An Improved High-Performance Liquid Chromatographic Assay for the Determination of Pyridinoline in Connective Tissues

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(Received May 19, 1992)

Summary A high-performance liquid chromatographic (HPLC) method for determination of pyridinoline and its application to connective tissues are described. The chromatographic separation was accomplished by using Inertsil ODS-2 column and a mixture of 0.1 M sodium phosphate and acetonitrile (75:25, v/v) containing sodium dodecyl sulfate (SDS) and ethylenediamine tetraacetic acid (EDTA) as eluent. The chromatogram was developed isocratically and the eluted components were monitored with excitation at 295 nm and emission at 395 nm. The pyridinoline in crude hydrolysate of connective tissues can be determined in 5 min, with as little as 1 pmol of sample. The present method is rapid, simple and can be used for the routine analysis of connective tissues such as cartilage, bone, Achilles tendon and aorta.

Key Words pyridinoline, HPLC, connective tissues

Pyridinoline, also called hydroxylysyl pyridinoline, is a trivalent crosslink of collagen fibers. It is formed presumably through condensation of two hydroxylysinoaldehyde residues and one hydroxylysine residue (1–3) and is widely distributed in hard connective tissues such as cartilage, bone and tendon (1–10). Recently, much attention is being denoted to the urinary excretion of pyridinoline and an analogue of pyridinoline, deoxypyridinoline, as a sensitive and specific marker of bone metabolism in vivo (11–14). As pyridinoline is one of the predominant crosslinks in a mature collagen, it is important to investigate the physiological role of pyridinoline with aging. Prior to examining the biosynthesis of pyridinoline, we described here a simple and rapid HPLC method for determination of pyridinoline, the major crosslink in connective tissues.

Molecular sieve and ion-exchange chromatographic methods for the determination of pyridinoline are often troublesome in terms of the loss of samples, and insufficient for the routine analysis of various animal tissues. A more sensitive HPLC method, using a gradient system has been recently introduced (8,11); however, it is time-consuming and its procedures are complicated. We have developed a reversed-phase HPLC method with an isocratic elution system to
facilitate the analysis, which enables a rapid quantification of pyridinoline in crude hydrolysates of animal connective tissues.

MATERIALS AND METHODS

Materials. Insoluble collagen from bovine Achilles tendon was obtained from Sigma Chemical Co. Sodium phosphate, acetonitrile, SDS, and EDTA were purchased from Wako Pure Chemical Industries, Ltd.

Isolation of pyridinoline standard. The pyridinoline standard was prepared from bovine Achilles tendon collagen according to a method described by Fujimoto et al. (1). Briefly, one gram of bovine Achilles tendon collagen was hydrolyzed in 6 M HCl (50 ml) at 110°C for 24 h in sealed tubes. The hydrolysate was dried up in vacuo and dissolved in 25 ml of water. It was applied on a p-cellulose column (H⁺ form, 1.8 × 25 cm) pre-equilibrated with water. Elution was performed with a linear gradient at a flow rate of 0.7 ml/min, starting with 400 ml of water in the mixing chamber and 400 ml of 0.5 M HCl in the reservoir. The fluorescent fractions were collected and applied on the second column of p-cellulose (H⁺ form, 1.2 × 10 cm), and then further purified by passing through a Sephadex G-10 column (1.6 × 29 cm) using 0.5 M acetic acid as eluent. The acetic acid was removed by a rotary evaporator at 50°C. The dried samples were dissolved in 0.1 M HCl.

Spectroscopic observation. The fluorescence of pyridinoline standard was measured at 395 nm with excitation at 295 nm using a Shimadzu RF-540 spectrofluorophotometer. Measurements of the ultraviolet (UV) absorption and ¹H NMR were carried out by using a Hitachi 100-20 and JEOL JNM-GX 270, respectively.

Preparation of tissues for analysis. Cartilage, bone, tendon and aorta samples, from male albino guinea pigs fed commercial chow diet, were cut into small pieces, washed in saline and defatted with methanol:chloroform (1:2). The tissue samples were hydrolyzed with 6 M HCl in sealed tubes at 110°C for 24 h and evaporated to dryness in vacuo. Bone samples were hydrolyzed after demineralization in 0.5 M EDTA, pH 7.5. For the HPLC analysis the dry residues of the hydrolysates were dissolved in 2 ml of water and filtered through a chromatodisc (pore size 0.45 μm).

High-performance liquid chromatography. A Shimadzu 6A Liquid Chromatography RF-535 spectrofluorophotometer was used. A stainless steel column (25 cm × 4.6 mm i.d.) was packed with an Inertsil ODS-2 (C₁₈, 5 μm; GL Sciences Inc., Japan). For mobile phase, a mixture of 0.1 M sodium phosphate, pH 3.5 and acetonitrile (75:25, v/v) containing 1 g SDS and 25 mg EDTA per liter was used at a flow rate of 1 ml/min. Ten microliters of each sample was injected. The eluate was monitored by fluorescence with excitation at 295 nm and emission at 395 nm. Pyridinoline content was determined by a comparison with an external standard and expressed as mol per mol collagen.

Determination of collagen. The collagen was measured from its hydroxyproline content, assuming that the hydroxyproline content was 0.11 mol per mol collagen (4). Hydroxyproline contents of the resulting hydrolysates were deter-
DETERMINATION OF PYRIDINOLINE

RESULTS AND DISCUSSION

The pyridinoline standard prepared from bovine Achilles tendon collagen was characterized by UV absorption, fluorescence, and \(^1\)H NMR spectroscopy. The UV absorption and fluorescence spectra of the purified compound are shown in Fig. 1. The UV absorption maximum was at 295 nm in acid solution (Fig. 1a). As shown in Fig. 1b, the fluorescence maximum at 295 nm with excitation was at 395 nm. The 270 MHz \(^1\)H NMR spectrum of the standard dissolved in D\(_2\)O ((\(\delta_{DDO}\)): 8.22 and 8.26 (two \(\alpha\) protons), 2.4 and 3.4 (4H), 1.0–2.1 (6H), 3.7–4.7 (6H)) is in good agreement with data reported previously (1). The pyridinoline standard in 0.1 M HCl was frozen as a stock solution. No deterioration was observed in the working standard frozen and thawed over several months.

Figure 2 shows a typical chromatogram of the standard solution (10 pmol) and cartilage extract from guinea pigs. Pyridinoline standard peak was clearly identified at 5 min by a reversed-phase chromatography using isocratic elution system on an Inertsil ODS-2 column. Each sample of connective tissues was analyzed within about 12 min, while the HPLC method using a gradient system required 30 min for elution (1). This isocratic elution system was useful to determine the pyridinoline content in hydrolysates of crude tissue without purification by molecular sieve and ion-exchange chromatography (1) and to greatly reduce the analytical time compared to that of linear gradient system (1). Furthermore, it was useful for holding the stable baseline and allowing for accurate quantification of pyridinoline.

The standard curves for pyridinoline in the range of 5 to 40 pmol are illustrated in Fig. 3. A linear relationship between the amounts of pyridinoline and peak height was obtained. Over 30 pmol, it deviated slightly from this calibration line. Hence the optimum concentration of samples is recommended to be less than 30 pmol.

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Fig. 1. Ultraviolet absorption and fluorescence spectra of purified pyridinoline. (a) Ultraviolet absorption spectrum. (b) Fluorescence spectrum; excitation was at 295 nm. The purified pyridinoline was dissolved in 0.1 M HCl.

Vol. 38, No. 4, 1992
Table 1. Pyridinoline concentration in tissues of guinea pig.

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Pyridinoline (mol/mol of collagen)</th>
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</thead>
<tbody>
<tr>
<td>Larynx cartilage</td>
<td>1.06±0.04 (6)</td>
</tr>
<tr>
<td>Achilles tendon</td>
<td>0.16±0.02 (6)</td>
</tr>
<tr>
<td>Bone</td>
<td>0.76±0.03 (5)</td>
</tr>
<tr>
<td>Aorta</td>
<td>0.53±0.05 (4)</td>
</tr>
<tr>
<td>Lung</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Skin</td>
<td>ND</td>
</tr>
</tbody>
</table>

Tissues were obtained from the guinea pig fed the commercial chow diet. Values are mean±SE.

pmol of pyridinoline.

Table 1 shows the results of pyridinoline measurements on various types of connective tissues in guinea pigs. The pyridinoline concentration was the highest in larynx cartilage among these tissues, whereas it was not detected in skin. These data support the previous observations that pyridinoline is present in collagens of hard tissues and absent in those of soft tissues (2,8), that is, type II collagen contained higher pyridinoline than type I collagen. Achilles tendon, which is rich in type I collagen, tended to have a rather low content of pyridinoline. These values
Table 2. Recovery of pyridinoline added to bone extracts.

<table>
<thead>
<tr>
<th>Pyridinoline added (pmol)</th>
<th>Pyridinoline found (pmol)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>14.5</td>
<td>—</td>
</tr>
<tr>
<td>10</td>
<td>23.5</td>
<td>93.1</td>
</tr>
<tr>
<td>20</td>
<td>33.4</td>
<td>92.4</td>
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<tr>
<td>20</td>
<td>33.0</td>
<td>89.7</td>
</tr>
<tr>
<td>30</td>
<td>43.6</td>
<td>93.8</td>
</tr>
<tr>
<td>30</td>
<td>44.3</td>
<td>98.6</td>
</tr>
</tbody>
</table>

were close to those of rabbit tissues (8), but higher than those of rats (4).

For six separate analyses, the average recovery of pyridinoline by HPLC assay was 93.5 ± 1.4% (Table 2). Bovine Achilles tendon collagen hydrolyzed for 12–24 h contained the maximum pyridinoline concentration (Data not shown). Hydrolyzation over 24 h resulted in the decreased pyridinoline concentration. These results indicate that collagen hydrolysis with the range of 12 to 24 h is required for a good recovery of pyridinoline.

The application of an isocratic elution system of HPLC for the determination of pyridinoline can be investigated. This assay is a rapid, simple, and sensitive method and can be applied to a routine analysis. This method is also appropriate to determine the pyridinoline content of a wide range of biological materials.

REFERENCES


