Rapid method for the isolation of the mature collagen cross-links, hydroxylysylpyridinoline and lysylpyridinoline

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Abstract

Hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP) are two non-reducible cross-links of mature collagen which are formed by a sequence of post-translational modifications. HP is a derivative of three residues of hydroxylysine and is present in almost all mature tissues (*e.g.*, tendons, vessel walls, cartilage, teeth and bone). LP is a derivative of two residues of hydroxylysine and one residue of lysine and is present only in dentine and bone. Neither cross-link is found in normal human skin. HP and LP were purified from commercially available bone gelatine ("ossein hydrolysate") by preparative reversed-phase HPLC and the degree of purity was verified by amino acid determination (>98% dry mass). Hydroxylysylpyridinoline and lysylpyridinoline are promising markers in urine of collagen resorption because their levels in urine should reflect only the breakdown of collagen fibres of skeletal tissues. The two components were used as external standards and the determination of HP and LP in urine provides a good means for the specific evaluation of pathological conditions associated with increased bone resorption, *e.g.*, high turnover post-menopausal osteoporosis.

1. Introduction

Collagen is the predominant structural protein of all forms of connective tissues. The covalent intermolecular cross-links between collagen molecules in macromolecular fibrils are essential in providing connective tissue matrices with the required physico-chemical properties and biomechanical stability. Hydroxylysylpyridino-line (HP) and lysylpyridinoline (LP) are the two non-reducible intermolecular cross-links of collagen derived from post-translationally modified hydroxylylsyl and lysyl residues of collagen chains [1,2].

HP is found in almost all mature tissues [2–5] whereas LP is present only in calcified tissues (bone and dentine [2,5]). During the process of collagen turnover the two cross-links are released into the blood and are subsequently excreted into the urine [6]. HP and LP are excreted in urine in two forms, one being the free form (about 40%) and the other being represented by a variety of peptide-bond components (about 60%). The total amount of both components can be measured by fluorimetry after reversed-phase high-performance liquid chromatography (HPLC) of hydrolysed urine [4,7,8].

The urinary hydroxylysyl- and lysylpyridi-noline are potentially more useful than is urinary

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hydroxyproline as a marker of the catabolism of collagen fibres from skeletal tissues, because urinary hydroxyproline has a lower specificity profile as it is found in all collagen types in all connective tissues. Moreover, a large proportion of hydroxyproline is metabolized in the liver, and escapes quantitative analysis of collagen breakdown by urinary measurements [9].

One fundamental problem is the current lack of synthetic standards of HP and LP for calibration so as to allow inter- and intra-laboratory comparisons with greater confidence [1,7,10,11]. In all instances questions arise about compound stability and comparability of molar fluorescence rates between HP and LP on the one hand and a reference compound on the other. Until now, adult bovine cartilage (for HP) and powdered and decalcified adult bovine bone (for HP and LP) have been used to prepare the reference standards. In this paper, we describe an alternative procedure for the isolation and purification of HP and LP and report on their use as external calibration standards. Thus, validity of the quantitative analytical data has been greatly improved.

2. Experimental

2.1. Materials

Gelatines from bone ("ossein") and other tissues were a gift from Deutsche Gelatine-Fabriken Stoess (Eberbach/Baden, Germany). Sephadex G-10 was purchased from Pharmacia–LKB (Freiburg, Germany). Fibrous cellulose powder (CF 1) was obtained from Whatman (Maidstone, UK). A Polyprop Econo-Column (4 × 0.8 cm I.D.) was supplied by Bio-Rad (Munich, Germany). A System 6300 amino acid analyser (Beckman, Munich, Germany) was used. Acetonitrile, n-heptafluorobutyric acid, glacial acetic acid and n-butanol were obtained from Sigma (Deisenhofen, Germany) and gelatine and hydrochloric acid (32%) from Merck (Darmstadt, Germany). All chemicals were of the highest analytical grade.

2.2. Hydrolysis of bone gelatine ("ossein") and batch adsorption chromatography on CF 1 cellulose

A 25-g amount of bone gelatine was dissolved in 1250 ml of 6 M hydrochloric acid using a glass bottle flushed with nitrogen for 5 min and sealed with a screw-cap and a PTFE liner. The sample was hydrolysed at 110°C for 24 h. The hydrolysate was filtered through a glass filter (porous glass G2) and evaporated to dryness at 50°C and the residue was dissolved in the mobile phase [n-butan-1-ol–glacial acetic acid–water (4:1:1)] to a final volume of 250 ml. A 250-g amount of cellulose powder (CF 1) was washed in 1000 ml of mobile phase and stirred for 2 h. The slurry was filtered to dryness through a glass filter (G2) and suspended in 1000 ml of mobile phase. The hydrolysate and CF 1 slurry were mixed and stirred for 1 h. Subsequently, the hydrolysate–slurry mixture was loaded on to a glass filter and washed with mobile phase (3 × 2 l). The pyridinium-containing fraction was eluted with water (3 × 1 l).

2.3. Molecular sieve chromatography

Following adsorption chromatography (CF 1), the HP- and LP-containing eluate was evaporated to dryness in a rotary evaporator at 50°C and the residue was dissolved in 10 ml of 10% (v/v) acetic acid. Subsequently the solution was applied to a Sephadex G-10 (90 × 2.5 cm I.D.). Elution was performed using 10% (v/v) acetic acid at a flow-rate of 20 ml/h at room temperature. Fractions of 6 ml were assayed for pyridinoline fluorescence (excitation at 297 nm, emission at 397 nm). The enriched pyridinium-containing fractions (32–54, see Fig. 1) were collected and dried (see above).

2.4. Preparative reversed-phase HPLC for isolation of HP and LP

The HPLC system consisted of a two-pump gradient system (Gynkotek Model M 480) equipped with a Gynkotek RF 1002 fluorescence

monitor. Chromatography was performed at room temperature and the flow-rate was 4 ml/min using two solvents: (A) 0.22% (v/v) n-heptafluorobutyric acid (HFBA) in water and (B) 0.22% (v/v) HFBA in 80% (v/v) aqueous acetonitrile. The resin (Gynkochrom Spherisorb ODS-II C_{18}; 5 μm; 250 × 8.0 mm I.D. column) was equilibrated with solvent B–solvent A (5:95, v/v) prior to the application of the sample [70 μl in 10% (v/v) HFBA in water]. The column was washed with solvent B–solvent A (5:95, v/v) for 5 min and developed with the following gradients: (1) from 5% to 27% solvent B in 5 min and (2) from 27% to 31% solvent B in 15 min.

The eluate was monitored for HP and LP fluorescence (excitation at 279 nm, emission at 397 nm, range 1 and sensitivity low). The individual peaks of the HP- and LP-containing fractions were collected (see Fig. 2) and evaporated to dryness at 50°C and the purity was checked by amino acid determination.

2.5. Amino acid determination

Hydroxylysyl- and lysylpyridinolines and potentially contaminating amino acids were determined on a Beckman Model .6300 automated analyser using postcolumn derivatization with ninhydrin. The eluate was monitored at 570 and 440 nm.

3. Results and discussion

We determined both the concentration of total cross-link components and the proportions of both HP and LP in a variety of gelatine and connective tissue samples. It is evident that gelatine specimens contain the cross-link compounds (HP and LP) in varying amounts depending on the tissue of origin. Table 1 summarizes the results from various types of gelatines and also from adult human vertebral bone, adult bovine cortical bone, adult bovine cartilage and adult bovine tendon. Clearly, human bone powder contains the highest proportion of LP followed by "ossein hydrolysate", the latter being gelatine made of bone. For reasons of convenience (no demineralization required) and because of its commercial availability (no ethical limitations), we used "ossein" as a raw material to isolate collagen cross-link components.

Black et al. [4] isolated 9.6 mg of HP and 1.5 mg of LP from demineralized sheep bone (80 g dry mass) by a modification of the previously described method [12,13]. Our results correspond with theirs. By a single series of the consecutive chromatographic steps, we isolated and purified from 25 g of ossein hydrolysate 2.5 mg of HP and 0.2 mg of LP. The fluorescence characteristics in dilute solutions of pyridinoline isolated by the methods described in here were identical with those described by Fujimoto et al.

Table 1
Concentration (dry mass) of hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP) in various gelatines and in a variety of adult tissues

| Material	HP:LP molar ratio	HP:LP concentration ratio (nmol/mg dry mass)	Species/tissue
Ossein hydrolysate	22:1	242:11	Adult bovine bone
Ossein gelatine	22.4:1	228:10.2	Adult bovine bone
Middle-layer hydrolysate	33:1	46:1.4	Adult bovine
Middle-layer gelatine	49:1	54:1.1	Adult bovine
Pig rind gelatine	15:1	24:1.6	Adult swine
Gelatine from Merck	17:1	205:12	Adult bovine
Bone	7.4:1	331:44.5	Adult human spine
Bone	33:1	237:18.5	Adult bovine cortex
Cartilage	933:−		Adult bovine
Tendon	55:−		Adult bovine
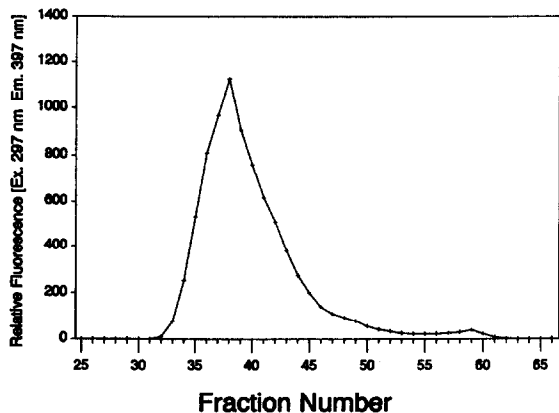
Fig. 1. Gel filtration chromatography (Sephadex G-10, 90 × 2.5 cm I.D. column) using 10% (v/v) acetic acid. The eluate was monitored by fluorescence detection with excitation at 297 nm and emission at 397 nm. Fractions of 6 ml (32—54) were collected and evaporated to dryness at 50°C.

[13]. The excitation maximum is at 297 nm and the fluorescence maximum (emission) is at 397 nm in acid. Using a two-step procedure we achieved virtually complete removal of concomitant impurities such as free amino acids. In the first step, a combination of adsorption and molecular sieve chromatography (Fig. 1) removed

Table 2
Purification of hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP) from "ossein"

Method	Purity (%)
CF 1 cellulose	25
Sephadex G-10	70
RP C_{18} preparative	98

about 70% of all amino acids. In the second step of chromatography, two consecutive linear gradients were applied: first, a gradient from 5% to 27% solvent B in 5 min, during which time all amino acids were eluted, and second, a gradient from 27% to 31% solvent B in 15 min to elute HP (16 min) and LP (18 min) (Fig. 2). Subsequently the column was washed free with solvent B. The HP and LP preparations recovered after the series of chromatographic steps had a purity higher than 98% as judged by amino acid determination. The purity results are summarized in Table 2.

The individual isolated peaks pooled after the HPLC each showed a single fluorescence signal and a unique ninhydrin-positive peak following
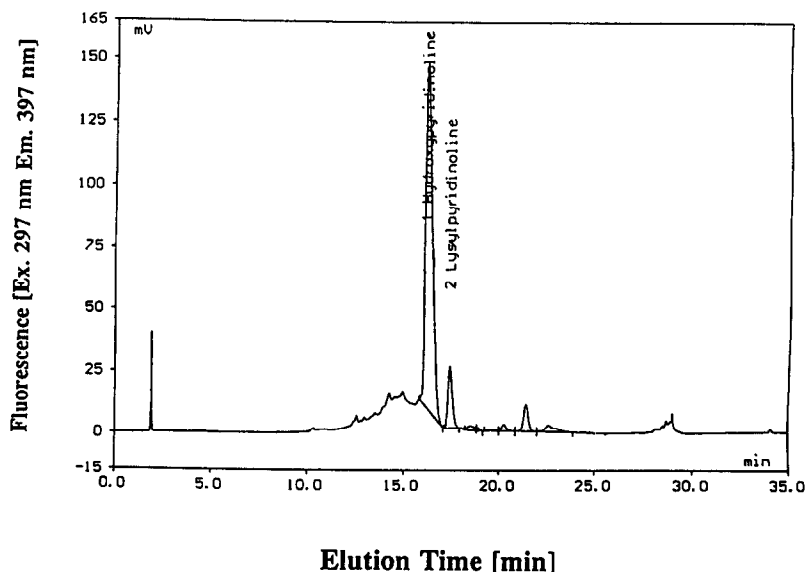

Fig. 2. HPLC of HP and LP with stepwise elution at a flow-rate of 4 ml/min at room temperature with solvent A = 0.22% (v/v) HFBA in water and solvent B = 0.22% (v/v) HFBA in 80% (v/v) aqueous acetonitrile. The pooled HP and LP were evaporated to dryness at 50°C and their degree of purity was checked by amino acid determination.
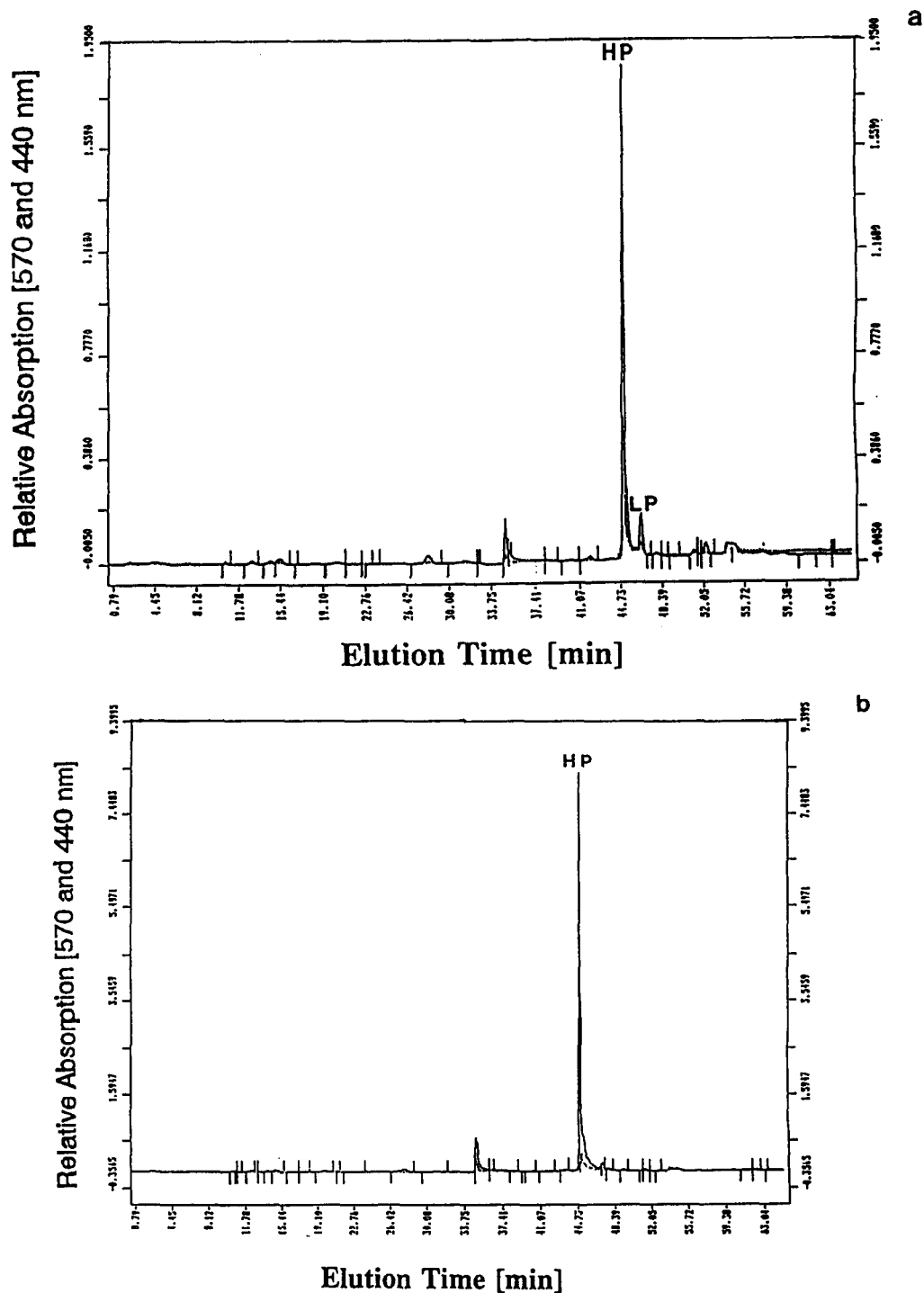
Fig. 3. Determination of purity of HP and LP standards by ion-exchange chromatography on an amino acid analyser. The HP and LP peaks were eluted at 45 and 47 min, respectively. (a) Amino acid analysis of pure HP and LP isolated from "ossein". (b) Pure HP isolated from "ossein".

ion-change chromatography on the amino acid analyser (see Fig. 3a and b).

We analysed serial dilutions of HP and LP in 0.22% (v/v) HFBA. The detector response was found to be linear up to the highest standard concentrations tested of 2250 nmol/ml HP and 1200 nmol/ml LP and gave good reproducibility throughout the assay range (see Fig. 4a and b). The ratio of HP to LP was identical when measured in aliquots of the same sample with ninhydrin on the amino acid analyser and by reversed-phase HPLC with fluorescence detection.

In conclusion, the efficient isolation of the highly purified collagen cross-links HP and LP from bone gelatine can greatly facilitate the determination of the breakdown products of tissue turnover and allows in particular the monitoring of human bone resorption rates in urine. Further, the HP-to-LP ratio is of diagnostic significance in rare connective tissue disorders, such as Ehlers–Danlos syndrome type VI.

Hydroxylysylpyridinoline [nmol/ml]
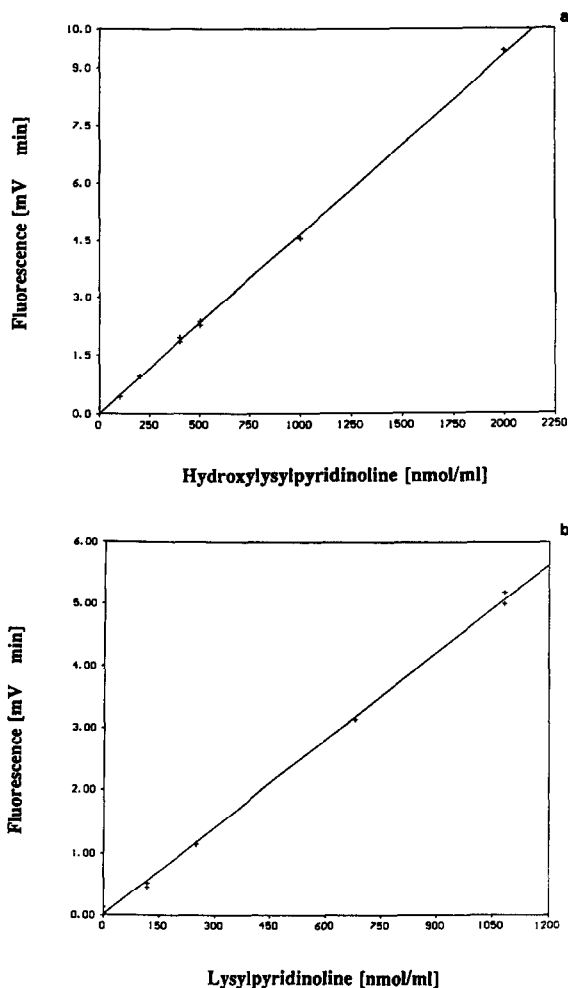

Lysylpyridinoline [nmol/ml]

Fig. 4. Calibration graphs for (a) HP and (b) LP. Serial dilutions of the HP and LP standards were analysed by HPLC and their fluorescence intensity was measured using a Gynkotek RF 1002 spectrofluorimeter with excitation at 297 nm and emission at 397 nm. Serial dilutions of the HP and LP standards showed a linear response of integrated fluorescence between 1.0 and 2250 nmol/ml.

4. Acknowledgements

5. References

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