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Measurement of pentosidine in human plasma protein by a single-column high-performance liquid chromatography method with fluorescence detection

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ABSTRACT

A rapid and sensitive single-column high-performance liquid chromatography method and application for the detection of protein bound pentosidine is described. Pentosidine, a cross-link between arginine and lysine, is a well-characterized advanced glycation endproduct. In order to detect protein-bound pentosidine, plasma proteins were hydrolysed in 6 N HCl. Detection of pentosidine is done based on its own fluorescence characteristics using fluorimetric detection (E_x = 325 nm, E_m = 385 nm). Separation is done, with a run-to-run time of 30 min, on a C_{18} Allspehere ODS-II column with a citric acid acetonitrile buffer. This detection enables sensitive and specific determination of protein bound pentosidine in plasma with a detection limit of 2.2 nmol/l or 0.02 pmol/mg protein (signal-to-noise: 6). The intra-assay coefficient variation is 6.5% at a plasma pentosidine concentration of 0.47 pmol/mg protein and 2.0% at a concentration of 1.27 pmol/mg protein. The inter-assay coefficient variation is 3.1% at a plasma pentosidine concentration of 0.43 pmol/mg protein and 1.6% at a concentration of 1.40 pmol/mg protein. Linearity is tested in 4 different plasma samples and showed linearity (0–200 nmol/l, $r^2 > 0.99$). Recovery of pentosidine in 4 different plasma samples at different concentration levels is 102 ± 10% (mean ± SD). Using this method protein bound pentosidine concentration is investigated in healthy controls (n = 24, age 67 ± 9 years) and patients with end stage renal disease (n = 24, age 65 ± 10 years). Higher plasma concentrations of protein bound pentosidine are measured in the patient group as compared with the control group 3.05 (2.03–3.92) pmol/mg protein and 0.21 (0.19–0.33) pmol/mg protein, respectively (median (interquartile range), $p < 0.00001$). These results are consistent with previously reported results.

1. Introduction

Glycation is the nonenzymatic reaction of glucose or other reducing sugars with amino groups of proteins. The amino groups of the side chains of arginine and lysine are the primary targets for this type of posttranslational modification. Over time, the initial glycation products may undergo intramolecular rearrangements and oxidation reactions and ultimately transform into stable, so-called advanced glycation endproducts (AGEs). AGEs represent a mixture of different products such as pentosidine and N^{ε}-(carboxymethyl)lysine (CML). AGEs have different biological functions: some are protein cross-links such as pentosidine and some, such as CML, are recognition factors for specific AGE-binding receptors. The physiological consequences of AGEs in ageing and in the aetiology of diabetic complications [1] and in the development of age-related diseases such as inflammation [2], atherosclerosis [3] and neurodegenerative disorders [4] has been described.

Despite progress in this field, a more comprehensive understanding of the putative effects of AGEs in the pathophysiology of these diseases is needed. In addition, the measurement of AGEs may also help to identify patients with a high risk for poor outcome and may thus help in risk stratification [5,6]. However, progress in the field is hampered by the lack of an easy method for the detection of AGEs in the laboratory. We need a rapid, simple and reliable method for the detection of AGEs.

AGEs have traditionally been detected by enzyme-linked immunosorbent assay (ELISA) [7–9]. For several reasons the use of antisera for quantitative immunoassays of protein-bound AGEs is questionable. Reproducibility and sensitivity of such an assay are not optimal, because the specificity of the antibodies is often difficult to define and, because of steric constraints, not all AGE epitopes on the protein may be available for interaction with the antibody. Thus, AGE measurements with immunoassays should be interpreted with care. A better approach for the quantitative determination of specific AGEs in proteins is the use of a specific

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analytical technique. For the determination of AGE levels in both tissue and blood samples, high-performance liquid chromatography (HPLC) measurements [10–16] and several mass spectrometry methods have been developed including gas chromatography mass spectrometry (GC–MS) [17], and liquid chromatography tandem mass spectrometry (LC–MSMS) [18,19]. Although the latter is considered to be the most accurate technique available at this moment for the detection of AGEs, a disadvantage is that this method is very expensive and not suitable for routine clinical use.

Pentosidine, one of the best-characterized AGEs, is a fluorescent cross-link between arginine and lysine. Because pentosidine is stable under the conditions used for acid protein hydrolysis and can be detected at very low concentrations based upon its fluorescence properties, pentosidine can be regarded as a biomarker for AGEs. Until now, pentosidine has been quantified by ELISA [7,9], by HPLC [10–16,20–25] and by LC–MSMS [19,28]. In addition to the restrictions to use immunoassays for the detection of AGEs as described above, antibodies against pentosidine or specific ELISA systems are not commercially available. Accurate quantification of pentosidine by HPLC could only be obtained with cumbersome double-chromatographic systems [10,13,14,20], with ion-exchange HPLC with long run-times and high variations in retention time [21], with RP-HPLC with the use of ion-pairing agent with low recoveries [10,12,26] or HPLC analysis with elaborate solid phase extraction (SPE) sample preparation [16].

We now describe a rapid, simple and sensitive one-column reversed-phase HPLC method for the detection of pentosidine in plasma protein hydrolysates.

2. Materials and methods

2.1. Instrumentation

Samples were analysed by reversed phase HPLC-fluorescence using an Allsphere ODS-2 (150 mm × 4.6 mm, 3 μm) analytical column fitted with an Allsphere ODS-2 (7.5 × 4.6, 5 μm) precolumn (Alltech/Grace, Breda, The Netherlands). Detection was carried out using a Jasco type 821-FP spectrofluorometer (Jasco Benelux, Maarssen, The Netherlands) set at an excitation and emission wavelength of 325 and 385 nm, respectively. HPLC analysis was performed using a binary high-pressure gradient at a flow of 1 ml/min using two Model PU-980 pumps (Jasco Benelux, Maarssen, The Netherlands). Solvent A was 25 mM citric acid and solvent B was (50/50, v/v) ACN/25 mM citric acid. A linear gradient was started at 99% solvent A which was changed within 15 min to 90% solvent A. After cleaning the column with 100% solvent B during 5 min the column was equilibrated for 8 min at the initial composition. Injection volume was 10 μl and column temperature was set at 20 °C using a Spark Mistral column oven (Spark Holland B.V., Emmen, The Netherlands). Samples where thermostatted at 6 °C using an auto-injector model 717 Plus Autosampler (Waters, Etten Leur, The Netherlands). Chromatograms were acquired and processed with Totalchrom (PerkinElmer, version 6.2.0.0.0:B27, Zoetermeer, The Netherlands).

2.2. Materials

HPLC-grade acetonitrile (ACN) was obtained from Chromanorm (Prolabo, Paris, France). Citric acid (GR for analysis), sodium hydroxide and hydrochloric acid (HCl) fuming (37%) were obtained from Merck (Darmstadt, Germany). Trichloroacetic acid, boric acid (99.5%) and trifluoroacetic acid (TFA) (99+%) were obtained from Sigma (SigmaUltra min. 99,0%) (Zwijndrecht, The Netherlands). Ultrapure waters was generated by a Super-Q system (Millipore, Amsterdam, The Netherlands) and sodium borohydride was obtained from Fluka (Buchs, Schweiz). Pentosidine standard was obtained from IMARS (International Maillard Reaction Society, http://imars.case.edu/). Levels determined with this standard are four times lower than published by Sell and Monnier [27] and agree with Dyer et al. [28].

2.3. Plasma samples

Heparinized plasma samples were obtained form 24 healthy volunteers (16 male, 8 female, mean age 67 ± 9 years) and 24 uremic patients on peritoneal dialysis (PD) (14 male, 10 female, mean age 65 ± 10 years).

2.4. Sample preparation

In a 10 ml glass tube with a Teflon-lined screw-cap 50 μl plasma was mixed with 100 μl water. To prevent a potential artifactual formation of pentosidine from early glycation products during sample preparation, plasma samples were reduced by 500 μl sodium borohydride borate buffer (200 mM, pH 9.2) before precipitation. This mixture was allowed to stand for 2 h at room temperature. Proteins were then precipitated by addition of 2 ml 20% trichloroacetic acid and centrifuged for 10 min (4 °C) at 4500 × *g*. The supernatant was carefully removed by aspiration with a Pasteur pipette. The protein pellet was washed once by adding 2 ml 5% trichloroacetic acid followed by centrifugation and removal of the supernatant as described above (The effect of this extra washing step on absolute peak area and recovery was tested negative). For recovery and linearity experiments 50 μl of pentosidine standard (0–200 nmol/l) was added. Samples were hydrolyzed by adding 50 μl 6 N HCl and incubation for 18 h at 110 °C. After hydrolysis, samples were evaporated to dryness at 80 °C under a stream of nitrogen gas and reconstituted in 200 μl 25 mM citric acid/l. This solution was centrifuged for 15 min (4 °C) at 14,000 rpm. Ten microliters of this solution (equals 2.5 μl plasma) was injected on the HPLC system.

2.5. Standard pentosidine

Stock standard pentosidine was prepared by dissolving pentosidine in 0.1% (v/v) TFA at a concentration of 20 μM. Standard was divided in portions and stored at −20 °C prior to use.

2.6. Determination of protein in plasma

Pentosidine concentration was expressed as pmol/mg protein in plasma. Protein concentration in plasma was analysed using Bradford reagent (Biorad Laboratories GMBH, München, Germany) [29]. The calibration curve was established using bovine albumin fraction V (Sigma, Zwijndrecht, The Netherlands).

2.7. Statistical analysis

The method validation data were expressed as mean and SD. The patient study data were expressed as median and interquartile ranges (IQR). To detect group differences we applied the Mann–Whitney *U* test.

3. Results

3.1. Reversed phase chromatography

During method development we tested first cation-exchange chromatography, as described by Mitsuru Saito et al. [21] and different ion-pair based HPLC systems. However, with these methods we observed retention time variations of >1 min with no baseline separation of pentosidine (data not shown). In contrast, with RP-HPLC, we found a retention time of 13.24 ± 0.07 min (CV 0.5%, *n* = 50

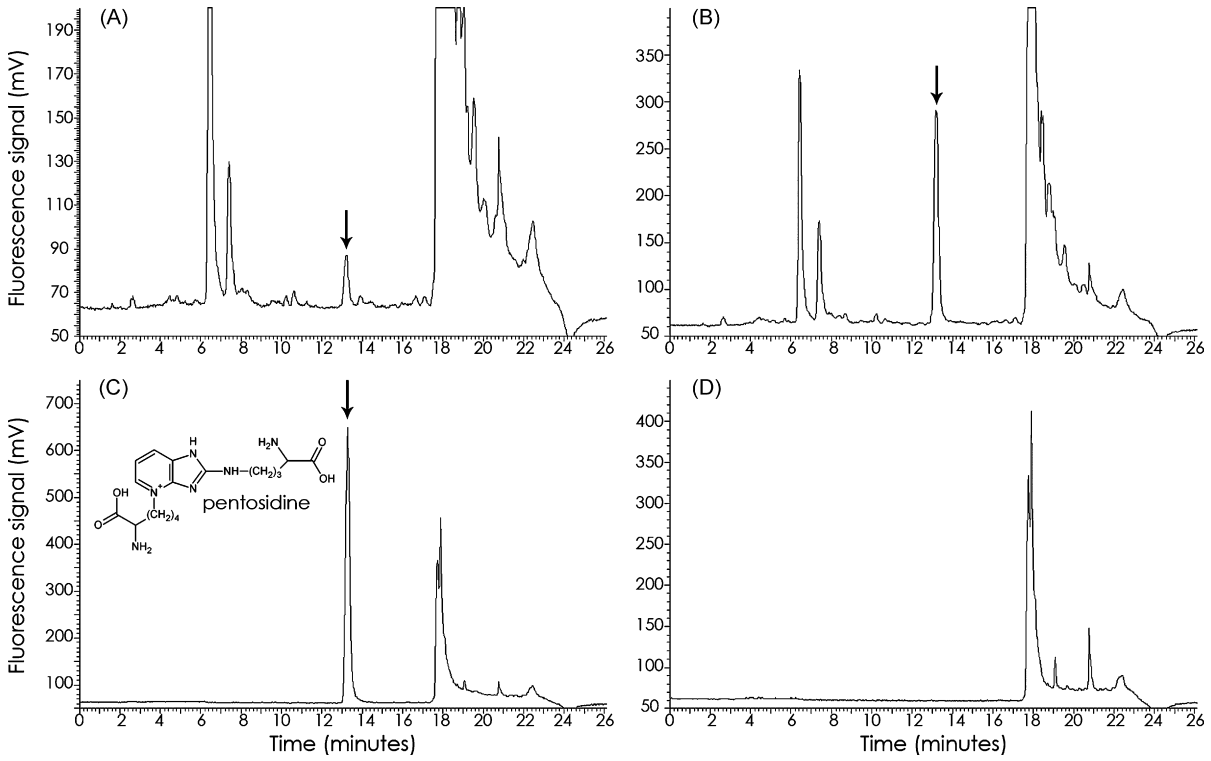

Fig. 1. Representative chromatograms of a standard solution of pentosidine (C), a plasma sample from a healthy control (A), a uremic patient on peritoneal dialysis (B) and a blank sample (D). The elution of pentosidine is indicated with an arrow mark with a peak area (and concentration) of pentosidine in A = 314,197 (0.60 pmol/mg protein), in B = 3,638,638 (6.06 pmol/mg protein), in C = 8,901,011 (460 nM) and in D = 0 (0 pmol). Pentosidine is clearly baseline separated which makes time consuming manual integration afterwards not necessary.

different plasma samples) and a baseline separated pentosidine peak. A chromatogram of a pentosidine standard, a typical chromatogram of a plasma sample from a healthy control and from a uremic patients on peritoneal dialysis are shown in Fig. 1. The baseline separated pentosidine peak in plasma makes automatic integration possible.

3.2. Stability of pentosidine

To make large number of measurements within one run possible, we tested the stability of pentosidine under different conditions. The stability of pentosidine was tested in solvents with neutral pH, acidic pH (pH 2.5) and basic pH (pH 10.6). Pentosidine was at least stable for 35 h in all the tested solvents when samples were stored in the auto-injector at 6 °C (Fig. 2).

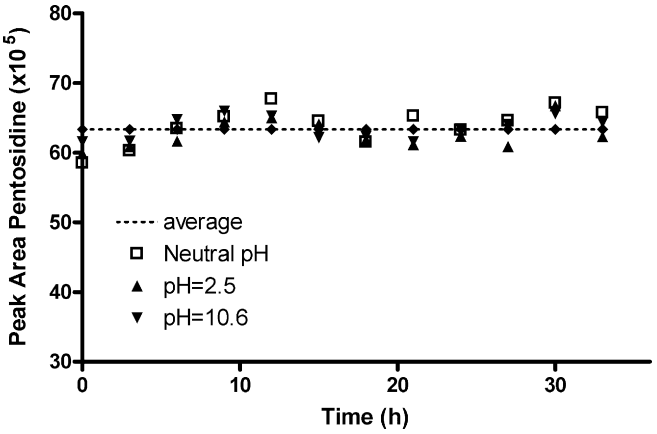

Fig. 2. The stability of pentosidine at different pH. Pentosidine was diluted in citric acid buffer (pH 2.5), in citrate buffer (pH 10.6) and in water to a final concentration of 32 nmol/l. Pentosidine concentration was measured upon a storage at 6 °C for 35 h.

3.3. Linearity and lower limit of quantification

Linearity of the detection of pentosidine was tested in water (with and without hydrolysis) and matrix by adding pentosidine standard during preparation of water and different plasma samples (Table 1 and Fig. 3). The slope, tested in 4 different plasma samples and in water measured on two different days, was 19,685 ± 3.4% (mean ± CV%). Acid hydrolysis had no effect on the peak areas of pentosidine, therefore calibration was carried out with non-hydrolysed standards. Also no matrix effect was observed as tested in different plasma samples (Table 1 and Fig. 3). The limit of detection (signal-to-noise: 6) of pentosidine was 2.2 nmol/l or 0.02 pmol/mg protein, corresponding to a concentration of 0.02 pmol/mg protein.

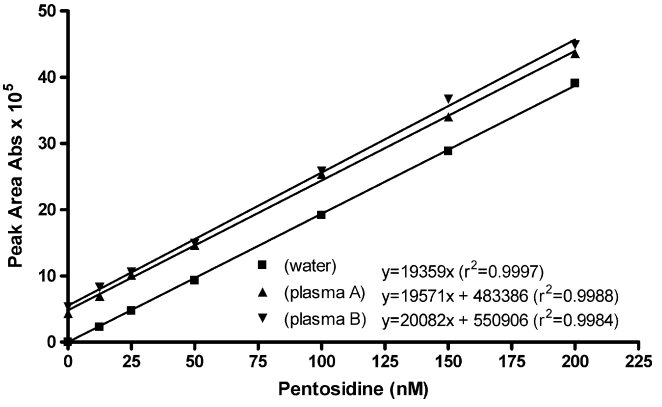

Fig. 3. Calibration curves of pentosidine in water and in plasma samples. Linearity of the detection of pentosidine was tested in water and in two different plasma samples by the addition of pentosidine standard (0–200 nM) during sample preparation. No matrix-effect was observed. *Y*-axis intercept in both plasma sample shows endogenous pentosidine (respectively, 24.7 nM in plasma A and 27.4 nM in plasma B).

Table 1
Calibration curves of pentosidine in different matrices.

Matrix	Slope	Y-intercept	r^2	Concentration range (nM)
Water (without hydrolysis)	19,359	–	0.9997	0–200
Water (with hydrolysis)	18,300	–	0.9991	0–200
Plasma A	19,571	483,386 (24.7)[a]	0.9988	0–200
Plasma B	20,082	550,906 (27.4)[a]	0.9984	0–200
Plasma C	19,849	1,027,646 (51.8)[a]	0.9983	0–75
Plasma D	20,630	473,838 (23.0)[a]	0.9959	0–75
Mean	19,731			
CV (%)	3.6			

[a] Endogenous pentosidine in plasma (nM).

3.4. Recovery and precision

The intra-assay variation of the method was determined in two different plasma samples analysed in one batch during 1 day. The intra-assay variation was 6.5% as determined in a plasma sample ($n = 9$) with a mean concentration of 0.47 pmol/mg protein and 2.0% in a plasma sample ($n = 10$) with a mean concentration of 1.27 pmol/mg protein. The inter-assay variation of the method was determined in two different plasma samples divided into batches and analysed during different days. The inter-assay variation was 3.1% as determined in a plasma sample ($n = 10$) with a mean concentration of 0.43 pmol/mg protein and 1.6% in a plasma sample ($n = 10$) with a mean concentration of 1.40 pmol/mg protein. Recovery of pentosidine was 102 ± 10% as determined in 4 different plasma samples (Table 2).

3.5. Comparison of pentosidine concentration between cases and controls

We analysed protein bound pentosidine in 24 healthy volunteers and in 24 uremic patients on peritoneal dialysis. The median (IQR) concentration of protein bound pentosidine was significantly higher in the peritoneal dialysis patients than in the healthy control group 3.05 (2.03–3.92) pmol/mg protein and 0.21 (0.19–0.33) pmol/mg protein, respectively, $p < 0.00001$ (Fig. 4).

Table 2
Recovery of pentosidine in 4 different plasma samples.

Matrix	Added amount (fmol[a])	Measured (fmol[a])	Recovery (%)
Plasma A	500	563	101
	375	439	102
	250	327	108
	125	189	106
	63	131	119
	0	58	–
Plasma B	500	580	102
	375	474	108
	250	333	106
	125	193	99
	63	136	108
	0	70	–
Plasma C	188	318	100
	125	261	104
	63	190	94
	31	166	110
	0	131	–
Plasma D	188	262	104
	125	186	96
	63	123	91
	31	89	71
	0	67	–
		Mean recovery (%)	102
		Recovery SD (%)	10

[a] Per injected volume of 10 μl.

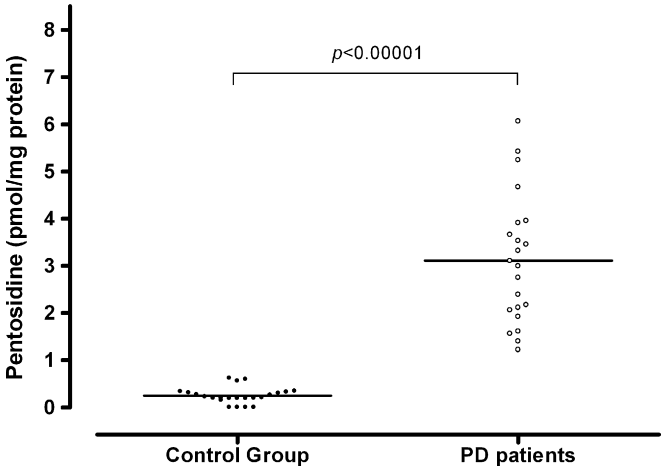

Fig. 4. Protein bound pentosidine concentration in plasma of healthy controls and in peritoneal dialysis (PD) patients. Data are presented as median (line) and separate datapoints.

4. Discussion

We describe here a rapid, simple and reliable method to determine protein bound pentosidine in plasma by a single step RP-based HPLC separation with fluorescent detection. The combination between a very simple sample preparation and a short run-time makes this method a strong and easy tool to determine protein bound pentosidine in a large number of plasma samples.

Many techniques have been used to quantify pentosidine [10–14,19,21–23,30] with some disadvantages such as long run-times [10,13,14,23], elaborate sample preparation [16] or analysis on expensive LC–MS/MS equipment [19,30].

Two main advantages of using "normal" reversed phase chromatography instead of the ion-pair based chromatography, which is commonly used in this field [9,10,12–15,23,25], is a very clean chromatogram and a very stable retention time. Indeed, we found very clean chromatographs, with baseline separation of pentosidine in plasma, which makes afterwards time-consuming manual integration unnecessary. With a retention time of only 13.24 ± 0.07 min and a run-to-run time of approximately 30 min, we can run, unattended, 48 samples a day.

Another advantage of the described sample preparation is that it is also suitable for the simultaneous analysis of protein-bound pentosidine with HPLC-fluorescence and for protein bound AGEs such as N^{ε}-(carboxymethyl)lysine (CML) and N^{ε}-(carboxyethyl)lysine (CEL) with LC–MS/MS technique [18]. Only 50 μl of plasma is needed for both methods. After hydrolysis the samples can be split; one part for the HPLC-fluorescence analysis of pentosidine and one part for the LC–MS/MS analysis.

With this method we measured protein bound pentosidine in healthy volunteers and patients on peritoneal dialysis with a limit of detection of 0.02 pmol/mg protein, which is comparable with limit

of detection as described in literature [15,20,22]. The mean plasma concentration of protein bound pentosidine in healthy controls as measured with the method described above was 0.21 pmol/mg protein. The absolute concentrations of protein bound pentosidine in healthy controls described in literature are divergent, ranging from 0.95 to 2.0 pmol/mg protein [10,31–33] and our value of 0.21 pmol pentosidine per mg protein is lower. Differences in the characteristics of the controls and a lack of an international pentosidine standard may explain the differences. Therefore, direct comparison of the absolute levels of pentosidine as measured in different studies is difficult. The 15-fold increase in plasma protein bound pentosidine in uremic patients in comparison to healthy controls is in agreement with previous results [9,32,34,35].

The detection of AGEs is currently of much experimental and clinical interest, in particular because data of few epidemiological studies have demonstrated associations of plasma levels of AGEs with vascular complications [5,6]. Although studies demonstrating a causal role of AGEs in the development of cardiovascular disease are limited, it might be that specific AGEs are risk factors of cardiovascular disease. In accordance with this, high serum levels of AGEs predict increased coronary heart disease mortality in non-diabetic women [5] and a recent study demonstrated that serum pentosidine concentration is an independent prognostic factor for heart failure [6]. However, AGEs in these studies were measured with immunoassays and AGEs measurements with immunoassays should be interpreted with care.

In conclusion, we describe a fast, simple and reliable method for the quantitation of pentosidine. This method may help to obtain a better understanding of the putative effects of pentosidine in the pathophysiology of different diseases such as vascular complications and to test whether pentosidine measurements can be used to identify patients with a high risk for poor outcome and may thus help in risk stratification.

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