Coated-tube radioimmunoassay for C-telopeptides of type I collagen to assess bone resorption

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We present a coated-tube RIA that is useful for assessment of bone resorption. The assay uses a monoclonal antibody raised against a linear 8-amino-acid sequence (EKAHDGGR) derived from the C-telopeptides of type I collagen. Within-run and total CVs were 4.4% and 5.3–6.2%, respectively, at concentrations of 1–7 mg/L (n = 4–20). Analytical recovery was 98% ± 8% and dilution 97% ± 7%. Values obtained in a group of 36 premenopausal women were 227 ± 89.6 mg/mol creatinine. In a group of 141 postmenopausal women, the values obtained were 429 ± 225 mg/mol creatinine, a highly significant increase of 89% (P < 0.001) over the premenopausal value. In a double-blind placebo-controlled clinical study of these postmenopausal women receiving five different doses of a bisphosphonate, a significant decrease of RIA-measured C-telopeptide values was seen in all bisphosphonate-treated groups, after just 3 months. Values in urine samples from postmenopausal women assayed with the RIA (y) and the CrossLapsTM ELISA (x) agreed well: slope = 0.98 (95% confidence interval, 0.94–1.01), intercept = 0.34 (0.25–0.43) mg/L, and $S_{xy} = 0.93$ mg/L (n = 678). We conclude that this RIA represents a valuable tool for assessing bone resorption.

INDEXING TERMS: biotin–streptavidin interaction • menopausal status • peptide fragments • urine

Remodeling of bone takes place continuously to maintain the strength of the organ. The bone remodeling process is reflected in body fluids by the presence of various molecules excreted from either the bone matrix or the cells actively involved in the bone resorption or formation. These biochemical markers can be measured and thus can serve as an estimate of the rate of bone turnover [1].

Pyridinoline (Pyr) and deoxypyridinoline (D-Pyr), crosslinks between mature collagen molecules, are primarily found in bone and cartilage but not in skin [2, 3]. D-Pyr is more specific for bone than Pyr, but neither is absolutely bone specific. Determination of the total excretion of Pyr and D-Pyr, however, can be used as an index of bone resorption. Pyr and D-Pyr currently are measured fluorometrically in hydrolyzed urine after separation by HPLC, a time-consuming and complicated method not suitable for routine use. Recently, an enzyme immunoassay for measuring the free D-Pyr in urine has been described [4] that appears to reflect bone resorption in certain situations [4, 5]. However, measurement of free D-Pyr by HPLC or immunoassay may not reflect the decrease in bone resorption known to follow treatment with bisphosphonate [6].

Enzyme immunoassays measuring a telopeptide-related fraction of the degradation products of type I collagen also have been reported [7, 8], and determination of a telopeptide-related fraction has been shown to constitute a sensitive and specific marker of bone resorption [9, 10].

Here, we describe an RIA based on a monoclonal antibody raised against an 8-amino-acid sequence derived from the C-telopeptides of type I collagen: EKAHDGGR. This sequence is derived from the same site as the sequence detected in the ELISA reported earlier [8]. Because a urinary C-telopeptide-related fraction of degradation products of type I collagen measured with the ELISA has proven to be a marker of bone resorption, an RIA also aimed at detecting such fragments represents a potentially valuable tool in the management of bone disorders.

Materials and Methods

The CrossLapsTM ELISA was obtained from Osteometer BioTech A/S (Herlev, Denmark). The absorbance of processed microtiter plates was read in an ELISA reader (Molecular Device Corp., Menlo Park, CA). A Roche (Basel, Switzerland) Cobas Mira analyzer was used to determine creatinine (Cr). Streptavidin and synthetic peptide EKAHDGGR were from

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1 Nonstandard abbreviations: Pyr, pyridinoline; D-Pyr, deoxypyridinoline; Cr, creatinine; BMD, bone mineral density; BSA, bovine serum albumin; and TFA, trifluoroacetic acid.
Kem-En-Tec (Copenhagen, Denmark). Biotinyl-N-hydroxysuccinimide was from Sigma Chemical Co. (St. Louis, MO). All chemicals used were of analytical grade or better and were from Sigma, Merck (Darmstadt, Germany), and Bio-Rad (Hemel Hempstead, UK).

SUBJECTS
To establish a reference value for the assay, we assayed urine samples from 36 healthy premenopausal women (mean ± SD age, 35.2 ± 5.6 years). All women had a history of regular vaginal bleeding, and none was taking any medication known to influence calcium metabolism. Likewise, we assayed samples from 141 postmenopausal women (ages 64.9 ± 5.6 years), participants in a double-blind placebo-controlled study of a bisphosphonate (ibandronate). The study group originally comprised 180 healthy, white postmenopausal women who were at least 10 years post natural menopause and <75 years old. The bone mineral density (BMD) in their distal forearms was at least 1.5 SD below the premenopausal mean for healthy white women.

Of these 180, 141 women completed the full 1-year study period. All received 1 g of calcium daily, 25 women received no further medication (placebo), 26 women an additional 0.25 mg of bisphosphonate daily, 22 women an additional 0.5 mg of bisphosphonate, 26 women an additional 1 mg of bisphosphonate, 23 women an additional 2.5 mg of bisphosphonate, and 19 women an additional 5 mg of bisphosphonate. All women were monitored for spine BMD, and second morning-void urine samples were taken at 3-month intervals (0, 3, 6, 9, and 12 months) during the study.

The study was performed according to the Helsinki Declaration II and the European standard for good clinical practice after approval by the regional research ethics committee. All women gave their written informed consent to participate in the study.

α-CROSSLAPS RIA
Balb/c mice were immunized with synthetic EKAHDGGR that had been conjugated to bovine serum albumin (BSA) in a two-step carbodiimide procedure [11]. Hybridomas were produced that were fusions of the murine spleen cells and myeloma cell line X63Ag8.653 [12]. Clone MabA7 was found to produce antibodies specific to the EKAHDGGR peptide and was thus used in the further development of the RIA. The RIA tubes were coated overnight with streptavidin (500 μL per tube, 10 mg/L) in a solution of 0.01 mol/L sodium phosphate and 0.143 mol/L NaCl, pH 7.2. Before use, these tubes were washed three times in 0.1 mol/L Tris (pH 8.0) containing 1 g/L BSA. To prepare biotinylated EKAHDGGR, we added 200 μL of 5 mmol/L biotinyl-N-hydroxysuccinimide ester to 1.0 mg of EKAHDGGR peptide in 1.0 mL of 0.1 mol/L phosphate-buffered saline, pH 7.2. After incubating the solution with gentle shaking for 2 h, we added 10 μL of 1 mol/L ethanolamine-HCl, pH 8.0, to destroy unreacted ester. The biotinylated EKAHDGGR was frozen at −18 °C until use [13].

The monoclonal antibody (MabA7) was iodinated according to routine procedures [14]. In short, MabA7 was labeled with 125I by a variation of the Chloramine T method, mixing 10 μL of MabA7 solution (0.8 g/L in 10 mmol/L sodium phosphate buffer, pH 7.4, containing 0.14 mol/L NaCl) with 250 μL of 0.3 mol/L sodium phosphate buffer, pH 7.4, containing 25 μg of Chloramine T and 1 mCi of Na125I. After 15 s we loaded the reaction mixture onto a 0.9 × 25 cm column of Sephadex G-300 (Pharmacia, Upsala, Sweden) equilibrated with 10 mmol/L sodium phosphate buffer (pH 7.4) containing 0.14 mol/L NaCl, 15 mmol/L NaCl, and 1.0 g/L BSA and then eluted the column with this buffer at a flow rate of 6 mL/h.

The principle of the RIA is as follows: 100 μL of biotinylated EKAHDGGR (usually diluted 1:50 000 in assay buffer: 300 mmol/mol/L Tris, pH 8.5, containing 1 g/L BSA and 1 mL/L Tween 20) is added to the precoated tubes and incubated with shaking at 200 rpm at room temperature for 15 min. During incubation the biotinylated EKAHDGGR binds to the immobilized streptavidin. After the tubes are washed three times with assay buffer, 100 μL of calibrator or urine sample is pipetted into the appropriate tubes. Next, 300 μL of the iodinated MabA7 (diluted to ~50 000 counts/min in assay buffer) is added to each tube. The immobilized peptide then competes with the breakdown products of the C-telopeptides of type I collagen in urine. As the peptide content of the solution increases, less antibody will bind to the immobilized biotinylated peptide, which leads to a decrease in counts/min bound to the tube. After a 3-h incubation at room temperature with shaking at 200 rpm, the tubes are washed three times with 2 mL of assay buffer. The radioactivity of each tube is measured in a gamma counter for 60 s.

We constructed the calibration curve on a log-linear graph paper by plotting the mean counts for the five calibrators (200–13 500 μg/L). The calibrators were prepared from HPLC-purified chemically synthesized linear peptide dissolved in assay buffer. The concentration of the synthetic peptide was determined by amino acid composition; its purity was checked by mass spectrometry. The concentration of EKAHDGGR equivalents in each patient's specimen was determined by interpolation on the calibration curve. Values obtained with the RIA (and the CrossLaps ELISA) were normalized for excretion rates by dividing by the corresponding Cr value.

CROSSLAPS ELISA
The ELISA was performed as described earlier [8, 10]. The within- and total-assay CVs were <8% in the range of concentrations covered by the calibration curve (100–6750 μg/L).

HPLC SEPARATION OF SYNTHETIC PEPTIDES
The chromatographic system (all from Waters, Milford, MA) consisted of two Model 510 pumps, a Model 717 autosampler, a scanning fluorescence detector, and an extended wavelength module equipped with a 214-nm cutoff filter. Separation was performed at room temperature with a reversed-phase Delta Pak™ C18 column (3.9 mm × 150 mm, 5 μm particle size, 30 mm pore size; Waters). Data were collected on computer and evaluated by using Waters's Maxima software. Effluents were monitored for peptide bonds at 214 nm. A mixture of EKAHDGGR and EKAHD-β-GGR was redisolved in 1 mL/L
trifluoroacetic acid (TFA) and was separated with a mobile phase of acetonitrile:1 mL/L TFA (5.995 by vol) at a flow rate of 1 mL/min. The effluent was monitored for peptide bonds, collected in 1-mL fractions, freeze-dried, redissolved in 1 mL of phosphate-buffered saline, and assayed in the RIA.

Results

The calibrators used in the assay define the measuring range from 200 to 13 500 µg/L. The detection limit (defined as the concentration corresponding to the mean absorbance of the zero calibrator minus 2SD) is 40 µg/L.

We determined within-run and total CVs (Table 1) based on 4 days of six determinations each of three urine samples with the same tracer preparation for the RIA. The average within-run and total CVs were 4.4% and 5.3%, respectively. Determination of the same urine sample each day for 20 days gave a value of 3.17 ± 0.20 mg/L (mean ± SD), for a CV of 6.2%. Analytical recoveries, measured after adding increasing amounts of synthetic peptide to three different urine samples, averaged 98% (Table 2).

The effect of dilution of samples with a high concentration in the RIA procedure is shown in Table 3. Samples were diluted in the zero calibrator (assay buffer). The overall average for different dilutions of the urine samples was 97% of the undiluted urine value.

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<th>Table 1. Assay imprecision.*</th>
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<td><strong>Sample</strong></td>
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* Six determinations each of three urine samples over 4 days with the same batch of tracer in the RIA.

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<th>Table 2. Analytical recovery of EKAHDGGR antigen added to urine samples* (n = 4).</th>
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<tr>
<td><strong>Added peptide, mg/L</strong></td>
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<td>0.25</td>
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<tr>
<td>0.75</td>
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* The range of EKAHDGGR concentrations in the urine samples was 0.53-1.26 mg/L.

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<th>Table 3. Effect of dilution on RIA of three urine samples.</th>
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<td><strong>EKAHDGGR in sample, mg/L (%)</strong></td>
</tr>
<tr>
<td><strong>Dilution</strong></td>
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<tr>
<td>None</td>
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<tr>
<td>1 + 1</td>
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<td>1 + 3</td>
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* % of concentration in undiluted sample.

Repeated freezing and thawing as many as 10 times before assaying did not substantially change the urine concentration in 10 samples tested (mean ± SD percent of initial value after 10 freeze–thaw cycles: 102% ± 11%). Furthermore, in another five samples of urine without any additives, the antigen measured was stable for at least 7 days at 25 °C (percent of initial value: 100% ± 16%).

Figure 1 shows the specificity of the RIA towards several different synthetic peptides, including the EKAHDGGR used for immunization.

The reactivity of the RIA toward EKAHDGGR and toward the synthetic isopeptide EKAHD-β-GGR, two molecules that differ in the linkage between an aspartate and a glycine, is shown in Fig. 2. The linkage in EKAHDGGR is a peptide linkage (via the α-carbonyl of aspartate), whereas the linkage in the isomerized peptide is via the β-carbonyl of aspartate.

The correlation between the values obtained by parallel measurements in the RIA and the ELISA (Fig. 3) was highly significant (r = 0.94, n = 678). The slope was 0.98 (95% confidence interval 0.935–1.007), the intercept was 0.34 mg/L.
(95% confidence interval 0.249–0.431 mg/L), the mean of the residuals was $7.3 \times 10^{-11}$ mg/L, and its standard deviation ($S_{\mu}$) was 0.93 mg/L.

The individual C-telopeptide values obtained for the group of premenopausal women and the group of postmenopausal women are shown in Fig. 4. The mean for the postmenopausal women was ~89% greater than for the premenopausal women. Figure 5 shows the effect of different doses of bisphosphonate on the RIA-measured excretion of degradation products of the C-telopeptides of type I collagen. The measured concentrations show a clear and significant decrease in each of the treatment groups, compared with the baseline measurements ($P < 0.001$).

BMD, after 12 months of daily oral administration of 2.5 mg of ibandronate or placebo, is shown in Fig. 6. The group receiving the bisphosphonate had a highly significant mean increase in BMD of 4.7% ± 0.7% (mean ± SE, $n = 23$, $P < 0.001$), whereas the placebo group had only a small, insignificant increase (0.7% ± 0.5%, $n = 25$, $P = 0.17$). Fig. 6 also shows the effect, after 3 months of treatment, on the excretion of degradation products of type I collagen measured with the RIA. Collagen breakdown products were highly signif-
significantly decreased, by 66% (n = 23, P < 0.001), in the group of women receiving 2.5 mg of Ibandronate per day.

Discussion
The present study shows that the RIA evaluated has a good performance in respect to precision, recovery, and dilution of urine samples (Tables 1, 2, and 3). With regard to the long-term handling and assaying of the urine samples, we have shown that the samples tested can be repeatedly frozen and thawed and that the antigen in these samples is stable for at least 7 days at 25 °C. Stability of urine samples is of particular interest in case patients' specimens have to be transported to a central laboratory. The data obtained with the RIA suggest that shipping of urine can take place even without refrigeration.

The specificity of the RIA towards various synthetic peptides, including the peptide used for immunization (EKAHDGGR), is shown in Fig. 1. Inhibition studies with a peptide with deletions from the N-terminus end (AHDDGGR) indicate that the epitope is located in the C-terminus of the 8-amino-acid sequence. Moreover, the presence of a free C-terminus arginine seems essential for the immunoreactivity; no reactivity was seen with a synthetic peptide prolonged by a tyrosine residue at the C-terminus, according to the sequence of the native C-telopeptide (EKAHDGGRY).

We recently showed that the CrossLaps ELISA exclusively recognizes an isomerized form of the same sequence, i.e., EKAHD β-GGR [15]. In the RIA, however, the monoclonal antibody used reacts almost exclusively (>98%) with the linear peptide form of the same amino acid sequence (EKAHDGGR), as seen in Fig. 2. Despite these obvious differences in the specificities of the two assays, values obtained in the ELISA are highly correlated with values obtained for the same samples in the RIA. The correlation data (Fig. 3) suggest that the two assays reflect similar aspects of the bone resorption process. The 95% confidence interval for the intercept, 0.25–0.43 mg/L, does not include the origin, for reasons we do not yet understand. However, further characterization of the possible differences in the fragments of type I collagen actually excreted into urine containing the isomerized or the nonisomerized form of the EKAHDGGR sequence will probably clarify the situation.

Assays for detection of type I collagen degradation reportedly give increased values in women after menopause [7-9], an effect we also saw in the RIA described here (Fig. 4). The 89% increase in the mean values measured indicates that menopausal status is reflected in an increased excretion of the C-telopeptide-related degradation products. The ability to reflect the changes in bone resorption known to occur at menopause is essential for an assay to be used as an index of bone resorption. Application of an assay as an indicator of risk of osteoporosis is also linked to such increase, given that increased bone resorption has been related to increased risk of osteoporosis [16]. Future studies will clarify the role of the RIA as an indicator of risk of osteoporosis and fracture in the skeleton.

After bisphosphonate administration in doses ranging from 0.25 to 5 mg/day, a significant decrease in collagen-related degradation was already evident after 3 months (Fig. 5). Values in the placebo group also decreased significantly (n = 25, P < 0.01), probably because all of the women received a calcium supplement of 1000 mg/day. Intake of this amount of calcium is known to decrease the values of markers of bone turnover [17]. Values in the group of women receiving 2.5 mg/day (n = 23) decreased by 66% in 3 months to a value less than the premenopausal mean (155 ± 162 vs 227 ± 89.6 mg/mol Cr). This dose (2.5 mg/day) therefore seems able to lower the excretion of degradation products of type I collagen to a level close to that excreted by women who are believed to be in a healthy and stable bone metabolic balance.

The effect of 2.5 mg/day bisphosphonate on the change of BMD of the spine after 12 months and the corresponding effect on the change in the excretion of the degradation products of type I collagen are shown in Fig. 6. In this treatment group, the 66% decrease in RIA-measured degradation products values after 3 months is followed by a significant increase (4.7%, P < 0.001) in the BMD of the spine after 12 months. In the placebo group, the RIA values are practically unchanged after 3 months, indicating the bone resorption continues at the same rate, i.e., a rate ~90% higher than that of healthy premenopausal women. The BMD of the spine in the placebo group after 12 months is also unchanged (mean ± SE 0.7% ± 0.5%), as would be expected from women age 65 who receive 1000 mg of calcium per day. Because the data obtained by the RIA thus seem to accord well with the actual changes observed in BMD, the RIA may serve as an early indicator of the effect of bisphosphonate. Such early information may increase the compliance with the therapy and thereby secure more-efficient disease management.

In conclusion, we have shown that the RIA has a good technical performance and is easy to use. Values obtained for a group of healthy postmenopausal women are highly correlated to an established marker of bone resorption (CrossLaps ELISA) and, as would be expected, are also significantly influenced by the menopausal status of the subjects. Moreover, the RIA reflects the changes in bone metabolism known to occur during bisphosphonate therapy and may be helpful in the management of such therapy. Further studies should reveal its potential utility in other relevant clinical situations.

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References
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