

Serum CrossLaps One Step ELISA. First application of monoclonal antibodies for measurement in serum of bone-related degradation products from C-terminal telopeptides of type I collagen

CHRISTIAN ROSENQUIST,^{1*} CHRISTIAN FLEDELIUS,¹ STEPHAN CHRISTGAU,¹ BRIAN J. PEDERSEN,¹ MARTIN BONDE,¹ PER QVIST,¹ and CLAUD CHRISTIANSEN²

We have developed a two-site ELISA for measurement in serum of bone-related degradation products derived from C-terminal telopeptides of type I collagen. The assay is based on the application of two highly specific monoclonal antibodies against the amino acid sequence of AHD- β -GGR, where the aspartic acid residue (D) is β -isomerized. In a one-step incubation procedure, the degradation products containing cross-linked diisomerized EKAHD- β -GGR peptides are captured by a biotinylated antibody and a peroxidase-conjugated antibody. The generated complex is then bound to the streptavidin surface via the biotin conjugate. Desalted urinary antigens are used for standardization, and parallelism is observed with serum samples. Results are obtained in <2.5 h, and both inter- and intraassay imprecision are <8%. The serum CrossLapsTM concentration was 1748 ± 740 pmol/L (mean \pm SD) in premenopausal women ($n = 65$) and 2952 ± 1325 pmol/L in a group of healthy postmenopausal women ($n = 169$). The Serum CrossLaps One Step ELISA was capable of detecting a highly significant ($P < 0.001$) effect of hormone replacement therapy in a retrospective study involving 22 postmenopausal women.

Type I collagen accounts for >90% of the organic matrix of bone (1). During the continued renewal of the bone

matrix throughout the skeleton, type I collagen is degraded, and small fragments are liberated into the bloodstream. Pyridinium cross-links (2, 3), cross-linked N-terminal telopeptides of type I collagen (4), and C-terminal telopeptides of type I collagen (5) are excreted into the urine, and these have all been reported to be markers of bone resorption. Particularly, the telopeptides of type I collagen have proven to be more sensitive markers than the pyridinium cross-links for detection of postmenopausal changes (6, 7).

Until now only two immunoassays detecting C-terminal telopeptide fragments of type I collagen in serum samples have been described (8, 9). Especially, the amino acid sequence EKAHDGGR (α CTX),³ found in the C-terminal telopeptides of the $\alpha 1$ chain of type I collagen, where the aspartic acid residue (D) is β -isomerized, has proven to be a specific and sensitive marker of bone resorption in serum (9). This was demonstrated using a competitive ELISA with an antiserum specific for the amino acid sequence AHD- β -GGR. Additionally, an immunoassay for measurement in serum of collagen type I N-telopeptides has also been reported (10).

Here we report new progress in the development of immunoassays for measuring bone resorption markers in serum, the Serum CrossLapsTM One Step ELISA. With this new assay we present the first application of monoclonal antibodies (mAbs) for the quantitative determination in serum of degradation products from C-terminal telopep-

¹ Osteometer BioTech A/S, Osteopark, Herlev Hovedgade 207, DK-2730 Herlev, Denmark.

² Center for Clinical and Basic Research, Ballerup Byvej 222, DK-2750 Ballerup, Denmark.

*Author for correspondence. Fax 45 44 94 89 40; e-mail christian_rosenquist@osteobio.dk.

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³ Nonstandard abbreviations: α CTX, EKAHDGGR amino acid sequence; β CTX, EKAHD- β -GGR amino acid sequence; mAb, monoclonal antibody; PBS, phosphate-buffered saline; BSA, bovine serum albumin; and BxNHS, biotinamido-caproate-N-hydroxysuccinimide ester.

tides of type I collagen. This ELISA method is based on the use of two highly specific mAbs against the amino acid sequence AHD- β -GGR. The molecules measured in the assay consist of two chains of EKAHD- β -GGR (β CTX) that are covalent cross-linked via the lysine residues. Our aim was to obtain a reliable, easily performing assay with a high potential for assessing the rate of bone resorption.

Materials and Methods

REAGENTS

All reagents were standard high-quality chemicals from either Merck or Sigma Chemical Co., unless otherwise stated. The acetonitrile was from Rathburn, the heptafluorobutyric acid was from Aldrich, and the trifluoroacetic acid was from Applied Biosystems. The synthetic peptides, β CTX and α CTX, were purchased from KJ Ross-Petersen ApS. Buffers for the Serum CrossLaps One Step ELISA were as follow: phosphate-buffered saline (PBS: 1.5 mmol/L KH_2PO_4 , 8.5 mmol/L $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 2.7 mmol/L KCl, 137 mmol/L NaCl, pH 7.4); calibrator buffer (PBS containing 10 g/L bovine serum albumin (BSA), 0.18 g/L Bronidox, 0.03 g/L bromphenol blue, pH 7.4); incubation buffer (7.5 mmol/L KH_2PO_4 , 42.5 mmol/L $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 2.7 mmol/L KCl, 137 mmol/L NaCl, 10 g/L BSA, 0.1 g/L Tween 20, 0.18 g/L Bronidox, 0.03 g/L bromphenol blue, pH 5.75); and washing buffer (25 mmol/L Tris, 50 mmol/L NaCl, 1 g/L Tween 20, pH 7.2).

PREPARATION OF mAbs

Production of mAbs. Female Balb/C \times CF1 mice (8–12 weeks of age) were immunized intraperitoneally with 200 μL of a emulsion of complete Freund's adjuvant and β CTX conjugated to thyroglobulin (100 mg/L) by a carbodiimide procedure (11). The conjugate and the adjuvant were mixed in equal volumes. Immunizations were repeated six times every 2 weeks using incomplete Freund's adjuvant. Three days before fusion the mice were boosted intraperitoneally with 100 μL of β CTX conjugated to thyroglobulin (100 mg/L). Spleen cells and ATCC P3-X63-Ag8.653 (12) myeloma cells were fused with 500 mL/L polyethylene glycol (PEG 4000 GK) as described previously, except that human endothelial culture supernatant (HECS, Costar) was used instead of feeder cells (12).

Screening of mAbs. Supernatants from hybridoma cells were diluted in 300 mmol/L Tris, 10 g/L BSA, 5 g/L Tween 20, pH 8.0, and incubated in microtiter wells (Nunc) coated with either β CTX conjugated to BSA by a glutaraldehyde procedure (13) or with collagenase-treated collagen from human bone (14). Bound antibodies were then detected using peroxidase-conjugated rabbit anti-mouse immunoglobulins (Jackson ImmunoResearch Laboratories). Hybridomas producing antibodies against both β CTX and collagenase-treated collagen were then cloned at least twice with limiting dilutions and propagated. The mAbs were purified using Protein A chroma-

tography in accordance with the manufacturer's instructions (Pharmacia). The subclass of each mAb was determined using the IsoStripTM Mouse Monoclonal Antibody Isotyping Kit (Boehringer Mannheim GmbH).

CHARACTERIZATION OF SERUM CrossLaps ONE STEP ELISA BINDING SPECIFICITY

The binding specificity of the Serum CrossLaps One Step ELISA was investigated using genuine cross-linked urinary fragments containing either two β CTX (β CTX-X- β CTX), one α CTX and one β CTX (α CTX-X- β CTX), or two α CTX (α CTX-X- α CTX) epitopes. The three different genuine cross-linked fragments (cross-link, galactosyl pyridinoline) were extracted from urine as by immunoaffinity chromatography and further purified by reversed-phase HPLC as described previously by Fledelius et al. (15). The fragments (α CTX-X- α CTX, α CTX-X- β CTX, and β CTX-X- β CTX) were mixed in equal amounts and separated by reversed-phase HPLC. The fragments were eluted from a Delta Pak C₁₈ column (3.9 \times 150 mm; particle size, 5 μm ; pore size, 30 nm; Waters) with a 0–125 g/L acetonitrile gradient containing 1 g/L trifluoroacetic acid over 45 min at a flow rate of 1 mL/min. The effluent was monitored for fluorescent material at 380 nm (emission) using 297 nm light for excitation (Waters 470 Scanning Fluorescence detector; Waters). The effluent was fractionated (fraction size, 0.3 mL), freeze-dried, redissolved in PBS, and assayed in the Serum CrossLaps One Step ELISA.

SIZE EXCLUSION CHROMATOGRAPHY OF SERUM FRAGMENTS OF THE C-TERMINAL TELEPEPTIDE α 1 CHAIN OF TYPE I COLLAGEN

Immunoaffinity chromatography. mAb F1103 was coupled to a CNBr-activated Sepharose 4B matrix (Pharmacia) in accordance with the manufacturer's instructions. Eighty milliliters of undiluted human serum was applied to the column. The column was washed with PBS, and bound material was eluted with 10 g/L trifluoroacetic acid and freeze-dried. The recovery of immunoreactive fragments was >75%.

HPLC size exclusion chromatography. The immunopurified fraction was redissolved in 0.2 mol/L NH_4HCO_3 and applied to a Superdex Peptide HR 10/30 column (Pharmacia) equilibrated with 0.2 mol/L NH_4HCO_3 . Fractions were collected in volumes of 0.5 mL. Each fraction was freeze-dried, redissolved in PBS, and assayed in the Serum CrossLaps One Step ELISA.

Calibration of the HPLC column. For the calibration of this HPLC size exclusion column, the following molecules were used as mass markers: kyotorphin (337 Da), α CTX (860 Da), purified urinary antigen (2036 Da) (15), aprotinin (6500 Da), and cytochrome C (13 337 Da). When the elution time (minutes) was plotted against the molecular

mass (\log_{10} scale), a calibration curve with an r value of -0.997 was obtained (data not shown).

PEROXIDASE LABELING OF mAbs

Horseradish peroxidase (Boehringer Mannheim GmbH) was conjugated to Protein A-purified mAb F12 as described by Nakane and Kawaoi (16).

BIOTINYLATION OF mAbs

Conjugation. A 2 g/L solution of Protein A-purified mAb F1103 (in PBS) was mixed with a solution of 0.3 mol/L Na_2CO_3 , 0.7 mol/L NaHCO_3 , pH 9.6, in the ratio 10:1. Biotinamidocaproate-*N*-hydroxysuccinimide ester (BxNHS) dissolved in dimethyl sulfoxide (4 g/L) was then added in the ratio of 5 mol BxNHS:1 mol IgG. The mixture was incubated at room temperature for 2 h with end-over-end rotation. The reaction was stopped by the addition of 0.2 mL of 0.2 mol/L ethanolamine and incubated for 1 h at room temperature. The solution was dialyzed (cutoff value: 12 000–14 000, Spectra/Por®; Spectrum Medical Industries) twice against 5 L of PBS for 2 days at 4 °C. Turbidity was removed by sterile filtration using 0.22 μm disposable syringe filter holders (Minisart NML, Sartorius). Aliquots were stored at -20 °C.

Determination of mol BxNHS/mol IgG. The degree of biotinylation was determined by a HABA kit from Pierce Chemicals and performed in accordance with the manufacturer's instructions. Briefly, the principle of this procedure is based on the formation of a complex between the HABA reagent (4-hydroxyazobenzene-2'-carboxylic acid) and avidin. When added, biotin will displace the HABA reagent, which will decrease the absorbance at 500 nm, and the unknown amount of biotin can be determined.

PREPARATION OF CALIBRATORS FOR THE ELISA

Desalting of urinary antigens. C₈ Sep-Pak Cartridge columns (Waters) were activated with 800 g/L methanol and equilibrated with 1 g/L trifluoroacetic acid. Urine from healthy adults, containing 10 g/L trifluoroacetic acid, was applied to the columns. The bound material was washed with 1 g/L trifluoroacetic acid, eluted with 400 g/L acetonitrile containing 1 g/L trifluoroacetic acid, freeze-dried, reconstituted in PBS, and stored at -20 °C. The desalted stock solution was quantified in an assay similar to that described previously by Bonde et al. (9). Briefly, this assay was a competitive ELISA using polyclonal antibodies specific for AHD- β -GGR. Additionally, the assay used the synthetic peptide βCTX , both for immobilization to the microtiter wells and for standardization. The exact concentration of the βCTX peptide was determined by quantitative amino acid analysis and expressed in pmol/L.

Calibrators. The desalted urinary antigens were diluted in the calibrator buffer. A line of calibrators was prepared covering a range from 500 to $\sim 15\,000$ pmol/L. The

calibrators were stored in brown glass vials at 4 °C. In this formulation the antigens were unaffected by stress treatment at 35 °C for 3 weeks.

SERUM CrossLaps ONE STEP ELISA

Fifty microliters of calibrators, controls, or unknown serum samples were pipetted into appropriate microtiter wells coated with streptavidin (MicroCoat), followed by 150 μL of a mixture of the biotinylated mAb F1103 and the peroxidase-conjugated mAb F12 in incubation buffer. The contents of the wells were incubated for 2 h at 20 °C on a mixing apparatus (330 rpm). The wells were then emptied and washed five times using the washing buffer. Then 100 μL of a 3,3',5,5'-tetramethylbenzidine solution (Kierkegaard & Perry) was added to each well and incubated for 15 min in darkness on a mixing apparatus (330 rpm). The color reaction was stopped by addition of 100 μL of 0.18 mol/L H_2SO_4 per well. The absorbance was measured at 450 nm, with 650 nm as the reference wavelength, using a microtiter plate reader (E_{max} Easy Microtiter Reader, Molecular Devices).

KNOWN INTERFERING SUBSTANCES

Interfering substances such as hemoglobin, bilirubin, lipids, and ascorbic acid were added to serum samples in accordance to the procedures described by Glick et al. (17). A stock solution of hemoglobin was prepared from fresh hemolysate, whereas the remaining substances were commercially available. Ditaurobilirubin, conjugated bilirubin, was purchased from Porphyrin Products, Inc.; IntraLipid®, 20% was from Pharmacia & Upjohn A/S; and ascorbic acid was from Merck.

ASSAYS FOR COMPARISON STUDIES

Urine CrossLaps ELISA. The commercially available CrossLaps ELISA from Osteometer BioTech A/S (Herlev, Denmark) was used for the method comparison. The CrossLaps ELISA uses an antiserum (rabbit) specific for the amino acid sequence AHD- β -GGR to measure breakdown products from C-terminal telopeptides of type I collagen in urine. Creatinine was determined by Cobas Mira (Roche).

ICTP RIA. The commercially available Telopeptide ICTP [^{125}I] RIA from Orion Diagnostics was used for head-to-head comparison of the clinical performance. The ICTP RIA uses an antiserum from rabbits for the measurement in serum of the carboxy-terminal cross-linked telopeptides of type I collagen.

PARTICIPANTS

Blood samples from 65 premenopausal (38 ± 5 years, mean \pm SD) and 169 postmenopausal (67 ± 7 years, mean \pm SD) women were used to determine the reference intervals. All the women were healthy and were not receiving treatments known to affect calcium metabolism.

Serum and urine samples ($n = 638$), originating from a

more comprehensive study (Alexandersen et al., submitted for publication), were used for the method comparison study toward the urine CrossLaps ELISA. A subpopulation of 22 early postmenopausal women (57 ± 3 years, mean \pm SD) undergoing hormone replacement therapy was selected for a head-to-head comparison between the Serum CrossLaps One Step ELISA and the ICTP RIA. Briefly, serum samples from postmenopausal women receiving either a sequential combination of 17β -estradiol (2 mg) and norethisteronacetat (1 mg) ($n = 10$) or a placebo treatment ($n = 12$) were measured at baseline and after 12 months of therapy.

STATISTICAL METHODS

SAS Institute procedures were used for statistical analysis (18). The significance of the mean difference between groups was assessed using the Student *t*-test for unpaired data. The significance of changes within groups was determined by the Student *t*-test for paired data. To assess longitudinal changes, we calculated the values for each group and expressed the changes as a percentage of the initial values.

Results

PREPARATION OF REAGENTS

mAbs. Two mAbs from two different fusions were selected, and they were designated F1103 and F12. Both antibodies were of subclass IgG₁. When similar specificity studies as described previously by Fledelius et al. (19) were used, these two mAbs were found to be highly specific for the amino acid sequence of AHD- β -GGR and with similar affinities. Furthermore, neither recognized the α form of the peptide, α CTX (data not shown).

Biotinylation. Approximately 2 mol of BxNHS were incorporated per mol of mAb F1103. In experiments where this ratio exceeded 10 mol BxNHS/mol F1103, destruction of the IgG was observed (data not shown).

DETERMINATION OF THE SPECIFICITY

The HPLC separation of three major urinary degradation products derived from the C-terminal telopeptide $\alpha 1$ chain of type I collagen is illustrated in Fig. 1. The detection by fluorescence revealed the presence of the three fragments in similar quantities. The results from the immunological profile indicate that the Serum CrossLaps One Step ELISA is specific for the measurement of degradation fragments of C-terminal telopeptide $\alpha 1$ chain of type I collagen, characterized by containing cross-linked diisomerized β CTX peptides (β CTX-X- β CTX). The two other breakdown products, containing either no isomerization (α CTX-X- α CTX) or one isomerization (α CTX-X- β CTX) were not detected by the ELISA.

INVESTIGATION OF THE SIZE OF SERUM FRAGMENTS

Fig. 2 shows that the majority of the degradation fragments of C-terminal telopeptide $\alpha 1$ chain of type I colla-

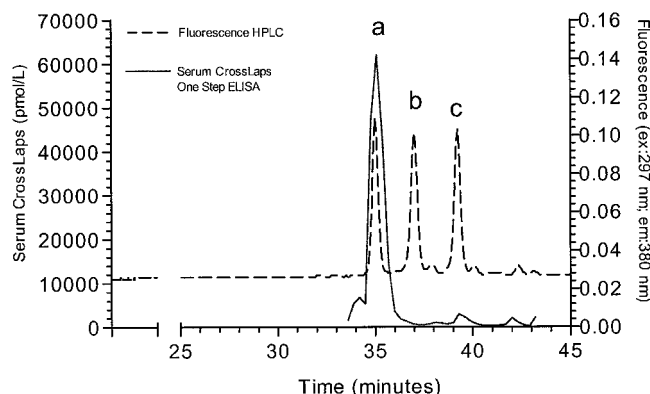


Fig. 1. Determination of the Serum CrossLaps One Step ELISA binding specificity.

An equal mixture of genuine cross-linked urinary fragments containing either β CTX-X- β CTX (a), α CTX-X- β CTX (b), or α CTX-X- α CTX (c) epitopes was separated by reversed-phase HPLC. The immunoreactive fractions were then quantified by the Serum CrossLaps One Step ELISA.

gen, which are found in serum and can be measured with the Serum CrossLaps One Step ELISA, have molecular masses in the range of 1000–10 000 Da. Additionally, the data indicate that $\sim 50\%$ of the immunoreactive material has a molecular mass < 3000 Da.

ASSAY DEVELOPMENT AND PERFORMANCE

The application of biotinylated mAb F1103 as the capture antibody in streptavidin-coated microtiter wells, together with peroxidase-conjugated mAb F12 in the Serum CrossLaps One Step ELISA, yielded a calibration curve that was approximately linear in the range 500–16 000 pmol/L when plotted on a linear scale (Fig. 3).

The detection limit, defined as the concentration corresponding to 2 SD above the mean of 21 determinations of the zero calibrator, was 80 pmol/L. The imprecision

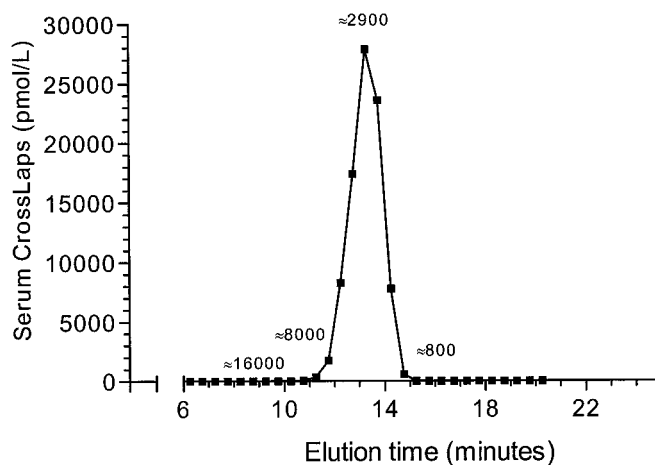


Fig. 2. Profile of the sizes of serum fragments of type I collagen after HPLC size exclusion chromatography.

Serum antigens were purified using a mAb against AHD- β -GGR. The effluent was further fractionated by HPLC size exclusion chromatography, and immunoreactive fragments were detected by the Serum CrossLaps One Step ELISA. The numbers indicate the calibration (in Da) of the HPLC size exclusion column.

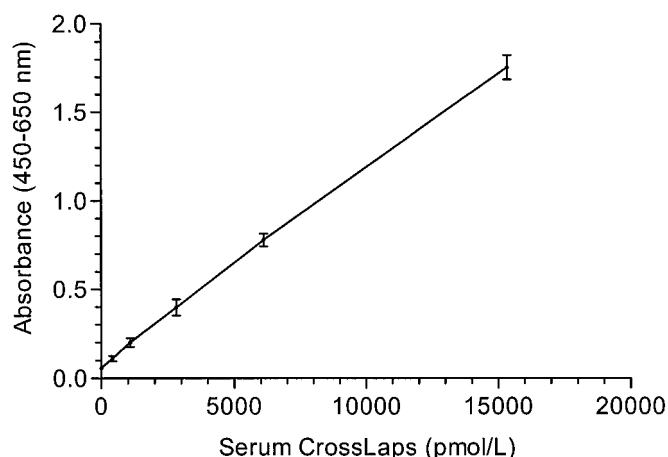


Fig. 3. Calibration curve for the Serum CrossLaps One Step ELISA. The absorbances at 450 and 650 nm were measured, and the difference (mean \pm SD) was plotted.

(CV) was assessed by the measurement of three serum samples in 12 consecutive analytical runs and 21 determinations of each of the three samples in the same analytical run; the overall CV was $<8\%$ (Table 1).

Serum samples from different populations (children, patients with renal failure, and healthy adults) were used to evaluate the linearity of serum samples. Samples were diluted in increments of 20% in calibrator buffer; the sensitivity of the assay to any serum matrix effects was then investigated (Table 2). Because the mean observed:expected ratio after dilution was $101\% \pm 2\%$ (mean \pm SD), we concluded that there was no interference from the serum matrix and that the assay detected degradation products from type I collagen C-terminal telopeptides in various serum samples with similar affinity. In support of this observation, the mean observed:expected recovery ratio was found to be $101\% \pm 4\%$ (mean \pm SD; Table 3). This result was obtained by mixing various calibrator solutions containing CrossLaps antigens isolated from urine with different serum samples in equal volumes. The mixtures were then measured in the Serum CrossLaps One Step ELISA (Table 3).

Serum samples from 638 healthy subjects were assayed in the Serum CrossLaps One Step ELISA, and the corresponding urine samples were measured in the CrossLaps ELISA and corrected for creatinine. Although different kinds of specimens were used, the two assays were highly

Table 1. Imprecision.

Mean CrossLaps, pmol/L	Interassay CV, ^a %	Intraassay CV, ^b %
1961	7.9	4.7
2818	5.4	4.9
3501	6.5	4.9

^a Determined by analysis of three serum samples in 12 consecutive analytical runs.

^b Determined by analysis of 21 determinations of three serum samples in the same analytical run.

Table 2. Dilution of serum samples.^a

Origin of serum sample	Dilution of serum, %	Observed pmol/L	Expected pmol/L	Observed/expected, %
Child	100	6301	6301	100
	80	5036	5040	100
	60	3900	3781	103
	40	2598	2520	103
	20	1315	1260	104
Patient with renal failure	100	7538	7538	100
	80	6040	6021	100
	60	4651	4514	103
	40	2983	3011	99
	20	1531	1503	102
Adult	100	4028	4028	100
	80	3134	3222	97
	60	2452	2415	101
	40	1572	1609	98
	20	797	807	99

^a Serum samples were diluted with standard buffer in increments of 20%. The undiluted serum sample was set to 100%.

correlated ($r = 0.856$; Fig. 4). Additionally, a high comparability was found between serum and plasma samples taken in parallel. Both EDTA- and heparin-treated plasma from nine volunteers correlated strongly with serum samples, giving r values of 0.973 and 0.949, respectively (data not shown).

The stability during storage of the antigens measured in the Serum CrossLaps One Step ELISA was examined. Sera from 10 healthy subjects were isolated within 2 h of blood collection. Aliquots of serum were incubated for various times at 4 °C and 20 °C and then frozen at -20 °C. The mean recovery after 7 days of storage was $93\% \pm 11\%$ and $60\% \pm 17\%$ (mean \pm SD) for 4 °C and 20 °C, respectively. The data indicate that the CrossLaps antigens are

Table 3. Analytical recovery.^a

Serum samples, pmol/L	CrossLaps calibrator solutions, pmol/L	Observed pmol/L	Expected pmol/L	Observed/expected, %
2433	1146	1746	1790	98
	2291	2525	2362	107
	4583	3597	3508	103
	9165	6173	5799	106
	18 330	10 613	10 382	102
3254	1146	2319	2200	105
	2291	2681	2773	97
	4583	4056	3919	104
	9165	5971	6210	96
	18 330	11 007	10 792	102
4198	1146	2667	2672	100
	2291	3202	3245	99
	4583	4124	4391	94
	9165	6768	6682	101
	18 330	11 663	11 264	104

^a Serum samples were mixed with equal volumes of five solutions of CrossLaps calibrator containing desalted urinary antigens.

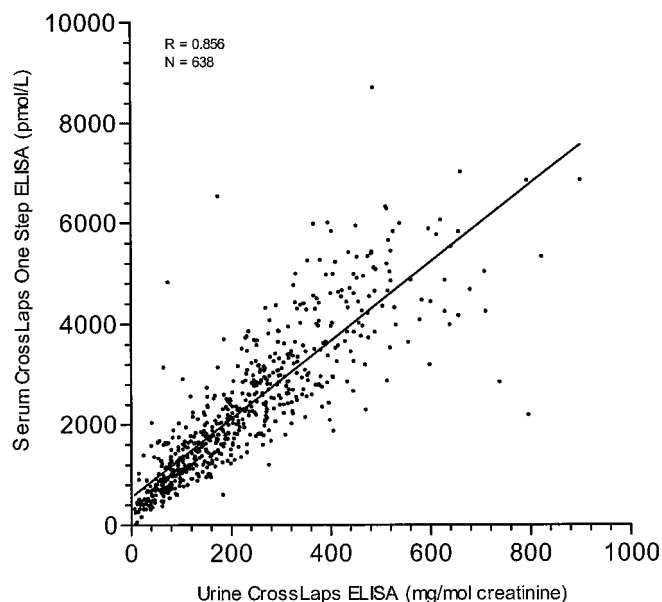


Fig. 4. Method comparison.

Serum and corresponding urine samples ($n = 638$) were run in parallel in the Serum CrossLaps One Step ELISA and in the urine CrossLaps ELISA. The urine measurements were corrected for creatinine.

very stable at 4 °C and resistant to ~2 days of storage at room temperature (Fig. 5).

Another important issue for routine clinical settings is the stability of the antigens during repeated freezing and thawing of serum samples. Sera from seven healthy subjects were collected and exposed to seven freeze-thaw cycles over a period of 5 days, and the serum CrossLaps concentration was then measured after 1, 2, 3, 5, and 7 cycles. No significant change in the mean concentration could be observed. The recovery, expressed as a percent-

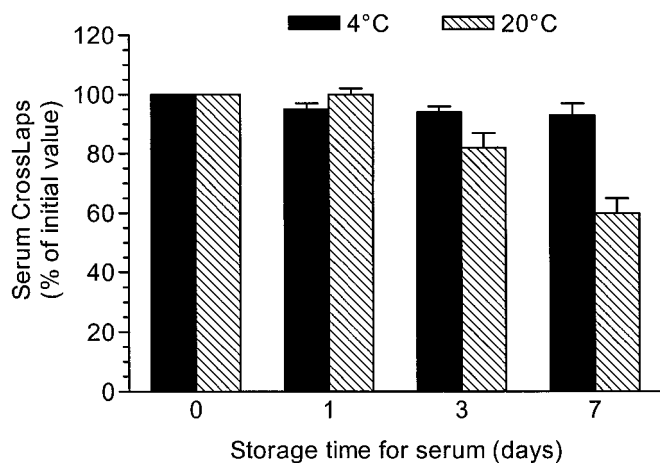


Fig. 5. Stability of the CrossLaps antigens during storage of serum samples.

Serum samples from 10 volunteers were incubated for various times at 4 °C and 20 °C and then stored at -20 °C. The serum concentrations were measured by the Serum CrossLaps One Step ELISA and expressed as a percentage (mean \pm SE) of the initial values.

age of the initial value, was $97\% \pm 5\%$ (mean \pm SD) after cycle 7 (data not shown).

KNOWN INTERFERING SUBSTANCES

Apparently, the concentrations of serum CrossLaps antigens were not susceptible to the presence of well-known interferents. These agents, known to be potential interferents in clinical chemistry analyses, were added to serum samples. The highest concentrations tested were as follows: ditau bilirubin, 600 g/L; hemoglobin, 10 000 g/L; IntraLipid, 1000 g/L; and ascorbic acid, 100 mg/L. The serum concentrations were then measured in the Serum CrossLaps One Step ELISA. No interference was found at any of the concentrations tested.

CLINICAL PERFORMANCE

The individual values obtained in the Serum CrossLaps One Step ELISA in groups of pre- and postmenopausal women are shown in Fig. 6. An increase of 69% ($P < 0.001$) in serum CrossLaps concentrations was observed after menopause. The mean serum concentration of CrossLaps was 1748 ± 740 pmol/L (mean \pm SD) in the premenopausal group and 2952 ± 1325 pmol/L (mean \pm SD) in the postmenopausal group. The t -score between the two groups (the difference between the mean of pre- and postmenopausal concentrations, expressed in number of SD from the mean of the premenopausal group) was 1.63.

Serum samples from postmenopausal women undergoing hormone replacement therapy were measured in the Serum CrossLaps One Step ELISA to assess the ability of the assay for monitoring antiresorptive treatment (Fig. 7). The mean serum concentration for the actively treated group decreased after 12 months of therapy by ~75%, thereby returning to a premenopausal concentration. Compared with baseline, the mean serum concentration for the placebo-treated group was unchanged after 12 months and was significantly different from the corresponding concentration of the actively treated group. In

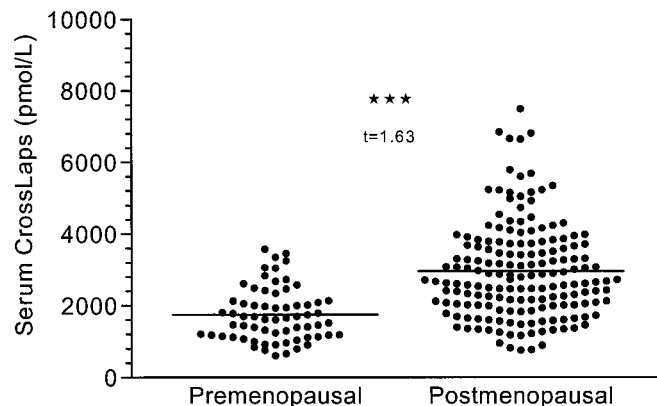


Fig. 6. Reference intervals for pre- and postmenopausal women.

Individual concentrations of serum CrossLaps in healthy premenopausal ($n = 65$) and postmenopausal ($n = 169$) women were measured by the Serum CrossLaps One Step ELISA. The mean serum concentration, the t -score, and the statistical significance (unpaired Student t -test) are shown. $***$, $P < 0.001$.

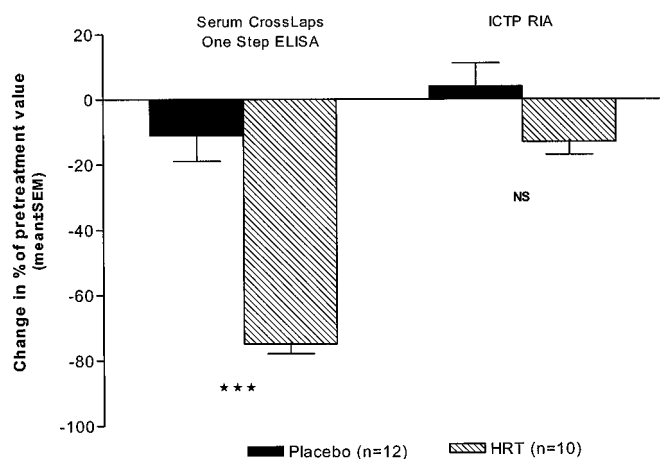


Fig. 7. Follow-up after hormone replacement therapy.

Serum samples from early postmenopausal women undergoing hormone replacement therapy (HRT) were measured in the Serum CrossLaps One Step ELISA and in the ICTP RIA. The women received either sequential combinations of 2 mg of 17 β -estradiol and 1 mg of norethisteronacetate ($n = 10$) or a placebo treatment ($n = 12$). Serum samples were collected at baseline and after 12 months of therapy. The results are expressed in the percentage of change (mean \pm SE) from the pretreatment value. A Student t -test for unpaired data was used to assess whether the difference between the HRT- and placebo-treated groups after 12 months of therapy was significant. ***, $P < 0.001$; NS, not significant.

contrast, the ICTP RIA, another serum assay measuring collagen fragments, lacked the capability to detect a significant difference between the two groups.

Discussion

The C-telopeptide fragments of type I collagen measured in the CrossLaps ELISA and the Serum CrossLaps One Step ELISA are generated in bone; however, they may also be generated by several tissues unrelated to bone, because of the widespread occurrence of type I collagen in the body (20). However, the study by Fledelius et al. (15) and the present study have shown that the various antibodies used in the CrossLaps ELISAs exclusively recognize an isomerized β -aspartate (D) form of the AHDGGR epitope. The nonenzymatic β -isomerization reactions occur spontaneously at this susceptible site at a low rate. Thus, this form is likely to accumulate over time in bone collagen because of the long residence time of bone collagen (21). Combined, these special conditions in bone may cause type I collagen from bone to have a substantially higher ratio of β -aspartate relative to the normal isomer, α -aspartate, compared with other type I collagen-containing tissues. However, this remains to be demonstrated by measurement of the degree of β -isomerization of collagen in various tissues and by determination of the equilibrium constants for the spontaneously occurring isomerization reaction.

Three types of breakdown products (β CTX-X- β CTX, α CTX-X- β CTX, and α CTX-X- α CTX) liberated from the C-terminal telopeptide $\alpha 1$ chain of type I collagen were used to evaluate the binding specificity of the Serum CrossLaps One Step ELISA. We found that this newly

developed ELISA procedure was specific for degradation fragments of collagen type I characterized by the presence of cross-linked diisomerized β CTX peptides. This is in accordance with the inability of the two mAbs used in the assay to measure the linear form of α CTX. The assay enables exclusive detection of the β CTX-X- β CTX fragments derived from mature bone tissue. This would be relevant in a population of relatively high age, such as the one investigated in this study, where a large proportion of the collagen molecules in the bone matrix is likely to be β -isomerized.

The procedure applied in the present study to investigate the size of the CrossLaps-reactive antigens in serum provides a reasonable estimate of the molecular mass of the collagen fragments measured in the Serum CrossLaps One Step ELISA. The findings indicate that the masses of the detectable antigens are similar but slightly higher than the major degradation products quantified in the urinary CrossLaps ELISA, where the major immunoreactive fragments have molecular masses of ~ 2000 Da (15). This observation was supported by the data from the method comparison, where a relatively high correlation ($r = 0.856$) was found. These findings could suggest that the serum and urinary CrossLaps assays measure similar and related populations of antigens but also that differences in specificity are likely to exist. A more detailed study of CrossLaps antigens in serum is currently being performed to answer these questions.

Various studies have shown that the circulating concentrations of biochemical markers of bone resorption increase by age, mainly because of the change in menopausal status (22, 23). This was also shown in the present study, where the passing of menopause produced a 69% increase in mean serum concentration. Approximately 37% of the samples from the group of postmenopausal women were >3228 pmol/L, corresponding to the mean concentration of the premenopausal group plus 2 SD. It is likely that this subpopulation of postmenopausal women might belong to the group that is characterized by losing substantial amounts of bone mineral, "the fast bone losers" (24). This issue was further investigated in the study of Christgau et al. (25), where Serum CrossLaps One Step ELISA was applied, to assess the future bone loss in a population of postmenopausal women; the findings suggested that high concentrations of serum CrossLaps for early postmenopausal women were associated with accelerated future bone loss. Future studies should reveal if serum CrossLaps measurements early in the menopause also have a predictive value for later development of fractures in the skeleton.

One of the most important features for biochemical markers for bone turnover is their ability to monitor the effect of antiresorptive therapy, such as hormone replacement therapy. In serum samples from postmenopausal women, the Serum CrossLaps One Step ELISA detected a significant reduction to premenopausal concentrations after 12 months of therapy. This observation, together

with the findings in the study of Christgau et al. (25), further supports that this new ELISA procedure reflects changes in bone metabolism known to occur during antiresorptive therapies.

Another serum assay for C-terminal telopeptide fragments of type I collagen, the ICTP assay (8, 26), has been described previously; this marker, however, has shown only a very limited response to bisphosphonate (27, 28) and hormone replacement therapy (29). This feature was also demonstrated in the present study, where the ICTP RIA failed to detect a significant effect of hormone replacement therapy after 12 months of treatment. The epitope recognized by the ICTP antibodies has recently been characterized and includes a phenylalanine-rich region of the C-terminal telopeptides of two $\alpha 1$ chains located between the triple helical domain and the lysine-derived trivalent pyridinoline/pyrrole cross-linkers. The latter is not directly involved in the binding of the ICTP antibodies, but the antibodies must connect to the three constituent chains for successful detection (30). This phenylalanine-rich region is located farther upstream from the β -isomerization-susceptible α CTX epitope of the C-terminal telopeptides. These data indicate that the ICTP assay measures another population of antigens, which probably are larger than the fragments detected by the CrossLaps assays.

We have described the development and characterization of a two-site ELISA procedure that uses two mAbs for the quantitative determination in serum of degradation products derived from the C-terminal telopeptide $\alpha 1$ chain of type I collagen. Only fragments characterized by the presence of cross-linked diisomerized β CTX peptides (β CTX-X- β CTX) were detected. This specificity ensures the specific measurement of degradation fragments that derive from matured bone tissue. The Serum CrossLaps One Step ELISA provides results in <2.5 h and with high technical precision (CV <8%). Furthermore, the CrossLaps antigen in serum is highly stable, being resistant to storage for >24 h at room temperature or to more than seven repeated freeze-thaw cycles. Additionally, this new ELISA procedure demonstrated a relative high discriminatory power between populations of pre- and postmenopausal women, as well as a high potential for monitoring the effect of antiresorptive treatment for postmenopausal women.

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