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Analytical strategy for the assessment of the protein glycation status in uremic patients by high-performance liquid chromatography

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Abstract

We propose a newly integrated procedure for the analysis of furosine (early glycation product) and pentosidine (glycoxidation end-product) in plasma proteins and the simultaneous assessment of advanced glycation end-product (AGE) peptides and free pentosidine in plasma. In order to determine furosine and protein-linked pentosidine, plasma proteins were hydrolyzed in 8 *M* HCl and each analyte was purified by solid-phase extraction. Furosine was determined by ion-pair RP-HPLC methodology with isocratic elution and spectrophotometric detection at 280 nm and pentosidine by ion-pair RP-HPLC by using gradient elution and fluorimetric detection at 335/385 nm. To assess free pentosidine concentration and simultaneously evaluate the AGE peptides, an aliquot of plasma sample was diluted and ultrafiltered by using Centricon 10 ($M_r \leq 10\,000$) ultrafiltration membranes. Free pentosidine and AGE peptides were analysed by ion-pair RP-HPLC, by using gradient elution and fluorimetric detection at 385 nm upon excitation at 335 nm. The HPLC methodology has been successfully used for the determination of glycation and glycoxidation protein status in uremic patients.

Keywords: Glycation; Proteins; Peptides; Pentosidine; Furosine

1. Introduction

Protein glycation and glycoxydation are currently of much experimental and clinical interest: the heterogeneous class of molecules that arise from these reactions, i.e. Amadori products and advanced glycation end-products (AGEs), have been implicated in tissue damage associated with the pathological complications of diabetes and renal failure on hemodialysis [1,2]. In renal failure patients, the loss of the normal clearance of low-molecular-mass (LMM) AGE products, arising from the metabolic degradation of AGE-proteins [3], results in a high circulating plasma level of AGE peptides [4], which are not efficiently removed by the current methods of hemodialysis. Some of these compounds are chemically reactive and responsible for an abnormal formation of AGE proteins (second generation AGEs) in plasma and tissues [5].

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In this context, it is important to check the glycation status of uremic patients by using furosine, as a marker of early protein glycation and pentosidine, as a marker of protein glycoxydation and finally AGE peptides, as markers of chemically-reactive molecules which exacerbate the accumulation of AGEs in uremic plasma.

The analytical methods, so far reported for furosine quantitation in different biological samples use ion-exchange chromatography with amino acid analysis and post-column ninhydrin derivatization [6,7], gas–liquid chromatography [8], and reversed-phase HPLC with detection at 280 nm [9,10]. The sensitivity, accuracy and precision of these methods differ and they are often time consuming, expensive and sometimes not sufficiently free of interference. More recently, reliable methods have been reported for the determination of furosine in milk proteins [11], in human serum and in dialysate proteins [12] by HPLC. These methods, although accurate, present several drawbacks in routine clinical studies: time-consuming analyses due to the use of the gradient elution mode; the high acidity of the sample which can damage either the chromatographic injector or the packing; the use of very expensive furosine-dedicated chromatographic column.

Protein-bound pentosidine may be assayed by the method described by Odetti et al. [13], its modification [14], or by the recently described HPLC method with column switching [15].

LMM AGEs, a class of uremic toxins [16], may be assessed in plasma ultrafiltrate by radioreceptor [17] or by a competitive AGE-enzyme-linked immunosorbent assay (ELISA) assay [18]. Another analytical procedure that uses HPLC for the measurement of low-molecular-mass AGEs in deproteinized plasma has recently been proposed [19]. These methodologies for protein-bound pentosidine and LMM AGEs determination appear poorly suitable for routine assays in clinical–metabolic studies.

In this paper we propose an integrated procedure for the determination of furosine (the early glycation product) and pentosidine (the main glycoxydation end-product) in plasma proteins, and the simultaneous assessment of AGE peptides and free pentosidine in plasma ultrafiltrate. Each determination is based on analyte-dedicated HPLC.

2. Experimental

2.1. Instrumentation

The chromatographic system consisted of the Model 920 Jasco chromatograph (Jasco, Milan, Italy), equipped with two Model 880-PU Jasco solvent delivery modules, a Model 7125 Reodyne injector, a Model 821-FP Jasco spectrofluorimetric detector, with two momochromators, for AGEs and pentosidine detection, a Model UV-975 Jasco spectrophotometric detector for furosine detection, and a Model HP 3394 Hewlett-Packard integrator.

Kromasil C_8, 5 μm particle size, 15×0.46 cm column was purchased from Alltech (Milan, Italy); Spherisorb ODS2, 5 μm, 15×0.4 cm column was from Waters (Milford, MA, USA).

2.2. Chemicals

The furosine standard was purchased from Neosystem Laboratories (Strasbourg, France); pentosidine, used as a chromatographic standard, was prepared according to the method described by Baynes [20]. The C_{18} bulk phase (40 μm) used for the preparation of solid-phase extraction (SPE) minicolumns was purchased from Alltech; ion pair octanesulfonic acid (OSA), sodium salt, and heptafluorobutyric acid (HFBA) were Sigma (Milan, Italy) ultrapure products. The water used for the preparation of buffers and standards was obtained with a Milli-Q purification system (Millipore, Milford, MA, USA). All other chemicals were analytical or HPLC-grade products by Sigma.

2.3. Biological samples

The study was approved by the local ethic committee and all subjects (patients and controls) gave their informed consent. Venous blood from ten healthy volunteers [six men, age 45±6 years and four women, age 47±6 years (mean±SD)] and eleven uremic patients on standard (4 h×3/week) hemodialysis [six men, age 53±8 years (mean±SD) and five women, age 55±7 years (mean±SD)], was collected into heparinized tubes and immediately centrifuged at 4°C for 10 min at 2700 *g* to separate

plasma. Blood samples were collected from patients immediately before the hemodialytic treatment. Unless immediately analyzed plasma samples were kept at −80°C.

2.4. Furosine and protein-bound pentosidine determination

The concentration of protein in the plasma samples was measured by the Biuret reaction on a Hitachi 717 multichannel analyser (Boeringer, Mannheim, Germany).

In order to assess furosine and protein-bound pentosidine, 2.25 ml of 6% (w/v) trichloroacetic acid solution were added to an aliquot (0.25 ml) of plasma sample into a tube with a PTFE-coated screw cap. All subsequent steps were performed in these tubes. After 5 min in ice, the mixture was centrifuged at 15 000 *g* at 4°C for 15 min. The bulk protein pellet was added with 2 ml of 8 *M* HCl and hydrolyzed at 110°C for 21 h. The sample was then diluted to 10 ml with HPLC-grade water and filtered through Watman filter paper.

To assess the furosine formed during acid hydrolysis of ϵ-deoxyfructosyl lysine, the Amadori compound of early protein glycation, 4 ml of the filtered hydrolyzed sample were poured into a C_{18} (100 mg) SPE minicolumn, previously conditioned with 3 ml of acetonitrile–water (70:30, v/v) and with 3 ml of 2 *M* HCl. The eluate was collected together with 3 ml of 2 *M* HCl used subsequently to elute the unretained furosine from the minicolumn. The sample was dried under vacuum by rotavapor, the residue dissolved in 1 ml of 0.05 *M* HCl solution and a 50-μl volume injected into the chromatograph. The chromatographic analysis was carried out on a Kromasil C_8 5 μm particle size, 15 cm×0.46 cm I.D. column, protected by a Spherisorb ODS2, 5 μm particle size, 1 cm×0.4 precolumn, equilibrated and eluted isocratically with 0.05 *M* sodium acetate buffer, pH 4.5, containing 3 m*M* OSA and 6% (v/v) acetonitrile, at 1 ml/min flow-rate. Furosine elutes at 13 min as monitored by spectrophotometric UV 975 detector set at 280 nm, 0.010 AUFS.

The quantitation of furosine in the sample was performed by external standard calibration. Results were expressed as pmol/mg protein.

To analyze pentosidine, 4 ml of the acid-hydrolyzed sample were dried under vacuum on rotavapor; the residue was reconstituted in 1 ml of 0.5 *M* potassium phosphate buffer, pH 6.5. A 0.5-ml volume of the sample was poured into a C_{18} (100 mg) SPE minicolumn, previously washed with 3 ml of acetonitrile–water mixture (70:30, v/v) and equilibrated with 3 ml of 0.5 *M* potassium phosphate buffer, pH 6.5. The eluate was collected together with 1 ml of 0.5 *M* potassium phosphate buffer, pH 6.5, used to quantitatively elute unadsorbed pentosidine from the minicolumn. A 50-μl volume of the purified sample was injected into a Spherisorb ODS2, 5μm particle size, 15 cm×0.4 cm I.D. column, protected by 1 cm×0.4 cm Spherisorb ODS2 precolumn, equilibrated with 0.1% (v/v) HFBA aqueous solution, used as phase A. Elution was carried out at 1 ml/min flow-rate by binary gradient elution using phase A and a solution of 50% acetonitrile in phase A, as phase B, according to the following program: 100% phase A at 0 min and 50% phase B in 20 min. The column was washed with phase B for 5 min and then with phase A for 10 min, before the next analysis. The native fluorescence of pentosidine was monitored by a spectrofluorimetric detection system set at 385 nm for emission and at 335 nm for excitation, bandwidth 10 nm. Analyte quantitation was carried out by external standard calibration. The concentration of protein-bound pentosidine was expressed as pmol/mg protein.

2.5. LMM AGEs and free pentosidine determination

To evaluate AGE peptides and simultaneously assess free pentosidine concentration, an aliquot (0.25 ml) of plasma sample was diluted 1:3 (v/v) with HPLC grade water and ultrafiltered at 3000 *g* for 2 h at 4°C using a Centricon 10 ($M_r \leq 10\,000$) ultrafiltration concentrator (Amicon, Beverly, MA, USA). A 50-μl volume was directly injected into the chromatographic apparatus. The analytical column and the chromatographic phases were the same used for the analysis of protein-bound pentosidine, but the following multigradient elution program was developed (proportion of phase B): 0–3 min, 0%; 3–10 min, 15%; 10–20 min, 20%; 20–30 min, 50% and

30–35 min, 100%. The column was then equilibrated with phase A for 10 min before the next injection. Free pentosidine and LMM AGEs were detected by fluorescence at 335 nm excitation and 385 nm emission. Plasma AGE-peptides (but not free pentosidine) may be also determined (not shown) by their fluorescence at 440 nm, on excitation at 370 nm, a typical property of a major fluorescent chromophore, which forms as an intermediate in the advanced glycation reactions [21].

Free pentosidine quantitation was carried out by external standard calibration and the results expressed as pmol/ml of plasma sample.

LMM AGE concentration was reported as arbitrary units (AU)/ml, calculated from native fluorescence by 335 nm excitation/385 nm emission or 370 nm excitation/440 nm emission detection, according to the following equation: $AU/ml = TIA \cdot D/10^6$, where TIA is the total integration chromatographic area and D is the dilution factor.

3. Results and discussion

3.1. Furosine assessment

The present method allows the chromatographic determination of furosine in plasma protein hydrolysates, after purification by SPE on C_{18} minicolumn (in which the analyte is not retained), in <20 min, under isocratic conditions. In the presence of the ion pair OSA, furosine is strongly retained on the analytical reversed-phase column. The C_{18} SPE minicolumn is meant to retain several contaminating UV-absorbing components without loss of furosine: this treatment minimises the contamination of the analytical column and makes it possible to carry out the chromatographic analysis under isocratic conditions. In addition, the injection of a low-acidity sample preserves from damage the chromatographic instrumentation (injector and column packing). Fig. 1 depicts the typical chromatographic profile obtained with a furosine standard sample (Fig. 1A) and plasma hydrolyzed protein samples from a normal subject (Fig. 1B) and a uremic patient on standard hemodialysis (Fig. 1C). When hydrolysates from the bulk plasma proteins are subjected to HPLC analysis, several peaks elute early which are not relevant to the analysis. The furosine peak appears as fully resolved from all the other contaminating components present in the chromatographic profile. Moreover, column washing with a high percentage of organic modifier shows the complete absence of adsorbed UV-absorbing material. We identified the analyte peak as furosine either by co-chromatography of hydrolyzed plasma proteins and different

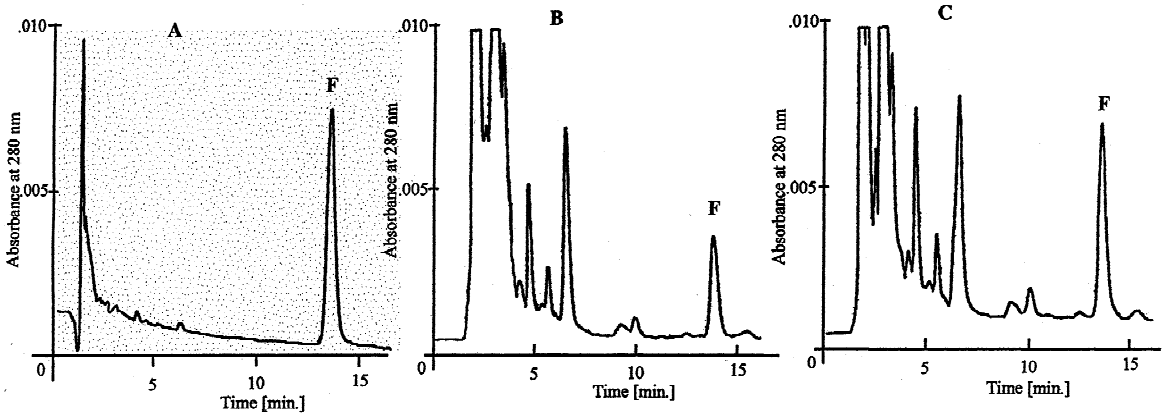

Fig. 1. Ion-pair reversed-phase chromatographic profiles of furosine analysis. (A) Furosine standard (100 pmol); (B) furosine in hydrolyzed plasma proteins from a normal subject; (C) furosine in hydrolyzed plasma proteins from a patient on standard hemodialysis. The injected biological samples corresponded to 200 μg of hydrolyzed protein. F=furosine (t_R=13 min). Detection: spectrophotometric at 280 nm, 0.010 AUFS. Chromatographic conditions: isocratic elution with 0.05 *M* sodium acetate buffer, pH 4.6, containing 3 m*M* OSA and 6% (v/v) acetonitrile, at 1 ml/min flow-rate.

amounts of standard furosine, or by a sample negative control from which Amadori product (and therefore furosine) had been removed by reduction of the plasma sample with sodium borohydride (not shown).

A major objective of the methodology presented in this paper was to develop a chromatographic system under isocratic conditions, suitable for routine analyses of furosine. The combined use of SPE of the hydrolyzed sample and the presence of an ion pair in the mobile phase permits a running time of about 20 min, making also automation possible.

The limit of furosine detection, defined as a signal-to-noise ratio of 3, was 10 pmol/mg of plasma protein, a value well below the level of the analyte present in plasma proteins. Linearity ($r=$ 0.999) of the detector response was observed over the range of calibration curve for furosine (50–500 pmol of injected standard furosine); moreover a linear correlation was observed between the peak area of furosine and the scalar amount of the analyte added to human plasma ($r=0.996$). The recovery obtained for furosine added to acid precipitated proteins, immediately before the hydrolysis step, was 97.2±3.6 (mean±SD, $n=7$). The method of analysis yields reproducible results in terms of retention time and integrated area, with RSD values of 0.3 and 5.7%, respectively, by running seven replicates from one plasma sample. Furthermore, duplicates from the same plasma sample assayed on five different occasions gave the intra-assay RSD values of 3.5 and 6.2% for retention time and integrated area, respectively. The sample hydrolysis in the presence of 8 *M* HCl and the analytical conditions described in this paper allow a higher furosine yield, in respect to 6 *M* HCl hydrolysis (not shown), used by other authors, [10,12,14], and permit a high longevity and performance of the chromatographic column and finally, a reduction of running time without column regeneration, admitting automation in routine analyses.

The concentrations of furosine in plasma proteins of ten healthy subjects and eleven uremic patients on standard hemodialysis were determined by the present method. The mean (±SD) concentrations of furosine were 865±98 pmol/mg protein for healthy subjects and 1460±148 pmol/mg protein for uremic patients. These values are in agreement with those reported by other authors [12,14]. The results we obtained are also in good agreement with those obtained for the same samples when applying (not shown) the method of Resmini et al. [11].

3.2. Protein-bound pentosidine determination

Pentosidine, a protein glycoxidation product, is present at a low concentration (about 1 pmol/mg protein) in proteins of physiological plasma samples. This is why the quantification of this analyte calls for a sensitive method. In addition, since hydrolyzed samples contain several products having a fluorescence property similar to pentosidine, a suitable high-resolution chromatographic system should be set up for routine clinical–biochemical analysis. Fig. 2 shows the analytical capability achievable with the sample preparation and chromatographic elution system described in this paper. The chromatographic profile of standard pentosidine (Fig. 2A) shows a single peak of homogeneous fluorescence material without contaminating products. Fig. 2B and C shows the chromatographic profiles of serum protein-bound pentosidine analyses of a healthy subject and a uremic patient on SHD, respectively. The native pentosidine peak, identified by chromatographic coelution with the synthetic analyte, is baseline separated and eluted in ≈20 min. The detection limit for pentosidine was about 150 fmol in the column. Linearity of the detector response ($r=0.998$) was observed over the range of calibration curve for pentosidine (1–10 pmol) injected in the column; a linearity ($r=0.996$) was also observed between the peak area of pentosidine and the scalar amount of the analyte added to the plasma. The recovery obtained for pentosidine added to acid precipitated proteins immediately before the hydrolysis step, was 90±3.8 (mean±SD, $n=7$). Analyte retention time and integrated area were highly reproducible with RSDs of 0.7 and 4.9%, respectively, in within-day analyses ($n=7$) and 2.9 and 5.3%, respectively, in between-day analysis ($n=7$). The concentration of protein-bound pentosidine in plasma of normal subjects and end-stage renal disease patients on hemodialysis is 1.4±0.3 pmol/mg protein (mean±SD) and 27.3±6.1 pmol/mg protein (mean±SD), respectively. These values are in good agreement with previously reported results [14,22,23] for pentosidine evaluated by chromatographic methodologies.

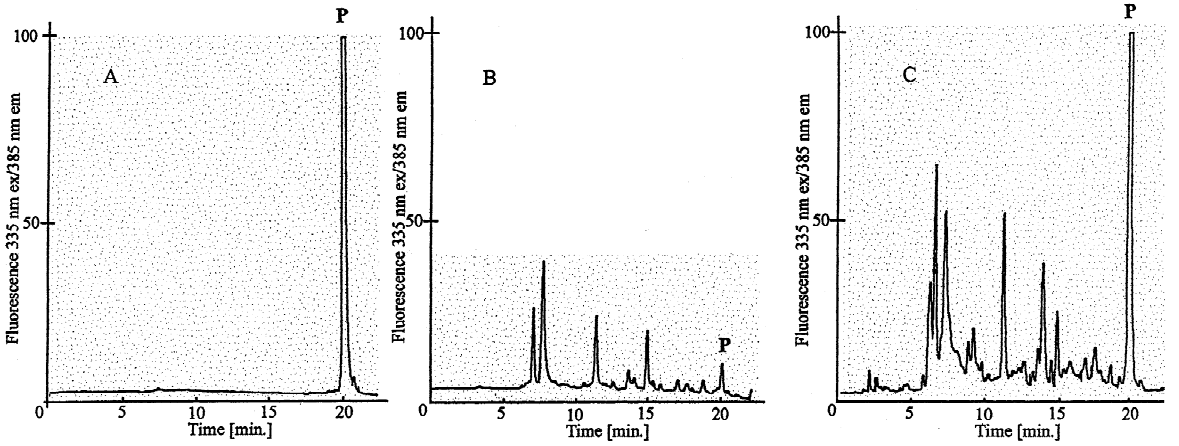

Fig. 2. Analysis of protein-bound pentosidine by ion-pair reversed-phase HPLC. (A) Pentosidine standard (10 pmol); (B) chromatographic analysis of pentosidine in a plasma protein hydrolysate from a normal subject; (C) analysis of pentosidine in a plasma protein hydrolysate from a patient on standard hemodialysis. P=pentosidine (t_R=20 min). Detection: fluorimetric at 335 nm excitation, 385 nm emission. Chromatographic conditions: gradient elution, at 1 ml/min flow-rate, according to the following program (proportion of phase B): 0–20 min, 50%.

3.3. AGE-peptides evaluation

So far, the important family of circulating peptides has been evaluated in plasma by immunological methodologies [17,18]. AGE peptides released from AGE proteins in skin samples by collagenase digestion were evaluated in arbitrary units by fluorescence measurement at 440 nm on excitation at 370 nm [24] of the clear supernatant containing digested material. In our integrated analytical scheme for the assessment of protein glycation status, a HPLC system for serum LMM AGEs has been developed, in order to check this glycation parameter in the clinical laboratory characterization of uremic patients. Fig. 3 depicts typical chromatographic profiles of standard pentosidine (Fig. 3A), normal serum ultrafiltrate (Fig. 3B), and uremic serum ultrafiltrate (Fig. 3C) with fluorescence detection at 335/385 nm. The

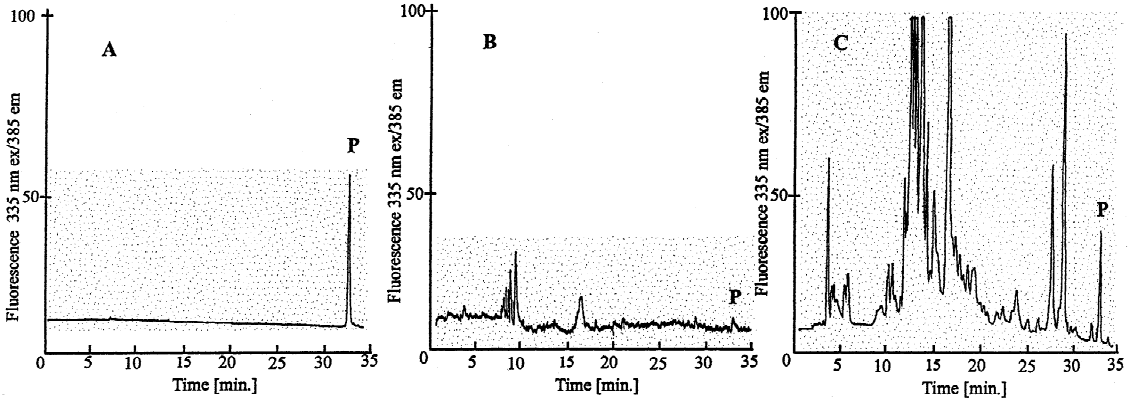

Fig. 3. Typical chromatographic profiles of AGE peptides and free pentosidine analyses. (A) Pentosidine standard (2 pmol); (B) Analysis of a plasma ultrafiltrate sample (50 μl) from a normal subject; (C) chromatrographic profile of a plasma ultrafiltrate sample (50 μl) from a patient on standard hemodialysis. P=pentosidine (t_R=32 min). Detection: fluorimetric at 335 nm excitation, 385 nm emission. Chromatographic conditions: gradient elution, at 1 ml/min flow-rate, according to the following program (proportion of phase B): 0–3 min, 0%; 3–10 min, 15%; 10–20 min, 20%; 20–30 min, 50% and 30–35 min, 100%.

standard pentosidine was eluted at a t_R of about 32 min, under the gradient elution program used for this analysis. The chromatographic profile of normal serum ultrafiltrate shows a few peaks relevant to AGE peptides fluorescing at 385 nm upon excitation at 335 nm, and the pentosidine peak cannot be assessed. Conversely, the chromatographic profile of serum ultrafiltrate of the uremic patient on hemodialysis appears very complex and shows both a striking increase in the number of AGE peptide peaks, and a remarkable quantity of free pentosidine. LMM AGE-peptides level was remarkably high in uremic patients, 1845±321 AU/ml plasma (mean±SD), in comparison with normal subjects, 30±9.5 AU/ml plasma (mean±SD). In physiological plasma samples the free pentosidine is not important, whereas in ultrafiltrate plasma samples of patients on standard hemodialysis the value of this final catabolic product of protein glycoxidation is 116.5±17.6 pmol/ml plasma (mean±SD).

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