Standardization of pyridinium crosslinks, pyridinoline and deoxypyridinoline, for use as biochemical markers of collagen degradation

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The collagen crosslinks, pyridinoline and deoxypyridinoline, have been developed as urinary markers of bone resorption but, despite wide clinical application of the technique, comparatively little attention has been paid to the standardization of these compounds. In this study, pyridinoline and deoxypyridinoline have been purified from bone and converted completely to monochloride trihydrochloride salts. In addition to mass spectrometry and NMR spectroscopy, the purity of the isolated materials was assessed by microelemental analysis including the chloride counterions. These purified compounds were used to establish individual molar absorptivity values as primary standardization criteria for the two crosslinks. For pyridinoline in 0.1 mol/L HCl, ε at 295 nm was 5490 L mol⁻¹ cm⁻¹; in 50 mmol/L sodium phosphate, pH 7.5, ε at 325 nm was 5785. The corresponding values for deoxypyridinoline at acid and neutral pH were 5160 and 5290 L mol⁻¹ cm⁻¹. The availability of standardization criteria for the crosslinks will allow more meaningful comparisons of clinical data between different laboratories.

INDEXING TERMS: bone resorption • osteoporosis • metabolic bone disease

Pyridinoline (Pyd) has been identified as a trifunctional collagen crosslink [1–3]. The discovery that this collagen-specific compound was present in urine [4–6] led to the development of assays to measure this component as a urinary marker of collagen degradation. An analogous compound, deoxypyridinoline (Dpd), identified by mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy [7], was shown to be formed by the reaction of two hydroxysine aldehydes with a lysine residue instead of the reaction with hydroxysine, which results in Pyd formation.

After the development of an HPLC assay for measuring both Pyd and Dpd [8], a modification involving the use of a prefractionation procedure facilitated quantification of both Pyd and Dpd in urine [9], a method that was subsequently automated by developing a synthetic internal standard [10]. Based on these procedures, a large number of studies from many different groups has established the utility of the pyridinium crosslinks as markers of collagen degradation [11–14]. More recently, direct immunoassays of the crosslinks have been developed [15, 16]. In most studies, the close correlation between Pyd and Dpd has indicated that both crosslinks in urine are derived mainly from bone, although changes in the Pyd/Dpd ratios in patients with active rheumatoid arthritis suggest that some Pyd may be derived from other tissues [17].

Published reference ranges for excretion of the pyridinium crosslinks have varied considerably between groups, particularly for Dpd [14]. Some of the variation between groups may be caused by differences in the standards used in the assays for quantifying the crosslinks. This would not be surprising because different criteria have been used to quantify the standards. Rather than attempt to quantify each crosslink directly [9], some groups have assumed identical characteristics for both crosslinks as well as equivalence with readily available fluorescent analogs [1, 8].

The aim of the present study was to establish criteria for the absolute quantification of the Pyd and Dpd that could be used to provide consistent standards for different assays of these markers of bone degradation. Our strategy was to use the measured absorptivity as the primary standardization criterion for each crosslink, having established purity by mass spectrometry and NMR spectroscopy. Not only did we take precautions to ensure that each of the isolated crosslinks was converted entirely to the chloride forms, but also, as an additional check on purity, we subjected the isolated compounds to elemental analysis including the chloride anions.

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3 Nonstandard abbreviations: Pyd, pyridinoline; Dpd, deoxypyridinoline; and NMR, nuclear magnetic resonance.

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**Materials and Methods**

**Isolation of Crosslinks from Bone**

Bovine diaphyseal bone was crushed and the marrow removed by extensive washing with water. After being reduced to powder in a hammer mill, the bone (1 kg) was decalcified by two treatments with 3 L of 2 mol/L HCl, each for 30 min. The bone powder was hydrolyzed in 6 mol/L HCl (3.5 L) under reflux for 24 h, after which the hydrolysate was evaporated to dryness under reduced pressure. We then reconstituted the hydrolysate with 0.4 mol/L acetic acid, removed any undissolved material by centrifugation, and chromatographed the supernatant on a 4.5 x 100 cm column of Sephadex G10, eluted with 0.4 mol/L acetic acid (Pharmacia Biotech, St. Albans, UK) at a flow rate of 120 mL/h. The fractions containing pyridinium crosslink fluorescence (excitation/emission wavelengths, 295/400 nm) eluted ahead of the bulk of the amino acids and were pooled and evaporated to dryness under reduced pressure. This fraction was taken up in 15 mL of 67 mmol/L trisodium citrate, pH 2, and 5-mL portions were subjected to ion-exchange chromatography on a 0.9 x 15 cm column of L48 resin (Locarte, London, UK) eluted with 67 mmol/L trisodium citrate buffer, pH 4.25, at 56 °C. Fractions of 2.5 mL were collected and the crosslinks were located by their absorbance at 295 nm. The fractions containing Pyd (eluted at 80–95 min) and Dpd (120–140 min) were pooled separately and further purified.

**Purification of the Crosslinks**

The isolated crosslink fractions were desalted and purified further by reversed-phase chromatography. After adding heptafluorobutyric acid (final concentration 10 mL/L), the sample was loaded onto a 2.5 x 10 cm preparative C18 column (PrepPak cartridge, Waters Chromatography Div., Watford, UK) and fractionated by elution at 4 mL/min with 10 mmol/L heptafluorobutyric acid in a linear gradient from 150 to 300 mL/L acetonitrile over 30 min. The crosslink-containing fractions (A295) were pooled and evaporated to dryness under reduced pressure. The yield of purified crosslink from two preparations, each starting with 1 kg of calcified bone, was ~56 mg of Pyd and 8 mg of Dpd.

To convert the crosslinks entirely to their chloride forms, we separately dissolved in 10 mL of 0.1 mol/L HCl portions containing 5–8 mg of each crosslink and applied each solution to a 0.9 x 5 cm column of phosphocellulose (P11; Whatman, Maidstone, UK) that had been freshly recycled according to the manufacturer's instructions. After the column was washed successively with 10 mL of 0.1 mol/L HCl and 20 mL of 0.2 mol/L HCl, the pyridinium crosslink was eluted with 10 mL of 0.5 mol/L HCl, and the solvent was evaporated under reduced pressure. The purified crosslink was finally chromatographed on a 1.3 x 95 cm column of Sephadex G10 with 50 mmol/L HCl, and 1.5-mL fractions were collected. Fractions containing the crosslinks, detected by absorbance at 295 nm, were pooled and lyophilized.

**Elemental Analysis**

All elemental analyses were performed by Butterworth Labs. (Teddington, UK), who provided preweighted crucibles for either C, H, N analysis after combustion, or Cl analysis directly in solution by chloride electrode. All analyses were performed in duplicate with ~1.5 mg of the compound for each C, H, N and Cl analysis. The purified compounds were dissolved in water, and 20 μL (~1.5 mg of material) was transferred to the crucible contained in an Eppendorf tube. Each tube and its contents were then placed in a vial containing water for freezing and lyophilizing the sample. Similar aliquots were dried to constant weight over P2O5 under reduced pressure for gravimetric analysis with a Cahn Electrobalance (Ventron Instruments, Paramount, CA) before ultraviolet spectroscopy and fluorescence measurements. Successful preliminary experiments with pyridoxine · HCl and pyridoxamine · 2HCl · H2O established the feasibility of the analyses with relatively small amounts of material. The precision of the balance in the range used for gravimetric analysis was 0.006 mg, giving a maximum error in weighing of <1%.

**Analytical Techniques**

Ultraviolet absorbance spectra were collected with a Cecil CE6700 double-beam recording spectrophotometer (Cecil Instruments, Cambridge, UK). High-field 1H-NMR were recorded on a Brucker AM-600 FT spectrometer at 600 MHz at the National Center for NMR Applications, Department of Chemistry, Colorado State University, Fort Collins, CO. All resonance values are listed in ppm downfield to external 2,2-dimethyl-2-silapentane-5-sulfonate, sodium salt (DSS), in D2O with use of a solvent suppression program (irradiation at the residual water peak). Liquid secondary ion mass spectrometry was performed with a Concept IIIH four-sector tandem instrument (Kratos Analytical, Manchester, UK), as described previously [18]. Amino acid analysis was performed with an AlphaPlus II analyzer (Pharmacia Biotech), a single-column separation method based on stepwise elution with lithium citrate buffers and ninhydrin detection.

**Results**

**Purity of the Isolated Compounds**

The NMR spectra recorded for purified Pyd and Dpd are shown in Fig. 1. In addition to the chemical shift data, 2-D proton–proton homonuclear coupling was performed at 500 MHz; this information was used to determine the peak assignments shown in the legend to Fig. 1. The structure and peak assignments confirm previous literature [1, 7] reporting NMR of Pyd and Dpd at lower magnetic strengths.

The high-resolution mass spectra for the crosslinks showed in each case the naturally occurring cation as the major species, with measured molecular masses of 429.37 and 413.34 Da for Pyd and Dpd, respectively. These values were within 0.04% of the calculated masses.

The absorbance and fluorescence spectra of each crosslink in 0.1 mol/L HCl or 50 mmol/L sodium phosphate, pH 7.5, are shown in Fig. 2. Amino acid analysis profiles of purified Pyd and Dpd in comparison with a buffer blank (Fig. 3) indicated that the purity of both crosslinks was >98% with respect to other amino acids.
**Elemental Analysis**

Values obtained for the composition of the purified crosslinks were as follows:

<table>
<thead>
<tr>
<th>Composition, %</th>
<th>C</th>
<th>H</th>
<th>N</th>
<th>Cl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyd · Cl · 3HCl · 2H2O</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Found</td>
<td>35.66</td>
<td>5.90</td>
<td>8.79</td>
<td>23.51</td>
</tr>
<tr>
<td>Expected for C18H36N4Cl4O10</td>
<td>35.42</td>
<td>5.95</td>
<td>9.18</td>
<td>23.24</td>
</tr>
<tr>
<td>Dpd · Cl · 3HCl · 2H2O</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Found</td>
<td>36.68</td>
<td>5.74</td>
<td>9.00</td>
<td>24.32</td>
</tr>
<tr>
<td>Expected for C18H36N4Cl4O9</td>
<td>36.38</td>
<td>6.11</td>
<td>9.43</td>
<td>23.86</td>
</tr>
</tbody>
</table>

Even though the samples were dried over P2O5, the elemental analyses in each case corresponded most closely with the retention of two molecules of water. These analyses provided further evidence for the purity of the crosslinks and indicated that the appropriate molecular masses for these preparations were 610.3 and 594.3 Da for Pyd · HCl and Dpd · HCl, respectively.

**Absorptivity Values**

The calculated molar absorptivities (ε) of the purified crosslink standards in both acid and neutral solution are given in Table 1.

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Fig. 1. Proton NMR spectra in D2O of purified hydrochloride salts of (a) Pyd and (b) Dpd. Proton structural assignments, established with a homonuclear proton-coupling experiment (2-D COSY), were as follows: Pyd · HCl: 8.822 (s, 1, ArH), 8.19 (s, 1, ArH), 4.6 [d, 1, NCH(H)CHOH], 4.25 [dd, 1, NCH(H)CHOH], 4.23 [t, 1, ring-4: CH2CHNH3(CO2)2], 4.01 [m, 3, ring-4: CH2CHNH3(CO2)2] and ring-N: CHNH3*(CO2)2 and NCHOH], 3.35 (m, 2, ring-4: CH2CH), 2.99 [m, 1, ring-5: CH(CH2)CHNH3(CO2)2], 2.87 [m, 1, ring-5: CH(CH2)CHNH3(CO2)2], 2.16 overlapped with 2.11 [m, 3, ring-5: CH(CH2)CHNH3(CO2)2] and ring-N: CHNH3*(CO2)2], 1.96 [m, 1, ring-N: CH(NH)CHNH3(CO2)2], 1.71 [m, 1, ring-N: CH(OH)CH(NH)CH2], 1.59 [m, 1, ring-N: CH(OH)CH(NH)CH2].

Dpd · HCl: 8.824 (s, 1, ArH), 8.19 (s, 1, ArH), 4.40 (t, 2, NCH2CH2), 4.18 [t, 1, ring-4: CH2CHNH3(CO2)2], 3.98 [t, 1, ring-5: CHNH3*(CO2)2], 3.89 [t, 1, ring-N: CHNH3*(CO2)2], 3.30 (m, 2, ring-4: CH2CH), 2.94 [m, 1, ring-5: CH(CH2)CHNH3(CO2)2], 2.82 [m, 1, ring-5: CH(CH2)CHNH3(CO2)2], 2.12 [m, 2, ring-4: CH2CHNH3(CO2)2], 1.95 (quintet, 2, NCH2CH2CH2), 1.86 [m, 2, ring-N: CH(NH)CHNH3(CO2)2], 1.41 [m, 1, ring-N: CH(NH)CHNH3(CO2)2], 1.32 [m, 1, ring-N: CH(OH)CH2CHNH3(CO2)2].
Fig. 2. (Top) Absorbance spectra and (bottom) uncorrected fluorescence excitation spectra with emission at 400 nm for (a) Pyd and (b) Dpd in 0.1 mol/L HCl (solid lines) or 50 mmol/L sodium phosphate, pH 7.5 (broken lines).

Pyd showed absorption maxima at 295 and 243 nm in acid and at 325 and 250 nm in neutral solution. Corresponding values for Dpd were 294 and 240 nm in acid and 324 and 251 nm at neutral pH. The primary fluorescence maximum for both crosslinks was at 295 nm in acid and 325 nm in neutral solution.

At 293 nm, the measured ε for pyridoxamine · 2HCl · H2O under the conditions of this experiment was 8610 L mol⁻¹ cm⁻¹, close to the literature value of 8500 [19].

Discussion

Despite extensive use of the pyridinium crosslinks, particularly Dpd, as urinary markers of bone resorption, comparatively little attention has been paid to the standardization of these components. The purpose of this study was to define primary standardization criteria for these crosslinks that would assist in the comparison of data from different groups. Standardization is particularly important for Dpd, in that reference values from different laboratories for the urinary excretion of this crosslink may vary by twofold [14].

After the initial characterization of Pyd, Fujimoto based quantification on the absorptivity of N-methylpyridoxine and the reaction with 2,4,6-trinitrobenzenesulfonic acid [20]. Eyre et al. used N-ethyl-3-pyridinol as the model standard compound but further assumed that the molar absorptivities of both Pyd (which they designated HP) and Dpd (LP) were the same [8]. Our previous studies, based on gravimetric analysis of isolated compounds, indicated that Pyd and Dpd exhibited differences in quantification by fluorescence [9]. More recently, several preparative procedures for isolating larger quantities of the
crosslinks have been described [21, 22] but without addressing the question of absolute standardization.

Although most chromatographic assays for the crosslinks use these compounds' natural fluorescence for quantification, temperature dependence [9], variation between instruments, and sensitivity of free pyridinium compounds to prolonged ultraviolet irradiation [23, 24] make this a poor basis for primary standardization. Ultraviolet spectroscopy provides a more suitable quantification, so we obtained data on the absorptivities of the compounds at 295 nm in acid and at 325 nm at neutral pH. Although photolysis of the pyridinium crosslinks can occur during fluorescence measurements, the much lower intensity of light sources used for ultraviolet spectroscopy ensures that losses of crosslinks during absorbance monitoring are negligible. ε for Pyd in 0.1 mol/L HCl (5490 L mol⁻¹ cm⁻¹) was much lower than the values for N-substituted 3-hydroxypyridinium compounds, which are generally in the 8000–8500 range [25] but can be affected markedly by substituents in the ring [19]. The ε values we obtained are based on gravimetric analysis of highly purified samples that had been converted to their monochloride trihydrochloride salts and gave no evidence by mass spectrometry and NMR of substantial impurities.

The absorptivity of Dpd was 6–8% lower than that of Pyd in both acid and neutral solution. For both crosslinks, the ratio between the absorbance at 295 nm in acid and at 325 nm at neutral pH may be used as an indication of purity, given that contaminants are unlikely to undergo the same absorbance shift with pH. The A₂₉⁵/A₃₂⁵ ratios for Pyd and Dpd were 0.950 and 0.975, respectively.

Standardization of the pyridinium crosslinks combined with some knowledge of the mean crosslink concentrations in bone allows expression of bone resorption in terms of bone collagen equivalent or as absolute amounts of bone [26, 27]. In recent years, several assays have been developed to measure telopeptide fragments of collagen type I [28, 29] and have been applied clinically as urinary markers of primarily bone degradation. It would be useful to be able to make direct comparisons between the different forms of bone resorption assay. For the telopeptide assays, the pyridinium compounds need not be present for reactivity, and the crosslinks cannot therefore be used to quantify the amount of bone resorbed. The concept of bone collagen equivalents provides some measure of comparison [30], but the lack of knowledge about the precise nature of the analytes measured in urine by the telopeptide assays confounds any direct comparison between the methods.

In conclusion, this study provides analytical criteria for standardization of the pyridinium crosslinks for use as bone resorption markers. A facility for direct comparison of clinical data between centers is important for the establishment of more clearly defined reference ranges.

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**References**


