Monoclonal antibody assay for free urinary pyridinium cross-links

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The pyridinium cross-links of collagen, pyridinoline (Pyd) and deoxypyridinoline (Dpd), provide structural integrity and rigidity to collagen fibrils in bone. During bone degradation (resorption), the cross-links are released into the circulation and eventually excreted in urine. Pyridinium cross-link measurements in urine have been shown to be sensitive and specific indicators of resorption by both established HPLC and newer enzyme immunoassay (EIA) techniques. We have developed a monoclonal antibody that preferentially binds to the non-peptide-bound free forms of Pyd & Dpd. We have incorporated the antibody conjugated to alkaline phosphatase in a competitive EIA by using Pyd-coated microtiter strip wells. After a 3-h incubation of sample and antibody–enzyme conjugate, color is developed for 1 h with p-nitrophenyl phosphate as the substrate. The intraassay (n = 52) CVs were 3.0–7.6%, and interassay (n = 8) CVs were 6.1–7.4%. Comparisons of the assay (y) with HPLC (x) and a polyclonal antibody–based EIA (x') gave regression equations of $y = 0.46x + 4, r = 0.96$, and $y = 0.56x' + 8, r = 0.96$. The EIA detected increased Pyd & Dpd concentrations in urine from postmenopausal women and patients with osteoporosis, hyperthyroidism, hyperparathyroidism, and Paget disease of bone. EIA concentrations also reflected the reduction in Pyd&Dpd excretion resulting from estrogen replacement in surgically menopausal women. Measurement of pyridinium cross-links with this simple EIA appears to provide an accurate index of the rate of resorption and may be useful for metabolic bone disease assessment and monitoring the effects of antiresorptive therapy.

INDEXING TERMS: bone resorption • deoxypyridinoline • enzyme immunoassay • metabolic bone disease • pyridinoline • collagen

Bone collagen derives its rigidity from molecular cross-linking between adjacent collagen fibrils. Trifunctional pyridinium cross-links form between hydroxylsine or lysine residues at the telopeptide ends of one collagen molecule and the helical portion of a neighboring one during collagen maturation [1]. When bone is resorbed, osteoclastic degradation of the bone matrix releases these cross-links, pyridinoline (Pyd) and deoxypyridinoline (Dpd), into circulation.4 They cannot be reused in new collagen synthesis and are cleared by the kidneys. Dpd is present almost exclusively in bone, though it has been detected in aorta, dentine, and ligament. In addition to these tissue sources, Pyd is also present in cartilage [1]. However, the far greater mass and metabolic turnover rate of bone suggests that urinary concentrations of Pyd&Dpd are essentially bone-derived [2, 3]. This is borne out by the observation that the molar ratio of Pyd to Dpd in urine is between 3 and 4 [4], very similar to that in bone [5].

Urinary Pyd&Dpd have been validated as markers of resorption by correlation with histomorphometry of bone biopsy [6] and radioisotope kinetics [7], and by their increase in conditions that are characterized by increased resorption. Pyd&Dpd are higher in children, in whom high bone remodeling rates occur during growth, than in adults [4]. Concentrations increase in postmenopausal women as a result of estrogen deficiency, which can lead to the bone loss causing postmenopausal osteoporosis. This effect can be reversed with estrogen replacement therapy [8, 9]. Concentrations of Pyd&Dpd are also increased in metabolic bone diseases including osteoporosis, hyperthyroidism, hyperparathyroidism, Paget disease, and certain metastatic cancers [10-16].

Pyd&Dpd have been measured in urine by HPLC with fluorescence detection [17, 18]. The procedure requires pre-

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Nonstandard abbreviations: Pyd, pyridinoline; Dpd, deoxypyridinoline; EIA, enzyme immunoassay; FMV, first morning void; KLH, keyhole limpet hemocyanin; EDC, N-(3-dimethylaminopropyl)-N-ethylcarbodiimide; PBS, phosphate-buffered saline; and BS5, N-succinimidyl suberate.
treatment of urine samples by hydrolysis and cellulose chromatography, and measures total cross-links, including free and all peptide-bound forms of Pyd&Dpd. This cumbersome and demanding 2-day process is not suitable for routine clinical use. More recently a polyclonal antibody-based immunoassay, which correlates highly with HPLC methods, has been commercially available [15, 19]. This enzyme immunoassay (EIA), which measures Pyd&Dpd both in their free and small peptide-bound forms, requires sample filtration and an overnight incubation. In the present study, we describe the development of the Pyrlinks® assay, a simple, monoclonal antibody-based EIA for the combined measurement of free Pyd and Dpd in urine, and report its analytical characteristics and results in various clinical conditions.

Materials and Methods

ANTIBODY-RELATED MATERIALS

Tissue culture reagents (DMEM, NCTC-109, l-glutamine, and gentamicin) were purchased from Life Technologies (Gaithersburg, MD). Oxaalacetic acid, insulin, hybridoma drug-selection reagents (hypoxyanthetic monoamine oxidase), and J774A.1 conditioned media were purchased from Sigma Chemical Co. (St. Louis, MO). The fetal bovine serum equivalent for hybridomas, FetalClone®, was purchased from Hyclone Laboratories (Logan, UT). Polyethylene glycol 1500 was purchased from Boehringer-Mannheim (Indianapolis, IN). The adjuvant used was MPL+TDM+CWS Emulsion from RIBI ImmunoChem Research (Hamilton, MT). The mouse nonscreening myeloma fusion partner (P3X63Ag8.653) and the mouse monocye-macrophage cell line J774A.1 were purchased from American Type Culture Collection (Rockville, MD). Incomplete Freund's adjuvant was purchased from Pierce Chemical Co. (Rockford, IL). The Protein A affinity matrix used for antibody purification was purchased from biOProcessing (Durham, UK).

OTHER MATERIALS AND METHODS

1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide (EDC), bis(sulfosuccinimidyl)suberate (BS²), N-succinimidyl-2-(2-pyridylthio)propionate, succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylic, keyhole limpet hemocyanin (KLH), and goat anti-mouse IgG+M(H+L)-alkaline phosphatase conjugate were obtained from Pierce. Streptavidin was obtained from Scripps Labs. (San Diego, CA), alkaline phosphatase from Biozyme Labs. (Blänavon, UK), biotin-X-2,4-dinitrophenyl-X- l-lysine succinimidyl ester from Molecular Probes (Eugene, OR), and N-ethyl maleimide, p-nitrophenyl phosphatase, bovine and porcine serum albumin, and amino acid mixture from Sigma. Regenerated cellulose membrane tubing of molecular mass cutoff 1 kDa, 6–8 kDa, and 10–14 kDa were used for dialysis and obtained from Spectrum Medical Industries (Houston, TX). Sephacryl S-300HR filtration gel and Sephadex G-25 desalting columns were obtained from Pharmacia Biotech (Uppsala, Sweden). Demineralized bovine bone powder was obtained from Collagen Corp. (Palo Alto, CA). MaxiSorp™ Nunc-Immuno™ microtiter plates were purchased from VWR Scientific (San Francisco, CA). The microtiter plate reader used was a ThermoMax™ from Molecular Devices (Santa Clara, CA). The analytical HPLC system consisted of a Hewlett Packard (Palo Alto, CA) Series 1050 injector and pump system linked to a Shimadzu (Columbia, MD) RF 551 fluorescence monitor, set at 295 nm excitation and 395 nm emission, and a Rainin (Emeryville, CA) C₁₈ 4.6 × 100 mm reversed-phase column. A flow rate of 1.00 mL/min of 120 mL/L acetonitrile was used. For preparative HPLC, a Waters (Milford, MA) Nova-Pak HR C₁₈ 25 × 100 mm reversed-phase column was used with a flow rate of 10 mL/min and a 20–40 mL/L acetonitrile gradient over 60 min. HPLC measurement of total Pyd&Dpd has been previously described [19, 20]. HPLC of free Pyd&Dpd was performed as previously described [20] at Rowett Research Institute, Aberdeen, UK. Urinary creatinine measurements were made by a modified Jaffe method, which yields intra- and interassay precision CVs of 1–2% and 2–7%, respectively.

URINE SAMPLES

First morning voided (FMV) urine samples were collected from 118 healthy men, ages 25–55 years (mean ± SD 36 ± 7) and 301 healthy premenopausal women, ages 25–44 years (35 ± 5). These subjects had no bone, endocrine, or other chronic disorders, and were not currently taking any medication known to influence bone metabolism. The women were not pregnant or breast-feeding. FMV urine samples from 115 postmenopausal women and individuals with postmenopausal (n = 123), male (n = 20), and drug-induced osteoporosis (n = 23); hyperthyroidism and hyperparathyroidism (n = 14); and Paget disease (n = 99) were obtained from Mayo Clinic (Rochester, MN), Henry Ford Hospital (Detroit, MI), INSERM Unit 234 Hôpital Edouard Herriot (Lyon, France), New Mexico Arthritis Clinic (Albuquerque, NM), Meese Clinic (Dunedin, FL), St. Joseph's Hospital–Creighton University (Omaha, NE), Jewish Hospital–Washington University Medical Center (St. Louis, MO), Miami Veterans Administration Hospital (Miami, FL), Western Nephrology and Metabolic Bone Disease (Lakewood, CO), Columbia University (New York, NY), Rush Presbyterian-St. Luke's Medical Center (Chicago, IL), Osteoporosis Research Center (Portland, OR), John Wayne Cancer Institute (Santa Monica, CA), and Sam Miller (San Antonio, TX). Urine samples from a double-blind placebo-controlled trial of transdermal 17β-estradiol in 88 surgically menopausal women [21] were obtained from Mayo Clinic. All studies were performed according to the Helsinki Declaration of 1983 and had passed each institutional review board.

PURIFICATION OF PYD AND DPD

Purification of urinary Pyd, as use as calibrators, and urinary Dpd, for use as an immunogen and in cross-reactivity determinations, has been previously described [19]. Pyd, used for plate coating, was isolated from demineralized bovine bone powder based on a modification of the procedure by Black et al. [17]. The bone powder was washed, dried, and then hydrolyzed in refluxing 6 mol/L hydrochloric acid for at least 8 h. The hydrolysate was filtered through activated charcoal, pH-adjusted to 2, desalted through a column of Sephadex G-10 (25 cm × 100 cm), and eluted with 0.2 mol/L acetic acid. Fractions that exhibited fluorescence (excitation 295 nm, emission 395 nm)
before a large increase in conductivity were lyophilized and reconstituted with 67 mmol/L sodium citrate pH 2.5, filtered, pH-adjusted to 2, and eluted two times through Aminex A-7 cation-exchange resin (2.5 cm × 500 cm). Pyridinium cross-link-containing fractions were identified by absorbance at 295 nm and fluorescence (excitation 295 nm, emission 395 nm). The fractions were pooled and further purified by preparative HPLC with a reversed-phase C18 column, monitoring the fractions by absorbance. The final purified Pyd-containing pool was lyophilized and reconstituted in 0.2 mol/L acetic acid. Analysis of the final product consisted of first testing by using a trinitrobenzenesulfonic acid assay for primary amines [22]. Then, analytical HPLC was performed with samples diluted into 2% heptfluorobutyric acid and eluted with 110 mL/L acetonitrile, monitoring by fluorescence.

**PREPARATION OF IMMUNOGEN**

The immunogen used to generate the anti-Pyd monoclonal antibody was prepared by covalently coupling Dpd to KLH by using EDC. To a cloudy, pale blue solution of the protein (20 mg) in phosphate-buffered saline (PBS; 10 mmol/L sodium phosphate, 0.15 mol/L sodium chloride, pH 7.0) was added 19 µmol of Dpd in water. The solution was adjusted to pH 5, and 313 µmol of EDC was added in two portions. After reaction for 4 h at room temperature, the solution was exhaustively dialyzed against PBS.

**PREPARATION OF PLATE-COATING CONJUGATES**

Biotin-labeled porcine serum albumin and streptavidin–Pyd conjugate were used in microplate coating. Porcine serum albumin was biotinylated by reacting 0.61 µmol in PBS with 5.8 µmol of biotin–X-2,4-dinitrophenyl–X-lysine succinimidyl ester dissolved in dimethylformamide. After reacting 2 h at room temperature, the solution was desalted through Sephadex G-25, eluting with PBS. Spectrophotometric analysis showed between four and eight biotin molecules incorporated per mole of porcine serum albumin.

The streptavidin–Pyd conjugate was prepared in a one-step procedure with the water-soluble homobifunctional cross-linker BS3. To 0.15 µmol of diazotized streptavidin mixed with 13 µmol of Pyd, both in 0.1 mol/L sodium phosphate buffer, pH 7.5, was added 1.9 µmol of BS3 and the solution was reacted for 2 h at room temperature. Any residual cross-linker was quenched with 10 mmol/L glycine, and the final solution was exhaustively dialyzed against PBS. Spectrophotometric analysis exhibited between 1.5 and 3.0 Pyd molecules incorporated per mole of streptavidin.

**PREPARATION OF MONOCLONAL ANTIBODIES TO PYD AND DPD**

Mice were initially immunized intraperitoneally with 83 µg of a Dpd–KLH immunogen emulsified in RIBI adjuvant. Mice were reimmunized approximately biweekly thereafter with 100 µg, 100 µg, and 75 µg, respectively. The mouse with the highest serum titer was then administered a final 200-µg intravenous boost 4 days before fusion. The immunized spleen cells were fused with the P3X63Ag8.653 fusion partner in the presence of polyethylene glycol according to the technique of Köhler and Milstein [23]. When sizable colonies of hybridoma cells were grown (10–14 days later), the supernatant antibodies were assayed by ELISA. The antibodies were captured by Pyd-coated microtiter plates. Goat anti-mouse IgG+M(H+L)–alkaline phosphatase was subsequently added to the plate, and antibody binding to the enzyme was detected by measurement of the color development of p-nitrophenyl phosphate substrate. Antibodies exhibiting acceptable absorbance at 405 nm were subsequently checked for their sensitivity, ability to generate a calibration curve, and their cross-reactivity with amino acids and Pyd. Antibodies exhibiting the desired sensitivity and minimal cross-reactivity with amino acids were subcloned by limiting dilution five times. After establishing a stable cell line, the cells were frozen and expanded in culture and mouse ascites. The antibodies were purified by Protein A affinity chromatography for assay development.

**PREPARATION OF PYD-COATED MICROTI TER PLATES**

We prepared the Pyd-coated plates by modifying the procedures described by Seyedin et al. [19]. Briefly, microtiter plates were coated with biotin-labeled porcine serum albumin followed by capturing the streptavidin–Pyd conjugate onto plates. The plates were washed, dried at 37°C, and stored under desiccant at 4°C until use.

**PREPARATION OF ALKALINE PHOSPHATASE-ANTIBODY CONJUGATE**

The detection conjugate was prepared by first thiolating alkaline phosphatase by using 10 mol of N-succinimidyl-2-(2-pyridylthio)propionyl, purifying by dialysis, and exposing the protected thiol by reduction with dithiothreitol. A yield of four to five thiol groups per enzyme was determined spectrophotometrically from the released pyridine-2-thione [24]. Simultaneously, the monoclonal antibody was maleimidated by reacting with 10 mol of succinimidyl 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate and purifying by dialysis. The modified antibody and enzyme were reacted in a 1:2 ratio, respectively, for 18 h, and any excess thiol was then reacted with N-ethyl maleimide. The final solution was purified through Sephacryl S-300HR size exclusion gel (1.6 × 88 cm) with 50 mmol/L sodium phosphate buffer containing 150 mmol/L sodium chloride and 0.5 mL/L Tween 20, pH 7.5. Individual fractions were tested for enzyme activity, mouse IgG–alkaline phosphatase activity, and actual recognition activity against a Pyd-coated plate. The fractions active to all three tests were pooled and lyophilized for storage.

**EIA OF PYD&DPD**

Calibrators and samples were diluted 1:10 in assay buffer (100 mmol/L sodium phosphate, 150 mmol/L sodium chloride, 1 g/L bovine serum albumin, 0.5 mL/L Tween 20, and 0.5 g/L sodium azide, pH 7). In each well of the Pyd-coated plate, 50 µL of the diluted calibrator or sample and 100 µL of alkaline phosphatase–antibody conjugate were added and incubated at 2–8 °C for 3 h. After the plate was washed, 150 µL of substrate solution (2 g/L p-nitrophenyl phosphate in 1 mol/L diethanolamine, 1
mmol/L magnesium chloride, pH 9.8) was added and incubated for 60 min at room temperature. The reaction was stopped with 100 µL of 1 mol/L sodium hydroxide, and the absorbance was read at 405 nm.

STATISTICAL METHOD
Reference intervals for healthy adults were calculated as suggested by the National Committee on Clinical Laboratory Standards [23]. We used a nonparametric method to estimate the reference interval with 90% confidence. Comparisons between subjects with metabolic bone disease or conditions known to increase bone resorption and the healthy population were calculated by using the Mann–Whitney U-test because of the nonparametric distribution of all groups. Comparisons between placebo and estrogen-treated postmenopausal women were calculated by using the unpaired Student’s t-test. Statistical calculations were performed with StatView® software (Abacus Concepts, Berkeley, CA).

Results

ASSAY PERFORMANCE
A typical calibration curve with a four-parameter curve fit is shown in Fig. 1. The minimum detection limit defined by the separation of 3 SD from zero is 7.5 nmol/L. Precision studies showed within-run and between-run CVs of 3.0–7.6% and 6.1–7.4% (Table 1). The accuracy of the assay is proven by analytical recovery studies. A linearity of dilution study of four samples serially diluted 1:2, 1:4, and 1:8 demonstrated dilution recoveries for each sample ranging from 95% to 104%. The addition of 68.2 nmol/L Pyd to 19 samples resulted in an average analytical recovery of 109% ± 7%.

ANTIBODY SPECIFICITY
The monoclonal antibody demonstrated 100% cross-reactivity with Dpd when a known concentration of Dpd was treated as unknown and quantified by the Pyd assay. Pyd&Dpd peptides with molecular mass >1000 Da were isolated by dialysis with a 1000-Da molecular mass cutoff membrane. The antibody had 2.5% cross-reactivity with these peptides. A physiological mixture of amino acids at 0.15 mmol/L showed <5% cross-reactivity in the EIA. Evidence that the antibody recognizes only the free forms of Pyd&Dpd and not peptide-bound forms

<1000 Da was obtained by performing comparisons with free and total Pyd + Dpd measured by HPLC as described in Method Comparisons.

METHOD COMPARISONS
We compared the EIA with HPLC measurements of total Pyd + Dpd with 175 samples from the estrogen replacement therapy study (Fig. 2). The EIA (y) was highly correlated with HPLC (x) (r = 0.96, P = 0.0001), the regression equation was $y = 0.46(±0.01)x + 4(±26)$, and $S_{yx}$ was 26. This slope is consistent with the observed free-to-total cross-link ratio of 40–50% in urine of healthy and diseased subjects [3, 26–28]. The EIA was equally highly correlated with HPLC measurement of total Pyd alone (r = 0.97, data not shown). We compared the EIA with HPLC measurements of free Pyd + Dpd made in 35 samples from healthy individuals (individual data not shown). The EIA-determined free Pyd&Dpd was highly correlated with free Pyd + Dpd by HPLC (r = 0.99, P = 0.0001), the regression equation was $y = 1.12(±0.03)x - 21(±58)$, and $S_{yx}$ was 54. This slope provides further evidence that the antibody recognizes the free forms of Pyd&Dpd. We also compared the EIA (y) with a polyclonal antibody-based EIA (x) that measures Pyd&Dpd

Table 1. Precision of Pyd&Dpd measurement by EIA in urine.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Pyd&amp;Dpd, nmol/L</th>
<th>SD</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Within-run (n = 52)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>60</td>
<td>4.6</td>
<td>7.6</td>
</tr>
<tr>
<td>B</td>
<td>151</td>
<td>7.1</td>
<td>4.7</td>
</tr>
<tr>
<td>C</td>
<td>418</td>
<td>12.5</td>
<td>3.0</td>
</tr>
<tr>
<td>Between-run (n = 8)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>61</td>
<td>4.0</td>
<td>6.6</td>
</tr>
<tr>
<td>B</td>
<td>145</td>
<td>10.7</td>
<td>7.4</td>
</tr>
<tr>
<td>C</td>
<td>393</td>
<td>24.1</td>
<td>6.1</td>
</tr>
</tbody>
</table>

EIA was performed as described in Materials and Methods. HPLC of total Pyd&Dpd was performed after acid hydrolysis and prefractonation of urine samples as previously described [19, 20].

**Fig. 1.** Typical calibration curve of the EIA for measuring Pyd&Dpd.

**Fig. 2.** Comparison of the EIA with an HPLC method.
both in their free and small peptide-bound forms [19] with 123 osteoporotic subjects (individual data not shown). The two EIA values were highly correlated \((r = 0.96, \ P = 0.0001)\), the regression equation was \(y = 0.56(\pm 0.01)x + 8(\pm 34)\), and \(s_{\text{Yx}} = 34\). This slope is consistent with the observed distribution of free and small peptide-bound cross-links in urine [19].

**PYD&DPD VALUES IN HEALTHY ADULTS**

We analyzed urine samples from a group of healthy adults with the EIA to establish preliminary reference intervals. The mean \(\pm\) SD PYD&DPD values divided by creatinine were \(18.5 \pm 4.4\) nmol/mmol for 118 men, and \(25.5 \pm 7.5\) nmol/mmol for 301 premenopausal women. The distribution of values for each gender was skewed and non gaussian. The nonparametric reference interval for men was \(12.8 - 25.6\) nmol/mmol, with the 95% confidence interval for the upper limit being \(23.1 - 30.1\) nmol/mmol. The nonparametric reference interval for women was \(16.0 - 37.0\) nmol/mmol (95% confidence interval for the upper limit, \(35.0 - 42.8\) nmol/mmol).

**CLINICAL PERFORMANCE**

We analyzed urine samples from individuals with bone disease or conditions that cause metabolic bone disorders with the EIA and compared them with the healthy adult groups (Fig. 3 and Table 2). The creatinine-corrected PYD&DPD values of all groups tested (postmenopausal, male, and drug-induced osteoporosis, hyperthyroidism, hyperparathyroidism, and Paget disease) were significantly increased in comparison with the appropriate reference group.

Markers of bone metabolism may be increased after menopause, in osteoporosis, and certain endocrine conditions, but the markers are not diagnostic for those conditions. The same is true of Paget disease; however, in patients with paetic lesions, bone markers are usually increased to such a great degree that it is possible to assess their diagnostic sensitivity and specificity with the receiver-operating characteristic technique [29]. Most of the Paget patients in this study were either undergoing treatment or were in remission. Even under these conditions, analysis of PYD&DPD measured by the EIA yielded relative sensitivity and specificity of 89% and 95%, respectively.

### Table 2. Pyd&Dpd concentrations measured by EIA in various subject groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Subjects</th>
<th>n</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Healthy men</td>
<td>118</td>
<td>18.5</td>
<td>4.4</td>
</tr>
<tr>
<td>B</td>
<td>Healthy premenopausal women</td>
<td>301</td>
<td>25.5</td>
<td>7.5</td>
</tr>
<tr>
<td>C</td>
<td>Women within 2 years of menopause</td>
<td>88</td>
<td>33.0</td>
<td>14.7</td>
</tr>
<tr>
<td>D</td>
<td>Women up to 35 years past menopause</td>
<td>115</td>
<td>26.8</td>
<td>9.1</td>
</tr>
<tr>
<td>E</td>
<td>Men with osteoporosis</td>
<td>20</td>
<td>30.5</td>
<td>14.0</td>
</tr>
<tr>
<td>F</td>
<td>Women with osteoporosis</td>
<td>123</td>
<td>32.5</td>
<td>19.9</td>
</tr>
<tr>
<td>G</td>
<td>Drug-induced osteoporosis</td>
<td>23</td>
<td>37.9</td>
<td>14.4</td>
</tr>
<tr>
<td>H</td>
<td>Hyperthyroidism and hyperparathyroidism</td>
<td>14</td>
<td>45.6</td>
<td>27.0</td>
</tr>
<tr>
<td>I</td>
<td>Paget disease</td>
<td>99</td>
<td>70.7</td>
<td>69.3</td>
</tr>
</tbody>
</table>

Cr, creatinine.

We assessed PYD&DPD concentrations retrospectively on urines from a clinical trial of women with surgically induced menopause randomized to three dosage treatment groups of transdermal 17β-estradiol (Fig. 4). Baseline creatinine-corrected PYD&DPD values were significantly increased above premenopausal values. Values in the two highest dosage groups, 0.05 and 0.01 mg/day, decreased significantly at 6 months and remained at concentrations near the premenopausal mean at 1 year. Values in the lowest dosage group, 0.025 mg/day, decreased nonsignificantly at 6 months but reached significant concentrations at 1 year.

**Discussion**

An increasing elderly population and rising healthcare costs have brought metabolic bone diseases, particularly osteoporosis, to the forefront of medical research. As awareness of management strategies to prevent bone loss grows and new therapeutic options become available, the need for sensitive and specific markers of bone metabolism has been growing steadily. The pathophysiology of bone loss leading to osteoporosis results
from an imbalance in bone metabolism, with the amount of bone resorption exceeding formation. Currently approved therapies to prevent bone loss or treat osteoporosis are antiresorptive. Thus, there is a significant need for accurate markers that reflect the bone resorption process.

The pyridinium cross-links of collagen have been investigated extensively over the past 10 years by HPLC [1, 4–14, 26, 28, 30–33] and immunoassay methods [15, 16, 19, 20, 27, 28, 30–35]. As products of collagen maturation, their release into circulation occurs only from the collagen degradation process. Dpd is found almost exclusively in bone. Pyd is present in significant quantity in bone but is also present in joint tissue. However, the much higher mass and rate of metabolism of bone and the similarity of the Pyd-to-Dpd ratio in bone and in urine suggest that Pyd in urine, except in cases of active joint catabolism, is essentially bone-derived [2, 3]. Thus, the pyridinium cross-links are very specific for the resorption process.

Simple immunoassay methods for measuring pyridinium cross-links in urine measure the non-peptide-bound free Dpd [20] or free and small-peptide-bound Pyd&Dpd [19]. The ratio of free to total (free plus all peptide-bound) cross-links, 40–50% free, has been observed to be consistent in healthy and diseased subjects [3, 26–28]. This is also confirmed by the high correlation of total Pyd and (or) Dpd measured by HPLC, with free cross-links measured either with HPLC [26, 30], our free Pyd&Dpd EIA, or a free Dpd EIA [20]. Recently, Garnero et al. [31] reported that the ratio of free to total cross-links changes after treatment with the bisphosphonate, pamidronate. After 3 days of intravenous administration to osteoporotic and pagetic subjects, no changes were noted for free cross-links, moderate changes were noted for total cross-links, and large changes were noted for peptide-bound cross-links. Although this suggests that the free-to-total cross-link ratio may not be stable under treatment with this drug, the effect may be an artifact of the timing of the analysis. Erdtieck et al. [35] reported significant changes of free Dpd after 3 months of daily treatment with an oral preparation of the same drug in osteoporotic subjects (~a 25% drop from baseline). Blumsohn et al. [32] reported a significant response with the EIA we have described here in subjects with mild Paget disease treated with another bisphosphonate, etidronate. In that study, Pyd&Dpd concentrations measured every 2 months dropped steadily, changing by −35% after 6 months. Additional research will be required to determine the optimal posttreatment testing interval for the purposes of monitoring these therapies.

The EIA described here exhibited excellent analytical performance characteristics in a simple, microtiter plate format. Evidence that the monoclonal antibody recognizes only the free forms of Pyd&Dpd is derived from sizing dialysis and method comparisons with both free and total Pyd&Dpd measured by HPLC. The high degree of correlation (r = 0.96) with HPLC methods suggests that the discriminative power of the EIA is comparable with HPLC. Indeed, the EIA differentiated all metabolic bone disease populations tested from healthy reference populations. Pyd&Dpd concentrations were also significantly increased in postmenopausal women. These findings are consistent with investigations of increased bone resorption made with both HPLC and other EIA methods for pyridinium cross-link measurement [1, 3, 6, 8–12, 13, 16, 20, 27, 28, 30–32, 34]. Longitudinal variation of Pyd&Dpd concentrations have been determined with this EIA in healthy men and women [33, 36]. Average within-subject CVs reported to range between 12% and 16% ensure that meaningful information will be obtained in serial measurements in individuals.

Menopause results in a period of rapid bone loss in many women, resulting from the loss of the bone-protective effects of estrogen [37]. The loss occurs as a result of higher rates of bone resorption exceeding those of formation [37]. Pyridinium cross-link concentrations rise during the perimenopausal period and remain increased at least during the first several years after menopause [8, 15, 34]. Estrogen replacement therapy restores the increased Pyd&Dpd to normal, premenopausal concentrations [8–10], corresponding to a retention of bone density [9], as measured by HPLC methods.

We have reproduced these findings with this EIA. In a 2-year, placebo-controlled, double-blind trial of transdermal 17β-estradiol [21], bone mineral density of the lumbar spine was retained in the two groups of surgically menopausal women given the highest dosages, 0.05 or 0.1 mg/day. Women receiving only 0.025 mg/day lost 3.0% of their lumbar bone density, and women receiving a placebo lost 6.4%. The average baseline Pyd&Dpd values, assessed with the EIA in samples from women in this study, were significantly increased compared with premenopausal women. Adequate estrogen replacement achieved with the two highest dosages reduced increased Pyd&Dpd to normal premenopausal concentrations. This reduction, which occurred by 6 months, predicted the prevention of lumbar bone density loss that occurred after 2 years. On average, women taking a placebo or insufficient dose of estrogen continued with high Pyd&Dpd concentrations and lost significant amounts of bone density.

This Pyd&Dpd EIA appears to provide an accurate index of the rate of resorption as compared with HPLC methods. The EIA is simpler and easier to perform than current HPLC techniques, which measure free or total Pyd&Dpd separately,
and should serve as a practical substitute for them. This EIA can identify increased rates of resorption in individuals with metabolic bone disease and may be useful for assessing the risk of bone loss, and monitoring the effect of therapies to prevent it.

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References

