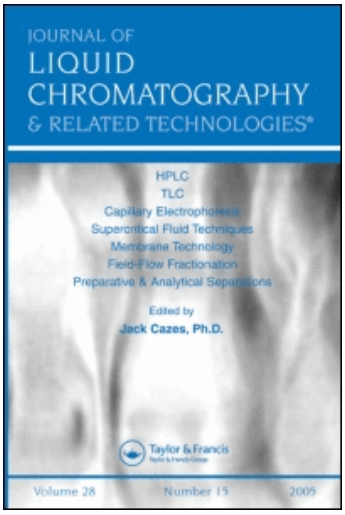

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HPLC METHOD FOR PENTOSIDINE DETERMINATION IN URINE, SERUM, AND TISSUES AS A MARKER OF GLYCATION AND OXIDATION LOADING OF THE ORGANISM

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ABSTRACT

A sensitive reverse phase HPLC method for pentosidine (PEN) determination in urine, serum, and tissues has been elaborated on utilizing a SHIMADZU liquid chromatograph, type CLASS VP, version 5.0. Silica C18 and water–heptafluorobutyric acid (HFBA)-acetonitrile (ACN) as stationary and mobile phase, was used. The fluorescence monitor setting was 335/385 nm as excitation and emission wavelengths, respectively. PEN standard was synthesized utilizing a simple polymer analogous reaction with no requirement for purification of the reaction product. Urine and serum required 250 μL and tissue (cartilage, synovium, granular tissue) required 2–4 mg (dry weight) sample sizes. Twenty five μL of urine and tissue sample volumes were injected into the HPLC column, whereas serum samples required only 10 μL. Reproducibility of the HPLC determination, alone, was slightly above 1% (RSD), and of the

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whole method (i.e., including sample hydrolysis and purification with cellulose) was 4.44%, respectively. Recovery of the whole method was $77 \pm 3.5\%$ with a sensitivity limit of 17.6 femtomols.

Urine PEN (U-PEN) concentrations have been determined in a group of patients with osteoarthritis (OA, $N = 58$, age 66.97 ± 9.89 years, mean $\pm$ S.D.) and compared with control individuals ($N = 15$, age 30.01 ± 8.67 years, U-PEN 1.65 ± 0.46 nmol/mmol creatinine). PEN-age dependence between both groups has been eliminated mathematically using a known measured PEN-age relationship. It was found, that U-PEN concentrations in OA were significantly higher as compared with (corrected) healthy controls (7.7 ± 7.8 vs. 2.1 ± 0.5 nmol/mmol creatinine, $P = 0.00002$). Additionally, a small correlation was noted between U-PEN and measured urinary pyridinoline (U-PD) in OA (U-PD = 2.3111* U-PEN + 55.475, $r = 0.50$). This may be partially related to the immune response of the organism caused by the toxic action of molecular domains containing PEN, secondarily leading to accelerated resorption kinetics and, thus, to a higher pyridinoline level.

INTRODUCTION

Especially during the last 10 years, many specialists and scientists have investigated an interesting chemical reaction that spontaneously occurs in many tissues within an organism. This reaction has been found to be independent of the functions of studied tissues and on the substrate species, including proteins, lipids, lipoproteins, phospholipids, glycoproteins, proteoglycans, etc., provided these components contain an amino-group in their molecular structure. The active agents are always present in the organisms oxo-groups (aldo-, keto-) of the reducing sugars (pentose, hexose). This type of reaction is known as glycation or, more exact, glycoxidation, and the resulting products are so-called AGE derivatives (advanced glycation end-products). Maillard[1] first described the glycation reaction while studying proteins in foodstuffs. It took another 50 years before this reaction was used for clinical follow-up of diabetes. Recently, it has been intensely studied in connection with the regards to the aging process.[2,3] During the research, relation to renal insufficiency,[4] inflammation processes during rheumatoid arthritis (RA) and osteoarthritis (OA),[5,6] as well as to eye,[7] nervous,[8] vascular,[9] and other diseases[10] has been found.

For the most part, the structural characteristics of these derivatives have not been characterized. Therefore, a non-selective method based on measurement of their natural integral fluorescence emitted at 440 nm (excited at 370 nm) for glucose-modified proteins was used.[11] For certain characteristics of the AGE

group, antibodies suitable for their sensitive immunochemical determination have been established.[12–14] Clinically, it is very important to investigate urine, serum, and tissue AGE levels, as their presence is pathologic, i.e., can be toxic in the organism. Accumulation of these toxins can be seen with the natural aging process, and their levels rapidly increase, especially, above the age of 70 in humans. In younger individuals, their increased level can indicate various pathological conditions, e.g., diabetes, renal, or inflammatory diseases.

As glycoxidation is a cascade of purely chemical reactions, the speed of the AGE formation depends on various conditions, such as pH, temperature, reaction time, and the concentration of the substances taking part in the reaction (i.e., sugars and proteins). As temperature, pH, and protein concentration are strictly controlled in vivo, the results of the reaction are influenced mainly by the reaction time and the concentration of sugars, namely glucose. In normo-glycemic subjects, relatively low concentrations of glucose result in a very slow accumulation of AGE derivatives, which may later serve for objective evaluation of the aging process. It can be said, that non-enzymatic glycoxidation reactions are not a direct cause of aging. However, a long-term accumulation of these modified proteins can evoke a number of troubles attributed to aging. In hyperglycemic patients, the reaction is accordingly faster and the determination of AGE derivatives is of great clinical importance. Levels of these substances are also increased in tissues and body fluids in patients with advanced renal insufficiency (a reduced clearance of the substrates used for their formation), namely at its final stage.[15] It was also reported, that immune-activation of macrophages, caused by the glycation modified proteins, produce reactive elements, a so-called "second AGE generation" that may lead to an accelerated secondary protein glycoxidation.[2]

Pentosidine (PEN), is one of the best structurally defined AGEs,[16] and is available in pure form. Therefore, it is often used as an indicator of the overall affection of the organism by the products of glycoxidation reactions. In vitro PEN is formed, e.g., by binding of amino acidic residues lysine and arginine with ribose, which is most reactive, but also by xylose, arabinose, lyxose,[16] as well as by glucose (after the transient highly reactive dicarbonyl is cleaved by oxidative degradation). Oxygen radicals in vivo play a positive role in the later reaction stages of AGE formation. It has been noted, that patients with the inflammatory forms of rheumatoid arthritis have significantly lowered oxygen-quenching capacity, causing higher PEN levels.[17] Significantly higher urine and serum pentosidine levels (about three times and two times, respectively)[18] were found using the HPLC method in patients with RA. For PEN determination, the HPLC method is commonly used and its concentration is monitored with fluorescence detector at $\lambda_{EX}/\lambda_{EM} = 335/385$ nm.[19]

The aim of this work is to elaborate a sensitive HPLC method for PEN determination in the urine, serum, and tissues, to characterize its substantial limits and to apply it in groups of healthy subjects and in patients with osteoarthritis (OA).

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EXPERIMENTAL

Materials and Chemicals

Heptafluorobutyric acid p.a. (HFBA), acetonitrile HPLC grade (ACN), poly-L-lysine hydrobromide M.W. 30–70 kDa, PBS buffer pH 7.4, *D*-ribose, *N*-α-acetyl-arginine, DETAPAC (diethyl triamine pentaacetic acid, chelatone), dialyzing tube, and spherical cellulose CC31 have been purchased from Sigma-Aldrich, Prague, Czech Republic. Normal ammonium sulfate, acetic acid 99.8%, methyl alcohol, *n*-butyl alcohol, and hydrochloric acid, all substances in the p.a. quality, have been received from LACHEMA a.s., Czech Republic. Demineralized water is produced at the Rheumatological institute, Prague, Czech Republic.

PEN-standard, being commercially unavailable, has been prepared in our laboratory using a slightly modified procedure kindly provided by Prof. V. Monnier, and its quantitative determination has been performed by Dr. D. Sell (both from Case Western Reserve University, Cleveland, OH 44106, by private e-mail communications). The synthesis results in poly pentosidine formation that may be separated from excessive non-reacting and ballast low-molecule components by dialysis, followed by hydrolysis, making time consuming purification unnecessary. Briefly, 500 mg of poly-L-lysine · HBr (2.39 mmol), 75 mg of *D*-ribose (0.50 mmol), 324 mg of *N*-α-acetyl-arginine (1.5 mmol), and 3.93 mg of DETEPAC (0.01 mmol, chelatone) reacted (air-bubbled) for seven days at 37°C in 10 mL of 0.2 M phosphate buffer having pH 9.0. The reaction mixture was first dialyzed (cellulose, retention >12 kDa) for two days against PBS buffer pH 7.4, then for seven days against demineralized water. The solution was dried, hydrolyzed in 67 mL of 6 M HCl for 16 hours at 105°C; the hydrolysate was evaporated and reconstituted in 10 mL of 0.1% trifluoroacetic acid. HPLC quantitative analysis determined 1130 nmol/mL very pure pentosidine, without any other peaks.

Apparatus

The HPLC SHIMADZU chromatograph, type CLASS VP, version 5.0, composed of an autosampler SIL-10ACvp, HPLC quarternary pump LC-10Advp, column thermostat CTO-10ACvp, fluorescent monitor RF-10Axl, and system controller SCL-10Avp has been used. The entire system, as well as the course of the chromatography, was controlled on-line by a computer using a special software CLASS-VP/Windows 98. The compact glass column, CGC Separon SGX C18, 150 × 3 mm was filled in by spherical silica with particles of $d_p = 7$ μm with the surface modified by octadecyl silane (manufacturer: Tessek s.e.o., Prague, Czech Republic).

Sample Preparation

SPE Column Preparation

For preliminary purification of samples containing pentosidine, selectively controlled sorption and desorption on an ion exchanger (Sephadex SP) governed by different HCl concentrations is often used, while for purifying samples for pyridinolines detection (collagen crosslinks), a selective sorption and desorption on cellulose using butanol and water is usually used.[20] As intended for the future simultaneous preparation of samples for determination of both crosslinking elements, we used a modified method[21] enabling such a preparation. Briefly, the following reserve media were prepared: medium A: *n*-butyl alcohol–acetic acid–water (8/1/1, v/v/v), medium B: *n*-butyl alcohol–acetic acid (8/1, v/v), 10% CC31 cellulose suspension in medium A (w/v), and 0.05 M HCl. Five mL of cellulose suspension were placed in a 6 mL polypropylene (PP) filtration column (LUER) equipped with a polyethylene (PE) frit at the bottom. After vacuum sedimentation (vacuum extractor Supelco, Sigma-Aldrich, Prague, Czech Republic), the top of the bed (about 1 mL) was stopped by a PE frit.

Urine and Serum Preparation

The HPLC method of PEN determination was tested on model urine and serum samples taken from one patient on the same day (male, 62 years of age, with no clinical signs of diabetes, renal insufficiency, or arthritis). One mL of the sample (urine, serum) was mixed with 1 mL of concentrated HCl (35%) and hydrolyzed (after previously washing it with N_2) for 16 hours at 105°C in a hydrolysis tube stopped with a teflon seal. An aliquot of 0.5 mL hydrolysate, (i.e., 250 μL of the sample) was mixed with 0.5 mL of cellulose suspension and 4.5 mL of medium B and loaded on a SPE column preliminarily conditioned with 5 mL of medium A. The fluid was vacuum drained (Supelco), returned onto the column, and drained again. Sorbed PEN was slowly washed (vacuum) with 30 mL of medium A. One mL of methanol was then loaded on the SPE column, slowly drained, and, possibly without interruption and air penetration into the cellulose bed, pentosidine was desorbed from cellulose column by 3 mL of 0.05 M HCl. This fraction, as well as the methanol fraction, was collected together in a PE tube and evaporated to dryness in a rotary vacuum evaporator (SpeedVac). The evaporation residue was reconstituted in 250 μL of mobile phase. The injected volume for the HPLC column was 10 μL and 25μL for serum and urine, respectively. This preparation caused no PEN concentration changes due to the volume changes during treatment.

Tissue (Cartilage, Synovial Tissue, Granular Tissue) Preparation

Five to 10 mg of dry, finely crushed tissue (scalpel, mortar) was dispersed in 2 mL of 6M HCl (35% HCl/H_2O, 1/1 v/v). Hydrolysis and all the following steps, were identical with those utilized when preparing urine and serum samples, except that the evaporation residue was reconstituted in 500 μL of mobile phase. For injection onto the HPLC column, 25 μL of the reconstituted solvent was used, corresponding to 62.5–125 μg dry weight of the tissue sample. The determined PEN concentration corresponds to PEN concentration in the hydrolysate.

HPLC

The HPLC separation in the revere phase is methodically linked to our previous works.[22,23] dealing with pyridinolines and desmosines determination. In this case, 0.02 M heptafluorobutyric acid as mobile phase, was used as an ion-pairing agent with 0.01 M of diammonium sulfate ($(NH_4)_2\ SO_4$), mixed with various concentrations of acetonitrile as an organic modifier. To obtain optimal separation, both isocratic (12.5%, 13.75%, 15%, and 17.5% ACN) and linear gradient runs (10 → 30%, 12.5 → 30%, 15 → 30%, 10 → 25%, 12.5 → 20%, 12.5 → 25% ACN in 20 min, 20 → 21 minutes linear return to original % ACN, 21 → 30 minutes isocratic original % ACN) was tested (see e.g., Fig. 1).

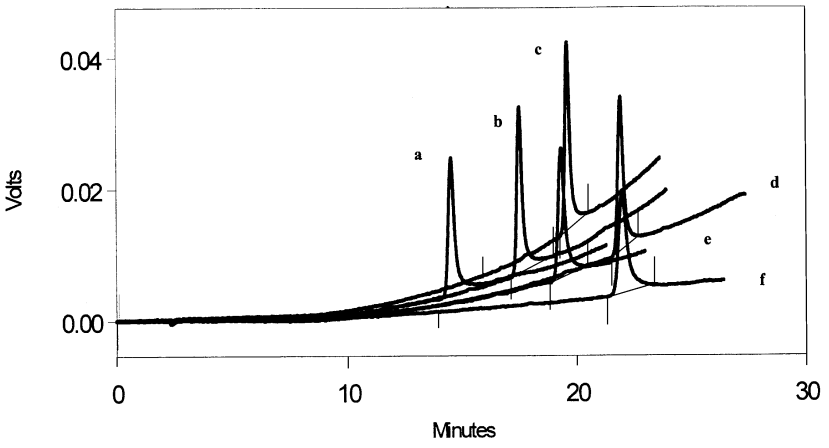

Figure 1. Gradient separation, choice of the optimal conditions: a) 15–30% ACN, b) 12.5–30% ACN, c) 10–30% ACN, d) 10–25% ACN, e) 12.5–25% ACN, f) 12.5–20% ACN. Linear grad. in 20 min, then linear in 1 min to initial % ACN, injected 10 μL 37 nM PEN, oven temp. 40°C, flow 0.5 mL/min, mean area 450520 $\mu V \cdot s \pm 1.43\%$ (RSD).

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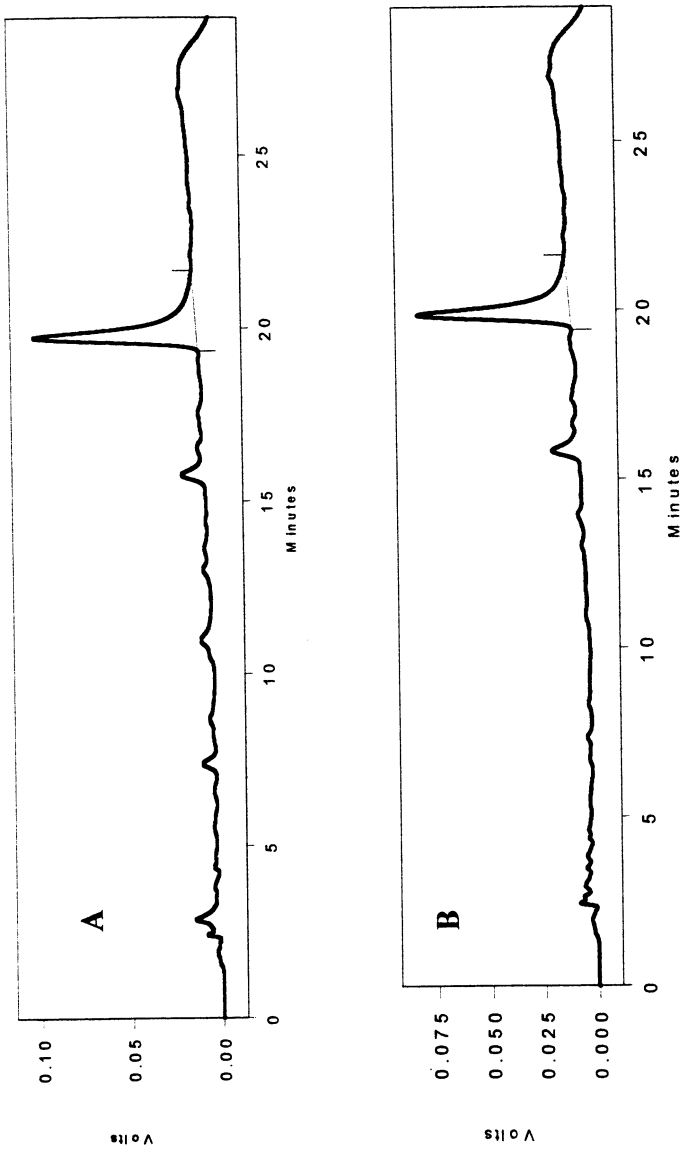
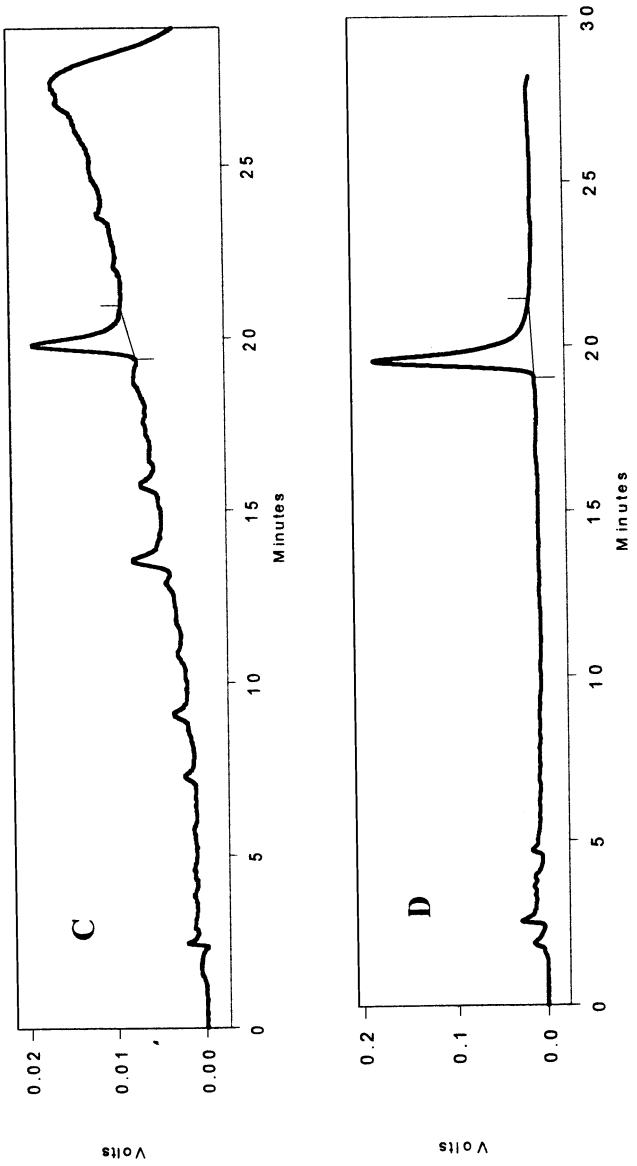


Figure 2. Illustrations of PEN separation in: (A) cartilage (110 pmol/mL), (B) synovium (86 pmol/mL), (C) granular tissue (12.7 pmol/mL), (D) urine (170 pmol/mL), (E) serum (117 pmol/mL). For details, see text.

(*continued*)



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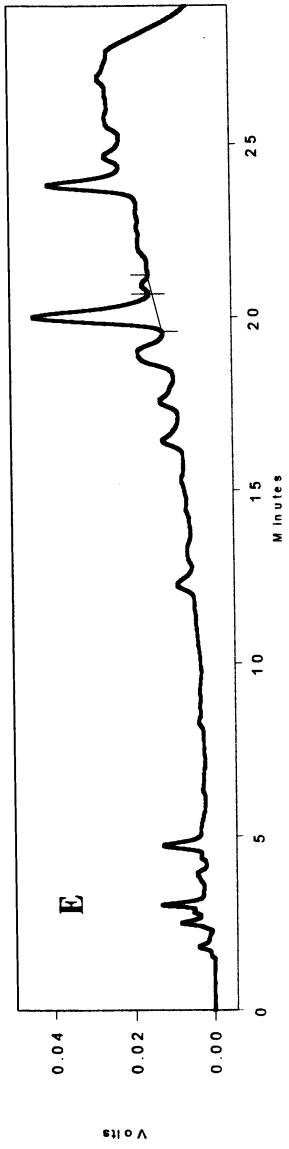

Figure 2. Continued.

Necessary ACN concentrations were achieved by programmed mixing of primary solutions containing 5% and 30% ACN, respectively. As an optimal linear gradient 12.5% to 25% ACN in 20 min was selected. The column was thermostated at 40°C, mobile phase flow rate was 0.5 mL/min, and pressure drop along the column was 6 MPa. The PEN concentration was measured by monitoring the light emitted at $\lambda_{EM} = 385$ nm and excited at $\lambda_{EX} = 335$ nm. One analysis takes 30 min, including the time necessary to achieve concentration equilibrium along the column (see Fig. 2).

RESULTS AND DISCUSSION

For method calibration, i.e., for the dependence of integrated peak area and relevant sample concentration, samples of 1190, 595, 395, 298, 197, 149, 99, 74, 49, and 37 nmol PEN/L were prepared. The sequence with a linear gradient from 15 to 30% ACN in 20 min and a 10 μL injection volume were used. The column temperature was 40°C, pentosidine retention time (RT) was approximately 14.5 min. The slope of the straight calibration curve (correlation coefficient 0.999) was 13244 μV · s/nM (microvoltsecond/nM). Hence: conc. PEN (nM/L) = (Area (μV · s)/13244)/0.77, where coefficient 0.77 takes method recovery (see below) into account.

A detection limit was calculated as the minimal detectable PEN peak measured for the expected signal/noise ratio = 3/1. For the PEN sample concentration of 35 nM/L, 10 μL injection, isocratic mobile phase of 17.5% ACN, RT of approximately 10.9 min, the peak height of 0.493043 V (volts) was found. For the detected noise level, 0.008270 V, and, thus for a threefold higher signal, i.e., 0.02481 V, this corresponds to a concentration of 1.76 nmol PEN/L, which represents a minimal measurable quantity of 17.6 femtomols.

Method reproducibility and recovery were determined by comparing PEN concentration in pure standard (136 nM/L) with concentrations of the same standard solutions ($N = 8$) treated in the same manner as was used for the preparation and purifying urine and serum samples. Detected differences, i.e., decrease of the original PEN concentration to 77.25%, can be considered as a direct indicator of PEN losses during hydrolysis and preliminary purification with cellulose. The whole method reproducibility was found to be 4.44% (RSD), and the reproducibility RSD of the HPCL analysis alone (independent HPLC determinations of the same sample, $N = 10$), was 1.2%.

The influence of washing during preliminary sample purification, was described in the published version of the method.[21] PEN, selectively sorbed on cellulose sorbent, was desorbed with the relatively large amount (20 mL) of 0.05 M HCl. Acid has to be subsequently evaporated, which may evoke unfavorable corrosive effect on the rotation vacuum evaporator. Therefore, testing

was done to see if it would be possible to considerably reduce HCl volume, or to replace it with water, analogous to sample preparation for pyridinoline determination.[22] Due to the fact that butanol used for washing out ballast impurities is only partially miscible with water, a relatively large required volume of diluted acid may be a result of its limited miscibility with butanol. Therefore, as an interphase, methanol, completely miscible both with butanol and water, was used. Therefore, the fraction containing PEN, was rinsed first with 1 mL of methanol, which was subsequently followed by 3, 8, 12, and 16 mL of 0.05 M HCl. HPLC analysis showed that the necessary washing volume for PEN desorption from cellulose may be reduced to 4 mL, including 1 mL methanol. On the other hand, when 0.05 M HCl was replaced by pure water, the determined PEN concentration was lowered to 63.1% of the value reached when diluted acid was used. Therefore, such a replacement was considered to be unsuitable (see Fig. 3).

Application in OA

The method has been applied for pentosidine determination in urine (U-PEN) in a group of patients with osteoarthritis, ($N = 76$, age 66.97 ± 9.89 years, mean $\pm$ S.D.). The results (related to urine creatinine) have been compared with a group of healthy individuals ($N = 15$, age 30.01 ± 8.67). The dependence

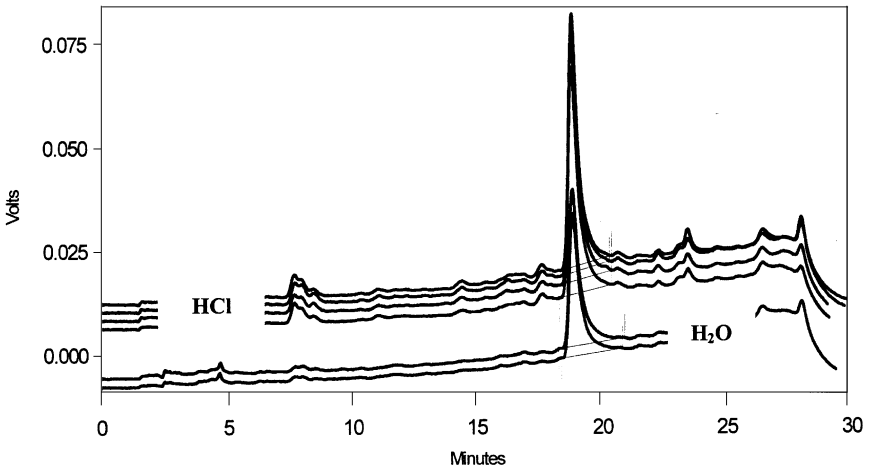

Figure 3. Influence of washing (water resp. diluted HCl) during PEN desorption from cellulose. Traces are mutually shifted along *Y*-axis.

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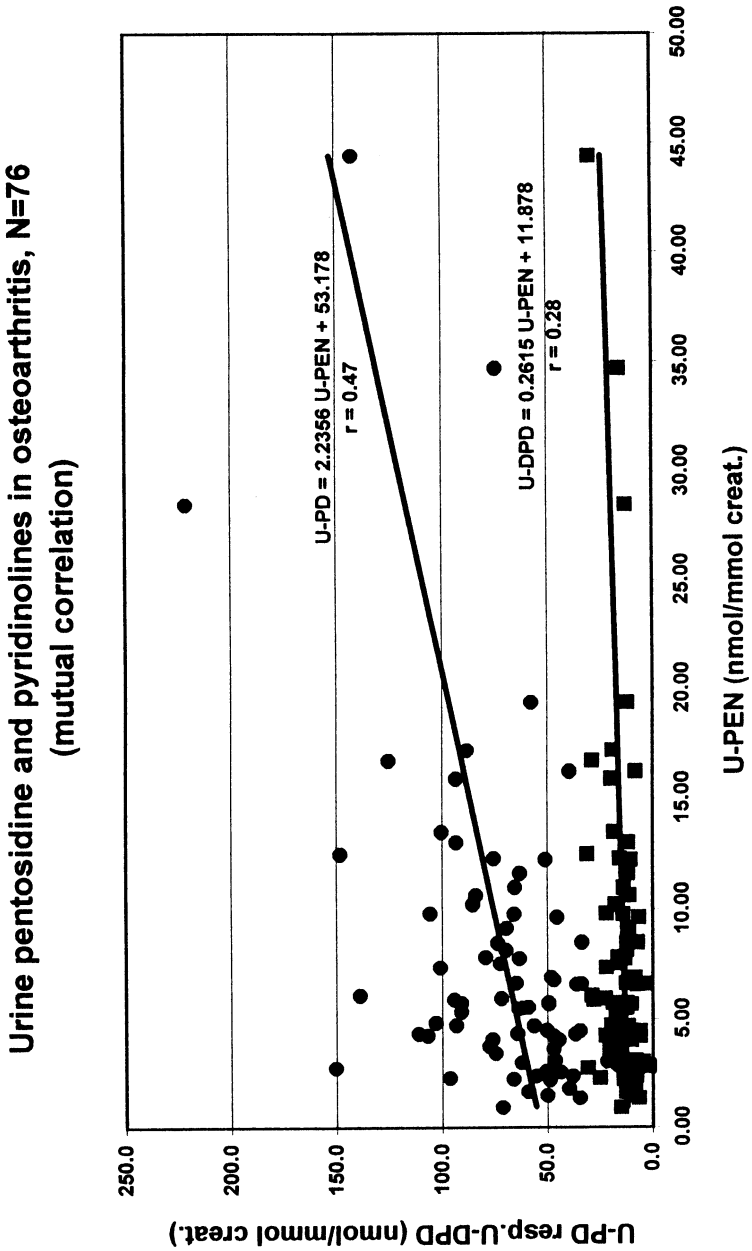

Figure 4. Dependence of the U-PD and U-DPD on U-PEN in osteoarthritis.

of U-PEN on the age of healthy individuals was eliminated by using a mathematical extrapolation according to published results.[18] It was found that U-PEN concentration in patients with OA is significantly higher than the extrapolated values[18] in healthy individuals (7.7 ± 7.8, and 2.1 ± 0.5 nM/mmol creatinine, respectively, $P = 0.00002$). An analogous extrapolation of the same data of healthy individuals made according to data found by other authors,[21] yields somewhat higher (4.17 ± 1.31 nM/mmol creatinine), but still statistically significant ($P = 0.00171$), results.

It was also found (see Fig. 4) that in patients with OA, there is a slight correlation between U-PEN and independently determined[22] urine pyridinoline (U-PD): U-PD $= 2.2356 *$ U-PEN $+ 53.178$, $r = 0.47$, and deoxypyridinoline (U-DPD): (U-DPD $= 0.2615 *$ U-PEN $+ 11.878$, $r = 0.28$). This correlation may be as a consequence of an immune response of the organism caused by toxic action of the PEN containing domains in the tissues.[2,24] This may lead to a secondary increase in the collagen tissue resorption kinetics, resulting in increased levels of both urine pyridinolines.

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REFERENCES

1. Maillard, L.C. C. R. Sc. Acad. Sci. **1912**, *154*, 66–68.
2. Vlassara, H. Diabetes **1997**, *46*, S19–S25.
3. Odetti, P.; Arogno, I.; Garibaldi, S.; Valentini, S.; Pronzato, M.A.; Rolandi, R. Gerontology **1998**, *44*, 187–191.
4. Miyata, T.; Ueda, Y.; Horie, K. Kidney Int. **1998**, *53* (2), 416–422.
5. Miyata, T.; Ishiguro, N.; Yasuda, Y.; Ito, T.; Nangaku, M.; Kurokawa, K. Biochem. Biophys. Res. Commun. **1998**, *244*, 45–49.
6. Newkirk, M.M.; LePage, K.; Niwa, T.; Rubin, L. Cell. Mol. Biol. (Noisy-Le-Grand) **1998**, *44*, 1129–1138.
7. Zhao, H.R.; Nagaraj, R.H.; Abraham, E.C. J. Biol. Chem. **1997**, *272*, 14465–14469.
8. Pappolla, M.A.; Alzofon, J.; McMahon, J.; Theodoropoulos, T.J. Rur. Arch. Psychiatry Neurol. Sci. **1990**, *239*, 314–319.

9. Meng, J.; Sakata, N.; Takebayashi, S.; Asano, T.; Futata, T.; Nagai, R.; Ikeda, K.; Horiuchi, T.; Myint, T.; Taniguchi, N. Atherosclerosis **136,** *136*, 355–365.
10. Seftel, A.D.; Vaziri, N.D.; Ni, Z.; Razmjouei, K.; Fogarty, J.; Hampel, N.; Polak, J.; Wang, R.Z.; Ferguson, K.; Block, C.; Haas, C. Urology **1997**, *50*, 1016–1026.
11. Katayama, Y.; Celic, S.; Nagata, N.; Martin, T.J.; Findlay, D.M. Bone **1997**, *21*, 237–242.
12. Nakamura, H.; Taneda, S.; Kuwajima, S.; Aoki, S.; Kuroda, Y.; Misawa, K.; Nakagawa, S. Biochem. Biophys. Res. Commun. **1989**, *162*, 740–745.
13. Horiuchi, S.; Araki, N.; Morino, Y. J. Biol. Chem. **1991**, *266*, 7329–7332.
14. Makita, Z.; Vlassara, H.; Cerami, A.; Bucala, R. J. Biol. Chem. **1992**, *267*, 5133–5138.
15. Sell, D.R.; Monnier, V.M. J. Clin. Invest. **1990**, *85*, 380–384.
16. Sell, D.R.; Monnier, V.M. J. Biol. Chem. **1989**, *264*, 21597–21602.
17. Chen, J.R.; Takahashi, M.; Suzuki, M.; Kushida, K.; Miyamoto, S.; Inoue, T. J. Rheumatol. **1998**, *25*, 2440–2444.
18. Takahashi, M.; Suzuki, M.; Kushida, K.; Miyamoto, S.; Inoue, T. Brit. J. Rheumatol. **1997**, *36*, 637–642.
19. Saito, M.; Marumo, K.; Fujii, K.; Ishioka, N. Anal. Biochem. **1997**, *253*, 26–32.
20. Takahashi, M.; Ohishi, T.; Aoshima, H.; Kushida, K.; Inoue, T.; Horiuchi, K. J. Liq. Chrom. **1993**, *16*, 1355–1370.
21. Yoshihara, K.; Nakamura, K.; Kanai, M.; Nagayama, Y.; Takahashi, S.; Saito, N.; Nagata, M. Biol. Pharm. Bull. **1998**, *21*, 1005–1008.
22. Špacek, P.; Hulejová, H.; Adam, M. J. Liq. Chrom. & Rel. Technol. **1997**, *1*, 1921–1930.
23. Špacek, P.; Hulejová, H.; Adam, M. J. Liq. Chrom. & Rel. Technol. **1999**, *22*, 823–842.
24. Sullivan, R. Arch. Physiol. Biochem. **1996**, *104*, 797–806.

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