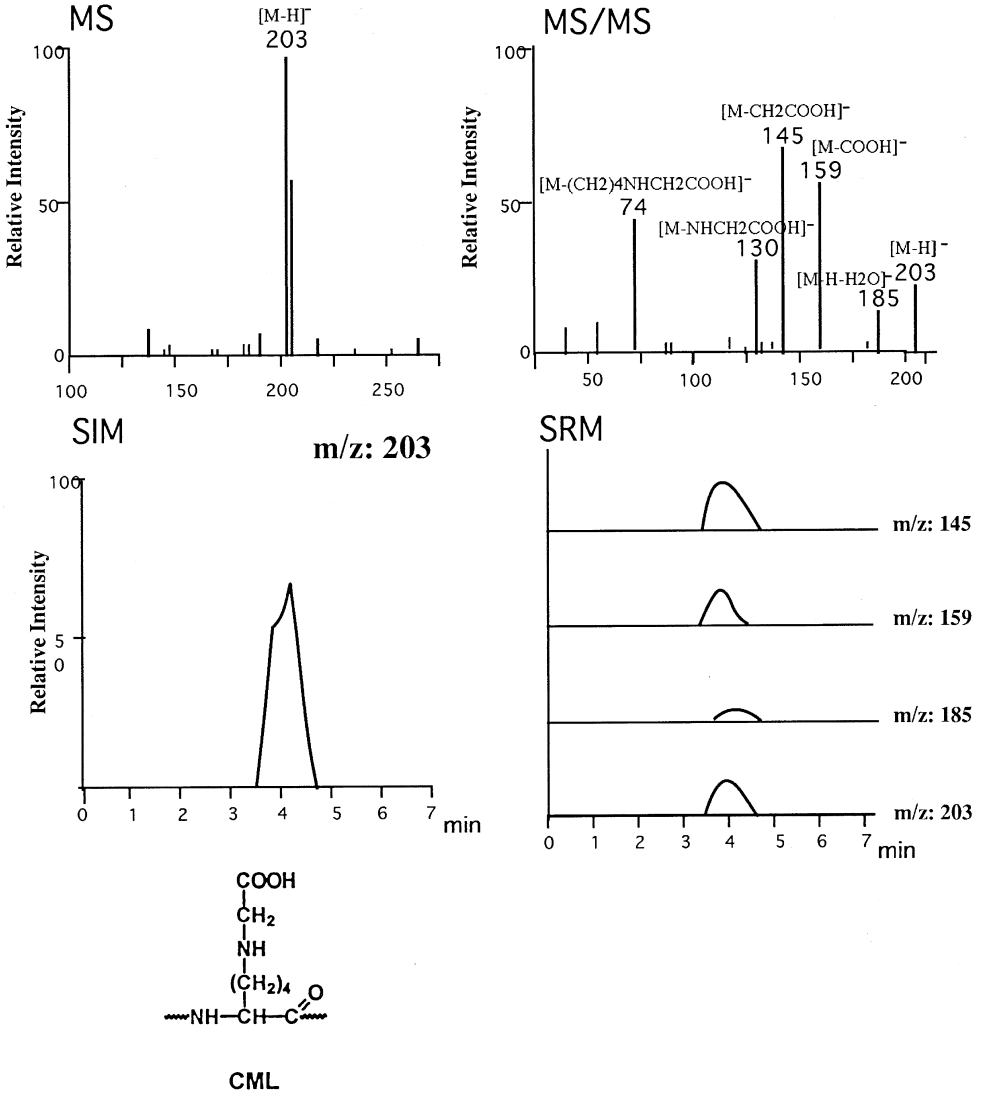


Fig. 3. ESI-LC–MS, MS–MS, SIM and SRM spectra of standard CML.

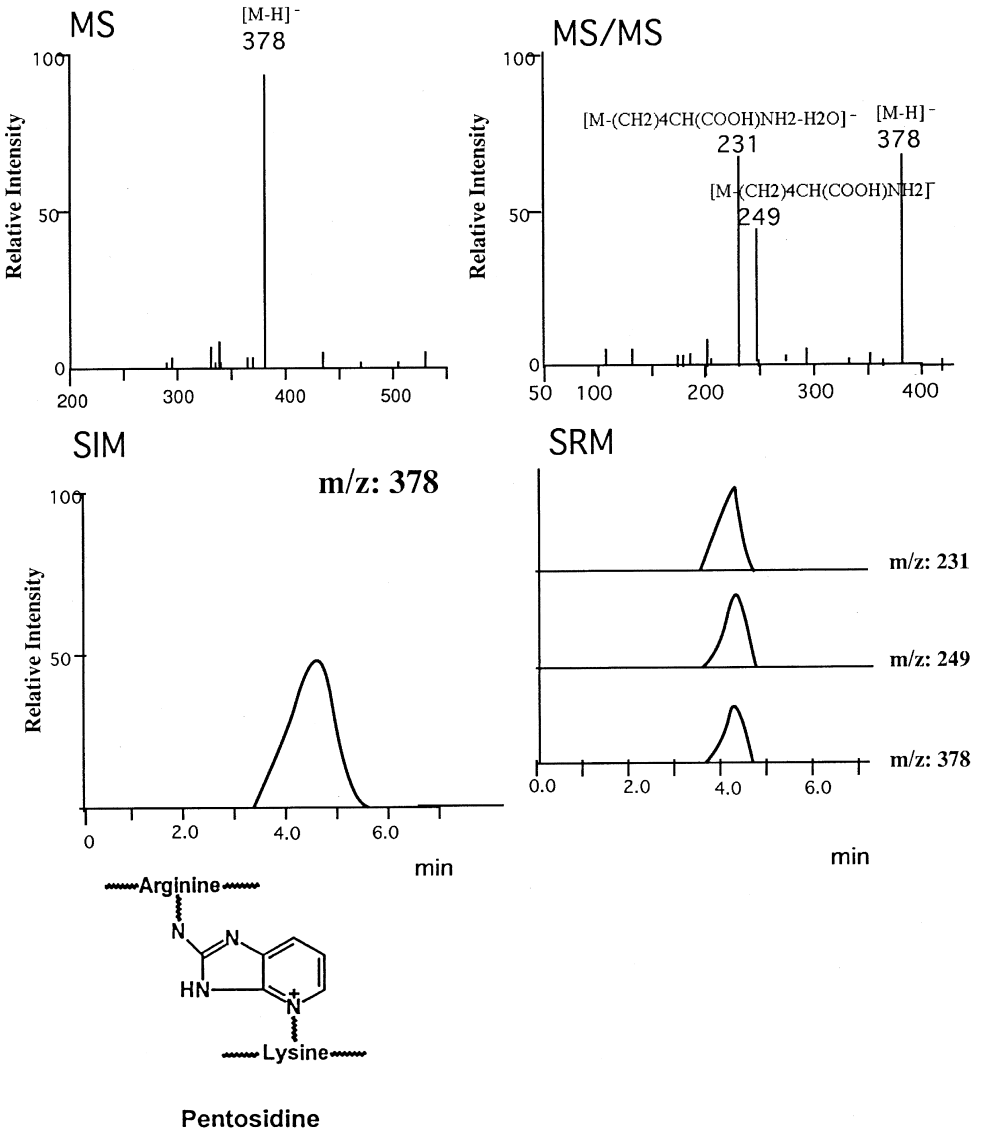


Fig. 4. ESI-LC–MS, MS–MS, SIM and SRM spectra of standard pentosidine.

$C(COOH)(CH_2)_4NH_2]^-$, $m/z$=124; and [M–H–$C(COOH)(CH_2)_4NH_2$–$CH_2OH$–H]$^-$, $m/z$=94 (Fig. 2). CML and pentosidine also gave deprotonated molecular ion peaks, $m/z$=203 and $m/z$=378, respectively (Figs. 3 and 4). GOLD and MOLD gave stable protonated ion peaks, $[M+H]^+$, $m/z$=327; and $m/z$=341; and they gave stable collision-induced stable daughter ion peaks, [M+H–C(COOH)–$(CH_2)_4NH_2]^+$, $m/z$=198, and $m/z$=213, respectively (shown in Figs. 5 and 6). To confirm their structures in human serum samples, each daughter ion was trapped and monitored by the ESI-LC–MS–MS and SRM system as shown in Section 2. Despite some differences in relative ion intensities resulting

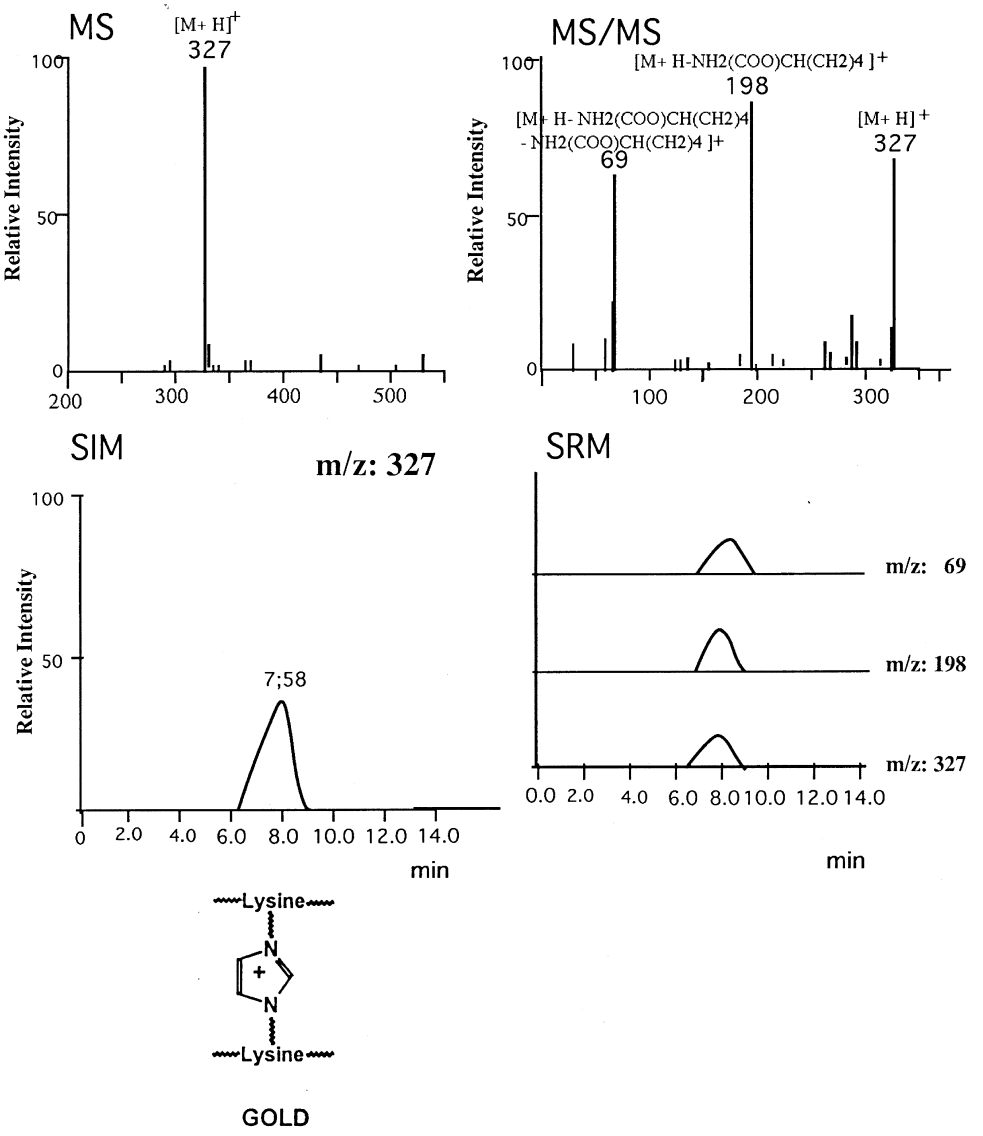


Fig. 5. ESI-LC–MS, MS–MS, SIM and SRM spectra of standard GOLD.

from differential ion suppression, daughter ion spectra characteristics of these five AGEs standards were readily apparent in products detected in uremic and normal serum. Quantitative analyses of CML, GOLD and MOLD were performed by calculating a peak area ratio of each deprotonated or protonated molecular ion peak intensity (CML, $m/z$=203; GOLD, $m/z$=327; and, MOLD $m/z$=341) to each protonated or deprotonated molecular internal standard ion peak intensity ($D_4$-CML, $m/z$=207; $^{15}$N-GOLD, $m/z$=331; $^{15}$N-MOLD, $m/z$=345) based on their ESI-SIM spectra. Quantitation of pyrraline and pentosidine was done according to each deprotonated molecular ion peak area ratio obtained by ESI-SIM spectra. The correlation coefficient between the added pyrraline standard concentration and the peak

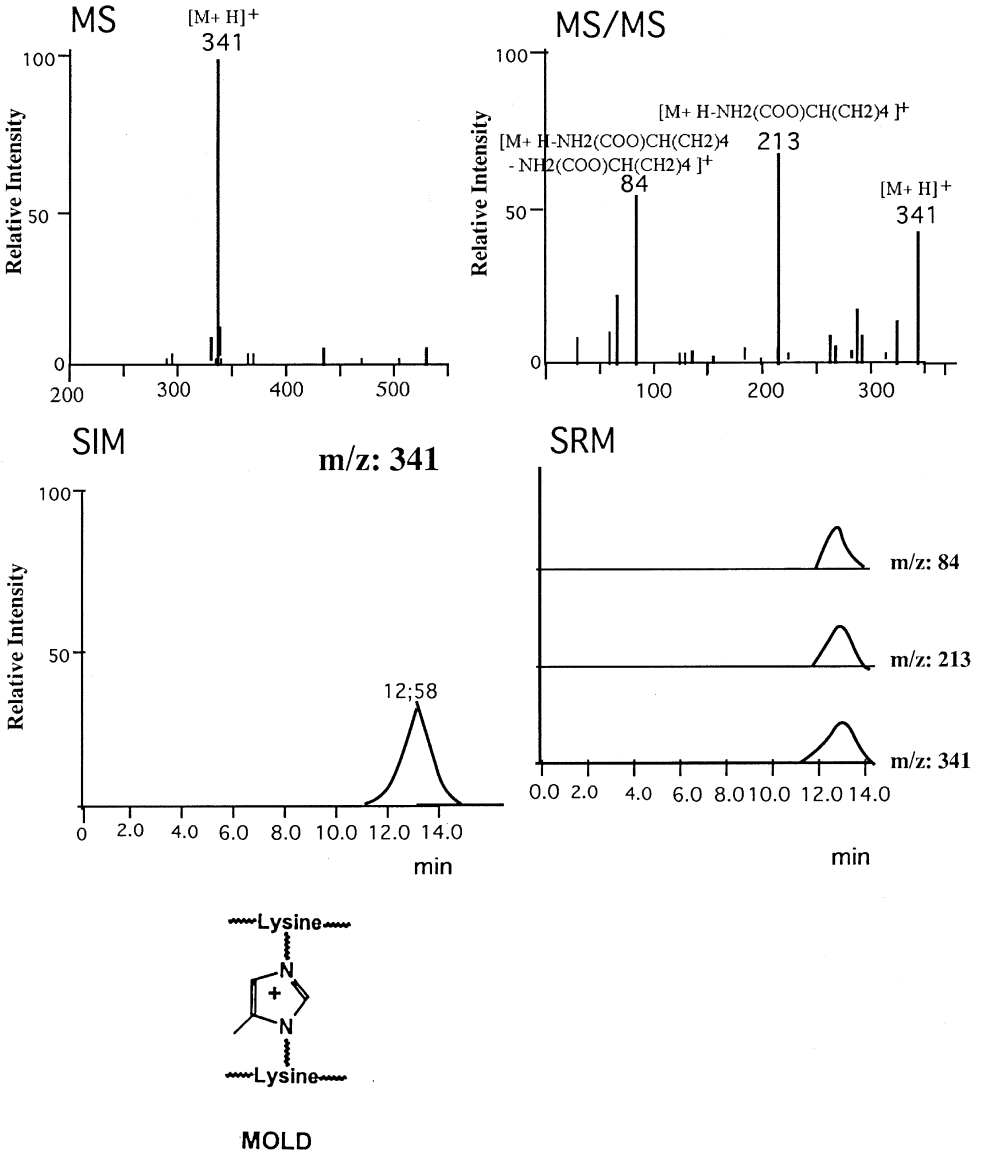


Fig. 6. ESI-LC–MS, MS–MS, SIM and SRM spectra of standard MOLD.

area ($m/z$=253) ratio was 0.998 (regression equation: $y$=2.756$x$−0.004049, with a standard concentration range from 100 pmol ml$^{-1}$ to 50 nmol ml$^{-1}$); pentosidine standard concentration and the peak area ($m/z$=378) ratio was 0.995 (regression equation: $y$=0.9544$x$−0.003738, with a standard concentration range from 1 nmol ml$^{-1}$ to 500 nmol ml$^{-1}$). The C18 RP-HPLC method described in Section 2 yielded recoveries of more than 80% and good reproducibility from human serum samples (data not shown). As shown in Fig. 7, quantitative analyses by ESI-SIM showed that these AGEs were present at similar levels in serum proteins of controls (20.4±10.8 to 49.5±15.3 pmol mg$^{-1}$ protein) and

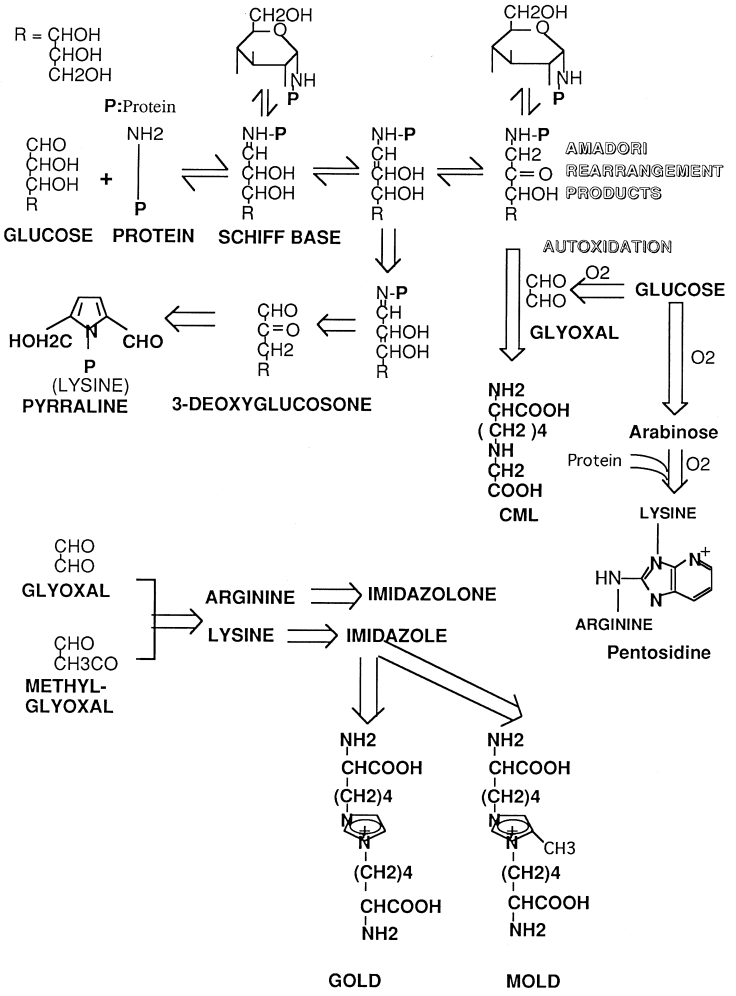


Fig. 7. Schematic representation of potential pathway leading to AGE formation during the Maillard reaction.

uremic patients (98.9±38.5 to 185.2±117.5 pmol $mg^{-1}$ protein), and that they were comparably increased by 2–9-fold in uremic patients [21–23].

## 4. Discussion

AGEs have been detected in vivo according to immunohistochemical methods [16–18]. To our knowledge, this is the first mass spectrometric study demonstrating the accumulation of several AGEs at the same time in uremic patients. Fig. 8 represents the AGEs formation scheme in advanced Maillard reactions. In this study, we here elucidated the involvement of pyrraline, CML, pentosidine, GOLD and MOLD in uremia. Pyrraline is produced from an advanced glycation reaction through 3-deoxyglucosone in vivo and in vitro. Recent studies show that CML and pentosidine are produced not only by advanced glycation reaction but oxidation reaction in vitro [24]. The production mechanisms of GOLD and MOLD could conceivably result from the reaction of methylglyoxal and glyoxal in glucose autoxidation with lysine residues in protein. Thus, it seems that their main chemical origins are different and they are produced by various pathways in vitro and in vivo. Mass spectrometric analysis is superior to the immunochemical method in accuracy and

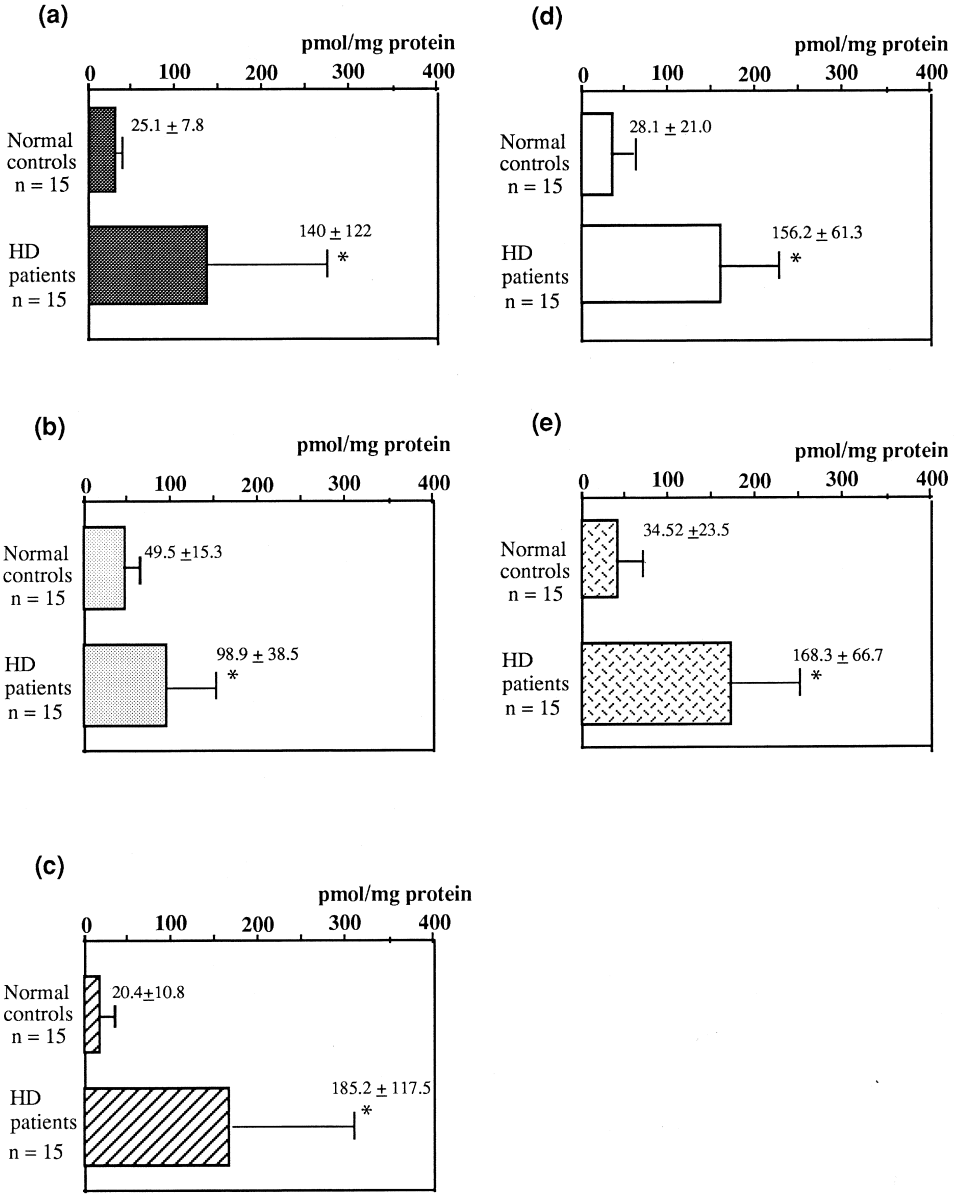


Fig. 8. Serum levels of AGEs in uremic patients with nondiabetic nephropathy and normal subjects. Data represent means ±SD of each 15 patients. * Denote $p<0.001$ for the comparison with normal subjects.

selectivity, and it enables quick analysis in mixture samples at the same time. We have shown that the serum levels of all AGEs in this study were significantly increased in uremic patients without diabetes compared to those in normal controls. Although our result is in agreement with previous reports [15,25], this is the first study of the effects of advanced glycation and oxidation on chemical modification of proteins by estimating serum levels of five different AGE compounds at the same time. Our results suggests a general increase in oxidative stress in uremia, independent of changes in blood glucose or lipids. The use of ESI-LC–MS–MS for measurement of these and other Maillard products in serum

proteins will be important for accurate, sensitive and specific measurement of these and other chemical modifications of plasma proteins during aging and in disease.

## 5. Conclusion

Although the immunochemical and HPLC methods could not be applied to the identification of several serum AGEs at the same time, ESI-LC–MS–MS with daughter scan mode, which is a rapid, sensitive and selective analysis method, made it possible to distinguish each material and establish a technique for identification and quantitation, with the following results. (1) All AGEs in this study gave stable precursor ions, $[M+H]^+$ in positive mode and $[M-H]^-$ in negative mode. (2) In ESI-LC–MS–MS analysis, all AGEs with lysine residues or modified lysine residues in their chemical structures, give $[M-H-C(COOH)(CH_2)4NH_2]^-$ in pyrraline, CML and pentosidine, or $[M+H-C(COOH)(CH_2)_4NH_2]^+$ in GOLD and MOLD as the product ions. (3) The method can reliably identify these AGEs at a concentration of 1 ng $ml^{-1}$ to 500 ng $ml^{-1}$ in human serum samples at the same time. (4) From comparison with previous studies, our quantitation results matched their results.

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## References

[1] M. Brownlee, A. Cermi, H. Vlassara, N. Engl. J. Med. 318 (1988) 1315.
[2] J.W. Baynes, V.M. Monnier, Prog. Clin. Biol. Res. 304 (1989) 1.
[3] M. Kirstein, J. Brett, S. Radoff, S. Ogawa, D. Stern, H. Vlassara, Proc. Natl. Acad. Sci. USA 87 (1990) 9010.
[4] H. Vlassara, M. Brownlee, K.R. Manogue, C.A. Dinarello, A. Pasagia, Science 240 (1988) 1546.
[5] A.M. Schmidt, S.D. Yon, J. Brett, R. Mora, R. Nowygrod, D. Stern, J. Chin. Invest. 92 (1993) 2155.
[6] J.L. Wautier, M.P. Wautier, A.M. Schmidt, Proc. Natl. Acad. Sci. USA 91 (1994) 7742.
[7] E.Y. Skolnik, Z. Yang, Z. Makita, S. Radoff, M. Kirstein, H. Viassara, J. Exp. Med. 174 (1991) 931.
[8] T. Doi, H. Vlassara, M. Kirstein, Y. Yamada, G.E. Striker, L.J. Striker, Proc. Natl. Acad. Sci. USA 89 (1992) 2873.
[9] P.J. Beisswenger, L.L. Moore, T. Brink-Johnsen, J. Clin. Invest. 92 (1993) 212.
[10] F. Hayase, R. Nagaraj, S. Miyata, F.G. Njoroge, V.M. Monnier, J. Biol. Chem. 264 (1989) 3758.
[11] J.A. Dunn, J.S. Patrick, S.R. Thorpe, J.W. Baynes, Biochemistry 28 (1989) 9464.
[12] D.R. Sell, V.M. Monnier, J. Clin. Invest. 85 (1990) 380.
[13] M. Portero-otin, R.H. Nagaraj, V.M. Monnier, Biochem. Biophys. Acta 1247 (1995) 74.
[14] P. Odetti, J. Forgarty, D.R. Sell, V.M. Monnier, Diabetes 41 (1992) 153.
[15] H. Odani, T. Shinzato, Y. Matsumoto, I. Takai, S. Nakai, M. Miwa, N. Jwayama, I. Amano, K. Maeda, Biochem. Biophys. Res. Commun. 224 (1996) 237.
[16] S. Miyata, V.M. Monnier, J. Chin. Invest. 89 (1992) 1102.
[17] S. Taneda, V.M. Monnier, Chin. Chem. 40 (1994) 1766.
[18] Z. Makita, H. Viassara, A. Cermi, R. Bucala, J. Biol. Chem. 267 (1992) 5133.
[19] R. Miller, K. Olsson, Acta. Chem. Scand. B 39 (1985) 717.
[20] O.H. Lowry, N. Rosebrough, A.L. Farr, R.J. Randall, J. Biol. Chem. 193 (1951) 265.
[21] Z. Makita, R. Bucala, E.J. Rayfield, E.A. Fnieman, A.M. Kaufman, S.M. Korbet, R.H. Barth, J.A. Winston, H. Fuh, K.R. Manogue, A. Cerami, H. Viassara, Lancet 343 (1994) 1519.
[22] T.P. Degenhardt, L. Grass, S. Reddy, S.R. Thorpe, E.P. Diamandis, J.W. Baynes, Kidney Int. 52 (1997) 1064.
[23] T. Miyata, O. Oda, R. Inagi, Y. Iida, N. Araki, N. Yamada, S. Horiuchi, N. Taniguchi, K. Maeda, T. Kinoshita, J. Chin. Invest. 92 (1993) 1243.
[24] M.C. Well-Knecht, S.R. Thorpe, J.W. Baynes, Biochemistry 34 (1995) 15134.
[25] H. Odani, T. Shinto, J. Usami, Y. Matsumoto, E. Brinkmann Frye, J.W. Baynes, K. Maeda, FEBS Lett. 427 (1998) 381.