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Sensitive fluorimetric quantitation of pyridinium and pentosidine crosslinks in biological samples in a single high-performance liquid chromatographic run

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Abstract

A high-performance liquid chromatographic assay was developed for pyridinium crosslinks and pentosidine in mature collagen of a wide variety of connective tissue hydrolysates by a simple two-step isocratic assay using a reversed-phase column. The crosslinks (including the internal standard pyridoxine) were optimally detected by their native fluorescence by switching wavelengths of the detector during the assay. The method resulted in highly sensitive and accurate measurements, without need for precleaning of the samples: crosslink levels in 200 μm thin slices of the various zones of articular cartilage were easily quantified. The detection limit was as low as 0.4 pmol for the pyridinolines and 0.05 pmol for pentosidine. The intra-assay and inter-assay coefficients of variation were as low as 2% (pyridinolines) and 5% (pentosidine); calibration curves for all compounds were linear over a concentration range larger than two orders of magnitude. With our chromatographic system, the diglycosylated form of hydroxylysylpyridinoline in unhydrolyzed urine was separated as well.

Keywords: Pyridinium; Pentosidine; Collagen

1. Introduction

Collagen crosslinks with fluorescent properties are derived from two different pathways: those initiated by the enzyme lysyl oxidase and those derived from the non-enzymatic glycation of lysine and hydroxylysine residues. The enzymatic pathway starts with the oxidative deamination of ε-amino groups of telopeptidyl hydroxylysyl (Hyl) residues by lysyl oxidase to aldehydes, eventually leading to the fluorescent crosslinks hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP; Fig. 1). HP is the maturation product of two Hyl residues from the telopeptides with a Hyl from the α-helix, whereas the analogue LP contains a lysine residue from the α-helix [1–4]. Furthermore, a glycosylated analogue of HP has been reported: glucosylgalactosyl–hydroxylysylpyridinoline (GGHP) [5]. This crosslink is the maturation product of two Hyl residues from the telopeptides with a glycosylated Hyl from the α-helix (Fig. 1). The nonenzymatic glycation pathway involves the condensation of a sugar aldehyde or ketone with the ε-amino group of lysine or hydroxylysine. The resulting Schiff base undergoes a series

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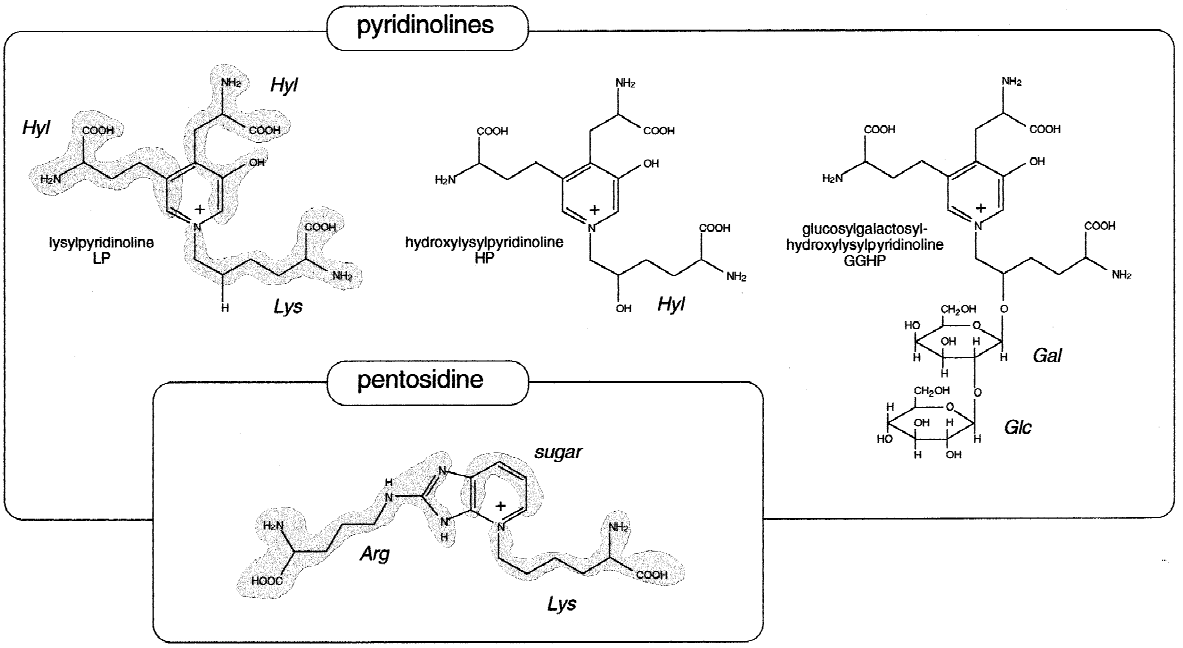

Fig. 1. Structure of hydroxylysylpyridinoline (HP), lysylpyridinoline (LP), glucosylgalactosyl-hydroxylysylpyridinoline (GGHP) and pentosidine. HP is derived from three hydroxylysine (Hyl) residues (two from the telopeptides and one from the triple helix); the disaccharide of GGHP is linked to the hydroxy-group of triple helical hydroxylysine. LP is derived from two telopeptidyl hydroxylysine residues and a lysine (Lys) residue from the triple helix. Pentosidine is a non-enzymatic glycation reaction product involving a lysine, arginine (Arg) and a reducing sugar such as glucose.

of reactions, producing fluorophores as well as browning pigments [6–8]. These reaction products are collectively known as advanced Maillard products. The only fluorescent NEG product of collagen characterized so far is pentosidine, a difunctional crosslink comprising arginine, lysine and pentose (Fig. 1) [8,9].

Crosslinks play an important role in the maintenance of the tensile strength and mechanic stability of the collagen network [10,11]. Tissue-specific variations of absolute amounts, as well as in the ratios of the pyridinolines HP and LP are reported [1–4]. Furthermore, changes in crosslink patterns are related to pathologic conditions, such as lathyrism, Ehlers–Danlos syndrome type VI, occipital horn syndrome and Menkes disease [1,3,12]. Determination of pyridinoline crosslinks in urine serve as sensitive markers of the degradation of collagen fibrils from musculoskeletal tissues [5,13–17]. Pentosidine has been used as a marker of non-enzymatic glycation. Increased levels have been reported in tissues in relation to aging, diabetes and/or uremia [8].

For investigations of micro-anatomical variations in tissue distribution of mature crosslinks [18–21], a sensitive assay is needed that requires minimal amounts of biopsy material. Earlier developed methods for the determination of HP, LP and pentosidine in tissues required precleaning on SP-Sephadex C25, two sample preparations, and two injections onto the HPLC system [22]. Recently, a one-injection method without sample precleaning was reported [23]. However, an internal standard was not included and the wavelength used (307/390 nm) is suboptimal for the crosslinks under consideration, due to the different fluorescence spectra of pyridinolines (295/400) and pentosidine (328/378).

Here we describe the simultaneous quantification of HP, LP and pentosidine in a single reversed-phase HPLC run in crude hydrolysates of a wide variety of connective tissues including an internal standard. The excellent separation between the pyridinolines

and pentosidine allows a wavelength switch with a programmable fluorimeter, resulting in optimal sensitivities. The same chromatographic procedure was used for the measurement of pyridinoline crosslinks in native as well as hydrolyzed urine pre-extracted on fibrous cellulose.

2. Experimental

2.1. Reagents and preparation of crosslink standard

Heptafluorobutyric acid (HFBA) and pyridoxine were purchased from Fluka (Buchs, Switzerland) and Sigma (St. Louis, MO, USA), respectively. HPLC-grade methanol and acetonitrile was obtained from Rathburn (Walkerburn, UK). CF-1 cellulose was supplied by Whatman (Maidstone, UK), Bio-Gel P-2 was obtained from Bio-Rad (Richmond, CA, USA). Pentosidine was a kind gift of Prof. V.M. Monnier (Cleveland, OH, USA).

HP and LP crosslinks were partially purified from demineralized bovine bone either with cellulose CF1 column chromatography [13] or Biogel P-2 gel permeation chromatography [2]. The purified fractions were dissolved in water and stored at −70°C; the concentration of HP and LP was measured with reversed-phase high-performance liquid chromatography (HPLC) using the Metra Biosystems (Palo Alto, CA, USA) HP/LP standard as the reference. With the calibrated samples a standard was prepared in 0.5–1.0% (v/v) HFBA in 10% (v/v) acetonitrile containing 510 pmol pyridoxine per ml (internal standard) and 30 pmol pentosidine per ml; the selected concentrations of HP and LP were typically around 500 pmol and 100 pmol per ml, respectively. No deterioration in fluorescence signals was observed after storage for six months at −20°C.

2.2. Instruments

The HPLC system consisted of a Gynkotek (Munich, Germany) High Precision Pump Model 3000, a Solvent Selection Valve (Waters, Milford, MA, USA), a Promis (Spark Holland, Emmen, Netherlands) autosampler, a Jasco (Tokyo, Japan) Model 821-FP fluorimeter and a Lab-Quatec Model Gastorr GT-103 degasser. Data Control (Harley Systems, Buckinghamshire, UK) software was used for data acquisition and processing.

2.3. Tissue treatment

Tissue samples (10–20 mg) were hydrolyzed in an oven with 800 μl 6 *M* HCl at 110°C for 20 h in 5 ml glass vials fitted with PTFE-lined screw caps. After drying (Speed Vac SC 110, Savant, Farmingdale, USA) samples were dissolved in water containing 10 nmol pyridoxine/ml and 2.4 μmol homoarginine/ml; for every mg (dry weight) soft tissue (e.g., cartilage) 95 μl water was used. For mineralized tissue, 50 μl water was used. For crosslink analysis, samples were diluted five-fold with 0.5% (v/v) HFBA in 10% (v/v) acetonitrile; 100 μl of the diluted sample was injected in the HPLC system. In this way, 210 μg soft tissue (or 400 μg mineralized tissue) is applied onto the column, together with 200 pmol pyridoxine (internal standard).

For hydroxyproline analysis, an aliquot of the above diluted sample was diluted another 50 times with 0.1 *M* sodium borate buffer pH 8.0; 200 μl was derivatized with 9-fluorenylmethyl chloroformate and analyzed according to Bank et al. [24]. Homoarginine was used as internal standard for amino acid analysis. Crosslinks are expressed as moles per mole collagen, assuming 300 hydroxyproline residues per collagen molecule.

2.4. Urine treatment

Native urine was treated with CF-1 cellulose as described by Black et al. [13]. In brief, neat samples are applied in acidic butanol to 6 ml packed cellulose columns. Interfering fluorophores are removed by washing with an eluent consisting of butanol–glacial acetic acid–water (4:1:1). The crosslinks are then eluted with water, lyophilized and resuspended in 0.5% (v/v) HFBA in 10% (v/v) acetonitrile.

2.5. Chromatography

The column (Micropak ODS-80TM, 150×4.6 mm I.D., packed with 5 μm, spherical silica particles with 80 Å pores; Varian, Sunnyvale, CA, USA) was equilibrated with 0.15% (v/v) HFBA in 24% (v/v)

methanol (solvent A). Elution of the crosslinks and the internal standard pyridoxine was achieved at ambient temperature at a flow-rate of 1.0 ml/min in two isocratic steps: time 0–17 min solvent A; time 17–30 min 0.05% (v/v) HFBA in 40% (v/v) methanol (solvent B). The column was washed with 0.1% (v/v) HFBA in 75% (v/v) acetonitrile (solvent C) for 10 min and equilibrated for 10 min with solvent A, resulting in a total analysis time of 50 min per sample. Fluorescence was monitored with a programmable fluorimeter: 0–22 min, 295/400 nm; 22–45 min, 328/378 nm (gain 100; band width 18 nm).

3. Results and discussion

3.1. Elution conditions

Since as pyridinolines and pentosidine are positively charged compounds at neutral or acidic conditions they are poorly retained on octadecylsilane (ODS) reversed-phase columns. To overcome this problem, the hydrophobic ion-pairing reagent heptafluorobutyric acid has been used to retain the compounds on the ODS-column. The less hydrophobic trifluoroacetic acid turned out to be unsuitable, since no retention could be achieved at concentrations below 0.2% (v/v). HFBA-complexed crosslinks are strongly retained on the column and require the addition of an organic modifier (in this case methanol) to the mobile phase to achieve elution.

For simplicity reasons, the pyridinolines and pentosidine were eluted with two isocratic steps instead of gradient elution. The isocratic elution results in a stable baseline, allowing accurate quantitation of crosslinks at low picomole levels. In the first isocratic step pyridoxine (internal standard) is the earliest eluting compound, followed by HP and LP (Fig. 2A). After 17 min the eluent is switched; around 10 min later pentosidine elutes (Fig. 2). It is not possible to use only solvent A for the elution of the three mentioned crosslinks; under these conditions, HFBA-complexed pentosidine is strongly retained (it was still bound to the column 1 h after LP was eluted). In order to achieve elution of pentosidine, the HFBA concentration must be lowered in association with an increased methanol concentration (solvent B).

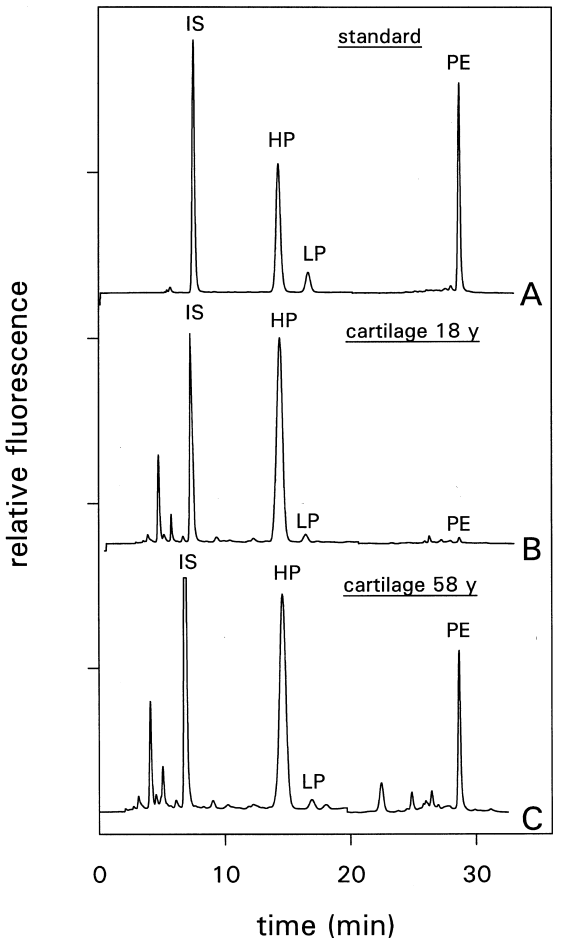

Fig. 2. (A) Elution profile of a crosslink standard containing 51 pmol pyridoxine (I.S.=internal standard), 58 pmol HP, 10 pmol LP and 3 pmol pentosidine (PE). (B) and (C) crosslink profiles in hydrolysates of human articular cartilage (femoral condyle) aged 18 and 58 years, respectively. Note the increase in pentosidine level with age.

The retention, as well as the separation of HP and LP, can easily be manipulated by adjusting the concentration of HFBA of solvent A. A small increase in the amount of HFBA results in increased retention times and a better separation between HP and LP (Fig. 3). However, the longer HP and LP are retained on the column, the broader the peaks become, resulting in lower detection limits. The HFBA concentration had a less pronounced effect on the retention of the internal standard pyridoxine (Fig. 3). The elution time of pentosidine was not effected

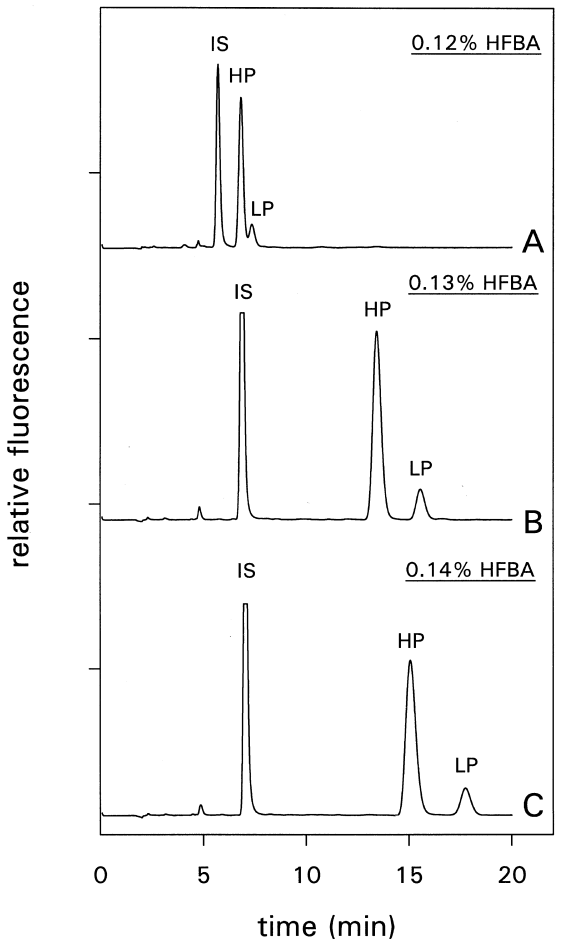

Fig. 3. Effect of HFBA concentration in 24% (v/v) methanol on the elution time and peak shape of pyridoxine (I.S.=internal standard), HP and LP. (A) 0.12% (v/v) HFBA; (B) 0.13% (v/v) HFBA; (C) 0.14% (v/v) HFBA. Increasing the amount of HFBA results in increased retention times and broader peaks for HP and LP whereas the elution profile of pyridoxine is relatively unaffected.

by the HFBA concentration of eluent A. However, the concentration of HFBA of eluent B affected pentosidine elution in a similar way as observed for HP and LP with eluent A (data not shown).

3.2. Detection limits; reproducibility

A standard (5.10 n*M* pyridoxine, 5.76 n*M* HP, 1.04 n*M* LP and 0.33 n*M* pentosidine in 0.5% HFBA in 10% acetonitrile) was serially diluted with 0.5% HFBA in 10% acetonitrile. Peak areas of pyridoxine (0–250 pmol), HP (0–576 pmol), LP (0–104 pmol) and pentosidine (0–33 pmol) per injection (100 μl) were proportional to the amount injected, with correlation coefficients of >0.9997 for all compounds. The detection limits (signal-to-noise ratio= 4) were as low as 0.2, 0.4, 0.4 and 0.05 pmol for pyridoxine, HP, LP and pentosidine, respectively.

The intra-assay coefficients of variation (C.V.s) were 1.4% for pyridoxine, 0.5% for HP, 1.7% for LP and 2.1% for pentosidine (nine repetitive analyses). The inter-assay C.V.s were 2.0%, 0.9% and 3.8% and 4.9% for pyridoxine, HP, LP and pentosidine, respectively ($n=7$).

3.3. Analysis of crosslinks in tissue hydrolysates

Figs. 2 and 4 show representative chromatograms of crude hydrolysates covering a wide range of tissues. Note the increase in pentosidine levels with advancing age (Fig. 2B, C), and the large amount of LP compared to HP in bone (Fig. 4A). All hydrolyzed samples were diluted in 0.5% (v/v) HFBA in 10% (v/v) acetonitrile before injection into the HPLC system to eliminate adsorption of pentosidine on glass or plastic surfaces and to prevent loss of crosslinks due to premature elution. This reconstitution procedure also has a positive effect on the separation of HP and LP for it reduced peak broadening. No interfering peaks were present in the region were HP, LP and pentosidine elute. Although the chromatograms were noisy at the very beginning, quantification of the internal standard pyridoxine was without problems. It should be noted that pyridoxine should be added only after the removal of HCl from the samples (i.e., after drying of the hydrolysates), since it partially decomposes in 6 *M* HCl (data not shown). Routinely, 210 μg connective tissue (dry weight; approximately 100 μg collagen=333 pmol) was loaded on the column; this amount can easily be reduced to 20 μg tissue without loss of accuracy. Routinely, with an autosampler large series of samples were conveniently analyzed (26 samples/24 h).

The applicability of the method to the dissection of microanatomical variations in tissue distribution of crosslinks is shown in Fig. 5. A cored full-depth plug from a human femoral condyle (age 43 years) with a diameter of 6 mm was cut in 200 μm thick

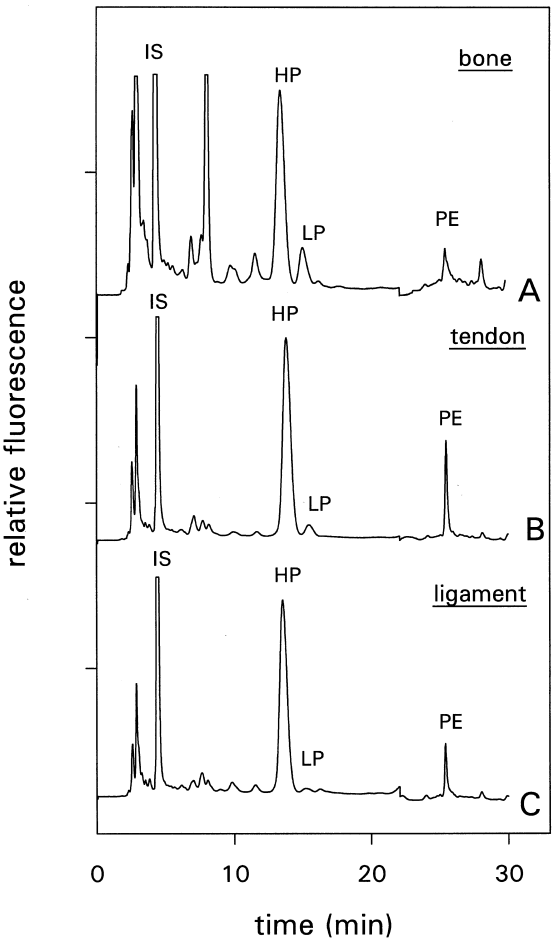

Fig. 4. Representative chromatograms of hydrolysates of bone (A), tendon (B) and ligament (C) obtained from a Rhesus monkey hip. Note the relatively large amount of LP compared to HP in bone; the noisy pattern in the beginning of the chromatogram can be improved by demineralizing the bone (e.g., with EDTA) before hydrolysis. I.S.=internal standard (pyridoxine), PE=pentosidine.

serial slices and analyzed for the amount of HP, LP and pentosidine per triple helix. Different profiles are seen of these crosslinks as a function of depth (Fig. 5). HP is lowest at the articular surface, and increased gradually with increasing depth. LP showed a pattern opposite to that of HP: it is highest at the surface and gradually decreased with depth. Pentosidine showed less marked differences with depth; on average it is slightly higher in the bottom half than in the upper half of cartilage. Until now, only the zonal variation of HP has been investigated in

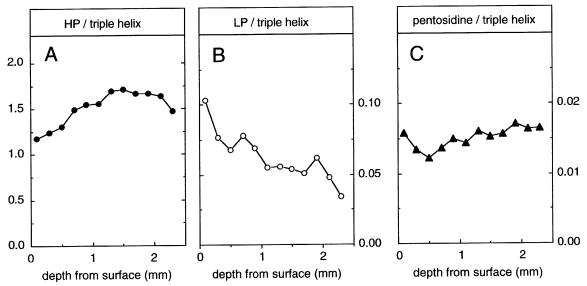

Fig. 5. Zonal variation of HP, LP and pentosidine in articular cartilage (human, femoral condyle, age 43 years) by slicing a 6 mm diameter core of full depth cartilage into 200 μm slices. The first slice is taken from the articular surface, the last slice from the deep layer close to the subchondral bone.

cartilage; the reported increase in HP with increasing depth [21] is consistent with our findings (Fig. 5).

3.4. LP in articular cartilage

Although LP is present in appreciable amounts in mineralized tissues such as bone and dentin (Fig. 4), its presence in cartilage is less documented. In our chromatograms, LP is consistently present in minor amounts as compared to HP (Fig. 2B, C); the molar ratio of HP/LP in human cartilage from femoral condyles is 25 ± 4.1 ($n=30$). To confirm the identity of the small LP peak, its excitation and emission spectra were determined at 400 nm and 290 nm, respectively. No differences were found between these spectra and the spectra obtained from LP purified from bone hydrolysates (data not shown). These results confirm the presence of LP in cartilage. Although bone is considered to contain relatively high levels of LP (i.e., ratio HP/LP$\approx$4), absolute amounts of LP in articular cartilage are almost as high as those in bone (0.05 and 0.1 LP per triple helix, respectively; $n=30$ and 6).

3.5. Analysis of crosslinks in urine

Hydrolyzed as well as native (non-hydrolyzed) urine contains large amounts of compounds with fluorescence characteristics similar to those of the pyridininoline crosslinks and pentosidine. Therefore determination of HP and LP in urine requires precleaning prior to the HPLC assay with e.g., partition chromatography on CF-1 or CC-31 cellu-

lose [13–16]. Unfortunately, pentosidine is lost with this procedure (Ref. [22]; own observations). Simultaneous determination of HP, LP and pentosidine can in principle be achieved in urine that is pretreated with the cation-exchanger SP-Sephadex C-25 [22]. In hydrolyzed urine cleaned by solid-phase extraction with CF-1 cellulose two peaks are seen (HP and LP) whereas in native urine three peaks are observed: HP, LP and a compound eluting in front of HP. This compound, only rarely mentioned in literature, has been identified as the diglycosylated form of HP, glucosylgalactosyl–hydroxylysylpyridinoline GGHP) [5]. During acid hydrolysis, both sugars are released (resulting in the formation of HP; see Fig. 6) [25], thus explaining why this compound is never observed in hydrolyzed tissues/urine. The tissue source of urinary GGHP has to be determined yet.

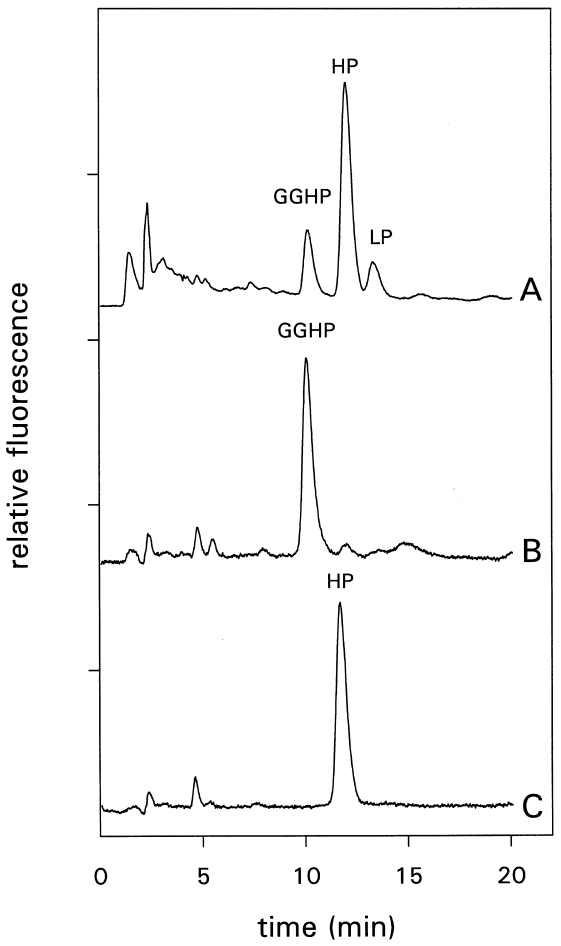

Fig. 6. (A) Chromatogram of native human urine pre-extracted with CF-1 cellulose. The purified glucosylgalactosyl-hydroxylysylpyridinoline (GGHP) (B) was subjected to acid hydrolysis, resulting in the destruction of the disaccharide and subsequently in the release of HP (C).

In conclusion, a sensitive method was developed to measure in a single run (without sample clean-up) the fluorescent collagen crosslinks HP, LP and pentosidine of a wide variety of connective tissues after acid hydrolysis. Pyridoxine was used as internal standard without significant interference of other fluorescent material. The detection limit for all compounds was below 1 pmol with a linear range larger than two-orders of magnitude. The diglycosylated form of HP, which is present in native urine, could be quantitated as well. LP was unequivocally identified in cartilage.

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