

Evaluation of plasma carboxy-terminal cross-linking telopeptide of type I collagen concentration in horses

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Objective—To evaluate a human assay for quantification of carboxy-terminal cross-linking telopeptide of type I collagen (CTX-I), assess the influence of age on plasma CTX-I concentration, investigate the relationship between plasma CTX-I and serum osteocalcin concentrations, and determine whether concentrations of plasma CTX-I or serum osteocalcin fluctuate in circadian manner in horses.

Horses—75 clinically normal horses.

Procedure—Cross-reactivity between equine serum CTX-I and CTX-I antibodies in an automated electrochemiluminescent sandwich antibody assay (ECLIA) was evaluated via a specificity test (ie, dilution test) and recovery calculation. Serum osteocalcin concentration was measured with an equine-specific osteocalcin radioimmunoassay. To analyze diurnal variations in plasma CTX-I and serum osteocalcin concentrations, blood samples were obtained hourly during a 24-hour period.

Results—Results of the dilution test indicated good correlation ($r > 0.99$) between expected serum CTX-I concentrations and measured serum CTX-I concentrations. The calculated CTX-I recovery was 97.6% to 109.9%. Plasma CTX-I and serum osteocalcin concentrations were correlated. Plasma CTX-I concentration was inversely correlated with age of the horse. No significant circadian variations in plasma CTX-I and serum osteocalcin concentrations were detected.

Conclusions and Clinical Relevance—Results suggest that the fully automated CTX-I ECLIA can be used for evaluation of plasma and serum samples from horses and may be a useful tool to monitor bone metabolism changes. Horses in this study did not have notable diurnal fluctuations in serum osteocalcin and plasma CTX-I concentrations. (*Am J Vet Res* 2004;65:104–109)

therefore required to monitor the response of the whole skeleton to developmental processes, nutrition, injury, treatment, or metabolic bone diseases. However, the assessment of the bones of horses by noninvasive methods is still limited.² Noninvasive techniques should be easy to perform without any risk for the horses and should not be influenced by nonosseous metabolism; analyses of samples of serum, plasma, or urine from horses should fulfill these criteria. Assessments of various biochemical bone markers in blood and urine (in combination with other noninvasive bone assessment methods) would be expected to provide better evaluation and monitoring of the status of bone in equids.

Bone is a dynamic tissue that undergoes continuous modeling and remodeling processes (bone turnover). In skeletons of healthy adults, the processes of bone formation and resorption are coupled and therefore in balance.³ Cells that are mainly involved in bone metabolism are osteoblasts and osteoclasts. An important function of osteoblasts is the synthesis of the organic matrix of bone, which is composed of type I collagen (approx 90%) and noncollagenous proteins (eg, osteocalcin).^{4,5} In contrast, osteoclasts resorb bone and degrade collagen into molecular fragments.³ Bone turnover can be assessed via monitoring of various biochemical markers in blood or urine, such as cellular enzyme activity (eg, bone alkaline phosphatase [ALP]) and concentrations of substances released during the formation or degradation of the organic bone matrix (eg, osteocalcin and procollagen type I carboxy propeptide [PICP]) or inorganic components of bone (eg, calcium and phosphorus).⁵⁻⁷ Biochemical bone marker assays are routinely used in human medicine to screen for metabolic bone diseases and evaluate the response to various treatments.^{6,8,9} Assessment of a combination of various serum biochemical bone markers that indicate different aspects of development and function of osteoblasts and osteoclasts might provide new insight into bone metabolism in horses.

Type I collagen telopeptides are released into the circulation during type I collagen degradation.^{5,8} Carboxy-terminal cross-linking telopeptide of type I collagen (CTX-I) can be quantified in serum or plasma by immunoassays that use antibodies against an octapeptide (EKAH β DGG) containing β -isomerized aspartic acid in the carboxy-terminal part.^{5,8,10,11} The CTX-I is supposed to be bone-specific because osteoclasts are not involved in the degradation of other type I collagen tissues.³ Furthermore, carboxy-terminal

Musculoskeletal diseases often occur in horses.¹ Noninvasive bone assessment techniques are

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cross-linking telopeptide of type I collagen generated by matrix metalloproteinases (CTX-MMPs [previously named ICTP]) can be quantified in serum by use of an immunoassay that involves a trivalent cross-link as the antigenic site.^{5,7,12} However, cathepsin K-mediated osteoclastic bone resorption was shown to destroy the CTX-MMP antigenicity.¹³

Osteocalcin is one of the most abundant noncollagenous proteins of bone; it is a small protein that contains γ -carboxyglutamic acid and binds calcium.¹⁴ Newly synthesized osteocalcin is incorporated into the bone matrix where it is tightly bound to hydroxyapatite or directly released into the circulation.^{6,15} At present, the exact function of osteocalcin is not fully understood. Serum osteocalcin concentration was correlated with bone formation rate volume referent as determined by histomorphometric evaluation of a transiliac biopsy sample in humans.¹⁶ Nevertheless, fragments of the osteocalcin protein are also thought to be released during osteoclastic bone resorption.¹⁷ Osteocalcin is an indicator of osteoblast activity¹⁸; however, to date, its role in the regulation of bone turnover has not been well-defined.¹⁹

The objectives of the study reported here were to evaluate an assay for quantification of human serum CTX-I, assess the influence of age on plasma CTX-I concentration, investigate the relationship between plasma CTX-I and serum osteocalcin concentrations, and determine whether concentrations of plasma CTX-I or serum osteocalcin fluctuate in a circadian manner in horses.

Materials and Methods

Biochemical assays—Carboxy-terminal cross-linking telopeptide of type I collagen was quantified with a commercially available automated electrochemiluminescent sandwich antibody assay (ECLIA).⁴ The assay uses monoclonal antibodies specific for the EKAH β DGGR octapeptide with β -isomerized aspartic acid in the carboxy-terminal of type I collagen. The ECLIA sample volume was 50 μ L of plasma or serum. Serum and plasma CTX-I values were expressed as nanograms of CTX-I per milliliter. The assay's sensitivity was 0.01 ng/mL. The intra- and interassay coefficients of variation (CVs) at 0.3 ng/mL were 5% and 5.2%, respectively. Serum osteocalcin concentrations were determined by use of a recently developed equine-specific osteocalcin radioimmunoassay (RIA).²⁰ The sensitivity of the osteocalcin RIA was 0.2 ng/mL. Serum osteocalcin concentration was expressed as nanograms of osteocalcin per milliliter; the intra- and interassay CVs at 43 ng of osteocalcin/mL of serum were 4.7% and 7.3%, respectively. Serum creatinine concentrations were analyzed by use of a commercially available test kit.^b The test is based on the Jaffé reaction without deproteinization, in which alkaline picric acid forms a colored solution in the presence of creatinine. Serum creatinine concentrations were expressed in micromoles per liter. **Gamma-glutamyl transferase (GGT) activity** was evaluated in serum samples with a commercially available test^c and expressed as units per liter.

Evaluation of the CTX-I ECLIA in horses—Multiple blood samples from a 2.5-year-old Warmblood gelding and a 2-year-old Warmblood-cross stallion were collected in 10-mL tubes^d for serum separation. Potential nonspecific effects of equine serum with the CTX-I ECLIA were evaluated by a dilution test, and the CTX-I recovery was calculated. For the dilution test, both equine serum samples were serially diluted with equine CTX-I-free serum samples. Furthermore, both equine serum samples were serially diluted with the

manufacturer's diluent.^e Equine CTX-I-free serum samples were obtained by pooling sera from 4 horses (a 16-year-old Warmblood gelding, 15-year-old Warmblood-cross gelding, 16-year-old Warmblood mare, and 4-year-old Thoroughbred gelding); the sera from these horses had signal values below the measurement range.

Effect of age on plasma CTX-I concentration—Fifty-four clinically normal horses (age range, 0.5 to 35 years) were used in this investigation. Between 9:00 and 10:00 AM, a blood sample from each horse was collected via right jugular venipuncture into 5-mL tubes containing EDTA.^f Within 1 hour of collection, all samples were centrifuged (1,000 \times g at 4°C for 15 minutes). The plasma was stored in aliquots of 1 mL at -21°C until assayed.

Correlation of plasma CTX-I and serum osteocalcin concentrations—Sixty clinically normal horses (age range, 0.5 to 35 years) were included in this investigation; 54 of these horses were also used to assess the effect of age on plasma CTX-I concentration. Between 9:00 and 10:00 AM, 1 blood sample from each horse was collected via right jugular venipuncture into 5-mL tubes with and without EDTA. Within 1 hour of collection, all samples were centrifuged (1,000 \times g at 4°C for 15 minutes). Serum and plasma samples were stored in aliquots of 1 mL at -21°C until assayed.

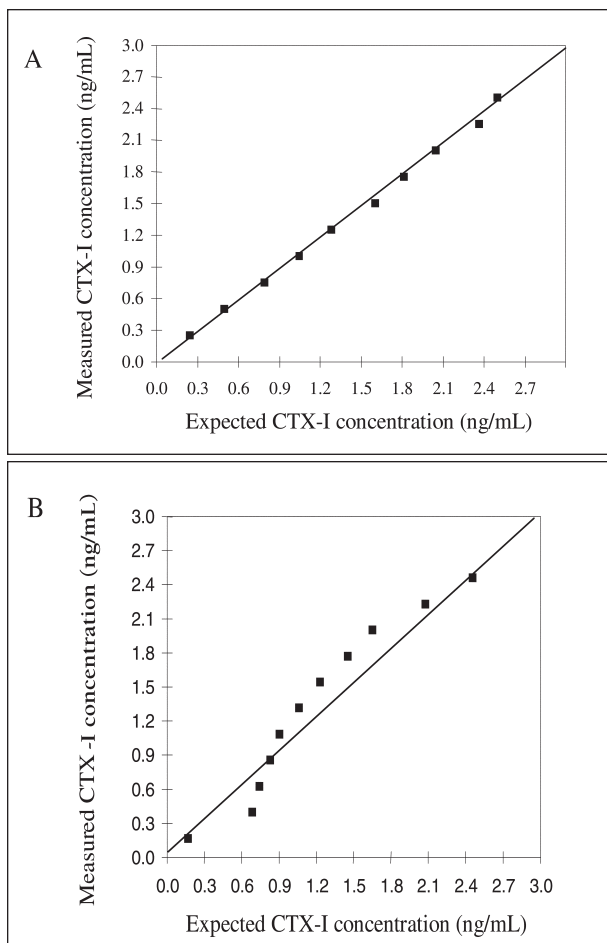


Figure 1—Evaluation of an automated electrochemiluminescent sandwich antibody assay (ECLIA) developed to quantify human carboxy-terminal cross-linking telopeptide of type I collagen (CTX-I) for use in analysis of equine sera. Plots represent serial dilutions of serum from a horse with unspiked equine serum samples (A) and assay diluent (B) to establish cross-reactivity between antibodies of the CTX-I ECLIA and markers in equine serum.

Assessment of circadian variations of serum osteocalcin and plasma CTX-I concentrations—Nine healthy horses were included in this 24-hour study; there were 6 mares, 2 geldings, and 1 stallion (mean age \pm SD, 7.2 ± 5.14 years, range, 2 to 16 years). These horses were used only in this study. The horses were assigned by age to 1 of 2 groups: group I included horses that were 2 to 6 years of age ($n = 6$), and group II included horses that were 12 to 16 years of age (3). All horses were confined to stalls during the study and were not exercised. The animals had free access to water and were fed good-quality hay at 8:30 AM and 5:00 PM. During the morning of the sampling day, a 14-gauge (80-mm) IV catheter^a was placed in the right jugular vein of each horse. Blood samples were collected at hourly intervals for 24 hours (starting at 3:00 PM and continuing until 2:00 PM of the following day). Blood samples were collected in 5-mL tubes with and without EDTA. To obtain sera, blood samples were allowed to clot for 1 hour at 4°C. Samples of serum and plasma were centrifuged ($1,000 \times g$ at 4°C for 10 minutes) and stored in aliquots at -21°C until analysis.

Statistical analyses—The data were analyzed with a commercially available computer program.^b Values of $P < 0.05$ were considered significant. The skewness and kurtosis of the distribution of all variables were controlled by the Kolmogorov-Smirnov test for normal distribution. Pearson's product-moment coefficient was used to calculate the correlation when distribution analysis fitted the normal curve. For the nonparametric variables, the Spearman distribution-free rank correlation was used to calculate the correlation of CTX-I and age. The Mann-Whitney U test was used to assess differences in 24-hour plasma CTX-I and serum osteocalcin concentrations.

Results

Creatinine and GGT analyses—Serum creatinine concentrations in all horses used in the investigations were within normal limits ($< 178 \mu\text{mol/L}$; reference range, 44 to $179 \mu\text{mol/L}$). Horses used in the evaluation of the CTX-I ECLIA and the assessment of circadian variations of serum osteocalcin and plasma CTX-

I concentrations had serum GGT activities within normal limits ($< 28 \text{ U/L}$; reference range, 5 to 28 U/L).

Evaluation of the CTX-I ECLIA in horses—Serial dilutions of equine serum samples with equine CTX-I-free serum had good correlation ($r > 0.99$) between expected and measured serum CTX-I concentrations (Fig 1). Serial dilutions of equine serum with the manufacturer's diluent had low correlation between expected and measured CTX-I concentrations (Fig 1). Recovery values (%) for CTX-I were calculated (Table 1).

Effect of age on plasma CTX-I concentration—There was a significant inverse correlation ($r = -0.46$; $P < 0.001$) between the horses' age (mean age \pm SD, 9.8 ± 9.3 years) and plasma CTX-I concentrations (mean concentration, $0.11 \pm 0.08 \text{ ng/mL}$; Fig 2).

Correlation of plasma CTX-I and serum osteocalcin concentrations—Data from all horses were pooled for analysis. A low but significant correlation ($r = 0.3$;

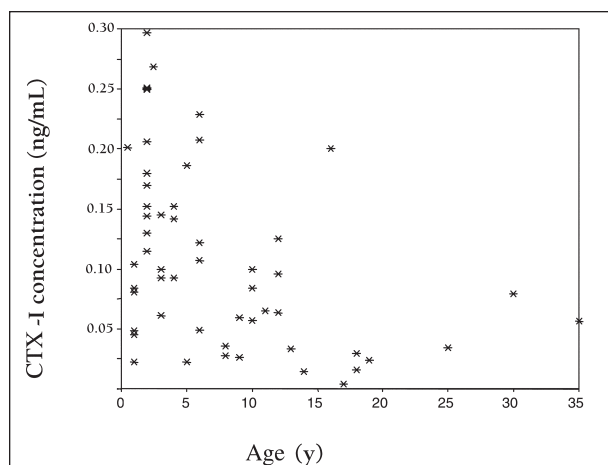


Figure 2—Scatter diagram of plasma CTX-I concentration versus age in 54 horses.

Table 1—Concentration of carboxy-terminal cross-linking telopeptide of type I collagen (CTX-I) in serum samples (diluted serially with known quantities of equine CTX-I-free serum) from 2 horses detected by use of an automated electrochemiluminescent sandwich antibody assay (ECLIA) for human CTX-I and recovery of CTX-I calculated as a percentage of the expected CTX-I concentration at each dilution

Sample	Expected CTX-I concentration (ng/mL)	Observed CTX-I concentration (ng/mL)	Recovery (%)
Serum 1	0.25	0.244	97.6
	0.50	0.498	99.6
	0.75	0.796	106.1
	1.00	1.049	104.9
	1.25	1.285	102.8
	1.50	1.605	107.0
	1.75	1.814	103.6
	2.00	2.047	102.3
	2.25	2.367	105.2
Serum 2	0.13	0.146	108.4
	0.27	0.296	109.9
	0.54	0.575	106.7
	0.67	0.700	103.9
	0.81	0.837	103.6
	0.94	0.987	104.7
	1.08	1.114	103.4
	1.21	1.242	102.4

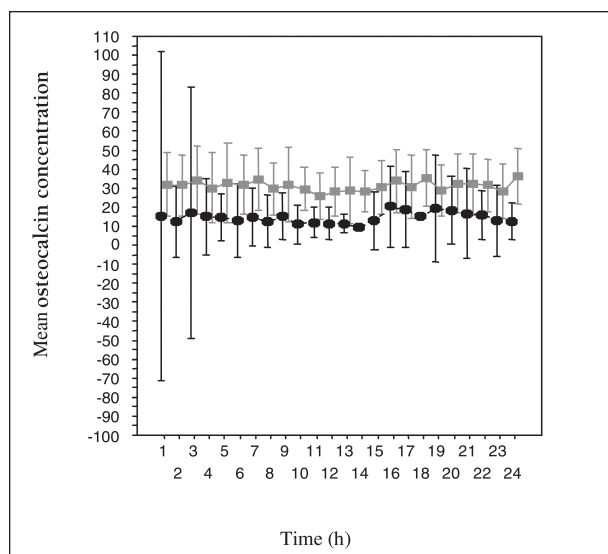


Figure 3—Mean serum osteocalcin concentrations (ng/mL) during a 24-hour period in 2- to 6-year-old horses (squares; $n = 6$) and 12- to 16-year-old horses (circles; 3). Bars represent the 95% confidence interval at each data point.

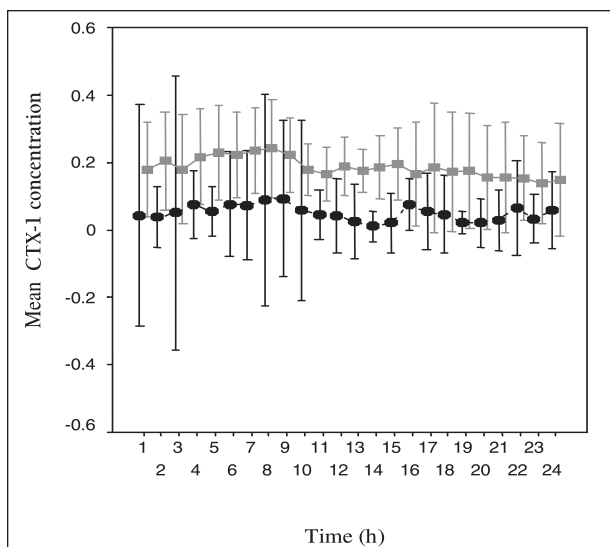


Figure 4—Mean plasma CTX-I concentrations (ng/mL) during a 24-hour period in 2- to 6-year-old horses (squares; $n = 6$) and 12- to 16-year-old horses (ovals; 3). Bars represent the 95% confidence interval at each datum point.

$P < 0.05$) was obtained between plasma CTX-I concentration (mean concentration, 0.08 ± 0.08 ng/mL) and serum osteocalcin concentration (mean concentration, 27.2 ± 23.8 ng/mL).

Assessment of circadian variations of serum osteocalcin and plasma CTX-I concentrations—Mean serum osteocalcin concentration did not significantly change during the 24-hour investigation. Horses had no notable diurnal variation in plasma CTX-I concentration. Neither age group had notable circadian variations in serum osteocalcin and plasma CTX-I concentrations (Fig 3 and 4).

Discussion

In the study of this report, serum and plasma CTX-I concentrations in horses were measured by use of an assay developed for the evaluation of bone resorption in humans.¹⁰ Our data indicated that the automated CTX-I ECLIA was appropriate for use in horses. When equine test serum was diluted with equine CTX-I-free serum, the assay indicated good correlation between expected serum CTX-I concentrations and measured serum CTX-I concentrations and an acceptable recovery range. However, results of the serial dilution test (and calculated CTX-I recovery values) were not acceptable when equine test serum was diluted with the manufacturer's universal diluent. These results were similar to those of another study²¹ in which the addition of gastrin-free serum samples to standards improved the recovery. The mechanism by which this occurs is not completely understood, but serum components might positively influence the test's accuracy (eg, as a result of the antigenic affinity of the analyte).

For evaluation of serum osteocalcin concentrations, an equine-specific osteocalcin RIA²⁰ was used, which is one of the few horse-specific bone marker assays available. Interspecies cross-reactivity between antibodies of human-specific metabolites of collagen

type I and serum or urine samples from horses have been described. For bone formation, the human-specific PICP RIA was validated for horse serum.²² For bone resorption, the human-specific RIA for CTX-MMP was validated for serum samples from horses.²² In addition, an ELISA for CTX-I was recently used in equine serum samples.¹ Equine pyridinolines can be measured by reversed-phase high pressure liquid chromatography²³; quantification of pyridinolines and deoxypyridinolines in urine and serum from horses has been described.^{24-26,1} Furthermore, the use of a bovine-specific osteocalcin RIA in horses has been described.²⁷

In humans, a strong correlation was obtained between CTX-I concentrations obtained with the serum CTX-I ELISA and sandwich CTX-I ECLIA.^{28,9} However, CTX-I concentrations in serum and urine samples from humans were less strongly correlated, which might be explained by analytical difficulties in the urine sample evaluation.¹ In our study, CTX-I assays were not compared, but there might be a correlation between results obtained with different CTX-I assays in horses.

Biochemical bone markers are mainly degraded and eliminated by the kidneys or liver.^{6,29} Concentrations of various biochemical bone markers in serum, plasma, and urine are known to be influenced by impaired kidney and liver function.^{17,29,30} The horses used in the study of this report had serum creatinine concentrations and GGT activities that were within normal physiologic ranges.

In our study, there was a modest inverse correlation between plasma CTX-I concentration and age. Findings of other studies^{22,31-33} indicate that young horses have high concentrations of bone ALP, PICP, CTX-MMP, and osteocalcin and that these values are decreased in adult animals. Compared with healthy adult horses, the high concentrations of various biochemical markers of bone resorption and formation in young horses might indicate greater bone modeling or remodeling; this phenomenon has also been observed in humans.

Our data indicated that plasma CTX-I concentration was significantly correlated (albeit modestly) with serum osteocalcin concentration. The bone resorption marker CTX-I contains an β L-aspartyl residue that is derived mainly from aging of extracellular proteins.^{34,35} In contrast, osteocalcin is liberated in the bloodstream during the mineralization phase of the organic matrix.⁶ The correlation between these 2 bone markers might indicate a balanced bone metabolism activity in the population of horses used in our study.

More than 3 decades ago, a diurnal regulation of skeletal growth was described.³⁶ However, the regulation and etiologic features of circadian fluctuations in humans and other animals are not completely understood. Results of studies^{36,37} of diurnal periodicity in metabolic activity of bones in rats and mice indicate a phase of highly active bone mineralization during the dark period. Several investigations³⁸⁻⁴³ to assess concentrations of serum and plasma bone markers in humans and other animals revealed biorhythmic profiles in which the highest concentrations were detected during the night. However, results of other studies^{41,44-47} indi-

cated only slight or no circadian fluctuations in serum or plasma bone marker concentrations of different species. In the horses of this report, no circadian pattern in plasma CTX-I concentration was detected. In humans, peak serum CTX-I concentration was detected between 1:30 and 4:30 AM and nadir values were detected between 11:00 AM and 3:00 PM⁴⁸; urine CTX-I concentration peaked during the night, and nadir values were detected at 5:00 PM.⁴⁹ In humans, food intake and fasting are known to influence bone marker concentrations^{9,50} and fasting significantly reduces the circadian variation in serum CTX-I concentration.⁹ The horses used in our study had free access to water and were fed daily with good-quality hay at 8:00 AM and 4:30 PM. Circadian variation of serum or plasma osteocalcin concentrations in horses is controversial.^{24,40,44-46} In our study, no significant diurnal fluctuations in serum osteocalcin concentration were detected; these findings are similar to those obtained by Hope et al⁴⁴ and Geor et al.⁴⁵ Additionally, a lack of significant fluctuations in serum osteocalcin concentration was reported in weanling colts,²⁴ although that was attributed to high interindividual variation in serum osteocalcin concentration. However, adult horses had highest serum osteocalcin concentration between 12:00 and 9:00 AM.²⁴ In another study,⁴⁰ a nadir in serum osteocalcin concentration was identified at 8:00 PM and peak serum osteocalcin concentration was detected at 5:00 AM after dividing the sample population into 2 age groups.

In our study, horses that were 2 to 6 years old and 12 to 16 years old did not have significant circadian variation in serum osteocalcin concentration. The difference in circadian profiles of the previous studies and the study reported here might be a consequence of dissimilar sampling conditions (eg, season and sample populations). Black et al²⁴ assessed serum osteocalcin concentrations in weanlings and adult horses (mean age, 12.8 years). Moreover, the sample population used by Lepage et al⁴⁰ was mainly composed of pregnant mares; furthermore, the influence of pregnancy on serum osteocalcin concentrations in horses is not understood, although variations in serum osteocalcin concentrations in pregnant women have been described.⁵¹ The discordant results obtained from studies investigating circadian variations of serum and plasma osteocalcin concentrations might be explained by the action of the assays' antisera that recognize osteocalcin as well as various osteocalcin fragments. Osteocalcin fragments that are released during the bone resorption process might therefore be included in the measurement result.⁵² Previous studies^{24,40} involving measurement of serum osteocalcin concentration in horses were performed with polyclonal bovine-specific osteocalcin assays. Equine osteocalcin has been recently isolated and characterized.⁵³ In the study reported here, an equine-specific polyclonal osteocalcin RIA was used. However, the osteocalcin fragments released during bone resorption in horses have not been precisely identified. Characterization of the osteocalcin fragments and the development of equine-specific assays that detect intact osteocalcin or its fragments might help to elucidate the circadian variation of osteocalcin in horses.

In our experience, the quantification of serum or plasma markers of bone metabolism is easy to perform. It is an inexpensive and noninvasive technique with which to evaluate dynamic changes in bones of horses. Our data suggested that the fully automated CTX-I ECLIA can be used for evaluation of serum and plasma samples obtained from horses; no notable diurnal fluctuations in plasma CTX-I and serum osteocalcin concentrations were detected in the horses used in our study.

^aElecsys β -CrossLaps/serum assay; all reagents supplied by Roche Diagnostics GmbH, Penzberg, Germany.

^b3385 Merckotest, Merck, Darmstadt, Germany.

^cGranustest, Merck, Darmstadt, Germany.

^dVenoject, Terumo Europe, Leuven, Belgium.

^eUniversal diluent, Roche Diagnostics GmbH, Penzberg, Germany.

^fVenoject containing 0.05 mL EDTA (K3), Terumo Europe, Leuven, Belgium.

^gIntraflon 2, Vygon SA, Brussels, Belgium.

^hSPSS for Windows 11.0, SPSS Inc, Chicago, Ill.

ⁱKellerhouse P, Brown C, Newhall K, et al. Assessment of bone resorption marker assays in thoroughbred horses. *J Bone Miner Res* 2000; 15:S526.

^jLeary ET, McLaughlin MK, Swezey D, et al. Performance of β -Crosslaps/Serum on the Elecsys 2010 in comparison with six serum and urine bone resorption markers in microtiter assay format (abstr). *Clin Chem* 2000;46:A145.

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