
Bone-Resorption Markers Galactosyl Hydroxylysine, Pyridinium Crosslinks, and Hydroxyproline Compared

P. Bettica,1 L. Moro,2 S. P. Robins,3 A. K. Taylor,1 J. Talbot,1 F. R. Singer,4 and D. J. Baylink4

We compared the clinical performances of four bone-resorption (BR) assays (hydroxyproline, HYP; galactosyl hydroxylysine, GHYL; deoxypyridinoline, DPD; and pyridinium, PYD) in subjects with different BR rates: normal (adult men and premenopausal women), mildly increased (postmenopausal osteoporotic women), high (Peget disease patients), and very high (children). The discrimination power (Z score) and the accuracy (estimated by receiver-operating characteristic analysis) for GHYL, DPD, and PYD were compared with those for HYP. Discrimination power and accuracy were similar for high- and very-high-BR groups for all four assays. However, in the mildly increased-BR group, DPD, GHYL, and PYD showed a higher discrimination power and accuracy than did HYP. The clinical performances of HYP, DPD, GHYL, and PYD are comparable for large changes in BR. For modest changes, DPD, GHYL, and PYD are more accurate and have a higher discrimination power than does HYP.

Three diagnostic procedures are available for evaluating metabolic bone diseases: bone density, bone biopsies, and biochemical markers of bone turnover. Bone density is essential for diagnosing osteoporosis but does not provide information on the dynamics of bone formation and bone resorption. Bone biopsies and biochemical markers provide this information. Bone biopsies, being invasive procedures, cannot be routinely included in the management of patients with osteoporosis. Accordingly, there has been a great effort in recent years to develop biochemical markers of bone turnover.

In the past decade assays to measure bone formation have been developed (i.e., assays of bone alkaline phosphatase, osteocalcin, and procollagen peptide). Bone resorption, which is frequently disturbed in the metabolic bone diseases and especially in osteoporosis, is still evaluated by measuring urinary excretion of hydroxyproline (HYP), a marker that is not specific for bone resorption (1, 2) and that undergoes extensive metabolism in the liver (3).6

In recent years new or improved assays to evaluate bone resorption have been developed. Accordingly, HPLC assays have been developed to measure galactosyl hydroxylysine (GHYL) and pyridinium crosslinks.

Additional Keyphrases: receiver-operating characteristic analysis

1 Jerry L. Pettis VA Hospital, Loma Linda, CA.
2 Dipartimento di Biochimica, Biologia e Chimica delle Macromolecole, Università degli Studi, Trieste, Italy.
3 The Rowett Research Institute, Aberdeen, Scotland, UK.
4 Cedars-Sinai Medical Center, Los Angeles, CA.
Received April 27, 1992; accepted June 18, 1992.

6 Nonstandard abbreviations: HYP, hydroxyproline; GHYL, galactosyl hydroxylysine; PYD, pyridinium; DPD, deoxypyridinoline; and ROC, receiver-operating characteristic.
Table 1. Age, Sample Size, and Sex Distribution of the Subjects Studied

<table>
<thead>
<tr>
<th>Group</th>
<th>Age, years*</th>
<th>n</th>
<th>Sex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control men</td>
<td>36.5 ± 10.3</td>
<td>11</td>
<td>M</td>
</tr>
<tr>
<td>Control women</td>
<td>35.5 ± 5.9</td>
<td>11</td>
<td>F</td>
</tr>
<tr>
<td>Osteoporotic women</td>
<td>65.6 ± 11.31</td>
<td>13</td>
<td>F</td>
</tr>
<tr>
<td>Paget disease patients</td>
<td>73.6 ± 5.18</td>
<td>12</td>
<td>F</td>
</tr>
<tr>
<td>Children</td>
<td>8.75 ± 2.21</td>
<td>4</td>
<td>M, F</td>
</tr>
</tbody>
</table>

* Mean ± SD.

(i.e., pyridinoline, PYD, and deoxypyridinoline, DPD). These markers are relatively specific for bone collagen, are released after collagen degradation, and appear not to be metabolized in the liver (4–8). Therefore, evaluation of the urinary excretion of GHYL, PYD, and DPD has been proposed to measure bone resorption. PYD, DPD, GHYL, and HYP have never been measured in the same set of samples nor have their performances been compared.

Here we evaluate and compare the clinical performance of HYP, GHYL, PYD, and DPD assays in subjects with normal (i.e., adult men and premenopausal women), mildly increased (i.e., postmenopausal osteoporotic women), high (i.e., Paget disease patients), and very high (i.e., children) bone-resorption rates.

Materials and Methods

Twenty-four-hour urine samples were collected from five groups of subjects. The groups selected were adult men, premenopausal women, postmenopausal osteoporotic women, Paget disease patients, and children. All the adult men, premenopausal women, and children were in good health and none was taking any medication known to affect bone metabolism. For postmenopausal osteoporotic women, the diagnosis of osteoporosis was based on clinical examination and quantitative computer tomographic measurements of spine density. For all patients the potassium phosphate value was <100 mg/cm², which is our threshold for increased fracture risk (9); the group mean ± SD was 61 ± 25 mg/cm². The patients with Paget disease of bone were established patients at the bone center at Cedars-Sinai Medical Center. No postmenopausal osteoporotic women or Paget disease patients were taking medications known to affect bone metabolism. The mean ages, sample size (n), and sex distribution of the groups are reported in Table 1.

To avoid any interference from the diet in measuring HYP, all subjects followed a collagen-free diet just before and during the urine-collection period.

HYP was measured by HPLC with fluorenylmethyl oxycarbonyl derivatization after reaction with o-phthal-
Table 2. Z Scores and Accuracy (%Acc) of the Four Assays in Osteoporotic and Paget Disease Patients and Children

<table>
<thead>
<tr>
<th>Group</th>
<th>PYP</th>
<th></th>
<th>DPD</th>
<th></th>
<th>GHYL</th>
<th></th>
<th>HYP</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Z</td>
<td>%Acc</td>
<td>Z</td>
<td>%Acc</td>
<td>Z</td>
<td>%Acc</td>
<td>Z</td>
<td>%Acc</td>
</tr>
<tr>
<td>Osteoporotic women</td>
<td>3.1</td>
<td>77</td>
<td>2.87</td>
<td>72</td>
<td>3.34</td>
<td>81</td>
<td>1.67</td>
<td>52</td>
</tr>
<tr>
<td>Paget disease patients</td>
<td>3.82</td>
<td>93</td>
<td>5.5</td>
<td>94</td>
<td>3.43</td>
<td>90</td>
<td>3.1</td>
<td>85</td>
</tr>
<tr>
<td>Children</td>
<td>10.4</td>
<td>—</td>
<td>14.3</td>
<td>—</td>
<td>7.8</td>
<td>—</td>
<td>4.8</td>
<td>—</td>
</tr>
</tbody>
</table>

dialdehyde (10). GHYL was measured by HPLC according to the method of Moro et al. (11), and GHYL standard was prepared from urine by the method of Moro et al. (12). PYD and DPD were measured by HPLC (13, 14). PYD and DPD standards were prepared as previously reported (13). The overall variation for all four bone-resorption assays was <10%. Creatinine was measured by the method of Heinegard and Tiderstroen (15). PYD and DPD urinary excretions were expressed as nmol/mmol of creatinine, and GHYL and HYP excretions were expressed as μmol/mmol of creatinine.

Paget disease patients and children were compared with adult men and premenopausal women grouped together as controls. Postmenopausal osteoporotic women were compared with premenopausal women alone.

The significance of the difference between means was evaluated by Student's t-test. To evaluate the performance of the four assays, we estimated the discrimination power and the accuracy of each assay in the different groups. The discrimination power of the four assays in all the different groups was estimated by calculating the Z scores.

In the osteoporosis and Paget disease groups, we evaluated the accuracy of the assays by selecting five arbitrary threshold values and estimating the specificity (as the ratio between true negatives and the sum of true negatives and false positives) and the sensitivity (as the ratio between the true positives and the sum of true positives and false negatives) for each value. The sensitivity was then plotted vs 1 – specificity to produce a receiver-operating characteristic (ROC) curve for every assay. The accuracy was estimated as the area under the ROC curve (16).

Results

The HYP, GHYL, PYD, and DPD values for children, osteoporosis patients, and Paget disease patients and their significance vs the corresponding control group are shown in Figure 1. For children and Paget disease patients, the urinary excretion of all four markers was severalfold higher than that for the control adult men and premenopausal women together, and the differences were highly significant. For these two groups, all four assays showed a high discrimination power (Table 2). For the Paget disease group, the Z scores ranged from 3.1 (P < 0.005, HYP) to 5.5 (P < 0.0001, DPD); for children, the Z scores ranged from 4.8 (P < 0.0001, HYP) to 14.3 (P < 0.0001, DPD). In the Paget disease group, the four assays showed a high accuracy, ranging from 83% (HYP) to 89% (PYD) (Figure 2).

In the postmenopausal osteoporosis group, only the urinary excretion of the three new markers was significantly increased (Figure 1). PYD excretion was 58% higher in postmenopausal osteoporotic patients than in the premenopausal control subjects (P < 0.01); DPD excretion was increased by 65.9% (P = 0.02) and GHYL excretion was increased by 63.9% (P = 0.006). In the postmenopausal osteoporosis group only PYD, DPD, and GHYL assays showed a good discrimination power (Table 2). The Z scores ranged from 2.87 (P = 0.004, DPD) to 3.34 (P < 0.002, GHYL) for the three new assays, whereas the HYP assay Z score was only 1.67.
Table 3. Correlation Coefficients between the Four Assays (n = 51)

<table>
<thead>
<tr>
<th></th>
<th>GHYL</th>
<th>PYD</th>
<th>DPD</th>
</tr>
</thead>
<tbody>
<tr>
<td>HYP</td>
<td>0.823</td>
<td>0.901</td>
<td>0.827</td>
</tr>
<tr>
<td>GHYL</td>
<td>-</td>
<td>0.896</td>
<td>0.859</td>
</tr>
<tr>
<td>PYD</td>
<td>-</td>
<td>-</td>
<td>0.948</td>
</tr>
</tbody>
</table>

(NS). Finally, in the postmenopausal osteoporosis group, HYP assay accuracy was only 52%, whereas the accuracies of DPD, PYD, and GHYL assays were 72%, 77%, and 81%, respectively (Figure 2).

Table 3 shows the correlation between the four assays when the pooled data from all subjects were used. All four assays were highly correlated, and the correlation coefficients ranged from 0.823 (GHYL and HYP) to 0.948 (PYD and DPD).

Discussion

We evaluated the clinical performances of four different bone-resorption assays in subjects with different predicted bone-resorption rates. Table 4 summarizes the characteristics expected from an ideal bone-resorption assay and compares them with the characteristics of the assays for HYP, GHYL, DPD, and PYD.

The ideal assay to evaluate bone resorption should use a simple and reproducible technique and should measure a molecule that is present only in bone, is released only during bone resorption, and is not altered before being excreted. An assay with such characteristics would show a relatively small variability in populations with similar rates of bone resorption and would accurately distinguish subjects with different bone-resorption rates (i.e., would show a good clinical performance).

HYP is present not only in bone but is a major component of all types of collagen and is present in all the molecules that contain a collagen-like structure, such as the C1q component of complement (4). GHYL, DPD, and PYD are largely localized in bone. GHYL is present in different types of collagen but is five- to sevenfold more concentrated in type I collagen of bone than in type I collagen of skin (bone and skin being the major sources of collagen in the human body) (6). DPD has been detected only in bone and dentine. PYD is present in different collagens but is mainly concentrated in type II collagen of cartilage and in type I collagen of bone (7). None of these three markers is present in molecules other than collagens.

HYP is not specific for bone resorption because it is present in procollagen propeptides and is also released during collagen deposition (2). None of the other three molecules is present in procollagen peptides; thus, the amount of DPD, PYD, and GHYL released in the blood is derived from collagen degradation.

A potentially serious problem with the HYP assay is that ~80% of the HYP released during collagen metabolism is metabolized in the liver and only ~20% is excreted in urine (17). GHYL, DPD, and PYD do not seem to undergo liver metabolism (18).

Regarding the analytical techniques used, all four assays studied use an HPLC method that is relatively simple, and all have relatively good reproducibility. Different clinical performances were expected and found. To test the clinical performance of the four assays, we tested their power to discriminate and their accuracy in subjects with different rates of bone resorption. Adult men and premenopausal women were selected as having representative normal bone-resorption rates, whereas postmenopausal osteoporotic women, Paget disease patients, and children were chosen as having representative mildly increased, high, and very high bone-resorption rates, respectively.

The discrimination power (Z score) defines how well a certain assay can distinguish between two groups of subjects with different characteristics. The accuracy of an assay defines how frequently the assay outcome provides a correct diagnosis (i.e., given a specific threshold value, the accuracy depends on how often the non-

Table 4. Characteristics of the Ideal Assay for Bone Resorption Compared with Characteristics of Assays for HYP, GHYL, DPD, and PYD

<table>
<thead>
<tr>
<th>Molecule assayed</th>
<th>Ideal</th>
<th>HYP</th>
<th>GHYL</th>
<th>DPD</th>
<th>PYD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Location</td>
<td>Bone</td>
<td>Many tissues</td>
<td>Mainly bone</td>
<td>Bone, dentine</td>
<td>Cartilage, bone</td>
</tr>
<tr>
<td>Released only during collagen degradation</td>
<td>Yes</td>
<td>No</td>
<td>Yes*</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Liver metabolism</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>No*</td>
<td>No*</td>
</tr>
<tr>
<td>Method</td>
<td>Simple</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Reproducible</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Clinical performance</td>
<td>Low population variability</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>Discrimination between subjects with normal and high bone resorption</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>Discrimination between subjects with normal and mildly increased bone resorption</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

* Not definitively established.
diseased subjects have values below the threshold (true negatives) and how often the diseased subjects have values above the threshold (true positives)). The ideal assay would have an accuracy of 100%; an assay with an accuracy of 50% is valueless (19).

The most important factors influencing the estimate of the accuracy of an assay are how the true disease status of the subjects is established and the prevalence of the disease and decision biases (20). Our diagnosis of Paget disease of bone was made with standard techniques, and an increase in bone resorption is always present when this disease is active (21). For osteoporosis the accuracy of the assays is more difficult to ascertain because there are no unequivocal diagnostic indexes. Nevertheless, diagnosis was made by evaluating spine density. As a group, patients with postmenopausal osteoporosis have increased bone resorption (22). We estimated the accuracy of the assays as the area under the ROC curve. This estimate of the accuracy is independent of the disease prevalence and of decision biases (20).

If the patients have bone-resorption rates that are much higher than those of normal subjects, even an assay exhibiting modest performance can discriminate between patients and normal subjects. This is indeed the case with Paget disease of bone and childhood. Paget disease of bone is characterized by big focal increases in osteoclastic activity coupled to increased bone formation (21), whereas the high bone resorption seen in children is part of the physiological growth process. As a result of the increased bone turnover, all the markers of bone resorption and formation are particularly increased in these two groups of subjects (6, 23–28). Similarly, the urinary excretion of GHYL, PYD, DPD, and HYP was severalfold higher in Paget disease patients and children than in normal adult control subjects.

As expected, in these two groups, all four assays showed a high power to discriminate and a high accuracy: the Z scores for all the assays were, in fact, highly significant and, in Paget disease of bone, all the assays were highly accurate, with an accuracy ranging from 83% (HYP) to 89% (DPD).

In postmenopausal osteoporosis, in contrast, the increase in bone resorption is relatively modest. In a study on a group of postmenopausal osteoporotic women in which histomorphometric indexes of cancellous bone turnover were measured, Eriksen et al. (22) showed that the rate of bone resorption was increased by 67% (compared with age-matched control subjects). Accordingly, in postmenopausal osteoporosis, only modest increases in the excretion of markers specific for bone resorption (i.e., PYD, DPD, or GHYL) have been reported (29, 30).

For our postmenopausal osteoporotic patients, values for the newer bone-resorption markers were significantly different from those for control subjects. Urinary excretion of PYD, DPD, and GHYL was 58–66% higher in these patients than in premenopausal control subjects. Urinary excretion of HYP, although increased, was not significantly different from that for premenopausal control subjects. Only the three newer assays showed significant discrimination power for postmenopausal osteoporotic subjects.

Similar to the results for discrimination power, only DPD, PYD, and GHYL showed high accuracy in distinguishing the osteoporotic patients from premenopausal control subjects, with the accuracies ranging from 72% (DPD) to 81% (GHYL), with HYP showing poor accuracy (52%).

We chose the postmenopausal osteoporotic patients as being representative of moderately increased bone resorption. However, to evaluate the accuracy of the new bone-resorption assays in the diagnosis of osteoporosis, further studies with age-matched control subjects are required.

Finally, in data pooled from all groups, all four assays were highly correlated, showing that despite the differences in performance, the information provided by the four assays was similar. In particular, results for PYD and DPD were closely correlated, meaning that the two assays provided almost the same information.

In summary, this study of the clinical performance of the DPD, PYD, GHYL, and HYP assays in the same subjects shows that these assays are comparable when a high bone-resorption rate is expected. When only modest increases in bone resorption are expected, the GHYL, PYD, and DPD assays are more accurate and show a higher discrimination power than does the HYP assay. These data therefore support the use of GHYL and pyridinium crosslinks for evaluating metabolic bone disease.

References
11. More L, Modricky C, Stagni N, Vittur F, de Bernard B. High performance liquid chromatography analysis of urinary hydroxy-

CLIN. CHEM. 38/11, 2318–2321 (1992)

Two-Site Assays of Bone Gla Protein (Osteocalcin) Demonstrate Immunochemical Heterogeneity of the Intact Molecule

Leonard J. Deftos,1 Robert L. Wolfert,2 Craig S. Hill,2 and Douglas W. Burton1

We developed a panel of monoclonal antibodies to human bone gla protein (BGP; osteocalcin) peptides that span the linear sequence of the molecule, specifically BGP 1–12 (N-terminal), BGP 15–30 (midregion), and BGP 38–49 (C-terminal). These antibodies were evaluated in various combinations of two-site formats in studies of serum BGP concentrations. For clinical studies, we selected from a panel of antibodies the two most sensitive antibody pairs for the intact molecule (N-C); we also used a polyclonal RIA based on BGP-C. For the two-site format, we used two N-terminal antibodies, 029 and 052, adsorbed to polystyrene beads, and radiodinated a C-terminal antibody, 663. The standard for each of the assays was purified human BGP. The following BGP serum concentrations (μg/L, mean ± SE) were measured with the various assays: by the 029–663 assay, results for normal subjects were 7 ± 3, for patients with renal failure 25 ± 8, and for patients with Paget disease 12 ± 4; by the 052–663 assay, the respective results were 22 ± 4, 44 ± 12, and 31 ± 7; by the polyclonal assay, the results were 3 ± 0.2, 13 ± 2, and 5 ± 1. The two intact (N-C) assays were significantly (P < 0.01) correlated (r = 0.94), but their serum values differed by more than twofold in terms of the same BGP standard. The polyclonal assay significantly correlated with each of the intact assays (r = 0.83, 0.77), but it, too, gave different serum values for BGP. These studies demonstrate the immunochemical heterogeneity of circulating BGP, heterogeneity that is manifest even in immunoassays specific for the same region of the molecule.

Additional Keyphrases: immunoadiometric assay · radioimmunoassay · monoclonal antibodies

1 Department of Medicine, San Diego VA Medical Center, the University of California, San Diego, CA. Address for correspondence: 3350 La Jolla Village Drive, Mail Code V-111C, San Diego, CA 92161.
2 Hybritch, Inc., San Diego, CA. Received May 28, 1992; accepted July 30, 1992.

2318 CLINICAL CHEMISTRY, Vol. 38, No. 11, 1992