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Quantification of immature and mature collagen crosslinks by liquid chromatography–electrospray ionization mass spectrometry in connective tissues

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ABSTRACT

We describe a novel high performance liquid chromatography–electrospray ionization mass spectrometry (HPLC–ESI-MS) method for the simultaneous quantification of enzymatic immature (dihydroxylysinonorleucine DHLNL, hydroxylysinonorleucine HLNL) and mature (pyridinoline PYD, deoxypyridinoline DPD) collagen crosslinks in connective tissues. The crosslinks were separated on a C18 Atlantis® T3 reversed-phase column with heptafluorobutyric acid (HFBA) as volatile ion-pairing reagent in an acetonitrile–water mobile phase. Detection was carried out by electrospray ionization mass spectrometry in a positive ion mode with selected ion recording (SIR). This method is more sensitive and selective than ion exchange chromatography with post-column ninhydrin detection which is the reference method used for the simultaneous quantification of collagen enzymatic divalent and trivalent crosslinks. The intra and inter-day precision errors were less than 3.4 and 7.7%, respectively for DHLNL, 3.5 and 5.9%, respectively for HLNL, 4.0 and 5.2%, respectively for PYD, 8.2 and 10.7%, respectively for DPD. This novel technique should be useful to quantify simultaneously DHLNL, HLNL, PYD and DPD in connective tissues and to evaluate the maturation of collagen by determination of the ratio between immature and mature enzymatic crosslinks.

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1. Introduction

In connective tissues, type I collagen is stabilized by the formation of lysyl-oxidase mediated intermolecular crosslinks. Two pathways of crosslinking formation have been described, depending on the type of precursors. The pathway that uses lysine aldehydes as precursors predominates in the skin, whereas in bone, tendon and most other connective tissues hydroxylysine aldehyde is utilized. The intermediate immature divalent ketoimine crosslinks derived from the hydroxylysine pathway are spontaneously converted into more stable mature trivalent pyridinium and pyrrolic crosslinks with aging [1]. These modifications of collagen are age and disease dependent and may impair the mechanical properties of connective tissues including bone, cartilage and tendons [2–7]. It is therefore important to develop reproducible and sensitive techniques allowing a simultaneous measurement of immature and mature crosslinks. Trivalent mature pyridinium crosslinks, pyridinoline (PYD) and deoxypyridinoline (DPD) are naturally fluorescent, resistant to acid hydrolysis and are usually measured in tissue hydrolysates by high performance liquid chromatography (HPLC) using a reversed-phase C18 column and sensitive fluorescence detection [8–10]. Specific immunoassays based on monoclonal antibodies have also been developed to measure free and peptide-bound pyridinium crosslinks in biological fluids [11–15]. In contrast to pyridinium crosslinks, immature divalent ketoimine crosslinks are non-fluorescent, acid labile and need to be stabilized by reduction with sodium borohydride ($NaBH_4$) to form stable dihydroxylysinonorleucine (DHLNL) and hydroxylysinonorleucine (HLNL) molecules prior acid hydrolysis and analysis. Ion exchange chromatography with post-column ninhydrin detection on amino acid analyser method is usually used for quantification of divalent crosslinks and trivalent crosslinks on a same chromatogram [16,17]. Tritiated $NaBH_4$ may be used to facilitate identification and location of reducible crosslinks [18]. A Biogel P2 size exclusion chromatography or a CF-1 cellulose partition chromatography preliminary fractionation step is suitable to remove the majority of common amino acid residues interfering with ninhydrin detection of crosslinks [16,17]. Saito et al. [19] has also proposed a cation-exchange HPLC method using fluorescent *o*-phthalaldehyde (OPA) post-column derivatization to detect immature crosslinks and common amino acids. However with this technique PYD and DPD can not be detected because

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OPA derivatives co-migrated with phenylalanine and hexosyllysine [19]. Pre-column derivatization followed by reversed-phase HPLC separation is not recommended for measurement of divalent reducible crosslinks, because the multiple derivatives generated by derivatization procedure are then eluted as separate peaks on the reversed-phase HPLC column [20].

All above described methods have limitations for routine analyses of connective tissues. Pyridinium crosslinks can be easily and sensitively quantified because of their natural fluorescence using reversed-phase HPLC but this technique cannot be applied to non-fluorescent divalent immature crosslinks. Ion exchange chromatography measurements require long equilibration time, lengthy run and the use of corrosive saline buffers. Crosslinks present in smaller amount than common amino acids in collagen are not selectively detected by OPA or ninhydrin derivatization. The use of radioactive isotopes is selective but not easy to apply for routine analysis. PYD and DPD immunoassays are specific and sensitive but these methods have been specifically developed for biological fluids analyses and not tissue extracts and are not available for immature divalent crosslinks.

Kindt et al. have published a highly sensitive and specific method using liquid chromatography tandem mass spectrometry for measuring PYD and DPD in arterial tissue and urine. However this method is restricted to mature pyridinium crosslinks quantification [21].

The HPLC methods usually employed to measure immature and mature crosslinks are based on ion exchange chromatography with ninhydrin post-column derivatization. In this paper, we describe the development and validation of a new HPLC–electrospray ionization mass spectrometry method for the simultaneous quantification of DHLNL, HLNL, PYD and DPD in connective tissues which is more sensitive and selective than those post-column derivation methods. In addition, this novel technique allows evaluating the maturation of collagen in connective tissues by determination of the ratio between immature and mature enzymatic crosslinks.

2. Experimental

2.1. Chemicals and reagents

HPLC gradient grade acetonitrile was purchased from Fisher Scientific (Loughborough, UK). All chemicals were all of analytical grade. Acetic acid and butanol-2 were obtained from Carlo Erba-SDS (Val de Reuil, France) and heptafluorobutyric acid (HFBA) was purchased from Apollo Scientific Ltd. (Stockport, UK). $NaBH_4$ and the following amino acids: hydroxyproline (Hyp), serine (Ser), aspartic acid (Asp), glycine (Gly), threonine (Thr), glutamic acid (Glu), cysteine (Cys), alanine (Ala), proline (Pro), histidine (His), hydroxylysine (Hyl), valine (Val), lysine (Lys), methionine (Met), arginine (Arg), tyrosine (Tyr), isoleucine (Ile), leucine (Leu) and phenylalanine (Phe) were purchased from Sigma (St. Louis, MO, USA). CF-11 cellulose powder was supplied by Whatman (Maidstone, UK) and 18 ohms water was obtained using Purelab UHQ (Veolia Water STI, Anthony, France). HPLC assay for Hyp was purchased from Bio-Rad (Hercules, CA, USA). SPE Chromabond® Crosslinks columns were supplied by Macherey-Nagel (Düren, Germany), PYD, DPD calibrators and the actetylated pyridinoline (Ac-PYD) were purchased from Quidel Corporation (San Diego, CA, USA).

2.2. Preparation and calibration of crosslink standards from bone tissue

2.2.1. Preparation of crosslink standards from bone tissue

Crosslinks (DHLNL, HLNL, PYD and DPD) calibrators were prepared from foetal bovine cortical diaphyseal femurs. Bone sample (100 g) were cut in small pieces and powered in liquid nitrogen-cooled freezer mill (Spex Centriprep, Metuchen, USA), demineralised with daily changing of 0.5 M EDTA in 0.05 M Tris buffer, pH 7.4 for 96 h at 4 °C and extensively washed with deionized water. The demineralized bone residue was suspended in phosphate-buffered saline (0.15 M NaCl in 20 mM sodium phosphate buffer, pH 7.4) and reduced with NaBH4 at room temperature as previously reported [19]. Briefly, $NaBH_4$ at an initial concentration of 10 mg/ml in 1 mM NaOH was added to give a reagent/bone tissue extract weight ratio of 1:30 (w/w) for 2 h at room temperature. The reduced samples were then extensively washed, freeze-dried and hydrolyzed in 6 M hydrochloric acid at 110 °C for 24 h.

One hundred ml of bone hydrolysate was added to 100 ml acid acetic and 400 ml of butanol-2. Then 100 g of CF-11 cellulose powder was mixed with the solvent preparation. The slurry was passed through a filter and the cellulose was suspended in a wash buffer prepared with butanol-2, acetic acid and deionized water in the respective proportion of 4:1:1 (vol/vol/vol). After repeating this washing step 10 times, the cellulose was suspended in water to release crosslinks and the eluate was concentrated. The DHLNL, HLNL, PYD and DPD peaks were located on the chromatogram by HPLC–ESI-MS (see below). This preparation was used as standard for the measurement of DHLNL, HLNL, PYD and DPD crosslinks in connective tissues.

2.2.2. Calibration of crosslinks

PYD and DPD were calibrated in the standard preparation by comparing peak fluorescence obtained with the commercial PYD and DPD calibrators from Quidel Corporation (San Diego, CA, USA).

DHLNL and HLNL were purified by HPLC from a part of the standard preparation and then calibrated using the ninhydrin reference method and leucine equivalence [21].

2.3. Bone tissue sample preparation

2.3.1. Sample demineralization

Human bone and bovine bone and cartilage samples were powdered in liquid nitrogen-cooled freezer mill (Spex Centriprep, Metuchen, USA) and defatted in methanol/chloroform, extensively washed with deionized water and freeze-dried. Bone samples were then demineralized with 0.5 M EDTA in 0.05 M Tris buffer, pH 7.4 for 96 h at 4 °C with daily changing of the demineralization solution. Demineralized bone powder was then extensively washed with deionized water and freeze-dried. It is recommended to powder a minimum of 10–20 mg of tissue to obtain enough material for the different steps before acid hydrolysis.

2.3.2. Sample reduction

Demineralized bone powder was suspended in phosphate-buffered saline (0.15 M sodium chloride, 0.1 M sodium phosphate buffer, pH 7.5) and reduced with $NaBH_4$ as already described. The reduced samples were then washed, freeze-dried and hydrolyzed in 6 M hydrochloric acid at 110 °C for 24 h (2 mg of reduced bone per ml) to quantify collagen crosslinks.

2.3.3. Sample pre-treatment

Bone hydrolysates were pre-fractioned on SPE Chromabond® Crosslinks to remove interfering molecules using a Vac Elut 20 Manifold apparatus (Varian, Inc., Palo Alto, CA, USA) and a N840FT.18 LABOPORT® pump (KNF Neuberger, Inc. Trenton, NJ, USA). Briefly, 400 μl of Ac-PYD previously diluted 5 times in 90% of acetic acid were added to 400 μl of hydrolysate samples and 2.4 ml of acetonitrile in 5 ml glassed tube. After mixing, the solution was transferred to a SPE column previously placed inside a vacuum box and equilibrated with 2.5 ml of wash buffer prepared with acetonitrile, acid

acetic and deionized water in the respective proportions 8:1:1 (vol/vol/vol). 2.5 ml of washing buffer were used for rinsing the 5 ml glass tube and applied to the SPE column. Columns were extensively washed with 4×2.5 ml of washing buffer and 100 μl of deionized water were applied on the column. The columns were completely drained and DHLNL, HLNL, PYD, DPD and Ac-PYD crosslinks were eluted with 600 μl of 1% HFBA directly in 750 μl HPLC vials. A portion of the eluate (20–60 μl) containing about 10–20 μg of collagen was analysed by HPLC–ESI–MS.

2.4. Chromatographic and mass spectrometric conditions

2.4.1. Chromatographic conditions

The DHLNL, HLNL, PYD, DPD extracted from bone tissue and added Ac-PYD were separated using an HPLC on an Alliance 2695 separation module equipped of with Waters Micromass® ZQ™ Single Quadrupole Mass Spectrometer, a 2647 Multi λ fluorescence detector and Empower2 chromatography data software (Waters Corp. Milford, MA, USA). Crosslinks were separated using an Atlantis® T3, 3 μm, 4.6×100 mm reversed-phase column protected by an Atlantis® T3, 3 μm, 4.6×20 mm guard cartridge (Waters Corp., Milford, MA, USA) maintained at 25 °C. The column flow rate was 1 ml/min. After the analytical column, the flow was split in half between fluorescence (0.6 ml/min) and MS (0.4 ml/min) detectors to avoid a too high flow rate in the ion source of the MS detector and to compare MS and reference fluorescence detection for PYD and DPD measurement. Solvent A consisted of 0.12% of HFBA in 18 ohms pure water, and solvent B was 50% of acetonitrile. The column was equilibrated with 10% of solvent B prior to use and chromatographic separation was achieved with a gradient elution from 10 to 20% solvent B in 40 min. PYD, DPD and Ac-PYD were monitored for fluorescence at an emission of 395 nm and an excitation of 297 nm. The concentration of HBFA was optimized at 0.12% to obtain optimal performances of both separation and sensitivity (data not shown).

2.4.2. Mass spectrometric conditions

Crosslinks were detected using a Waters Micromass® ZQ™ Single Quadrupole Mass Spectrometer (Waters Corp., Milford, MA, USA). The electrospray ionization (ESI) source was operated in the positive ion mode. The target ions were $[M+H]^+$ at m/z 308 for DHLNL, 292 m/z for HLNL and $[M]^+$ m/z 429 for PYD, m/z 413 for DPD and m/z 471 for Ac-PYD, respectively. The optimized ionization conditions were as follows: capillary voltage of 1.0 kV, cone 30 kV, source temperature 120 °C and desolvation temperature 350 °C. Nitrogen was used as a desolvation gas at a flow rate of 650 l/h and cone gas at a flow rate of 75 l/h. Data collected in selected ion recording (SIR) mode were acquired and processed using Empower2 chromatography data software (Waters Corp., Milford, MA, USA).

3. Results and discussion

3.1. Selectivity and matrix effect

To assess the efficacy of the SPE procedure for removing common amino acid contaminants and the performance of HPLC condition to separate the crosslinks from main amino acid residues, a mixture of 19 of the most common collagen amino acids (0.1 μmol/ml each) was analysed by HPLC–ESI-MS and compared with the same mixture pre-treated by SPE. Recovery was calculated by comparing the area of the chromatographic peak of each amino acid before ($n = 3$) and after SPE extraction ($n = 3$). Matrix effects (ME) on the ionization of crosslinks was evaluated by comparing crosslinks peak areas of a bone hydrolysate sample and a blank sample (solution of HCl 6N). Each sample was spiked with the same amount of DHLNL, HLNL, PYD, DPD calibrators and internal standard, pre-treated by SPE procedure and separated using the HPLC–ESI-MS conditions as described above. For the ME test, we selected a foetal bone sample which was not reduced by NaBH4 before acid hydrolysis in order to destroy immature crosslinks and which contained only very small amounts of mature crosslinks. Each experiment was repeated 4 times. The ME was calculated by using the ratio (spiked bone hydrolysate sample/spiked 6N HCl solution $\times$ 100)%. If the ratio was comprised between 85% and 115%, matrix effect was considered acceptable.

Representative SIR chromatograms for DHLNL, HLNL, PYD, DPD and Ac-PYD internal standard were shown in Fig. 1. DHLNL, HLNL, PYD, DPD and Ac-PYD peaks on the chromatogram had retention times of 20.7, 24.0, 29.8, 34.5 and 39.5 min, respectively and were well resolved from others amino acids. Indeed, the SPE procedure allowed to remove 85–100% of the 19 collagen amino acids analyzed and no interference was observed at the retention times of the molecules of interest. The peaks of Ile, Leu and Phe that eluted the closest of those of the collagen crosslinks were very efficiently eliminated by the SPE pre-treatment procedure (99 ± 1%). We observed no significant matrix effect. ME ratios being 95 ± 2% for DHLNL, 94 ± 2% for HLNL, 96 ± 2% for PYD, 100 ± 2% for DPD and 98 ± 2% for Ac-PYD.

3.2. Extraction recovery

The extraction recovery of crosslinks on the SPE column was evaluated by comparing peak areas obtained from foetal bovine bone hydrolysate samples spiked with known amounts of analytes before SPE extraction (DHLNL (36, 14 and 7 μM), HLNL (6, 2 and 1 μM), PYD (6, 3 and 1 μM) and DPD (1, 0.4 and 0.2 μM)) with those from hydrolysate samples to which analytes were added after SPE extraction. The procedure was repeated 4 times for each concentration. The recovery rates (RE %) were calculated using the following equation: RE (%) = response (bovine pool spiked with analytes before SPE treatment)/response (bovine pool spiked with analytes after SPE treatment) $\times$ 100.

As MS detection was less sensitive than fluorescence detection, we used, during the SPE pre-treatment procedure, Ac-PYD internal standard solution diluted 5 times with 90% acetic acid (see before) instead of 20 times recommended for fluorescence quantification of pyridinium crosslinks by Quidel Corporation. The extraction recovery of Ac-PYD was evaluated by using three different concentrations of the internal standard solution (pure, diluted 5× and 10×) added to foetal bovine bone hydrolysate sample before SPE extraction and compared to internal standard solution directly injected on reversed-phase HPLC column. The procedure was repeated 4 times for each dilution.

The recovery rates were the following: 97 ± 11%, 106 ± 6% and 107 ± 9% for DHLNL at 36, 14 and 7 μM, respectively, 96 ± 12%, 102 ± 6% and 100 ± 10% for HLNL at 6, 2 and 1 μM, respectively, 94 ± 10%, 98 ± 5% and 93 ± 7% for PYD at 6, 3 and 1 μM, respectively, 102 ± 11%, 84 ± 5% and 88 ± 10% for DPD at 1, 0.4 and 0.2 μM, respectively and 93 ± 11%, 95 ± 5% and 100 ± 8% for Ac-PYD internal standard solution used pure, diluted 5 and 10 times, respectively. These data indicate that the bone collagen crosslinks were efficiently recovered by using the SPE pre-treatment procedure and accurately estimated by the use of Ac-PYD as internal standard in this procedure.

3.3. Linearity of calibration curves, lower limit of detection (LOD) and lower limit of quantification (LOQ)

Calibration curve were prepared by assaying standards in duplicate at seven concentrations ranging from 0–7.5 μM for DHLNL, 0–2 μM for HLNL, 0–5 μM for PYD and 0–2 μM for DPD. The linear-

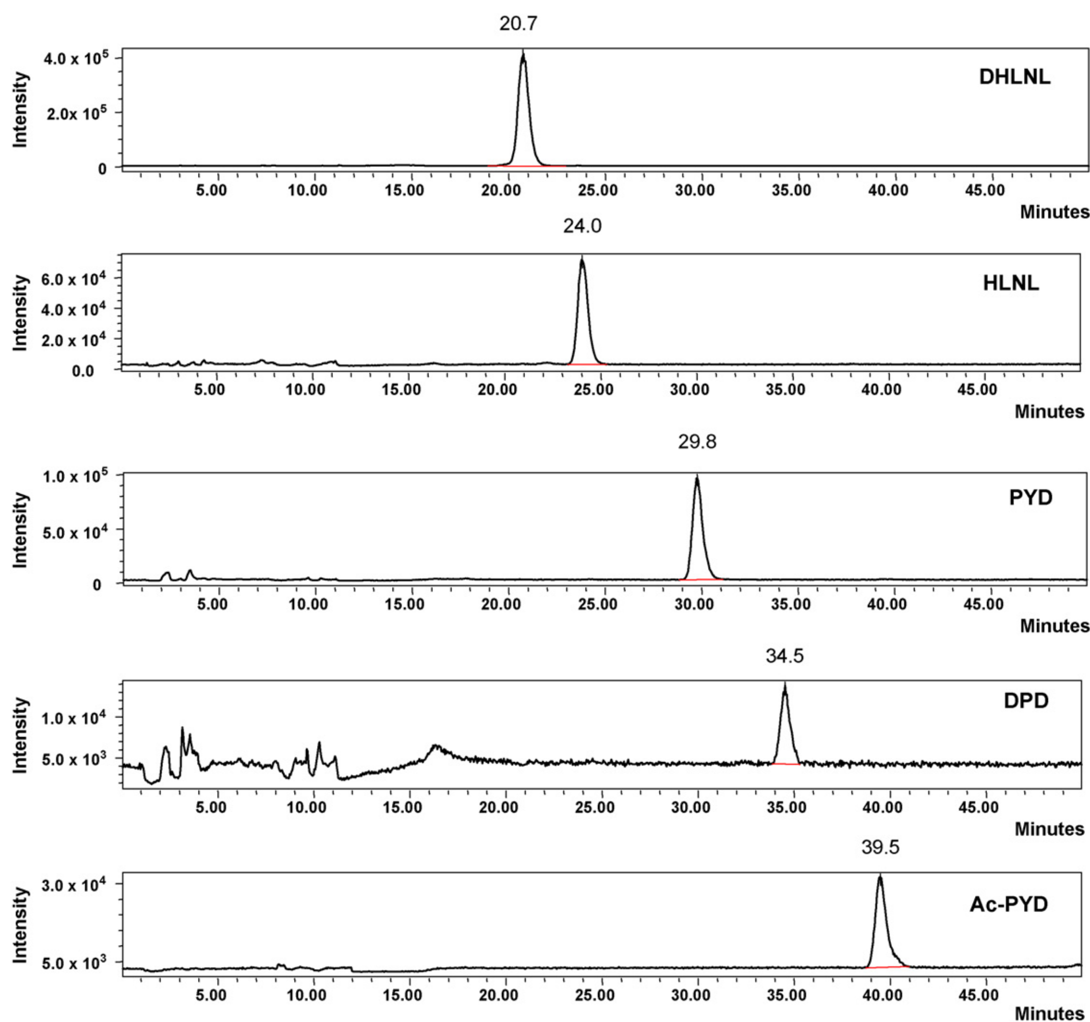

Fig. 1. Typical SIR chromatograms of DHLNL, HLNL, PYD and DPD calibrators (170, 26, 30 and 5 pmol injected, respectively) and Ac-PYD used as an internal standard (4 μl injected).

ity of each calibration curve was determined by linear regression analyses. Limit of detection (LOD) was defined as a signal to noise ratio of 3. The limit of quantification (LOQ) was defined as 3 times the limit of detection as previously proposed [22].

The calibration curve of DHLNL, HLNL, PYD, DPD were described by the equations $y = 2670443x$, $y = 2654938x$, $y = 2736510x$ and $y = 1998851x$, respectively. The assay was linear over the validated concentration range of 0–7.5 μM for DHLNL, 0–2 μM for HLNL, 0–5 μM for PYD, 0–2 μM for DPD. Determination coefficients (r^2) of the calibration curves were >0.996 for all the compounds. The LOD and LOQ expressed in pmol injected in the column were respectively 0.2 and 0.6 for DHLNL, 0.3 and 0.9 for HLNL and PYD and 0.4 and 1.2 for DPD.

3.4. Precision and accuracy

The precision and accuracy of the assay were assessed by repeated measurements of a foetal bovine bone sample not reduced by NaBH4 added with low (a), medium (b) and high (c) concentrations of calibrator. Intra-day precision and accuracy were calculated from seven runs of the same SPE pre-fractioned bone samples on the same day. The inter-day precision and accuracy were determined by repeated analysis on 7 consecutive days using calibrators prepared on the same day. Precision was expressed as relative standard deviation (RSD %) and accuracy as relative error [((calculated concentration − true concentration)/true concentration) × 100%].

The intra- and inter-day precision and accuracy for DHLNL, HLNL, PYD and DPD are presented in Table 1. The intra- and inter-day precision errors were lower than 3.4% and 7.7%, respectively for DHLNL, 3.5% and 5.9%, respectively for HLNL, 4.0% and 5.2%, respectively for PYD, 8.2% and 10.7%, respectively for DPD. Accuracies precision errors were below 2.2% for all crosslinks.

3.5. Application of the method to measure collagen crosslinks in connective tissues

The HPLC–MS-ESI method we developed was used to quantify divalent immature and trivalent mature crosslinks in trabecular bone of human lumbar vertebrae ($n = 6$), in foetal and 2 years old bovine cortical bone and cartilage ($n = 3$). To induce collagen maturation and crosslinking, foetal bovine cortical bone specimens were incubated at 37 °C in phosphate-buffered saline 0.1 M, pH 7.4 at 37 °C for 0, 60, 90 and 120 days as previously described ($n = 3$ for each incubation) [23].

DHLNL, HLNL, PYD and DPD were measured in trabecular bone of human lumbar vertebrae using HPLC–ESI-MS method (Fig. 2, Table 2). The same samples were concurrently analysed by a fluorescence reference method for PYD and DPD. We found comparable PYD and DPD contents between MS and fluorescence detection

Table 1
Precision and accuracy of the novel HPLC–ESI-MS crosslinks assay.

	DHLNL concentration (nM)		Intra-day RSD (%)	Inter-day RSD (%)	Accuracy RE (%)
	Spiked	Calculated			
A	720	730 ± 25	3.4	7.6	1.4
B	144	147 ± 4	3.1	6.3	2.2
C	72	70 ± 2	2.4	7.7	−2.2
	HLNL concentration (nM)		Intra-day RSD (%)	Inter-day RSD (%)	Accuracy RE (%)
	Spiked	Calculated			
A	108	107 ± 3	3.1	5.9	−0.9
B	21.6	21.3 ± 0.8	3.5	3.7	−1.5
C	10.8	10.6 ± 0.3	2.9	4.0	−2.1
	PYD concentration (nM)		Intra-day RSD (%)	Inter-day RSD (%)	Accuracy RE (%)
	Spiked	Calculated			
A	127	129 ± 5	4.0	3.2	1.7
B	25.4	25.8 ± 0.7	2.8	3.3	1.7
C	12.7	12.8 ± 0.2	1.9	5.2	0.7
	DPD concentration (nM)		Intra-day RSD (%)	Inter-day RSD (%)	Accuracy RE (%)
	Spiked	Calculated			
A	20.9	20.7 ± 0.9	4.4	5.1	−0.88
B	4.18	4.21 ± 0.11	2.5	10.6	0.83
C	2.09	2.13 ± 0.17	8.2	10.7	1.82

The precision was expressed as relative standard deviation (RSD) and accuracy as relative error (RE).

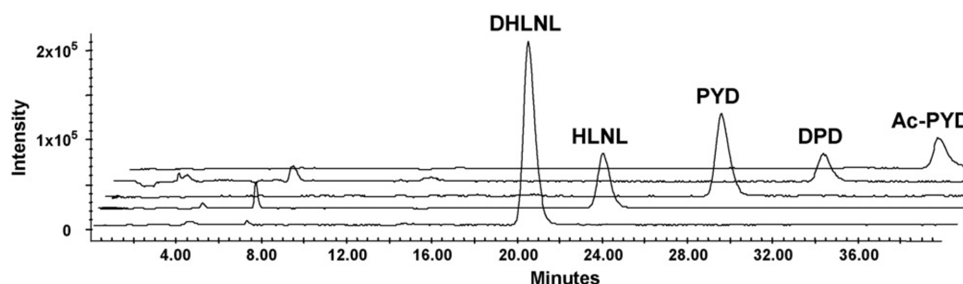

Fig. 2. Typical elution profiles of immature and mature enzymatic crosslinks from trabecular bone of human lumbar vertebrae. The chromatograms correspond to the injection of 16 μg of collagen.

(Table 2). With this method the concentrations of DHLNL, PYD and DPD were relatively consistent with those previously determined by the ninhydrin reference method [24,25] or by OPA method [20]. However, we found lower HLNL content compared to these studies. The discrepancies could be explained by the small sample size and differences in the type (trabecular lumbar vertebrae versus cortical diaphyseal femurs) and age of bone tissues analysed.

DHLNL, HLNL, PYD and DPD were measured in fetal and 2-year-old bovine cortical and cartilage femurs (Table 3). With this method the concentration of collagen crosslinks are comparable with those previously published [24,26]. Fig. 3 shows that as expected [23] the concentrations of trivalent non reducible mature crosslinks increase with incubation time of a fetal bovine bone specimens whereas those of divalent reducible immature crosslinks decreases.

Table 2
Enzymatic divalent immature and trivalent mature crosslink contents in trabecular human bone tissue measured by the novel assay (HPLC–ESI-MS) and a reference HPLC-FLUORESCENCE technique.

n	Age	HPLC–ESI-MS				HPLC-FLUORESCENCE	
		DHLNL	HLNL	PYD	DPD	PYD	DPD
6	79 ± 13	0.345 ± 0.063	0.136 ± 0.022	0.254 ± 0.037	0.111 ± 0.022	0.247 ± 0.036	0.114 ± 0.017

Crosslinks mol/mol collagen.

Table 3
Enzymatic divalent immature and trivalent mature crosslink contents in cortical bovine bone tissue expressed in mol/mol collagen.

Tissue (age)	*n*	DHLNL	HLNL	PYD	DPD
Bovine bone (foetal)	3	2.40 ± 0.15	0.50 ± 0.05	0.07 ± 0.01	0.011 ± 0.001
Bovine bone (2 years)	3	1.51 ± 0.11	0.43 ± 0.06	0.29 ± 0.02	0.07 ± 0.01
Bovine cartilage (foetal)	3	1.42 ± 0.10	–	0.72 ± 0.05	–
Bovine cartilage (2 years)	3	0.057 ± 0.05	–	1.86 ± 0.09	–

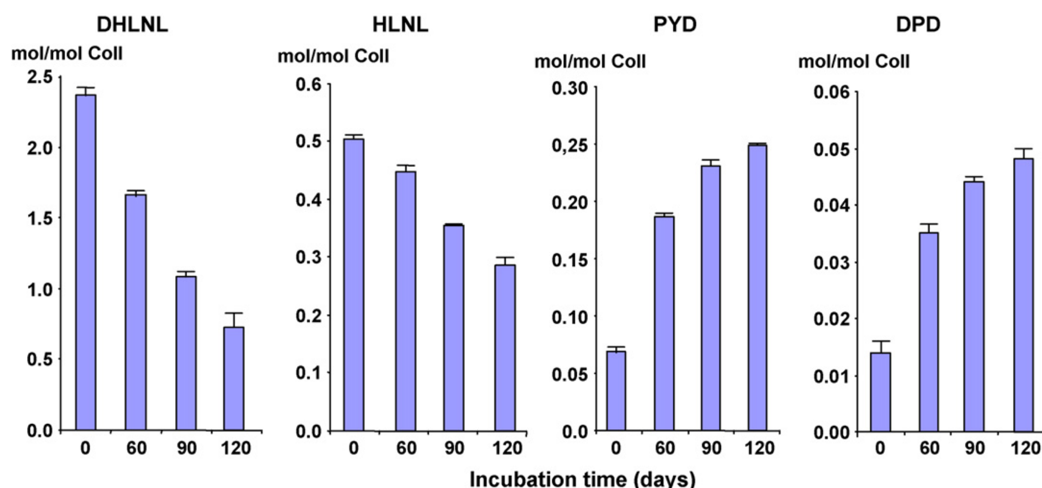

Fig. 3. Change in collagen crosslinks during incubation of foetal cortical bone at 37 °C in phosphate-buffered saline 0.1 M, pH 7.4 for 0, 60, 90 and 120 days.

4. Conclusion

Divalent immature and trivalent mature crosslinks of bone type I collagen are usually concomitantly determined using a modified method of amino acid analysis by ion exchange chromatography with ninhydrin post-column derivatization [16,17]. This technique, however, is time-consuming and lacks sensitivity and specificity.

We have developed and validated a novel method based on a single reversed-phase HPLC and a specific MS-ESI detection which is more sensitive and specific than the reference method. This method allows the simultaneous quantification of collagen divalent immature and trivalent mature enzymatic crosslinks in connective tissues and could be useful to evaluate their change with maturation, ageing and disease.

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