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Quantitative analysis of collagen and elastin cross-links using a single-column system

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ABSTRACT

The separation of both the immature and mature cross-links present in collagen together with the stable cross-links of elastin has been achieved on a single ion-exchange column. This technique avoids the current necessity for two different systems, ion-exchange and high-performance liquid chromatography–fluorescence techniques. The value of the method is illustrated by the comparison of the cross-link contents of ageing bovine skin, from foetal to old age.

INTRODUCTION

It is now well established that the collagen fibre is stabilised by a series of intermolecular covalent cross-links between the collagen molecules making up the fibre, and that the chemistry of these cross-links is dependent on both the nature of the collagenous tissue and the age of the tissue [1–3]. Thus, the intermediate cross-links present in immature tissue, dehydrohydroxylysinonorleucine (deH-HLNL) and hydroxylysino-keto-norleucine (HLKNL), vary considerably between tissues, *e.g.* rat-tail tendon and skin contain deH-HLNL whilst cartilage and bone contain predominantly HLKNL. These intermediate divalent cross-links are converted to stable trivalent cross-links which accumulate in the tissue as the turnover decreases during maturation [3,4]. The proportion of the known mature cross-links, hydroxylysylpyridinoline (HYL-PYR), lysylpyridinoline (LYS-PYR) and histidinohydroxylysinonorleucine (HHL), again vary with age and with the type of tissue. For example, HHL is the major known cross-link component of mature skin [5] and pyridinoline the major cross-link of mature bone and cartilage [6], whilst tendon contains a mixture of both cross-links. It is therefore important in any study of the changes in pathological tissue to understand the nature of the normal age-related changes of the cross-links of the particular tissues under investigation. It is also important to have a reproducible technique to measure all the different cross-links.

The intermediate cross-links must be stabilised by reduction with borohydride prior to acid hydrolysis, deH-HLNL being reduced to hydroxylysinonorleucine (HLNL), and HLKNL to dihydroxylysinonorleucine (DHLNL). The mature cross-links are stable to acid and can be analysed directly from acid hydrolysates. Sensitive techniques are required to detect these cross-linking amino acids since a maximum of one cross-link per two collagen molecules would require the detection of this one cross-linking amino acid

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among the six thousand other amino acid residues from the two collagen molecules. Currently the intermediate cross-links may be determined by radioactivity following reduction with tritium-labelled borohydride [7] or by using ninhydrin or similar post-column derivatisation after separation of the acid hydrolysate on an ion-exchange column using either volatile buffers or standard salt buffers [7,8]. The mature cross-link (HHL) may be analysed by amino acid analysis on ion-exchange columns [5] but HYL-PYR and LYS-PYR are generally determined separately by highly sensitive fluorescent detection on HPLC [9]. The total immature and mature cross-links have therefore to be determined on two completely different systems.

The other major connective tissue protein, elastin, is stabilised by lysine-derived cross-links based on the same enzymic mechanism but yielding the two tetravalent pyridinium compounds, desmosine and isodesmosine [10]. Both these compounds can be detected by ninhydrin or similar reagent after elution from ion-exchange columns or high-performance liquid chromatographic (HPLC) systems [11,12].

In this paper we describe a modification of the ion-exchange column procedure to allow the detection of all these cross-links on one chromatogram using the same detection system thus allowing direct comparison of results.

EXPERIMENTAL

Chemicals

CF1-cellulose was purchased from Whatman (Maidstone, UK) and all stock solutions and chemicals for the LKB autoanalyser from Pharmacia (Milton Keynes, UK). Sodium borohydride was purchased from Sigma (Poole, UK). All other chemicals were purchased from Fisons (Loughborough, UK). The cross-linking amino acids were isolated and purified in our laboratory.

Preparation of the sample

The tissue was homogenised and suspended in phosphate-buffered saline (0.15 *M* sodium chloride, 0.1 *M* sodium phosphate buffer, pH 7.4) and reduced with sodium borohydride as described previously [7]. The reduced material was then washed and freeze-dried, and approximately 5–50 mg were hydrolysed in 6 *M* hydrochloric acid at 110°C for 24 h in a 15-ml screw-top hydrolysis tube (Corning Laboratory Service, New York, NY, USA). The excess acid was removed by rotary evaporation and the residue dissolved in 1 ml of distilled water.

Preparation of a pre-separation column

The sample was initially separated on a CF1 cellulose (Whatman) column to remove the non-cross-link amino acids based on the technique originally described by Starcher and Galione [13] and Black *et al.* [14]. The CF1-cellulose was washed prior to packing the column by shaking a weighed quantity of CF1-cellulose in water, the slurry was allowed to settle and the supernatant decanted. This washing procedure was repeated a further four times. The washed cellulose was then poured into a sintered-glass funnel and equilibrated in an organic mobile phase consisting of butanol–acetic acid–water (4:1:1). The CF1 was re-suspended in the organic phase at a concentration of approximately 5% dry weight per volume.

A mini-CF1 column was then prepared from a 3-ml disposable plastic Pasteur pipette (Sterilin, Hounslow, UK) by inserting a plug of glass wool into the lower end of the pipette. The CF1 slurry was then poured into the Pasteur pipette and the cellulose allowed to settle to a bed height of 8 cm, using the 3-ml graduation mark on the pipette as a guide. The mini-column was then washed with 8 ml of the mobile phase to settle the bed.

Analysis of the sample

(i) CF1 column. To the sample in 1 ml of water, 1 ml of glacial acetic acid and 4 ml butanol were added to give a final butanol–acetic acid–water ratio of 4:1:1 and a total volume of 6 ml. The sample (6 ml) was then applied to the CF1 column followed by two 2-ml washings of the hydroysis tube using the organic phase. The non-cross-linking amino acids were then eluted from the column with a further 16 ml of the mobile

TABLE I

ELUTION PROGRAMME

Step	Buffer	Time (min)	Temperature (°C)
1	0.2 *M* Sodium citrate pH 4.25	14	45
2	0.2 *M* Sodium citrate pH 5.25	33	83
4	0.5 *M* Borate–citrate pH 8.6	8	83
5	0.4 *M* Sodium hydroxide	5	83
6	0.2 *M* Sodium citrate pH 2.85 + 2% isopropyl alcohol	6	83
7	0.2 *M* Sodium citrate pH 2.85 + 2% isopropyl alcohol	13	45

phase in four 4-ml aliquots. Under these conditions the standard amino acids were eluted and the cross-linking amino acids remained adsorbed to the cellulose. The cross-linking amino acids were then eluted from the column with 8 ml of distilled water. This aqueous eluent was freeze-dried and redissolved in 100–200 μl of either 0.01 *M* hydrochloric acid or 0.2 *M* sodium citrate buffer, pH 2.2. After thorough agitation to ensure complete dissolution of the sample it was centrifuged briefly (approximately 30 s) to bring the solution to the bottom of the tube and thus ensure maximum recovery. The solution was then filtered through a 0.2-μm filter (Gelman Sciences, Northampton, UK). The percentage recovery of individual cross-links from CF1-cellulose was determined and found to be all in the range 90–95%. These results are consistent with those obtained by Black *et al.* [14].

(ii) Ion-exchange column. The sample (20–80 μl) was then analysed on an LKB 4400 amino acid analyser (Pharmacia, Loughborough, UK) configured for the separation of the collagen cross-links as shown below.

Modified gradient for cross-link analysis. The column consisted of Aminex A9 11-μm cation exchange resin (BioRad Labs., Hemel Hempstead, UK) and measured 270 mm × 4 mm I.D. The resin was equilibrated in 0.2 *M* sodium citrate buffer, pH 2.85, and after application of the sample it was eluted using the programme given in Table I.

Calculation of cross-link content. (1) Collagen. The cross-link peaks were verified by comparison with authenticated cross-link standards and were expressed as nmol/mg of collagen using the leucine equivalent values shown in Table II. The amount of collagen in the tissue was determined by hydroxyproline assay [15] of the acid hydrolysate, prior to chromatography on the CF1 column, using a continuous-flow autoanalyser (Cam-Lab, Cambridge, UK).

(2) Elastin. The elastin cross-links were determined as nm of cross-link per mg of tissue.

Reproducibility. The reproducibility of the measured concentrations and retention times are shown in Table III and the linearity of response to ninhydrin in Fig. 1 and the range in Table IV. Based on the figures of Tables III and IV it is

TABLE II

LEUCINE EQUIVALENCE FACTORS USED IN THE CALCULATION OF COLLAGEN AND ELASTIN CROSS-LINKS

Cross-link	Leucine equivalence factor
Hydroxylysinonorleucine (HLNL)	1.8
Dihydroxylysinonorleucine (DHLNL)	1.8
Hydroxylysylpyridinoline (HYL-PYR)	1.7
Lysylpyridinoline (LYS-PYR)	1.7
Histidinohydroxylysinonorleucine (HHL)	1.97
Desmosine (DES)	3.4
Isodesmosine (I-DES)	3.4

TABLE III

REPRODUCIBILITY OF THE MEASURED CONCENTRATION AND RETENTION TIMES

Replicate analyses were performed on a mixture of cross-link standards.

Cross-link	Concentration[a] (nmol)	C.V. (%)	Retention time[b] (min)	C.V. (%)
HYL-PYR	3.97 ± 0.027	1.51	25.09 ± 0.087	1.56
LYS-PYR	0.16 ± 0.002	3.13	26.48 ± 0.103	1.75
Isodesmosine	0.95 ± 0.006	1.37	33.85 ± 0.087	1.15
HHL	1.13 ± 0.006	1.17	35.06 ± 0.193	1.69
Desmosine	1.17 ± 0.005	1.04	36.60 ± 0.084	1.03
HLKNL	3.09 ± 0.016	1.15	38.98 ± 0.075	0.86
HLNL	4.56 ± 0.034	1.65	43.85 ± 0.089	0.91

[a] Results were obtained from five successive analysis of the cross-link mixture and the concentration is expressed as the mean ± standard error of the mean (S.E.M.).

[b] Results were based on twenty analyses and the retention time is expressed as the mean ± S.E.M.

possible to perform cross-link analyses on 2 mg of collagen and obtain peaks which lie within this linear range. This assumes an average cross-link content of 0.4 mol of cross-link per mol of collagen, though there are of course tissue, age and species variations; 20–30 mg of dry tissue is usually sufficient to provide enough material for triplicate analyses.

RESULTS AND DISCUSSION

The complete separation of all the known cross-links to allow integration of the individual

TABLE IV

LINEARITY OF RESPONSE TO NINHYDRIN

Each cross-link was analysed across a range of concentrations to determine the linearity of colour development with ninhydrin.

Cross-link	Linear range (nmol)
HYL-PYR	0.5–3.8
LYS-PYR[a]	0.04–0.15
HHL	0.3–2.2
HLKNL	0.3–3.0
HLNL	0.6–4.7
Isodesmosine	0.1–0.9
Desmosine	0.1–1.1

[a] A paucity of this cross-link prevented measurement across a wider range of concentration.

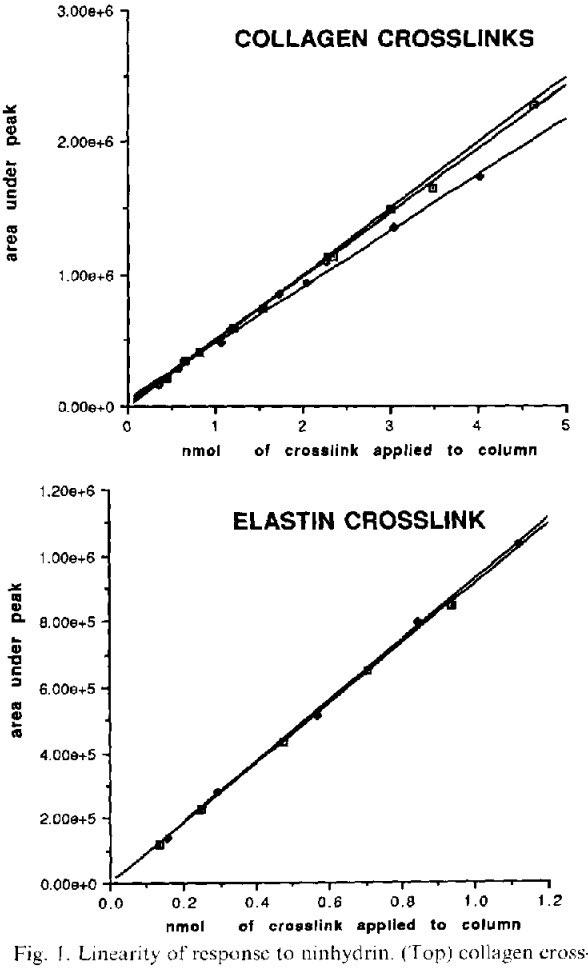

Fig. 1. Linearity of response to ninhydrin. (Top) collagen cross-links (□ = HLNL; ◆ = HYL-PYR; ■ = HLKNL; ◇ = HHL); (bottom) elastin cross-links (□ = isodesmosine; ◆ = desmosine.

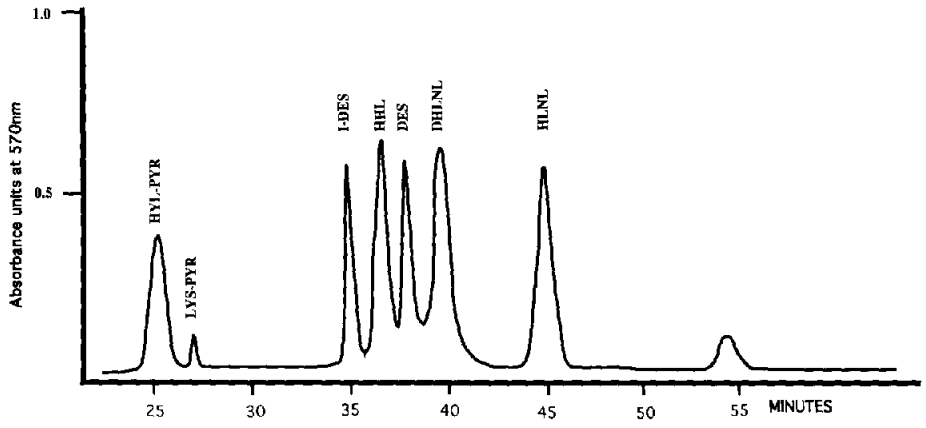

Fig. 2. Typical elution profile of cross-linking amino acids from collagen and elastin. For peak identification, see Table II.

peaks has been achieved on a single ion-exchange column (LKB 440 amino acid analyser) using the modified gradient system described in Table I. The use of the CF1-cellulose columns, in addition to removing the standard amino acids and permitting higher loadings of the cross-links, also removed the hexosyl-lysines which elute in this area of the chromatogram, thereby allowing resolution of HHL in older tissues where the hexosyl-lysine are also high [1]. A typical elution profile using authentic cross-linking amino acids is shown in Fig. 2. The difficulty of overlapping peaks has been overcome, particularly that of HHL and desmosine.

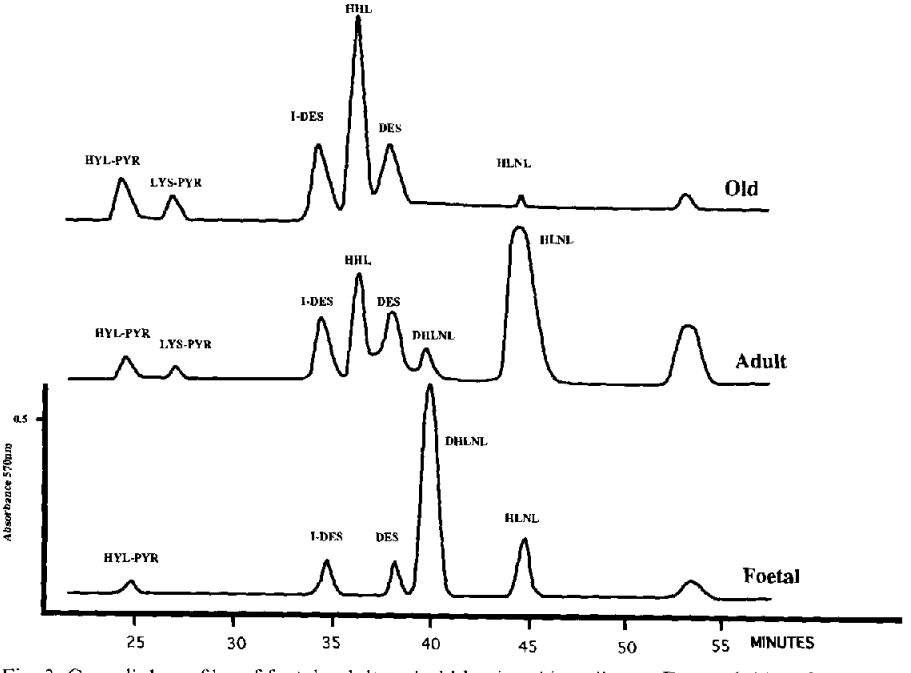

Fig. 3. Cross-link profiles of foetal, adult and old bovine skin collagen. For peak identification, see Table II.

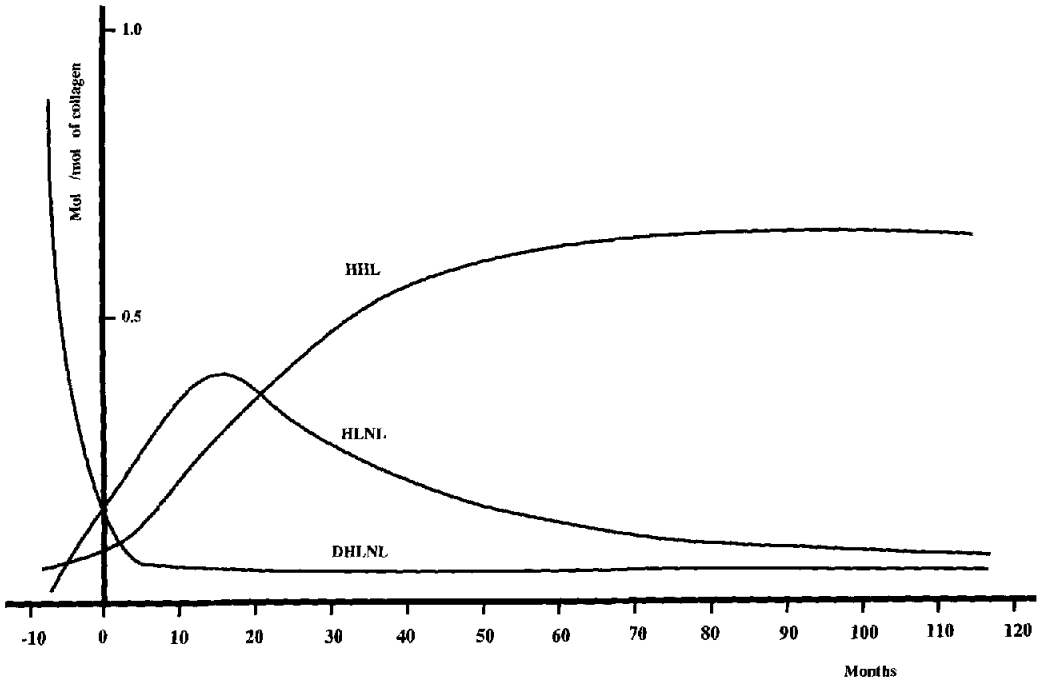

Fig. 4. Change in cross-link content in bovine skin with age. For abbreviations, see Table II.

We illustrate the ability of the system to resolve both the intermediate and mature cross-links by demonstrating the change in cross-link profile with age by comparison of foetal, adult and old bovine skin collagen. The early conversion of HLKNL in foetal skin to pyridinoline and the

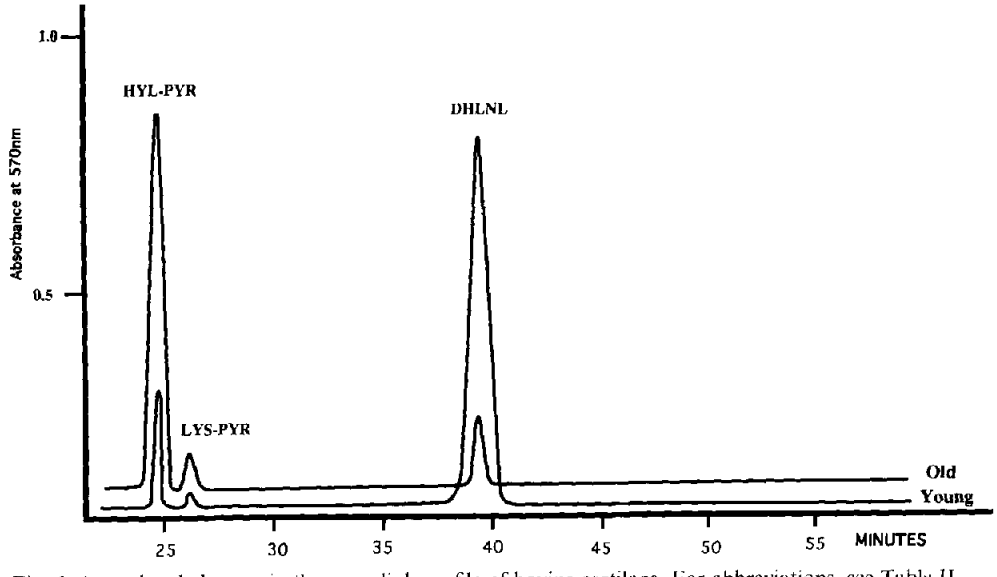

Fig. 5. Age-related changes in the cross-link profile of bovine cartilage. For abbreviations, see Table II.

subsequent rise of deH-HLNL in the adult and its conversion to HHL during maturation is clearly demonstrated (Fig. 3). The rate of this conversion is shown in Fig. 4.

Despite the small amount of elastin in bovine skin the desmosine and isodesmosine peaks are clearly resolved. The quantitation of these desmosine peaks can provide an estimate of the amount of elastin present in the tissue, assuming a desmosine content of 2.7%.

The pyridinoline peaks are the predominant cross-link components of bone and cartilage. The change in cross-links with age in bone and cartilage has previously been reported [7] using HPLC and fluorescent detection together with amino acid analysis, and we illustrate similar changes using our modified gradient on a single system (Fig. 5).

The method described can be used on any amino acid analyser and avoids the need for additional HPLC systems and alternative detection systems when determining the total cross-link content, *i.e.* intermediate and mature cross-links of normal and pathological tissues.

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