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Direct, Enzyme-Linked Immunoassay for Urinary Deoxypyridinoline as a Specific Marker for Measuring Bone Resorption

SIMON P. ROBINS,[1] HENNING WOITGE,[2] ROBERT HESLEY,[3] JULIA JU,[3] SAEID SEYEDIN,[3] and MARKUS J. SEIBEL[2]

ABSTRACT

Several studies in recent years have shown that the pyridinium crosslinks of collagen provide good urinary markers of collagen degradation, primarily reflecting bone resorption. Most studies, however, were based on time-consuming HPLC assays of the crosslinks. We now describe the development of an immunoassay (ELISA) based on a monoclonal antibody for free deoxypyridinoline (Dpd) and its use in healthy individuals and patients with bone-related disorders to measure the urinary excretion of Dpd as an improved assessment of bone resorption rate. The Dpd antibody exhibited less than 1% cross-reaction with free pyridinoline and was shown to react only with free Dpd in urine, having no significant interaction with peptide forms of the crosslinks. The intra- and interassay variations were less than 10 and 15%, respectively. A total of 402 urine samples from patients and healthy volunteers were analyzed by both the immunoassay and HPLC. The ELISA results were highly correlated with those for total Dpd measured by HPLC over the full range of sample groups ($r = 0.95$). In normal adults, the excretion of Dpd (mean ± SD) was 4.7 ± 1.6 nmol/mmol creatinine, with about fivefold higher excretion rates in children. For 31 osteoporotic patients, the ELISA Dpd values (median 6.7; range 3.0–13.5 nmol/mmol Cr) were significantly higher ($p < 0.0001$) than the corresponding values for age- and sex-matched controls (median 4.0; range 1.8–7.4). The difference between the groups was similar for total Dpd by HPLC (osteoporotic: mean 12.8, range 4.8–30.7; controls: 6.6, range 3.0–18.1; $p < 0.0001$). For other patient groups, comparisons of the Dpd excretion with healthy controls revealed similar differences for both the immunoassay and total Dpd analyses in primary HPT ($n = 23$: $p < 0.0001$), Paget's disease ($n = 28$, $p < 0.0001$), renal dysfunction ($n = 26$, no significant difference), and breast cancer ($n = 17$, $p < 0.0001$). We conclude that the immunoassay constitutes a simpler, more direct way of assessing bone resorption rates that provides similar information to the more cumbersome HPLC methods.

INTRODUCTION

THE COLLAGEN CROSSLINKS pyridinoline (Pyd) and deoxypyridinoline (Dpd) are formed during maturation of extracellular collagen fibrils, and measurement of these components in urine has been shown to provide valid clinical markers of collagen degradation.[1–4] Pyd is present in bone, cartilage, and many soft tissues, whereas Dpd has a much more restricted tissue distribution and is primarily located in bone collagen[5–7]: both crosslinks are absent from normal skin. In most individuals, the Pyd/Dpd ratio in urine is similar to that in bone,[8,9] suggesting that the majority of both crosslinks are normally derived from skeletal tissue. In some conditions, however, such as rheumatoid arthritis, there are significant increases in Pyd

[1]Rowett Research Institute, Bucksburn, Aberdeen, Scotland.
[2]Department of Internal Medicine, Division of Endocrinology, University of Heidelberg, Heidelberg, Germany.
[3]Metra Biosystems, Palo Alto, California.

from sources other than bone,[9] thus confirming the view that Dpd is a more specific and reliable indicator of bone resorption.

Following the development of high-performance liquid chromatography (HPLC) methods for measuring the total amounts of crosslinks in hydrolyzed urine,[10] these techniques have been applied to a wide range of clinical studies involving arthritic disorders, metabolic bone diseases, including osteoporosis, and malignancies affecting bone.[9,11–15] These methods have proved particularly valuable in longitudinal studies and monitoring the response to therapy. More recently, it has been shown that about 40% of the crosslinks are excreted in free form and that the proportion of free crosslinks was similar in many different diseases.[12,16] Measurement of free crosslinks therefore provides similar information to that from the total amounts. The development of a direct immunoassay based on a polyclonal antibody that recognizes both Pyd and Dpd was recently described,[17] together with its application to patients with Paget's disease[18] and osteoporosis.[19]

The present study describes the development and application of a direct immunoassay for free Dpd in urine. The assay is simple to use, with good precision and reproducibility. The results show that this assay provides discrimination between normal and increased bone resorption similar to that given by the measurement of total Dpd by HPLC.

MATERIALS AND METHODS

Tetrahydrofuran (HPLC grade) was obtained from Rathburn Chemicals, Ltd. (Walkerburn, Peeblesshire, UK), and heptafluorobutyric acid (Pierce Sequanal grade) was supplied by Life Science Laboratories, Ltd. (Luton, UK). 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide was obtained from Pierce (Rockford, IL). All other chemicals were of analytic reagent grade when possible and were obtained from BDH, Ltd. (Poole, Dorset, UK).

Preparation of antigen

Dpd was isolated from urine or from acid hydrolysates of decalcified bovine bone powder using methods similar to those described previously for Pyd.[10,17] Purity was assessed by comparison of the fluorescence and ultraviolet absorption characteristics with a highly purified compound standardized gravimetrically. Dpd was conjugated to a carrier protein, keyhole limpet hemocyanin, with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide to serve as immunogen.

Monoclonal antibody preparation and characterization

Female mice (5 weeks old) were immunized with 100 μg intraperitoneal (IP) immunogen in Ribi adjuvant (Ribi Immunochem. Research) three times, with 2 week intervals between immunizations.[20] The mouse was boosted with 200 μg intravenous immunogen in Hank's balanced salt solution 4 days before splenectomy. Spleen cells were fused with P3X63Ag8.653 mouse myeloma cells according to previously described techniques.[21] Hybridoma cells were cultured in modified Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum.

Hybridoma supernatant antibodies were screened for Dpd specificity using an enzyme-linked immunosorbent assay (ELISA) in which biotin-Dpd was attached to a microtiter plate via streptavidin. The antibody properties of sensitivity, selectivity, cross-reactivity, and recognition of urinary Dpd were tested by competition between added Dpd, Pyd, amino acids, or urine and the ELISA plate-bound Dpd. The bound antibody was detected by an enzyme-conjugated second antibody, goat antimouse alkaline phosphatase. The enzyme substrate, *p*-nitrophenylphosphate, was added to the plate, and the absorbance at 405 nm was measured.

The best Dpd monoclonal antibody (MAb) among several monoclonal antibodies was selected for high affinity, specificity to free Dpd, and low cross-reactivities with Pyd and amino acids. The subclass of this MAb was determined to be $IgG_{1,k}$ by Fisher Scientific kit. Ascites fluid was produced by Balb/C mice primed with incomplete Freund's adjuvant and IP injection of the hybridoma cell line. The MAb was further purified by a Prosep-A protein A column for direct coating of stripwells used in the ELISA protocol.

Preparation of Anti-Dpd-coated plates

The stripwell coating procedure entailed adsorption of rabbit antimouse IgG onto the stripwells overnight at 20–28°C. This was followed by three washes and the capture of MAb to Dpd overnight at 20–28°C. The stripwells were then washed three times and soaked in a sucrose preservative solution. The sucrose solution was aspirated, and the stripwells were dried overnight at 37°C. The strips were then stored at 4°C until use.

Immunoassay procedure

Samples or standard solutions were diluted 10-fold with assay buffer (phosphate-buffered saline, PBS, and Tween; 0.15 M NaCl, 10 mM sodium phosphate, pH 7.0, and 0.05% Tween 20). The diluted samples, standards, or controls (50 μl) were added to the antibody-coated stripwells, followed by 100 μl enzyme conjugate solution. The plate was incubated for 2 h at 4°C in the dark, after which time the wells were washed three times with PBS-Tween. The latter procedure was completed within 1 minute to prevent any reequilibration with changing temperature of the wash solution. After blotting dry on paper towel, 150 μl enzyme substrate solution (2 mg/ml of *p*-nitrophenylphosphate in 1.0 M diethanolamine and 1 mM $MgCl_2$, pH 9.8) was added to each well. Following incubation at room temperature for 45 minutes, 100 μl of 1 M NaOH was added to each well and the absorbance was measured at 405 nm. Assays were performed using a Dynatech System 7000 comprising an automated plate washer and reader (Dynatech Laboratories, Ltd., Billingshurst, UK). The results were calculated with the instrument software using a four-parameter curve-fitting procedure with standards ranging from 3 to 300 nM Dpd.

HPLC Analyses

Analyses of total Dpd were performed on urine samples hydrolyzed in 6 M HCl using a fully automated system with

prefractionation on cellulose CC31 columns and additional solvent extraction (tetrahydrofuran) to allow direct transfer to the HPLC column with *O*-acetylpyridinoline as internal standard.[22] Free Dpd in unhydrolyzed urine was measured directly by the same automated HPLC system.[22] The overall coefficient of variation for this assay was less than 6%.

Subjects

Urine samples were collected from a total of 402 individuals. Usually these were first morning voids, but in all cases the spot urine samples were collected between 7 and 11 a.m. without dietary restrictions. Samples were stored at −20°C for up to 6 months before analysis. Dpd excretions were expressed relative to creatinine measured by a Kone autoanalyzer (Lab Medics, Stockport, UK) using the Jaffe technique. The variations with age in urinary Dpd were assessed in 255 healthy individuals aged 1–81 years. Additional samples were collected from patients with various metabolic bone disorders attending the outpatient clinic at the Department of Internal Medicine, University of Heidelberg.

Osteoporosis: A group of (male to female, 31 patients M/F, 13:18) aged 40–80 years were diagnosed osteoporotic on the basis of having one or more typical vertebral fracture(s) and bone density measures (dual x-ray absorptiometry) below 2 standard deviations (SD) of the age- and sex-specific mean. This patient group was compared with age- and sex-matched controls having no sign of bone disorders.

Paget's Disease of Bone: A total of 28 patients (M/F 21:7) aged 42–72 years with polyosteotic bone disease were studied. None was receiving treatment at the time of sample collection, although some of the samples were from routine monitoring of patients without active disease.

Chronic Renal Dysfunction: A group of 26 patients (M/F 12:14) aged 28–76 years were studied. All individuals had a creatinine clearance of less than 50 ml/minute and no signs of secondary hyperparathyroidism (serum intact parathyroid hormone, PTH < 6.0 pg/ml).

Secondary Hyperparathyroidism: A group of 18 patients (M/F 7:11) aged 32–66 years with chronic renal failure and elevated levels of intact PTH (>6.0 pg/ml) were studied.

Primary Hyperparathyroidism (pHPT): A group of 23 patients (M/F 19:4) aged 17–73 years were diagnosed with pHPT on the basis of chronic hypercalcaemia, hypercalcuria, and elevated serum intact PTH levels. None of these patients had radiologic signs of pHPT.

Breast Cancer: A group of 17 patients aged 28–78 years were studied having histologically proven breast cancer and one or more bone metastases detected by radioisotopic bone scan. Areas of interest were further evaluated by x-ray examination. None of these patients had received antineoplastic treatment before sample collection.

Chromatographic Fractionation of Urine

Urine (2.0 ml) was fractionated by size exclusion chromatography on a column (1.7 × 140 cm) of Sephadex G-10 run at 22 ml/h with 0.4 M acetic acid as eluant. Fractions (5 ml) were collected and, after fivefold dilution with 0.15 M NaCl and 50 mM sodium phosphate, pH 7.5, aliquots equivalent to 10 μl of each fraction were quantified by ELISA. Appropriate fractions were pooled and lyophilized and were further chromatographed by HPLC using a Rosil C_{18} column (0.9 × 25 cm) run in 10 mM heptafluorobutyric acid with a gradient of 15–30% acetonitrile. The natural fluorescence of the pyridinium crosslinks was monitored, and aliquots (100 μl) of each fraction were dried in vacuo using a rotary concentrator (Gyro Vap; VA Howe Ltd., London, UK) and were redissolved in assay buffer for testing by ELISA.

Statistical analyses

Analyses were performed using either Minitab statistical software (Minitab, Inc., State College, PA) or Genstat V (Rothampstead Experimental Station, Harpenden, UK). All comparisons of the significance of difference in Dpd excretion between patient groups were made using a nonparametric test (Mann-Whitney).

RESULTS

Immunoassay specificity and reproducibility

Formulation of the assay using Dpd covalently attached to alkaline phosphatase as the competitive species produced a rapid, single-step assay for which a standard inhibition curve is shown in Fig. 1. The mean value for 50% inhibition was 20.9 nM Dpd, and the sensitivity of the assay (2.5 SD at zero Dpd concentration) was 2 nM Dpd. By testing standard compounds in the assay, the monoclonal antibody was shown to have less than 1% cross-reaction with Pyd and no detectable reaction with glucosyl-galactosyl-Pyd (at 2000 nM). Similarly, there was no significant reaction with hydroxylsine or other natural amino acids or with desmosine crosslinks or other related pyridinium

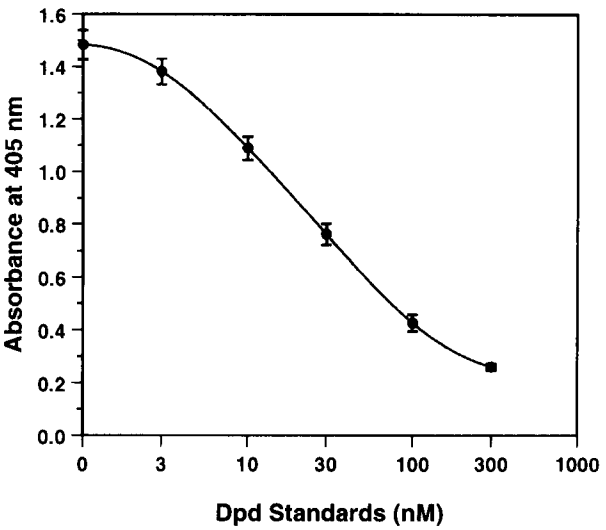

FIG. 1. Standard curve for the Dpd ELISA. Various concentrations of free Dpd were assayed as described in Materials and Methods, and the mean values of 10 replicates were plotted. The continuous line represents the best fit sigmoid curve, and the error bars are 1 standard deviation from the mean.

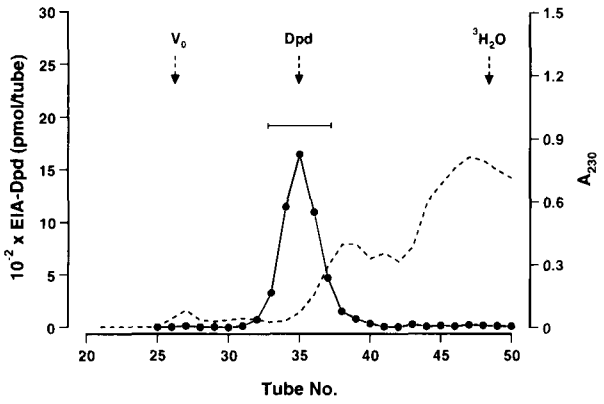

FIG. 2. Fractionation of urine by size exclusion chromatography. Whole urine was fractionated on a column of Sephadex G-10 and monitored by ELISA against Dpd (circles) and absorbance at 230 nm (dashed line). A single peak of Dpd immunoreactivity was obtained. The position of excluded material (V_0) having molecular masses of greater than 800–1000 daltons is indicated, as is the included volume measured using tritiated water. For further details, see Materials and Methods.

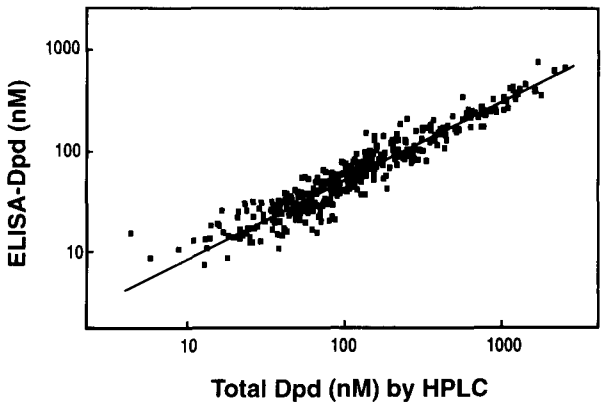

FIG. 3. Relationship between urinary concentrations of free Dpd measured by ELISA and total Dpd assayed by HPLC. The data, normalized by logarithmic transformation, showed a high correlation ($r = 0.95$) between the two methods with a regression equation of $\log_e y = 0.76 \pm 0.01 \log_e \times 0.42 \pm 0.06$, giving the relationship ELISA = $1.52(\text{total})^{0.76}$ between values for the two assay methods.

compounds. The antibody exhibited equal reactivity with urinary free Dpd and with the crosslink isolated from acid hydrolysates of bone. To confirm the immunoreactive species in urine, the assay was used to monitor fractions of whole urine separated on a calibrated size exclusion column (Fig. 2). A single, symmetrical peak of immunoreactive material was obtained, which was shown by preparative reversed-phase HPLC to cochromatograph with Dpd. There was no reaction with smaller peptides eluting earlier in the chromatogram.

The reproducibility of the assay was assessed by repeated measurements of urine samples with different Dpd concentrations. As shown in Table 1, the intraassay and interassay variations were less than 10 and 15%, respectively. In recovery experiments, additions of Dpd sufficient to increase the concentration by 50 nM to urine samples containing 26.7 and 59.6 nM endogenous Dpd resulted in recoveries of 96 and 99%, respectively.

Comparison with HPLC

All samples subjected to the direct ELISA were also analyzed by HPLC for total Dpd content after acid hydrolysis of the urine. Because of the very wide and skewed distribution of the raw data uncorrected for creatinine concentration, the relationship between the ELISA and HPLC results was plotted after logarithmic transformation, resulting in normally distributed data as judged by probability plots. The ELISA and HPLC measurements correlated with $r_p = 0.95$ ($p < 0.001$; Fig. 3). Analysis of individual groups of patients or healthy volunteers showed similar relationships, with no significant deviations from the gradient of the regression line for the total group. There was also a close correlation ($r_p = 0.97$) between the ELISA values and free Dpd measured by HPLC (data not shown). Overall, the urinary Dpd concentrations determined by ELISA were 48.5 ± 12.4% of the values for total Dpd measured by HPLC.

TABLE 1. INTER-AND-INTRAASSAY VARIATION IN DPD IMMUNOASSAY OF HUMAN URINE SAMPLES

Interassay (n = 14)		*Intraassay (n = 25)*	
Dpd (nM)	*CV*[a] *(%)*	*Dpd (nM)*	*CV (%)*
236.6	4.0	214.6	4.2
100.9	7.0	95.2	6.1
49.8	11.8	25.4	8.3
18.8	13.7		

[a]Coefficient of variation.

Variations with age

Values for free urinary Dpd measured by ELISA in a total of 255 individuals grouped according to age and sex are shown in Fig. 4. The combined mean value for adults aged 21–81 years was 4.7 ± 1.6 nmol/mmol creatinine. Values for women were on average about 15% higher than those for men ($p < 0.05$). In the adult age groups, there were no progressive, statistically significant variations with age in either sex. The interindividual variations in women over 50 years of age were much more marked than in the corresponding male age groups (Fig. 4). The Dpd excretion in children was up to fivefold higher than in adults and appeared to be dependent on the rate of growth, with the highest values recorded for the very young age group.

Dpd excretion in skeletal disorders

To evaluate the ELISA in monitoring bone resorption for patient groups, comparisons were made with the results obtained by total Dpd by HPLC. For a group of osteoporotic patients, the Dpd excretion measured by ELISA was significantly higher than for an age- and sex-matched control group (Fig. 5). The median value for the osteoporotic group was 1.8 times higher ($p < 0.0001$) than that for the controls. A similarly signif-

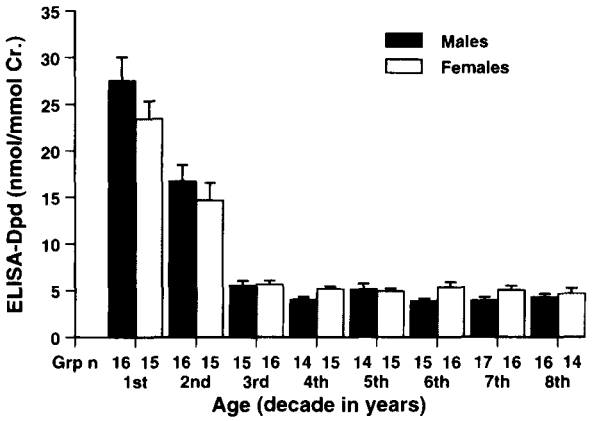

FIG. 4. Variations with age and sex in the urinary excretion of Dpd measured by immunoassay expressed relative to creatinine concentrations. The number of individuals in each group is indicated on the *x* axis (Grp *n*), and the bars represent standard error of the mean.

icance difference between groups was obtained for total Dpd measurements by HPLC in the same samples (Fig. 5), for which the corresponding ratio of median values was 1.9.

In Fig. 6, urinary excretion of Dpd measured by direct ELISA and by HPLC are plotted for a series of patient groups in relation to their respective normal ranges (2 SD) derived from measurements in healthy adults aged 30–81 years (n = 151). The results for the ELISA showed that there were highly significant elevations of urinary Dpd compared with the normal range for patients having breast cancer, Paget's disease of bone, and primary hyperparathyroidism, with less marked differences in secondary hyperparathyroidism and no significant difference for patients with renal impairment. The pattern of results for total Dpd excretion was very similar to that for the ELISA (Fig. 6). Thus,

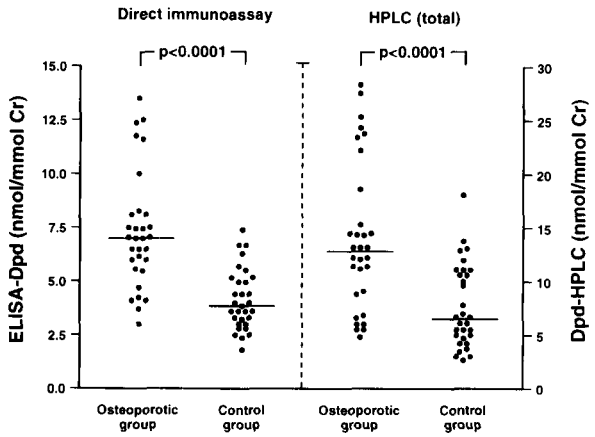

FIG. 5. Urinary excretion of Dpd in osteoporotic patients. The scatterplots show individual values for the osteoporotic patients compared with age- and sex-matched controls. The lines indicate median values, and the significance of difference between groups was by Mann-Whitney test. Note that similar differences between groups were found for both the free Dpd by ELISA and the total by HPLC.

for example, 14 of 17 (82%) of patients with breast carcinoma were above the normal range for the ELISA, in comparison to 13 of 17 (76%) for total Dpd measurement.

DISCUSSION

A considerable body of evidence has accumulated over the past few years that confirms the validity of the pyridinium crosslinks as markers primarily of bone resorption.[1–3,7] Although the concentrations in urine of the two crosslinks, Pyd and Dpd, are usually highly correlated, suggesting that both are mainly derived from bone, the results from several recent studies have indicated that Dpd is a more specific and sensitive marker of bone resorption.[19,23] This fact, together with the uncertainties introduced by disturbances in Pyd excretion caused by arthritic disease,[9,24] led to the development of an immunoassay for Dpd that would more accurately reflect bone resorption rates.

The present work shows that Dpd immunoassay was rapid (about 3 h to complete) and easy to use: no pretreatment of the urine was required, and the necessity to dispense small volumes of sample was avoided. Based on a high-affinity monoclonal antibody, the assay was shown to be specific for a single component in urine, the free Dpd crosslink. Free Dpd constitutes about 40–50% of the total crosslink, so that the current assay is measuring a major component in urine. The high correlation with total Dpd measurements by HPLC over the complete range of samples, including those from patients with various metabolic bone disorders (Fig. 3), confirms previous reports that the proportion of free crosslinks is consistent.[12,16] The measurement of a single, well-characterized urinary metabolite may therefore have advantages over a recently described method[25] involving an assay of a range of crosslink peptides for which standardization under different physiologic situations has not been established. From the known concentration of Dpd in bone and the proportion measured by ELISA,[7,12] the calculated equivalent of bone collagen excreted by adult males is 140 pmol per μmol creatinine, over threefold higher than that obtained with the peptide assay.[25]

Studies of the changes with age in urinary Dpd showed no significant increase during adult life in females. This is in contrast to the results for an assay measuring primarily Pyd, in which a small increase with age was observed.[18,19] This finding may indicate either an increase with age in the proportion of Pyd from nonbony sources or a slight increase in the Pyd/Dpd ratio in bone. These possibilities are currently being investigated. The excretion of Dpd relative to creatinine in women was about 15% higher than in men, a similar difference between sexes to that observed using an immunoassay recognizing both crosslinks.[19] In the present work, no systematic study was made of the changes in Dpd excretion caused by the menopause, because the number of individuals with confirmed menopausal status was insufficient for any conclusions to be drawn; further studies of well-characterized volunteer groups are currently in progress to resolve this point. Nevertheless, the more pronounced variations in crosslink excretion for women over 50 years of age may be an effect associated with the menopause. Dpd excretion in children was about fivefold higher than in

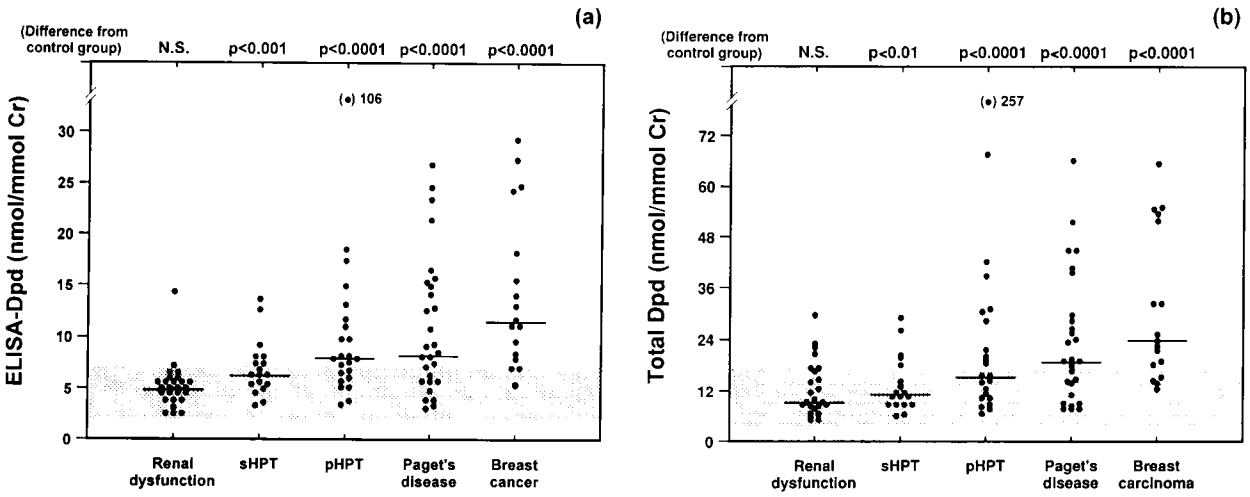

FIG. 6. Urinary Dpd excretion in bone-related disorders in relation to the normal range. The scatterplots show individual values for the different patient groups measured by direct ELISA (a) or by HPLC after acid hydrolysis (b) in relation to their respective normal ranges (2 SD), shown as the shaded areas. The median values for each group are indicated, together with the significance of difference (Mann-Whitney test) from the normal range.

adults, suggesting the applicability of this assay for monitoring growth.[26] Such applications have proved valuable in assessing growth abnormalities and the response to therapy.[27]

The Dpd immunoassay results for the osteoporotic patients gave similar differentiation with respect to their age- and sex-matched controls as the total Dpd assay by HPLC. There was an almost twofold difference in median values between the osteoporotic and control groups, which provides good evidence that this assay will have applications as part of a screening procedure for vertebral osteoporosis. It is unlikely, however, that a single marker will be able to be sufficiently discriminative,[19] and additional markers, particularly of bone formation rates, will be necessary to provide an assessment of net bone loss in these patients.

For a series of bone-related disorders, the patterns of free Dpd excretion measured by immunoassay were very similar to those for total Dpd (Fig. 6). These disorders covered a wide spread of values, ranging from breast cancer patients, for whom most were outside the normal range, to the relatively normal values for patients with renal impairment. The results therefore confirm the efficacy of the immunoassay for measuring bone resorption because the HPLC method can be regarded as the gold standard.

The relatively normal values for Dpd excretion in patients with renal dysfunction is in agreement with a previous finding that crosslink excretion is generally unaffected by changes in renal function.[15] This result also supports the use of urinary creatinine as a correction for urine volume and body mass in expressing the crosslink results.

In summary, therefore, a rapid ELISA for free Dpd in urine has been described that has good sensitivity and reproducibility and requires no pretreatment of the urine. This assay measures a single analyte in urine, and the results are highly correlated with those for total Dpd measured by HPLC. In a range of patient groups, the ELISA provided information on bone resorption similar to that given by the total amounts and therefore constitutes a much more convenient alternative to the HPLC assay.

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Address reprint requests to:
Dr. Simon P. Robins
Biochemical Sciences Division
Rowett Research Institute
Bucksburn
Aberdeen AB2 9SB, Scotland

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