Liquid-Chromatographic Measurement of Elastin

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We developed a method for estimating tissue elastin by measuring desmosine and isodesmosine in tissue hydrolysates by "high-performance" liquid chromatography, and we applied this method to 13 canine and 11 human aortic samples. Results agreed well with those obtained by automated liquid-chromatographic amino acid analysis. Precision of the assay was satisfactory. The method can detect as little as 100 pmol per sample and has advantages over other types of amino acid chromatography.

Additional Keyphrases: amino acids, desmosine, isodesmosine, aortic tissue, dogs

The principal strategy for measuring elastin in tissue has long consisted of sequential extraction, leaving elastin as the residue. The most popular methods are those of Lansing (1) and Harkness et al. (2), based on the method described by Lowry et al. in which milled tissue is extracted to remove lipids and dried; the dried residue is extracted with 10 g/L NaCl, to remove soluble proteins; autoclaved, to remove collagen; and finally extracted with hot 0.1 mol/L NaOH, to remove glycoproteins. Many modifications of this extraction procedure have been developed, including those involving the use of guanidine (4–6) and Clostridium histolyticum collagenase (EC 3.4.24.3) (6–10).

Techniques for quantitation of the final elastin residue have included gravimetry (1, 11, 12); treatment with elastase (EC 3.4.21.11), with estimation of the resulting solubilized material (13, 14); and acid hydrolysis followed by estimation of total nitrogen (15), hydroxyproline (16), or the desmosines (17).

These procedures are cumbersome and slow. Moreover, the results are affected by unknown degree by incomplete extraction of one or more glycoproteins (9, 16–21) and by degradation of some of the elastin during extraction (11).

To obviate these difficulties, one can eliminate sequential extraction and measure desmosine concentration as a measure of elastin content. Together, desmosine and isodesmosine—lysine-derived cyclic amino acids unique to elastin (22)—account for two or three of every 1000 amino acid residues (23).

The desmosines can be measured by conventional amino acid chromatography, for which satisfactory methods abound (24–29). If most of the other amino acids are first removed by paper chromatography, the sensitivity of the assay can be substantially improved (28). Recently a radioimmunoassay for desmosine has been described, which has a sensitivity of 1 to 50 pmol per sample (29).

We describe here a "high-performance" liquid-chromato-

graphic (HPLC) method for directly estimating the desmosines in tissue hydrolysates. The method can be used to measure both desmosine and isodesmosine, as little as 100 pmol each per sample. We compare this method with conventional amino acid chromatography for measuring aortic elastin in dogs and humans.

Materials and Methods

Specimens: We examined dog aortas and human aortas obtained at autopsy from persons of various ages. A generous portion of aorta (4–5 cm) adjacent to the great vessels was excised, cleaned, minced into a weighed beaker, reweighed, and dried under reduced pressure overnight at 50 °C to constant weight. The material was extracted sequentially with 95% ethanol, an equimolar mixture of ethanol and diethyl ether, and ether alone, all with gentle shaking. Again, the material was dried overnight as before. Aliquots of the fat-free dried material were weighed into hydrolysis tubes and 6 mol/L HCl was added in the ratio of 1 mL/75 mg of fat-free residue, dry weight. We carefully evacuated air from the tubes while heating them in a water bath, then sealed them under reduced pressure. The contents were hydrolyzed in a heating block at 110 °C for 48 h. After cooling and opening the tubes, we added charcoal (25–100 g/L) for decolorizing, gently warmed the mixture to 60 °C for 5 min with agitation, then filtered with suction through a Millipore filter (0.45-μm pore size; Millipore Corp., Bedford, MA 01730). The filtrate was evaporated under reduced pressure at 60 °C, and the residue was redissolved in distilled water three times and redried to remove all the HCl. After final drying, the material was dissolved in distilled water at about 30 mg aortic dry weight (equivalent) per millilitre.

Desmosine and isodesmosine standards: For each standard, commercially obtained desmosine or isodesmosine (Elastin Products, St. Louis, MO 63069), about 12 mg, was dissolved in 0.2 mol/L sodium citrate buffer, pH 2.20, and applied to a 27 × 2.5 cm jacketed column packed with Aminex MS “C” resin (Bio-Rad Laboratories, Richmond, CA 94804; the comparable resin is now Aminex Q150S, cat. no. 174-2103, a sulfonic acid resin with 8% crosslinks, diameter 28 ± 7 μm). Maintaining the temperature at 45 °C, we eluted the amino acid, first with 100 mL of sodium citrate buffer (0.2 mol/L, pH 3.25), then with 1 L of another sodium citrate buffer (0.38 mol/L, pH 4.00). The desmosine or isodesmosine was then eluted with a third citrate buffer (0.38 mol/L, pH 5.65) and detected by monitoring the absorbance of the eluate at 280 nm. After lyophilization, the residue was dissolved in a minimum volume of 0.1 mol/L HCl, and quantitatively transferred to a 10 × 2 cm column packed with 50–100 mesh Dowex 50W X8 cation-exchange resin in hydrogen form (Bio-Rad; cat. no. 745-6431). Salts were washed out with 3 L of de-ionized water, and the desmosines were eluted with 0.5 mol/L ammonium hydroxide. After lyophilization, the desmosine and isodesmosine were dissolved in water and their concentrations were determined from their absorptivities at 268 and 278 nm, respectively, using the molar absorptivity values ε = 4900 and 7850, respectively, as determined in our laboratory and elsewhere (22).
Fig. 1. Separation of isodesmosine (Ide) and desmosine (Des) in a canine aortic hydrolysate by amino acid chromatography

Amino acid analysis (AAA): We used a Model 121C Amino Acid Analyzer (Beckman Instruments, Inc., Fullerton, CA 92634). For determination of the desmosines, PA-35 resin was maintained at 56 °C and the following sodium citrate buffers were used sequentially: 0.2 mol/L, pH 3.45; 0.35 mol/L, pH 4.25; and 0.35 mol/L, pH 5.35. Figure 1 shows a typical example of separation of the desmosines in a canine aortic hydrolysate. About 1 nmol of each desmosine is required for quantitation.

Ion-paired, "high-performance" liquid chromatography:

Fig. 2. The effect of pH on the separation of desmosine and isodesmosine from other amino acids by reversed-phase, ion-paired HPLC
Mobile phase: methanol/water = 20/74 by volume, containing 10 mmol of heptane sulfonic acid per liter, with the pH varied

The columns used were either C-18 µ Bondapak, 300 x 4 mm (i.d.), 10-µm particle size (Waters Associates, Inc., Milford, MA 01757) or C-18 ODS 5, 150 x 4 mm (i.d.), 5-µm particle size (Bio-Rad). The amino acids were eluted with various proportions of methanol/water containing 10 mmol of sodium heptane sulfonate (Eastman Kodak Co., Rochester, NY 14650; cat. no. 10683) per liter. The pH of the eluting solution was adjusted to 3.0 with concentrated phosphoric acid (see Figure 2). The methanol concentration was varied between 19 and 30 mL/dL, depending on the particular column and the interval desired for detection of the desmosines. At lower methanol concentrations the desmosines are eluted slowly, but with better resolution.

Detection was at 205 nm with a variable-wavelength detector (M-440 or M-450 from Waters Associates, or LC-85 from Perkin-Elmer Corp., Norwalk, CT 06856). Figure 3 shows a typical example of separation of the desmosines in a hydrolysate of canine aorta. We calculated concentrations of the

Fig. 3. HPLC separation of desmosine and isodesmosine in a hydrolysate of canine aorta
Column monitored at 205 nm.

Fig. 4. Peak height at 205 nm vs concentrations of desmosine and isodesmosine

Fig. 5. pH calibration

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desmosines from peak heights, as compared with those of known standards (every fourth or fifth sample). Figure 4 shows the linear relation between peak height and concentration of standards.

**Results**

In Table 1 we compare data for desmosine, isodesmosine, and the calculated elastin content of canine and human aortas by both methods. Figure 5 compares, for canine aortas, the present and automated-analyzer results for the hydrolysates. Figure 6 shows the calculated elastin content for both canine and human aortas by both methods.

For canine aortas there is considerable variability in the analytical results, probably because the dogs were mongrels of different sexes and widely different ages and sizes. Nevertheless, results by the two methods agreed well. The same was true for human aortas. For the three human subjects younger than age 20, the mean value for aortic elastin, 41.3 g/100 g of dried fat-free aorta, significantly exceeded the mean value, 28.9 g/100 g, for the other human specimens (ages 34 to 90).

**Table 1. Isodesmosine, Desmosine, and Calculated Elastin Content of Dog and Human Aortas**

<table>
<thead>
<tr>
<th></th>
<th>Isodesmosine</th>
<th>Desmosine</th>
<th>Calculated elastin</th>
<th>% FFDW *</th>
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<tbody>
<tr>
<td></td>
<td>AAA HPLC</td>
<td>AAA HPLC</td>
<td>AAA HPLC</td>
<td>AAA HPLC</td>
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<tr>
<td><strong>Dog (n = 13)</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Mean</td>
<td>4.18</td>
<td>4.44</td>
<td>5.17</td>
<td>5.34</td>
</tr>
<tr>
<td>SD</td>
<td>0.69</td>
<td>0.70</td>
<td>0.85</td>
<td>0.88</td>
</tr>
<tr>
<td><strong>Human (n = 11)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>3.23</td>
<td>3.45</td>
<td>4.21</td>
<td>4.63</td>
</tr>
<tr>
<td>SD</td>
<td>0.93</td>
<td>0.75</td>
<td>0.89</td>
<td>1.12</td>
</tr>
</tbody>
</table>

* Elastin content was calculated on the basis of 1.3 g of (desmosine + isodesmosine) per 100 g of elastin, as given in reference 23. FFDW, fat-free residue, dry wt; AAA, automated amino acid analysis; HPLC, present method.

A decrease in aortic elastin with age has been noted previously (31).

To check the analytical recovery in our method, we added known quantities of desmosine and isodesmosine to samples of bovine serum albumin and aliquots of dried fat-free residue from canine aorta. Each sample was hydrolyzed and analyzed as described above. The final concentrations in the hydrolysates of the desmosine and isodesmosine added to bovine serum albumin were 22–78 and 13–40 μmol/L, respectively. Analytical recoveries for eight analyses varied between 97.0 and 102.8% (mean 99.6%, SD 2.35%). For the aortic hydrolysates...
Table 2. Precision of Present Assay (n = 10)

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<thead>
<tr>
<th></th>
<th>Within-day</th>
<th></th>
<th>Among-day</th>
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<tbody>
<tr>
<td></td>
<td>Ide</td>
<td>Des</td>
<td>Ide</td>
<td>Des</td>
</tr>
<tr>
<td>Peak height, cm</td>
<td>3.98 0.063</td>
<td>7.99 1.58</td>
<td>3.79 1.85</td>
<td>7.96 1.69</td>
</tr>
<tr>
<td>SD, cm</td>
<td>7.99 0.063</td>
<td>1.58 2.39</td>
<td>7.96 1.69</td>
<td>1.58 2.39</td>
</tr>
<tr>
<td>CV, %</td>
<td>99.9%</td>
<td>&lt;3%</td>
<td>94.4%</td>
<td>114.3%</td>
</tr>
</tbody>
</table>

\[
\text{Ide (isodesmosine)} = 56.9 \mu\text{mol/L}; \text{Des (desmosine)} = 55.1 \mu\text{mol/L}.
\]

(10 analyses), recoveries ranged from 94.4 to 114.3% (mean 99.9%, SD 5.23%).

Table 2 gives the within- and among-day variability found for assay of desmosine and isodesmosine standards by our method. The CV was <3% in all cases, but the among-day CV was almost twice the within-day CV.

In the one other brief report we found of adaptation of HPLC to measurement of the desmosines (32), the analytical conditions differed from ours and desmosine and isodesmosine were not resolved.

We conclude that our method is a useful one, combining ease, rapidity, accuracy, and reasonable sensitivity, and that it is probably superior to other chromatographic methods for determination of desmosines.

References