A new rapid method for the identification of reducible collagen cross-links in small tissue samples

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A normal-phase high-pressure liquid-chromatography system was used with amino-propyl-bonded silica as the column packing in order to resolve amino acids, reduced amino acid derivatives and reduced cross-links of collagen. The method utilized a solvent system comprising acetonitrile and 10 mM-potassium phosphate buffer, pH 4.3, as described by Schuster [(1980) *Anal. Chem.* **52**, 617–620]. With modifications to the original gradient elution it was possible to resolve fully the radiolabelled components of reduced collagen in one run of less than 80 min. The method is very sensitive, and small biopsy samples can readily be investigated. Although solute retention times gradually decreased with repeated use, little loss of resolution of the reducible cross-links of collagen occurred during a 30-day trial period.

The inherent strength of the newly formed collagen fibre is due to the presence of covalent intermolecular cross-links formed between lysine or hydroxylysine aldehydes at the non-helical terminal ends of one molecule and hydroxylysine residues at two helical sites in a second molecule (Piez, 1976; Light & Bailey, 1980). The two Schiff bases so formed are the aldimine hydroxylysinonorleucine and the Amadori-rearranged keto-imine hydroxylysino-5-oxonorleucine. The structures of both compounds have been long established, but during maturation the relative amounts of these cross-links, assayed as their tritiated reduced forms, decrease as the content of the unknown 'stable' non-reducible cross-links increases (Robins *et al.*, 1973).

Although a great deal of work has been carried out on the nature, distribution and extent of the reducible cross-links in many tissues (for reviews see Light & Bailey, 1980, 1982; Tanzer, 1976), the analysis of these compounds involves lengthy procedures and requires reasonably large samples. Analyses are generally performed by using the volatile buffer ion-exchange system described by Bailey and co-workers (for review see Light & Bailey, 1982), by citrate buffer ion-exchange chromatography, or by a combination of the two methods. Both techniques, although reliable, are extremely time-consuming and cannot be used for the analysis of small amounts of material. In such

cases losses and high background radioactivity make unequivocal assignment of peak identity and quantification impossible.

In the present paper we describe a completely new method for resolution and identification of collagen cross-links in small tissue samples. This method utilizes a simple and rapid h.p.l.c. procedure.

Materials and methods

Materials

H.p.l.c.-grade acetonitrile and methanol were obtained from Rathburn Chemicals (Walkerburn, Peeblesshire, Scotland, U.K.). All other chemicals were of AnalaR quality from BDH Chemicals (Poole, Dorset, U.K.) and all solvents were filtered and degassed at room temperature before use. Water was glass-distilled and filtered. KB^3H_4 was obtained from Amersham International (Amersham, Bucks., U.K.) with a specific radioactivity of 606 mCi/mmol and was diluted with solid KBH_4 to a final radioactivity of 1 mCi/3 mg. [^{14}C]Proline and [^3H]lysine were from the same source.

Preparation of reduced collagen and tissue samples

Samples (young bovine skin, bone and tendon, 8-month human bone, rat tail tendon and rat skin) were finely chopped and washed with phosphate-buffered saline (0.15 M-NaCl/20 mM-sodium phosphate buffer, pH 7.4) at 4°C before reduction with K^3BH_4 in the same buffer at room temperature for 1 h (100:1 wet weight of sample/borohydride). The reaction was stopped by the addition of 100% acetic

Abbreviations used: h.p.l.c., high-pressure liquid chromatography; HLNL, hydroxylysinonorleucine; DHLNL, dihydroxylysinonorleucine.

acid to give pH 3.0, and samples were washed in or dialysed against distilled water until free of soluble radioactivity. Samples were hydrolysed in 6 M-HCl at 110°C for 24 h under air, and were rotary-evaporated to dryness twice from water before being taken up in water. Hydrolysates were neutralized with either aq. NH_3 or NaOH before h.p.l.c.

Isolation and characterization of reduced collagen components

The reducible collagen components investigated in the present study were 6-hydroxynorleucine, 5,6-dihydroxynorleucine, the reduced aldol, chlor-norleucine, hexosyl-lysine, hexosylhydroxylysine, hydroxylysinonorleucine (HLNL), dihydroxylysinonorleucine (DHLNL) and histidinohydroxymerodesmosine. All of these compounds were isolated from acid hydrolysates of reduced collagen and purified by ion-exchange chromatography in volatile pyridine/formate buffers as previously described (for review see Light & Bailey, 1982). Samples were desalted into water on 90 cm $\times$ 1.6 cm columns of Sephadex G-10 (Pharmacia, Uppsala, Sweden) before h.p.l.c. These columns were standardized with a mixture containing tritiated hydroxylysinonorleucine (M_r 292), [^3H]lysine (M_r 147), pyridine (M_r 79) and tritiated water. Compounds prepared in this way were stored frozen at -20°C until use. Purified preparations were subjected to a second ion-exchange chromatography in citrate buffers on a 67 cm $\times$ 0.9 cm column of Beckman PA 28 resin run on a JEOL JLC-6AH amino acid analyser at 60°C (Light & Bailey, 1982), to confirm their identity.

H.p.l.c.

H.p.l.c. was performed on a Du Pont Series 8800 liquid chromatograph comprising a programmer, heated column compartment, u.v. spectrophotometer and pump. Detection of standard amino acids was at 205 nm, and chromatographs were recorded on a Spectrophysics SP4100 computing integrator.

Initial separations were performed on 25 cm $\times$ 0.46 cm columns of Du Pont Zorbax NH_2 (10 μm) at a temperature of 35°C and an eluent flow rate of 2 ml/min. Column life was considerably lengthened by use of a Du Pont ETH precolumn and a Du Pont Zipax SAX guard column in the solvent line. Later work was carried out on 3 cm $\times$ 0.46 cm columns of Zorbax NH_2 supplied by Technicol (Manchester, U.K.).

The gradient system comprised two solvents. Solvent *A* contained 10 mM-potassium phosphate buffer, pH 4.3, and solvent *B* contained h.p.l.c.-grade acetonitrile diluted 50:7 (v/v) with water. Amino acids could be separated by using a gradient programme similar to that of Schuster (1980), but

the programme was modified for separation of reduced collagen components.

Programme 1 (for amino acids) was as follows: 1, 95% solvent *B* for 5 min; 2, linear gradient 95%–70% solvent *B* over 15 min; 3, linear gradient 70%–50% solvent *B* over 15 min; 4, 50% solvent *B* for 10 min; 5, linear gradient 50%–95% solvent *B* over 5 min.

Programme 2 (for reducible collagen compounds) was as follows: 1, 80% solvent *B* for 15 min; 2, linear gradient 80%–60% solvent *B* over 40 min; 3, 60% solvent *B* for 10 min; 4, linear gradient 60%–50% solvent *B* over 6 min; 5, linear gradient 50%–80% solvent *B* over 9 min.

Scintillation counting

Fractions of volume 2 ml were collected during h.p.l.c. These were either added to 10 ml of Packard Instagel (Packard Instrument Co., La Grange, IL, U.S.A.) in 20 ml bottles or were collected directly into 7 ml scintillation-vial inserts, and 4 ml of Instagel was subsequently added. As some quenching by the organic phase was noted, radioactivities of samples with small incorporation of radiolabel often needed to be counted in the larger volume of scintillant. Counting was done in an LKB 1215 Rackbeta II instrument.

Manual quantification of cross-links

DHLNL and HLNL were purified as described above by ion-exchange chromatography in volatile pyridine/formate buffers, gel filtration on Sephadex G-10 columns (95 cm $\times$ 1.6 cm) in water and h.p.l.c. on the 25 cm $\times$ 0.46 cm Zorbax NH_2 columns. Finally, after assessment of purity by amino acid analysis, the two compounds were individually treated with ninhydrin and their molar concentrations calculated from a norleucine standard curve. Known amounts of each cross-link and norleucine were then treated at room temperature with fluorescamine (Pierce and Warriner, Chester, U.K.). Samples (0.2 ml, containing 0.2–1 nmol of amine) were each added to a 0.4 ml volume of 0.4 M-lithium borate, pH 9.3, and thoroughly mixed. Then 0.2 ml of 0.02% fluorescamine in acetone was immediately added to each mixture with mixing, and fluorescence intensity was observed at 475 nm with excitation at 390 nm. Measurements were made with a Perkin-Elmer 3000 fluorescence spectrophotometer. In this way, the molar fluorescence yields for HLNL-fluorescamine and DHLNL-fluorescamine reaction products in terms of norleucine equivalents were calculated.

For quantification of HLNL and DHLNL present in tissues, the hydrolysate of the reduced samples was filtered and approx. 2–4 mg of material in 50 μl of solvent *A* was chromatographed under the conditions stated above on a 25 cm $\times$ 0.46 cm

column of Zorbax NH_2 . HLNL and DHLNL were located by scintillation counting of the radioactivity of up to 1.0 ml of each 2 ml fraction (as described above), and then fluorescamine assay of 0.2 ml of each 2 ml fraction was carried out. Background correction was obtained by subtracting values for fluorescamine reaction with buffer mixtures at the same portion of the gradient collected on a blank run. Absolute amounts of HLNL and DHLNL were then calculated by using a norleucine standard curve.

Results

Separation of amino acids and collagen cross-links

By using a modification of the method of Schuster (1980) we were able to separate rapidly most of the amino acids occurring in collagen. Hydrophobic amino acids were eluted very early from the column, with phenylalanine and leucine in the first peak and isoleucine, tyrosine and methionine in the second. All other amino acids except arginine and cysteine were fully resolved (Fig. 1a). With this gradient system the basic amino acids were eluted late from the column, but it was found that the basic cross-link compounds HLNL and DHLNL were eluted with poor resolution by using this programme (programme 1; see the Materials and methods section), and so we modified the method to achieve good separation of the cross-link compounds. As is shown (Fig. 1b), with this system (programme 2; see the Materials and methods section) excellent resolution of HLNL and DHLNL was obtained. By using a combination of both programmes it was possible to retain separation of amino acids (detected by u.v. scanning) as well as cross-link compounds. However, in order to optimize the system for cross-link assays, we adopted programme 2.

Resolution of reduced components of collagen by h.p.l.c. on 25 cm columns of aminopropyl-silica

In an initial experiment to determine if HLNL and DHLNL co-migrated with any other radiolabelled components, crude preparations of reduced tissue were used. A sample of reduced rat tail tendon gave on h.p.l.c. analysis a single, clearly defined, peak eluted at 50–53 min. When this peak was re-chromatographed on the 67 cm Beckman citrate ion-exchange column, a single peak identified as HLNL was obtained. No other radioactive peaks were observed in this second chromatography. The elution pattern from the h.p.l.c. column for bone collagen showed a major peak at 55–57 min, which was sharp and well-defined. This component was pooled and re-chromatographed on the Beckman ion-exchange system and was positively identified as DHLNL. Again, this peak was the only radioactive compound present.

Fig. 1. Resolution of (a) amino acids and (b) HLNL and DHLNL by h.p.l.c. on a 25 cm $\times$ 0.46 cm analytical Zorbax NH_2 column

Conditions were 35°C and elution at 2 ml/min with gradient programme 1 (a) or 2 (b). For experimental details see the text. In (a) amino acids are identified by numbers: 1, phenylalanine; 2, leucine; 3, isoleucine; 4, methionine; 5, tyrosine; 6, valine; 7, proline; 8, alanine; 9, hydroxyproline; 10, threonine; 11, glycine; 12, serine; 13, histidine; 14, cysteine; 15, arginine; 16, lysine; 17, hydroxylysine; 18, glutamic acid; 19, aspartic acid. —, Relative absorbance at 205 nm; ----, concn. of solvent B. In (b) the bulk of standard amino acids were eluted in 20 min. —, Relative absorbance at 205 nm; ----, radioactivity.

Table 1 contains results obtained from repeated runs of pure preparations of all of the different reduced collagen components investigated. Data are shown for two separate Zorbax NH_2 columns. We

Table 1. Retention times of purified reduced amino acid components from collagen

For experimental details see the text. Data from two separate Zorbax NH₂ columns is shown. Retention times are shown for each component obtained in different runs on different days. Abbreviations used are as follows: 6-HNL, 6-hydroxynorleucine; LNL, lysinonorleucine; A, reduced aldol; CNL, chloronorleucine; AHL, the acid produced anhydride derivative of hexosyl-lysine and hexosylhydroxy-lysine; HL, hexosyl-lysine; HHL, hexosylhydroxy-lysine; HHMD, histidinohydroxymeresmosine.

Component	Retention time (min)	
	Column A	Column B
6-HNL	—	6
LNL	—	18, 18
A	27, 27	26, 26
CNL	—	36
AHL	—	39, 40
HL	38	40
HHL	40, 42	42, 42
HLNL	54, 54	45, 48, 47, 45
DHLNL	61, 59	50, 52, 53
HHMD	65, 64	56, 59.5, 54

noted that retention times of components that were eluted late varied on the different columns, but this was readily compensated for, since each new column was standardized with known pure compounds. It should be noted that, although there was some degree of variation in retention time for all components, no decrease in the resolution of any two compounds on different columns was observed.

H.p.l.c. of reduced collagen compounds from whole tissue

Fig. 2 compares the elution profile of an acid hydrolysate of reduced 4-day-old bovine achilles tendon on the standard pyridine/formate ion-exchange system (Fig. 2*b*) with the separation achieved on the new h.p.l.c. system (Fig. 2*a*). Peaks are identified as indicated. As can be seen, the overall separation of reduced cross-links is as good on the h.p.l.c. system as that obtained by using the pyridine/formate method. In addition, from the start of each run to re-equilibration the h.p.l.c. method is 10 times quicker than its ion-exchange counterpart.

The Zorbax NH₂ column was also found to be more sensitive in the analysis of small biopsy samples, as peaks representing cross-link components containing very small amounts of radioactivity could be analysed. Fig. 3(*a*) shows an analysis of old bovine bone in which the content of reducible components is known to be extremely low, yet we could resolve and identify peaks of HLNL and DHLNL with peak heights of only 300–400 c.p.m. About 5–10 times more material would have had to

Table 2. Retention times for amino acids on aminopropyl-bonded silica columns of 25 cm and 3 cm lengths

For experimental details see the text. Mean retention times were calculated from results obtained from two to five runs for each amino acid.

Amino acid	Mean retention time (min)	
	3 cm column	25 cm column
Phe	1.67	8.5
Leu	1.92	9.5
Ile	1.96	11.0
Met	2.19	11.5
Tyr	2.26	11.8
Val	2.95	13.8
Pro	3.23	16.1
Thr	4.97	19.3
Ala	5.1	17.6
Hyp	4.67	18.2
Gly	7.45	19.6
Ser	8.37	20.9
His	14.33	25.4
Glu	16.55	31.7
Asp	17.31	35.0
Arg	18.06	25.0
Cys	17.91	25.0
Lys	20.23	26.4
Hyl	21.0	27.5

be used in pyridine/formate ion-exchange analyses. Similarly, Fig. 3(*b*) shows a separation of HLNL and DHLNL by h.p.l.c. from a reduced 3-month human skin biopsy. A 12 mg (wet wt.) portion of the sample was reduced as described with tritiated KBH₄, and one-third of the acid hydrolysate of the sample was used for chromatography. As can be seen, clear peaks for HLNL and DHLNL were obtained by the new h.p.l.c. method. A much less clear profile was obtained by means of the pyridine/formate ion-exchange system, and peak designation was found to be impossible with such a small sample on this system.

H.p.l.c. of amino acids and reduced collagen compounds on the 3 cm aminopropyl-silica column

Table 2 shows a comparison of retention times of all the standard collagen amino acids after elution from both long (25 cm) and short (3 cm) aminopropyl-silica columns. In both cases the column packing was Zorbax NH₂. The order of elution of the amino acids (except glutamic acid and aspartic acid) was the same on both columns, and it is clear that the decrease in column length had not significantly altered the fundamental aspects of the chromatography.

Furthermore, resolution of reduced collagen cross-links was maintained on the 3 cm column (Fig. 4) when compared with the 25 cm analytical-size column. HLNL and DHLNL were eluted in the

Fig. 2. *H.p.l.c. of an acid hydrolysate of reduced 4-day bovine tendon*

(a) Resolution of radioactive compounds in an acid hydrolysate of reduced 4-day bovine tendon by h.p.l.c. on a 25 cm $\times$ 0.46 cm Zorbax NH_2 column. For experimental details see the text. —, Radioactivity; ----, concn. of solvent B. (b) Resolution of radioactive compounds from the same sample on a 70 cm $\times$ 1 cm column of Zerolit 225 in pyridine/formate buffers at 60°C. For details of the method see Light & Bailey (1982). Abbreviations are as defined in Table 1 legend.

same order in 37 and 43 min respectively at 2 ml/min flow rate, compared with 45–50 min and 50–55 min respectively for the 25 cm column at the same flow rate.

Column deterioration

It has been reported that aminopropyl bondings deteriorate with use (Verhaar & Kuster, 1981; Smolenski, 1983). We followed this deterioration quantitatively by recording the retention times of serine and alanine and HLNL and DHLNL at various times in the life of each column. Fig. 5 shows the results obtained for serine and alanine on two long columns, and Table 3 summarizes the changes in specific column characteristics with alanine, serine, HLNL and DHLNL as solutes. Although the

resolution time between alanine and serine decreased markedly and, over 21 days, the capacity factor for serine (k'_{ser}) almost halved, we noted little change in resolution of HLNL and DHLNL over 30 days' use. Most columns could be used for up to 3 months before unacceptable loss in resolution of the cross-link compounds was noted.

The rate of deterioration appeared to be dependent on the number of runs performed and particularly on the total length of time the bonded phase was exposed to high concentrations of polar solvent. We found that our initial procedure of washing columns in 100% inorganic phase (i.e. 0% solvent B) led to faster deterioration than when a 50% solvent B concentration was used for the column washing step.

Fig. 3. H.p.l.c. of samples containing small amounts of radioactivity

(a) H.p.l.c. on a 25 cm $\times$ 0.46 cm Zorbax NH_2 column of radioactive compounds from an acid hydrolysate of reduced old bovine bone. For experimental details see the text. (b) H.p.l.c. of an acid hydrolysate of 4 mg of reduced 3-month human skin on a 25 cm $\times$ 0.46 cm Zorbax NH_2 column. For experimental details see the text.

Quantification of cross-links by manual fluorescamine assay

By using norleucine equivalents of 1.75 and 1.84 for HLNL and DHLNL respectively (Robins *et al.*, 1973) we calculated the amount of each cross-link in standard preparations from ninhydrin assays. We were then able to calculate a similar norleucine equivalent value for both compounds when the fluorescamine assay procedure was used. Values of 0.86 and 0.93 were obtained for HLNL and DHLNL respectively, and these were used for calculation of the amount of each cross-link in unknown mixtures. By using these methods we were able to show that the yield of HLNL and DHLNL from Zorbax NH_2 columns was $95.1 \pm 4.4\%$ by fluorescence assays and $82.3 \pm 4.8\%$ by quantifying radioactivity. It should be noted that no significant

Fig. 4. H.p.l.c. of HLNL and DHLNL on a 3 cm Zorbax NH_2 column

—, Elution profile for a mixture of HLNL and DHLNL (4:1, by radioactivity). Column conditions and gradient elution were as indicated in the text. ----, Elution profile for a mixture of $[^{14}\text{C}]$ proline and $[^3\text{H}]$ lysine from the same column for reference.

Table 3. Deterioration of retention time, capacity factors and resolution of alanine, serine, HLNL and DHLNL. For experimental details see the text. HLNL and DHLNL, chromatographed as a mixture, were resolved on different days with slightly different programmes, and so retention times are not shown. However, it can clearly be seen that the resolution (α) of the two cross-link compounds did not seriously decrease over a 30-day period of constant column use.

Day no.	Retention time (min)				α				α	
	t_{Ala}	t_{Ser}	k'_{Ala}	k'_{Ser}	$\left(\frac{k'_{\text{Ser}}}{k'_{\text{Ala}}}\right)$	k'_{HLNL}	k'_{DHLNL}	$\left(\frac{k'_{\text{DHLNL}}}{k'_{\text{HLNL}}}\right)$	$\left(\frac{k'_{\text{DHLNL}}}{k'_{\text{HLNL}}}\right)$	$\left(\frac{k'_{\text{DHLNL}}}{k'_{\text{HLNL}}}\right)$
1	6.95	10.65	5.60	9.10	1.63	31.4	34.2	1.09		
5	6.35	8.60	5.05	7.24	1.41	—	—	—		
8	5.70	7.10	4.43	5.76	1.30	—	—	—		
9	5.85	7.30	4.50	5.95	1.32	38.1	41.9	1.10		
22	5.85	6.90	4.57	5.59	1.24	—	—	—		
30	—	—	—	—	—	59.0	62.8	1.06

Fig. 5. Deterioration in the resolution of alanine and serine on two 25 cm $\times$ 0.46 cm Zorbax NH_2 columns. The varying resolution times (i.e. time between peaks) for alanine and serine on column 1 (—) and on column 2 (---) are shown. For experimental details see the text.

difference in quench characteristics over the range of the phosphate gradient was observed.

Discussion

We have developed a simple, rapid and effective method for analysis of radiolabelled reduced collagen cross-links by h.p.l.c. Radioactive compounds eluted from the h.p.l.c. columns were collected and their identity was verified by re-chromatography on an ion-exchange column developed in citrate buffers. In this way we were able to show that both of the reducible cross-links of collagen could be resolved from one another and that no other radiolabelled compound co-migrated with either component. Chromatography of total acid hydrolysates of borohydride-reduced whole-tissue samples showed clear resolution of both reduced cross-link compounds in less than 60 min.

In the past it has been impossible to measure the cross-link content of very small tissue samples, such as punch biopsies of skin, by using the classical methods. Our present results show that even very small biopsies may be analysed by this h.p.l.c. technique. Samples containing 1.5–3 mg dry wt. of collagen (i.e. approx. 12–20 mg wet wt. of tissue) were reduced with tritiated borohydride and hydrolysed. An equivalent of 0.8 mg of hydrolysed collagen could be analysed by our h.p.l.c. technique (i.e. loading 2 mg of tissue hydrolysate). Such small amounts of material could not be handled by the larger-scale and less-sensitive ion-exchange methods. Recovery of radioactivity from aminopropyl-silica columns was similar to that seen with the pyridine/formate ion-exchange method. Typically, most preparations showed between 82% and 90% recovery.

The short 3 cm columns that we investigated produced a resolution of both purified reduced cross-link components and mixtures in whole-tissue hydrolysates equivalent to that of their 25 cm-column counterparts. Cross-link compounds were eluted in 45 min, shortening the overall run time to some 17 times less than the classical ion-exchange method while using less solvent than needed for 25 cm-column runs. These mini-columns would have an application if used infrequently, as their working life was found to be somewhat shorter than that of the 25 cm columns (approx. 3–4 weeks' use).

Disadvantages of the new h.p.l.c. system were that resolution of some minor reduced components was slightly less acceptable than that obtained with the pyridine/formate ion-exchange system. Also, columns deteriorated with extensive use over a period of 1–3 months, eventually resulting in an unacceptable decrease in resolution of components.

With respect to this latter criticism, we found that the apparent constant loss of amine groups from the columns resulted in a high background when fractions were assayed with the fluorescent amine reagent fluorescamine. The resulting loss of sensitivity meant that small amounts of cross-link compounds (<20 nmol) could not be reproducibly quantified after h.p.l.c. resolution on the current system. If the amine derivatives could be stabilized on the silica support, this would result in a longer life-expectancy for columns and the elimination of the high background of the eluent. This would mean that columns could be reliably used with a post-column detection system to allow accurate quantification of the amounts of cross-link compounds present in tissues. Further investigations are required with different types of amine-derived silica supports with a view to modifying the system to allow such quantifications to be made.

The advantages of the new method are, firstly, that the qualitative analysis of the reducible cross-

links of collagen present in very small biopsy samples could readily be performed by using this technique, as so little material was needed for each run. Secondly, the reduced cross-links, as well as most of the other radiolabelled collagen components, could be resolved in a single extremely fast chromatographic procedure. The method takes less than one-tenth the time of the classical pyridine/formate ion-exchange method. Finally, the solvent system is u.v.-transparent, and so standard amino acids can readily be detected and quantified spectrophotometrically at 205 nm.

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