Quantitative Determination of Collagen Crosslinks

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1. Introduction

The primary functional role of collagen is as a supporting tissue and it is now well established that the aggregated forms of the collagen monomers are stabilized to provide mechanical strength by a series of intermolecular crosslinks. These links are formed by oxidative deamination of the ε-amino group of the single lysine in the amino and carboxy-telopeptides by lysyl oxidase. The aldehyde thus formed reacts with an ε-amino group of a lysine at a specific point in the triple helix because of the quarter-staggered end-overlap alignment of the molecules in the fibers. The chemistry of these crosslinks is dependent on both the nature and age of the collagenous tissue (1,2). Differences in the crosslinks are because of the degree of hydroxylation of both the telopeptide and the specific lysine in the triple helix. Thus, the amounts of intermediate crosslinks present in immature tissue, dehydro-hydroxylysino-norleucine (Δ-HLNL), and hydroxylysino-keto-norleucine (HLKNL) may vary considerably between tissues, e.g., rat tail tendon and skin contain Δ-HLNL whereas cartilage and bone contain predominantly HLKNL.

These divalent crosslinks are only intermediates and are subsequently converted into stable trivalent crosslinks that accumulate in the tissue as collagen turnover decreases during maturation (2). The Schiff base aldimine Δ-HLNL is stabilized by reaction with histidine to form the trivalent crosslink, histidino-hydroxylysino-norleucine (HHL). The keto-imine HLKNL, on the other hand, reacts with a second hydroxylysyl aldehyde to form the pyridine derivatives, hydroxylysyl-pyridinoline (Hyl-Pyr), and lysyl-pyridinoline (Lys-Pyr), Fig. 1. The proportion of these three known mature crosslinks, again varies with age and with the type of tissue. For example, HHL is the major, known, mature crosslink of human and bovine skin (3), whereas the pyridinolines are the
Fig. 1. Schematic representation of the formation of collagen crosslinks. (A) The divalent Schiff base (aldimine) dehydro-hydroxylysinoonorleucine (Δ-HLNL) which subsequently reacts with histidine to give the trivalent crosslink histidinohydroxylysinoonorleucine. (B) The intermediate keto-imine crosslink (hydroxy-lysino-ketonorleucine; HLKNL) can react with hydroxylsine aldehyde to give hydroxylsyl-pyridinoline (Hyl-Pyr) or with lysine aldehyde to give the hydroxylsyl-pyrrole.
major, mature crosslinks of bone and cartilage (4); tendon, however, contains a mixture of both mature crosslinks. An additional mature crosslink may be formed if the keto-imine reacts with a lysine-aldehyde in which case a pyrrole structure is favored (see Fig. 1). Although the structure of the so-called “pyrrole” crosslink has not been confirmed there is mounting evidence for its presence in bone and tendon collagen (2). An accurate determination of the ratio of the immature to mature crosslinks provides a valuable indication of the degree of turnover of a collagenous tissue. It is, therefore, important in any study of changes to the collagen in a pathological tissue to understand the nature of the normal, age-related changes that occur in the particular tissue under investigation.

The intermediate crosslinks may be radio-labeled by reduction of the tissue with tritiated sodium borohydride, thus facilitating their location and identification during subsequent chromatography (5). However, their quantification requires either ninhydrin, or a similar post-column derivatization technique, following their separation from the acid hydrolysate of the tissue by ion-exchange chromatography. Precolumn derivatization of these polyvalent crosslinks for subsequent analysis by reversed-phase high performance liquid chromatography (HPLC) can produce multiple derivatives that elute as separate peaks throughout the subsequent analysis and is therefore not recommended. The mature crosslinks HHL, Hyl-Pyr, and Lys-Pyr can be simultaneously quantified using the same ion-exchange column (6). Hyl-Pyr and Lys-Pyr can also be determined, with greater sensitivity, by HPLC utilizing their natural fluorescence to facilitate their detection and quantification (7). It has not yet been possible to analyze the pyrrole crosslink by ion exchange or HPLC chromatography and until this is possible a rather nonspecific colorimetric method is employed (8).

The other major connective tissue protein, elastin, is also stabilized by lysine-derived crosslinks based on the same enzymic mechanism, but yields two tetravalent pyridine compounds, desmosine (DES) and iso-desmosine (I-DES) (10). Both of these compounds can be detected by ninhydrin after elution, under the same conditions, from the same ion-exchange column.

A second crosslinking mechanism occurs when the turnover of collagenous tissues decreases following maturation and involves the reaction of glucose with the ε-amino group of lysine and subsequent oxidation reactions (9). Generally known as glycation, the addition of glucose is nonenzymic, adventitious, and possibly random. Crosslinks formed by this mechanism, such as pentosidine, could provide good biomarkers of low metabolism and possible damage to the functional properties of collagen during aging and in diabetic subjects. However, to date, none of the glycation crosslinks has been related to changes in the functional properties of collagen, hence, we have only considered pentosidine.
2. Materials

Unless stated otherwise, all reagents should be of Analar grade.

1. Sodium borohydride should be dissolved in 0.01 \( M \) sodium hydroxide solution at 5\(^\circ\)C immediately prior to use. The dry solid is deliquescent producing an explosive gas (hydrogen) when wet, consequently, care with storage and handling is essential.

2. The hydrochloric acid used for protein hydrolysis is a constant boiling mixture. This can be purchased commercially (BDH, Poole, UK) or prepared in the laboratory by distillation of a 50\% mixture of hydrochloric acid with distilled water and collecting the distillate that separates at 110\(^\circ\)C.

3. Fibrous cellulose, CF-1, is a commercially available product from Whatman (Maidstone, Kent, UK).

4. Filters, for sample preparation, and for both HPLC and amino acid analyzer buffer filtration, are commercially available (HPLC Technology, Macclesfield, UK). 4 mm or 13 mm PVDF syringe filters are used for sample filtration.

5. A steel ‘mortar and pestle’. The ‘mortar’ consists of a cylindrical block of stainless steel (40 mm \( \times \) 40 mm) with a flat-bottomed 10-mm diameter hole drilled into it to a depth of 30 mm. The “pestle” is also made from stainless steel and measures 9.5 mm in diameter and 100 mm in length. These dimensions provide a close sliding fit into the mortar.

6. A source of liquid nitrogen.

7. An amino acid analyzer equipped with a post-column ninhydrin detection system.

8. A high performance liquid chromatography (HPLC) system linked to a fluorescence detector.

9. Ideally, both of the above should be equipped with computer-based chromatography data handling software or a computing integrator.

10. The reagents and buffers for use with the amino acid analyzer are best purchased from the equipment supplier. Any alteration to the concentration or pH of such buffers should be done with great care and any buffers modified in this way should be passed through a 0.2 \( \mu \)m filter prior to use to remove particulate matter. The buffers should incorporate 0.01\% phenol to prevent bacterial spoilage and should be stored at 15–20\(^\circ\)C.

11. All HPLC reagents need to be HPLC grade and 0.2 \( \mu \)m filtered prior to use.

12. A microtiter plate reader fitted with a 570 nm filter and a number of flat-bottomed 96-well microtiter plates are required for the pyrrole assay.

    The following reagents are also required for measuring the pyrrole crosslink, and can all be obtained from Sigma-Aldrich (Poole, UK).

13. TAPSO Buffer: 2.81 g of 3-[N-tris(hydroxymethyl)methylamino]-2-hydroxypropanesulfonic acid (TAPSO) is dissolved in 80 mL of distilled water, adjusted to pH 8.2 with 1 \( M \) sodium hydroxide and then made up to 100 mL.

14. TPCK/TAPSO enzyme inactivator: 1.6 mg N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) is dissolved in 100 \( \mu \)L of ethanol and then taken up to 5 mL in TAPSO buffer. This solution may be turbid, but the turbidity will be removed later in the assay by filtration.
Collagen Crosslinks

15. TPCK-Trypsin reagent: TPCK is added to trypsin to inactivate any residual chymotrypsin activity, which would otherwise destroy the pyrrole. This reagent should be freshly prepared on the day of use. Trypsin is dissolved in the TPCK inactivator solution (1000 U/200 µL) and left at room temperature for 25 min to inactivate any chymotrypsin.

16. DAB reagent: 500 mg of 4-dimethylaminobenzaldehyde (DAB) is dissolved in 4.4 mL 60% perchloric acid and made up to 10 mL with water. Reagent blank is prepared as above minus the DAB.

17. Pyrrole standards: A standard curve is prepared using 1-methyl pyrrole. This is obtainable as a liquid from Aldrich (Cat no. M7, 880-1). 11.1 µL is made up to 2.5 mL in ethanol from which 20 µL is diluted to 100 mL in TAPSO/TPCK reagent to give a final concentration of 10 µM pyrrole. This stock solution is used to produce a series of solutions in the concentration range 1–5 µM by dilution according to the following table:

<table>
<thead>
<tr>
<th>Pyrrole conc., µmol/L</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stock pyrrole, µL</td>
<td>20</td>
<td>40</td>
<td>60</td>
<td>80</td>
<td>100</td>
</tr>
<tr>
<td>TAPSO/TPCK buffer, µL</td>
<td>180</td>
<td>160</td>
<td>140</td>
<td>120</td>
<td>100</td>
</tr>
</tbody>
</table>

3. Methods (see Note 1)

3.1. Borohydride Reduction of Sample

1. The weighed sample is finely comminuted (see Note 2) and evenly dispersed in a volume of phosphate buffered saline (0.15 M sodium chloride, 0.05 M sodium phosphate pH 7.4) equal to between 5 and 10 times the volume of the sample (see Note 3).

2. A weight of sodium borohydride equal to 1% of the sample wet weight is dissolved in 0.001 M sodium hydroxide at 4°C and this is added to the sample (see Note 4).

3. The temperature of the reduction mixture is raised to approximately 20°C and reduction allowed to proceed for 1 h in a fume hood with occasional stirring. After this period the mixture is acidified to approximately pH 3.0 by addition of glacial acetic acid (pH paper accuracy is sufficient). (See Note 5.)

4. The acidified reducing reagents are now discarded either by filtration or after centrifugation, however, it can often be simply achieved by carefully decanting the reagents. The sample is then washed three times with distilled water in order to remove both the acetic acid and salts from the reduction mixture prior to freeze-drying (see Note 6).

3.2. Hydrolysis of the Sample

1. The weighed, dry sample is placed in a suitable vessel and hydrolysed in a volume of constant boiling hydrochloric acid to give a concentration of approx 5 mg of sample / mL of acid (see Notes 7 and 8).

2. Seal the hydrolysis vessel and heat to 110°C for 24 h (see Note 9).
3. After hydrolysis the sample is allowed to cool, the seal is broken and the sample then brought to between −20°C and −80°C prior to lyophilization to remove all residual 6 N hydrochloric acid (see Note 10).

4. After drying, the sample can be rehydrated in water (usually 0.5 mL) and divided according to the requirements of the subsequent analyses (see Note 11).

3.3. Measurement of Hydroxyproline (see Note 12)

1. A portion of the rehydrated hydrolysate, which is estimated to contain about 16 nmol of hydroxyproline (representing 15 µg of collagen), can be analyzed on the same ion-exchange column as that used for the analysis of the collagen crosslinks (see Subheading 3.6.), but using a different set of elution buffers.

2. The column is equilibrated in 0.2 M sodium citrate buffer pH 2.65 and held at 50°C throughout the run. After the sample has been applied, the column is eluted with 0.2 M sodium citrate buffer pH 3.20.

3. Hydroxyproline elutes early from the column (before aspartic acid) and reacts with ninhydrin to produce a yellow color that can be detected at 440 nm. The column and detection system are calibrated using an external standard consisting of a solution of pure hydroxyproline of known concentration.

4. Collagen is generally considered to contain 14% hydroxyproline by weight, so the collagen content of the sample can now be calculated from the measured hydroxyproline value.

3.4. Preparation of a CF-1 Prefractionation Column (see Note 13)

1. The CF1 cellulose powder (50 g) is first thoroughly wetted with 400 mL distilled water in a 2-L measuring cylinder, to which is subsequently added 400 mL glacial acetic acid, and finally 1600 mL of butan-1-ol. The resulting 2.5 L of thin slurry is shaken carefully to ensure complete mixing and suspension of the cellulose and then left to settle (about 20 min) until the bulk of the cellulose is below the 500 mL mark. The supernatant containing suspended cellulose fines is then poured to waste leaving approximately 600–800 mL of the 4:1:1 organic mixture of butan-1-ol:acetic acid:water containing the bulk of the original 50 g of CF-1. The slurry is now topped up to 1 L with fresh 4:1:1 organic mixture to yield an approximately 5% CF-1 slurry, which is decanted into a screw-cap container and stored at room temperature until required (see Note 14).

2. The prefractionation procedure requires the production of a minicolumn of CF-1. The top of a 3 mL plastic, Pasteur pipet bulb is cut off and the flow from the tip reduced, but not blocked, with glass wool or nonabsorbent cotton wool. The CF1 slurry is poured into the pipet through the cut bulb and the cellulose is allowed to settle, adding more slurry as necessary to produce a settled bed height of 8 cm. The 3-mL graduation mark on the pipet is a useful guide. Care should be taken to avoid fluid-filled cavities in the column bed, as this will adversely affect the chromatographic properties of the column. The newly prepared column should then be conditioned by passing 2 × 3 mL of fresh 4:1:1 eluant through the column. CF-1 columns made in this fashion do not readily dry out, although this should be guarded against.
3.5. **CF-1 Prefractionation**

1. The dried sample is rehydrated in 0.5 mL water followed by 0.5 mL glacial acetic, and finally 2 mL butan-1-ol. It is necessary to ensure thorough mixing of the sample by using a vortex mixer after the addition of each component of the solvent (see Note 15).

2. The 3 mL of sample is applied to the CF-1 column and its containment vessel washed with 2 x 1 mL of fresh 4:1:1 eluant, which is also loaded onto the column after the initial sample load has run to waste.

3. 6 x 3 mL of 4:1:1 eluant are now run through the column resulting in the elution of the bulk of the standard amino acids while the collagen crosslinks remain adsorbed to the cellulose. This portion of the eluate can therefore be discarded.

4. After the 4:1:1 eluant has passed through the column, a collection vessel is placed under the Pasteur pipette and 3 x 3 mL of water passed through the column to desorb the crosslinks from the cellulose. This aqueous eluate should now be taken to dryness (see Note 16).

3.6. **Ion-Exchange Chromatography with Ninhydrin Detection**

1. The freeze-dried aqueous phase eluate from the CF-1 column is reconstituted in 120 µL of 0.01 M hydrochloric acid by thorough vortex mixing of the tube to ensure complete dispersion of the solution around the walls of the vessel.

2. The tube is then centrifuged briefly (30 s) to bring the solution to the bottom of the tube and thus ensure maximum recovery.

3. The sample should be passed through a 4 or 13 mm 0.2-µm PVDF syringe filter to remove particulate matter. The sample is now ready for analysis on the amino acid analyzer (see Note 17).

4. The analytical column used for the analysis is the high resolution column supplied by Pharmacia (Uppsala, Sweden) measuring 270 x 4 mm and filled with their UltroPac 8 resin in the sodium form. The column should be maintained at 90°C throughout the analysis.

5. Prior to application of the sample, the column should be equilibrated in 0.2 M sodium citrate buffer pH 4.25.

6. After the sample has been applied, the column should be eluted with 0.4 M sodium citrate buffer pH 5.25 (see Note 18) for 46 min during which time data are collected (see Note 19). The column is then washed for 6 min in 0.4 M sodium hydroxide and regenerated for 23 min in 0.2 M sodium citrate buffer pH 4.25 when it is ready for running the next sample.

7. At the completion of the run, the area of each peak is computed from the collected data and the concentration of each crosslink is determined by comparison with the peak area of a leucine external standard of known concentration (see Note 20 for a detailed explanation of the calculations).

8. A typical elution profile using authentic collagen crosslinking amino acids is shown in Fig. 2.
3.7. HPLC Techniques

1. After hydrolysis and lyophilization each sample is rehydrated in an acidic, aqueous solution at a concentration of approximately 1 µg of collagen per µL. The HPLC system used for analysis dictates the solvent used for rehydration.

2. This laboratory originally used a 250 × 4.6 mm, 5 µm, octadecyl silane (ODS) column eluted with a 5–35% acetonitrile (MeCN) gradient in water at 1 mL/min over 70 min (0.5%/mL/min) (a modification of (4)). Both aqueous and organic solvents contained 0.05 M heptafluorobutyric acid (HFBA), ion pairing agent. Samples destined for this system were hydrated in 5% or 10% HFBA (see Note 21).

3. Currently, we use a Shandon (Runcorn, UK) Hypercarb S, 100 × 4.6 mm, graphitic carbon column eluted with a 0 – 12% tetrahydrofuran (THF) gradient in water at 1 mL/minute. Both aqueous and organic solvents contain 0.5% trifluoroacetic acid (TFA). Samples destined for analysis by this system are hydrated in 1% TFA (see Note 22).

4. Following rehydration the samples are 0.2 µm filtered into tapered glass sample vials (Chromacol, Welwyn Garden City, UK), sealed and stored at 4°C. An aliquot of up to 90 µL is loaded onto the analytical column via an autosampler or a larger volume can be manually loaded via a Rheodyne valve with a 500 µL sample loop (Anachem, Luton, UK).

5. Prior to use the HPLC buffers are degassed either under vacuum (for 10 min) or by helium sparging (1–2 min).
6. After an 8 min isocratic period in water, a 0–12% THF linear gradient in water is applied over 60 min at a flow rate of 1 mL/min (0.2%/mL/min). Hydroxylysyl and lysylpyridinoline elute at approx 26 and 29 min, respectively, and the glycation crosslink pentosidine elutes at 62 min.

7. The pyridinium crosslinks are detected by means of their natural fluorescence at 405 nm emission after excitation at 295 nm. Pentosidine is also naturally fluorescent but at 385 nm emission after excitation at 335 nm. We program a wavelength shift into our Perkin-Elmer LS-5 fluorimeter (Bucks, UK) to take place after the pyridinolines have eluted. A typical HPLC elution profile of pyridinoline and pentosidine standards is shown in Fig. 3.

8. In this laboratory, data are collected during the analytical run using a computing integrator and stored to disk at the end of the analysis.

9. The area under each peak of interest is calculated as a proportion of that derived from known concentrations of standards prepared within this laboratory or purchased commercially. Where possible, the concentration of the standards should be confirmed by amino acid analysis.

10. Column integrity and fluorimeter efficiency is confirmed by regularly running a standard mixture (every 8–10 samples) and calculating the fluorescence yield per pmol of each fluorophore.

Fig. 3. Relative elution positions of the standards, pyridoxamine, hydroxylysyl-pyridinoline (Hyl-Pyr); lysylpyridinoline (Hyl-Pyr) and the glycation crosslink pentosidine on a Hypercarb S reversed phase HPLC column using fluorescence detection.
11. In addition, pyridoxamine, a commercially available fluorophore not naturally present in protein hydrolysates, can be added to all samples. Fluorescing at the same wavelengths as pyridinoline, but migrating earlier, it acts as an internal fluorescent standard to provide a constant monitor of both column and fluorimeter efficiency.

3.8. Pyrrole Crosslink (see Note 23)

1. Powdered bone or tendon (see Note 2) is placed in a glass universal and repeatedly extracted to remove lipids by gentle agitation in acetone at room temperature until the acetone no longer forms an opaque emulsion when mixed with water.

2. 40–50mg of the bone powder defatted in this way is then suspended in 5 mL of 0.5 M acetic acid, sealed in a tube and left to decalcify for about 2 wk at 4°C. The acid is then decanted and the sample washed with 4 mL distilled water and then combined with 400 µL of TAPSO buffer pH 8.2.

3. The container should be resealed and the sample denatured by heating in an oven or dry-block at 110°C for 35 min, following which it is placed in a shaking water bath and left to equilibrate at 37°C.

4. 200 µL of the trypsin-TPCK solution is added to the sample tube and this digest mixture is shaken gently for 18 h at 37°C.

5. The digest is then centrifuged for 10 min at 10,000 g to remove particulate material.

6. 40 mL of DAB reagent is added to 200 µL of the digest sample and to each of the calibration standards. Similarly, 50 µL of reagent blank is added to further 200 µL of digest, to provide a sample blank. After 10 min the samples and blanks are passed through a 13 mm 0.2 µm filter and 180 µL of each sample, blank, and calibration standard pipeted into the wells of a 96-well microtiter plate.

7. The plate is scanned using a microtiter plate reader set at 570 nm and sample blank values subtracted from the test sample values. A standard curve is prepared from the calibration standard readings by plotting absorbance against concentration of 1-methyl pyrrole and corrected values for the test samples are read off this curve.

4. Notes

1. Before undertaking the collagen crosslink analysis of an extracellular matrix, consideration should be given to the amount of tissue available for the multiple analytical procedures and the likely collagen content of that tissue.

There exists an approximately 10-fold difference in sensitivity between HPLC using fluorescence detection and amino acid analysis using ninhydrin detection in favor of HPLC and, in addition, the HPLC procedure is nondestructive allowing complete recovery of sample for further analysis. However, the intermediate crosslinks cannot be readily quantified by HPLC without prior derivatization, with the previously discussed associated problems of multiple peaks for each component. The advanced glycation end-product pentosidine cannot be quantified by ion-exchange chromatography as it is retained on the column. Ideally both analytical procedures should be adopted, but occasionally samples are so
small, e.g., at biopsy, that the limitations of the HPLC procedure alone have to be accepted. In such a case, reduction of the sample with borohydride and CF1 pre-treatment can be ignored but it will not then be possible to quantify the intermediate crosslinks, deH-HLNL and HLKNL, as these are destroyed by acid hydrolysis. However, the mature crosslinks hydroxylsyl and lysyl pyridinoline, and the advanced glycation end-product pentosidine can all be quantified after hydrolysis without prior reduction.

In summary, amino acid analysis by ion-exchange chromatography using ninhydrin detection can be used to assay the following crosslinks:

- Hyl-Pyr, Lys-Pyr, HHL, DES, and I-DES.
- HLNL and HLKNL can only be assayed after reduction of the sample with borohydride prior to acid hydrolysis.

Reversed-phase HPLC using fluorescence detection can be used to assay Hyl-Pyr, Lys-Pyr, and pentosidine. (None of these crosslinks require prior reduction with borohydride, nor is it essential to use the CF1 clean-up procedure, though its use will remove other fluorescent compounds resulting in a chromatogram, which is easier to interpret.)

2. Various methods of homogenization are available and the one chosen should be appropriate to the tissue under analysis:

- **Skin or Hide.** These should first be cleaned of subcutaneous fat and adhering muscle, and any hair removed with a scalpel or razor blade. The cleaned skin can then be chopped very finely with a blade or alternatively, homogenized in phosphate-buffered saline (PBS) (see Subheading 3.1.). We use a Polytron (Kinematica AG, Lucerne, Switzerland) for this purpose, which works well for most soft tissues, except tendon, which has a tendency to accumulate at the end of the homogenizer probe. Tendon is best treated by being chopped finely with a blade.

- **Bone and cartilage.** These are probably best comminuted in a steel “mortar and pestle” (see Subheading 3.6.) at the temperature of liquid nitrogen. The sample, in a cryothermic container, is frozen in a bath of liquid nitrogen as is the steel mortar and pestle. This usually takes about 5–10 min. After removal from the nitrogen bath the sample is placed in the mortar and the pestle, hammered onto the sample, causing it to shatter. With a suitably sized mortar and pestle, very small samples such as biopsies can be handled in this way with good recovery of the powdered sample.

- **Fatty tissues.** Lipids are readily removed from tissues with a very high fat content by overnight extraction in 3:1 chloroform:methanol at 4°C. The chloroform mixture can then be decanted and the tissue rehydrated by several extractions in PBS.

- **Muscle.** The collagen content of muscle is very low (1–5%), therefore it is necessary to remove the bulk of the myofibrillar proteins prior to analysis. This can be achieved by brief ultrasonic homogenization in Hasselbach-Schneider buffer consisting of 0.6 M potassium chloride, 0.1 M disodium hydrogen phosphate, 0.01 M sodium pyrophosphate, 0.001 M magnesium
chloride, and 0.005 M dithiothreitol according to the method of Avery and Bailey (11). The insoluble collagenous network that remains is recovered by filtration through a 380-µM copper sieve.

3. This volume is not critical, although too much could result in under reduction because of over dilution, and too little could result in the sample being carried out of the reducing reagent by gaseous hydrogen.

4. The volume of hydroxide should be as small as possible (µL) to avoid altering the pH of the sample buffer. The weight of borohydride used for reduction assumes 30% dry matter in the sample and 30% collagen. As a consequence, the proportion of sodium borohydride to collagen will be 1 part to 10 parts of collagen. For convenience, with multiple reductions, the requisite amount of borohydride for all samples can be dissolved in a volume of the ice-cold sodium hydroxide and then appropriate volumes immediately pipetted into each sample as required.

5. At this point, excess sodium borohydride will rapidly release gaseous hydrogen with a potential risk of sample loss. This should be kept in mind when selecting the vessel for the reduction procedure.

6. If the sample is very small, it is better to carry out all the above procedures in a vessel suitable for subsequent acid hydrolysis to avoid loss of sample.

7. The hydrolysis vessel is classically of borosilicate glass and can be reused after cleaning with chromic acid.

8. The ratio of sample to volume of acid is not critical provided the sample concentration does not exceed 10 mg/mL when certain resistant peptide bonds may not be cleaved.

9. It is customary to perform hydrolysis under a barrier of nitrogen gas (elimination of oxygen), however, the presence of oxygen is not known to influence collagen crosslink assays, though certain other amino acids are affected.

10. Even at –80°C the hydrolysate will not be frozen, however, without prior chilling there is a considerable risk of sample loss owing to boiling in the reduced pressure of the dryer. The freeze-drying apparatus must be rigorously defended from attack by hydrochloric acid vapor that will destroy seals, welds, and pump valves very rapidly. We use a glass vapor trap at –110°C to protect the vacuum pump, which itself is of a specialist design to resist corrosion. We also maintain a strict monthly vacuum pump oil-change regime.

11. 50 µg of collagen is sufficient for measurement of crosslinks by HPLC, but for ion-exchange 1 mg of collagen or more is required with a CF-1 prefractonation step. It is important that the CF-1 column is not overloaded, so it is recommended that no more than 30 mg dry weight of sample be run on a CF1 column.

12. The amino acid hydroxyproline is almost unique to collagen; present in mammalian collagen at about 95 residues per 1000, it is used to determine the total collagen content. This determination is crucial to subsequent procedures because the final crosslink quantification is expressed as moles of crosslink per mole of collagen. The most accurate method to use is the ion-exchange column used for crosslink analysis, but using the standard buffer gradient for amino acid analysis as described in the text. However, other analytical techniques are available, for
example, an automated flow analyzer (Burkard Scientific, Uxbridge, UK) based on the method of Grant (12) or a microtiter plate method (13), employing the same chemistry, can also be used for rapid determination of multiple samples.

13. The collagen crosslinks represent about 1 mol per mole of collagen, consequently locating these novel amino acids among the excess of normal amino acids has historically proved difficult. Prefractionation is therefore carried out to enhance the relative proportion of the crosslinking amino acids. The preferred method used in this laboratory is fibrous cellulose although several other methods have been reported with varying success, Sell and Monnier, (14); Dyer et al. (15); Takahashi et al. (16); Avery (17).

14. It is important to record the date of the slurry production as prolonged storage, e.g., longer than 2 mo, gives rise to an aggregated product that is unusable and must be discarded. On long-term storage, the 4:1:1 eluant also tends to separate into two layers and must not be used once this has occurred as the two layers will not remix into a single phase.

15. Rehydration in this fashion prevents the formation of an “oily” residue that occasionally occurs if the samples are hydrated with the 4:1:1 eluant directly.

16. This is best done in a centrifugal evaporator as the sample is then maintained as a small volume at the bottom of the tube. However, if an evaporator is not available, then the sample can be freeze-dried in the following manner. The vessel containing the column effluent is capped and a small hole pierced in the lid. The vessel should then be frozen at an angle of 45° in a –80°C freezer. This minimizes the chance of sample loss resulting from its rising up the container during the freeze-drying process.

17. The technique is based on the use of an automatic amino acid analyzer; we use an AlphaPlus II (Pharmacia) as previously described (20), but the technique can be applied to other amino acid analyzers. The supplier of such equipment obviously provides instructions on its use, so detailed explanations will not be provided here except for information specific to the analysis of crosslinks.

18. This can be prepared by dilution of Pharmacia’s 1.2 M sodium citrate buffer pH 6.45 to a molarity of 0.4 M with water containing 0.1% phenol followed by adjustment to pH 5.25 with concentrated hydrochloric acid.

19. Our laboratory uses the AI-450 data handling software from Dionex UK Ltd. (Camberley, Surrey, UK) (their current version is called ‘PeakNet’), to collect and manipulate the data generated by the amino acid analyzer, although any chromatography data-handling software would do. A simple strip-chart recorder linked to the analyzer would suffice, however, integration of the peak areas would then have to be performed by manual measurement of the peaks or by cutting out and weighing the peaks.

20. The crosslink peaks should be identified by comparison with authenticated crosslink standards and expressed as moles of crosslink per mole of collagen or as the reciprocal of this value, i.e., 1 crosslink molecule every “×” molecules of collagen. The elastin crosslinks Desmosine and iso-Desmosine should be determined as nmols of crosslink per mg of tissue. The following equation is used
to calculate the amount of each collagen crosslink as moles of crosslink/mol of collagen.

\[
A \times RF_{(\text{Leu})} \times V_{(\text{HCl})} \div (V_{(\text{anal})} \times W_{(\text{coll})} \times L \times 3.3)
\]  (1)

\(A\) = Area under the crosslink peak (this value is obtained from the data-handling software or can be determined from a chart recorder connected to the analyzer by measuring the dimensions of the peak and calculating the sum of the peak height and the peak width at half the height).

\(RF_{(\text{Leu})}\) = Response Factor for leucine (obtained from the calibration of the analyzer using an external leucine standard of known concentration) and is calculated as follows:

\[
n\text{nmols of leucine run on the analyzer/measured area under the leucine peak} \tag{2}
\]

\(V_{(\text{HCl})}\) = Volume (in \(\mu\text{L}\)) of 0.01 \(N\) hydrochloric acid used to dissolve the sample after CF-1 chromatography.

\(V_{(\text{anal})}\) = Volume (in \(\text{mL}\)) of this sample solution run on the amino acid analyzer.

\(W_{(\text{coll})}\) = Weight of collagen (in \(\mu\text{g}\)) contained in the sample applied to the CF-1 column (this is calculated from the measured hydroxyproline content of the hydrolyzed sample prior to CF-1).

\(L\) = Ninhydrin Leucine Equivalence value for each crosslink; these are:

- HLNL 1.8; HLKNL 1.8; Lys-Pyr 1.7; Hyl-Pyr 1.7; HHL 1.97; I-DES 3.4; DES 3.4.

**Worked example:**

Let us assume that a sample of hydrolyzed bone was run on a CF-1 column and that this sample contained 11.4 mg of collagen \((W_{(\text{coll})})\) obtained from measurement of its hydroxyproline content. After CF-1 chromatography, the aqueous eluate was dried and redissolved in 120 \(\mu\text{L}\) \((V_{(\text{HCl})})\) of 0.01 \(M\) hydrochloric acid, of which 60 \(\mu\text{L}\) \((V_{(\text{anal})})\) was run on the amino acid analyzer. A peak was obtained on the analyzer for hydroxylysyl-pyridinoline (Hyl-Pyr) with an area of 681,731 arbitrary units \((A)\), as obtained from the data-handling software connected to the analyzer. A previous calibration run on the analyzer with a standard solution of leucine showed that 174,848 units of area were equivalent to 1 nmol of leucine. Therefore, using Eq. 2, \(RF_{(\text{Leu})} = 1/174,848 = 5.719 \times 10^{-6}\).

The leucine equivalence value \((L)\) for Hyl-Pyr is 1.7.

Therefore, using Eq. 1, we have:

\[
\frac{681,731 \times 5.719 \times 10^{-6} \times 120}{60 \times 11.4 \times 1.7 \times 3.3} = 0.122 \text{ mols Hyl-Pyr/mol of collagen}
\]

21. Silica-based columns are degraded by prolonged exposure to low pH solvents. The working life of such columns is very short (months) although they can be regenerated once or twice by a repacking procedure Avery and Light (18).
22. TFA and HFBA are strong (fuming) organic acids, additionally, HFBA has a pungent smell, MeCN and THF are both flammable, consequently, buffer preparation should be in a fume cupboard and care during handling is important.

23. The structure of the so-called pyrrole crosslink has yet to be determined and, at the present time, is quantified by a modification of the procedure for Ehrlich chromogens (9). Tryptic digests of the collagen are reacted with 4-dimethyl-aminobenzaldehyde (DAB) to give a pink/purple reaction product indicating the presence of pyrroles. The method is not specific, for pyrroles colored products also being formed with imidazoles, polyhydroxyphenols, and indoles (19).

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EXTRACELLULAR MATRIX PROTOCOLS

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