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SHORT SCIENTIFIC COMMUNICATION

Bone turnover markers are differentially affected by pre-analytical handling

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Abstract

Summary Given that bone turnover markers are often shipped to central laboratories, it is essential to be aware of factors that will affect stability. We have evaluated how sample type, time before separation of blood samples, and time between separation and analysis affect the stability of four bone turnover markers.

Introduction Bone turnover markers are often shipped to central laboratories for analysis, which require knowledge of the stability of the markers of interest in different sample materials. The aim of the current study was to evaluate how time before separation of blood samples and time between separation and analysis affect the stability of four bone turnover markers in serum and plasma samples.

Methods Serum, EDTA, and Lithium heparin (LiHep) plasma samples from seven osteoporosis patients and three healthy controls were collected and stored at room temperature for up to 72 h before separation and analysis. After separation, samples were stored at room temperature for up to 72 h and re-analyzed. The bone turnover markers N-terminal pro-collagen type 1 extension pro-peptide (P1NP), bone-specific alkaline phosphatase (BAP), C-terminal teleopeptide cross links of collagen type 1 (CTX), and osteocalcin (OC) were analyzed using the automated iSYS IDS platform.

Results P1NP and BAP were stable in both plasma and serum for 72 h before centrifugation. CTX levels were higher in EDTA plasma at all time points compared to LiHep plasma and serum. The use of EDTA plasma prolonged the stability of CTX as compared to LiHep plasma and serum. Osteocalcin showed high tendency to degrade in all sample types and concentrations were significantly lower after 24 h of storage.

Conclusions For the bone turnover markers P1NP and BAP, the use of both plasma and serum is recommended. Samples for CTX analysis should be taken as EDTA plasma. Samples for osteocalcin analysis can be taken in either type of plasma or serum, but should be analyzed within 3 h or preserved at − 18 °C.

Keywords BAP · Bone turnover markers · CTX · Osteocalcin · Pre-analytical · P1NP · Stability

Introduction

The diagnostic criteria for osteoporosis are based upon bone mineral density measurement assessed with dual energy X-ray absorptiometry (DXA) scanning. The diagnostic criteria being less than or equal to $-2.5 \times$ SD from the average of young adults, also termed T-score [1–3]. DXA is a valid diagnostic test, when the aim is to determine the exact level of bone mineral density. However, the DXA method is less sensitive for detection of smaller changes induced by disease progression or treatment response [4] over shorter terms. In contrast, peptides and proteins released during bone formation or resorption can be used as circulating biomarkers to follow changes in bone turnover. For many years, bone turnover markers (BTMs) have been measured in clinical studies.

The BTMs can be divided into two classes depending on which specific bone-related activity they reflect. The bone resorption markers originate from osteoclastic activity, leading to breakdown of collagen matrix. These include the collagen breakdown products C and N-terminal telopeptide cross links of collagen type 1 (CTX/ICTP/

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NTX-1) and Deoxypyridinoline (DPD). The bone formation markers are substances related to osteoblast activity or collagen synthesis and include bone-specific alkaline phosphatase (BAP), osteoblast-derived osteoprotegerin (OPG), receptor activator of Nf-kappa B ligand (RANKL), N-terminal procollagen type 1 extension pro-peptide (P1NP) and C-terminal pro-peptide of type 1 pro-collagen (P1CP), and osteocalcin (OC). The choice of markers to be used clinically has varied between medical centers and various assays have been developed for clinical platforms. Despite many reports on the use of BTMs, the variation in bone markers used, assays, and sample handling have made direct comparisons difficult. The joint working group on bone marker standards under the International Osteoporosis Foundation (IOF) and International Federation of Clinical Chemistry and laboratory medicine (IFCC) has recommended the use of CTX and P1NP as first choice resorptive and formative bone markers [5]. However, comparisons of these standard markers with other markers will aid in the evaluation of their clinical usefulness in different pathological situations.

The use of BTMs is expected to aid in evaluation of treatment response and fracture risk prediction. In addition to the variation in use of markers, the clinical use of the BTMs has also been delayed by challenges with great interlaboratory variation. Neither measurement levels nor pre-analytical handling of samples has been standardized [6, 7]. Reports describing factors affecting pre-analytical and analytical variation are accumulating, allowing the construction of standardized guidelines for sample handling and reference areas [8].

Immunodiagnostics Systems (IDS) offers automated immunoassays for a range of BTMs and recommends both serum and heparin or EDTA-containing plasma as acceptable sample material for analysis of P1NP, CTX, and OC. These samples are advised to be centrifuged immediately and stored at − 20 °C until analysis. For analysis of BAP, it is advised to avoid EDTA plasma as sample material and keep samples at 2–8 °C for up to 48 h before analysis. The aim of this study was to provide a more detailed picture of how differences in pre-analytical handling of blood samples affect the stability of BTMs measured with the automated chemiluminescense assay kits from IDS using an iSYS platform.

Materials and methods

The inter-assay intermediary precision was calculated from individual determinations in 20–56 separate assays at 3–4 concentration levels. The acceptable change limit for the individual analysis was calculated based on the highest CV percentage, as $\sqrt{2} \times 1.96 \times \mathrm{CV}$. Twelve venous blood samples each from seven healthy study participants and three osteoporosis patients were used for isolation of serum (4), lithium heparin (LiHep), plasma (4), and EDTA plasma (4). To evaluate the effect of time from sampling to centrifugation, samples were stored at room temperature for half, 3, 24, or 72 h to mimic transportation times from blood sampling facilities (blood sampling outpatient clinics or general practitioners' office) to the analysis laboratory. After centrifugation at 1860 g for 10 min, serum or plasma was collected and frozen at − 18 °C until analysis. In addition, the samples centrifuged after half an hour were distributed in three vials and frozen immediately or after incubation at room temperature for 24 or 72 h. All samples from the same patient were analyzed in one batch. Four BTMs were analyzed; C-terminal telopeptide cross links of collagen type 1 (CTX) was measured using the IDS-iSYS Crosslaps®assay and intact procollagen type 1 (N-terminal teleopeptide) was measured using the IDS-iSYS intact P1NP assay. Bone-specific alkaline phosphatase (BAP) was measured using IDS-i-SYS Ostase® BAP assay and OC was measured using the IDS-iSYS N-mid osteocalcin assay. All assays were run on a IDS-iSYS-automated analyzer (Immunodiagnostic Systems, plc, Tyne and Wear, UK).

Statistics

Comparisons between initial value and values measured in stored samples were performed with one-way repeated measures ANOVA with Dunnett's post hoc test. Comparisons between initial level in serum and LiHep or EDTA plasma were performed with a two-tailed Student's t test. p values < 0.05 were considered as significant.

Results

The inter-assay intermediary precisions expressed as coefficients of variation for CTX were 5.3% (at CTX concentration 213 ng/L), 3.4% (869 ng/L), and 3.5% (2113 ng/L). For P1NP, the inter-assay intermediary precisions were 5.4% (18.96 μg/L), 6.5% (48.48 μg/L), and 6.1% (122.10 μg/L). For osteocalcin, the inter-assay intermediary precisions were 3.0% (8.73 μg/L), 3.6% (27.6 μg/L), and 3.5% (68.7 μg/L). Finally, for BAP, the inter-assay intermediary precisions were 8.5%, 7.1%, 3.7%, and 6.3% at levels of 4.5, 13.2, 20.1, and 52.1 μg/L, respectively. The acceptable change limits based on the highest CV for the analysis are indicated in Table 1.

To evaluate stability in non-separated blood samples, blood collected for serum, LiHep, or EDTA plasma preparation was incubated at room temperature and centrifuged for half an hour, 3 h, 24 h, or 72 h after sampling. To evaluate stability after centrifugation, the half-hour samples were aliquoted and left at room temperature for 24 or 72 h before analysis.

CTX levels were significantly affected by handling time before centrifugation, when the sample material was serum or LiHep plasma. In serum, CTX was stable for 24 h,

Table 1 Analyte levels (SEM) normalized to initial concentration in the serum sample for each bone turnover marker. Boldface indicates significant difference from a half-hour sample within the same material; underlined data indicates difference between serum sample and lithium heparin (LiHep) or EDTA plasma sample at starting point (half hour)

		Half hour	3 h	24 h	72 h	ACL (%)
P1NP	Serum	100 (17)	95 (15)	99 (15)	102 (16)	18.1
	LiHep	101 (13)	105 (15)	101 (15)	102 (15)	
	EDTA	101 (14)	101 (16)	101 (15)	103 (16)	
CTX	Serum	100 (28)	92 (25)	67 (16)	**29** (7)	14.7
	LiHep	107 (27)	105 (27)	**62** (17)	**21** (4)	
	EDTA	115 (30)	116 (31)	119 (32)	117 (30)	
OC	Serum	100 (13)	96 (14)	**73** (8)	**56** (6)	10.0
	LiHep	98 (14)	100 (16)	**84** (13)	**60** (8)	
	EDTA	99 (14)	96 (14)	**80** (9)	**68** (9)	
BAP	Serum	100 (11)	100 (11)	100 (11)	100 (12)	23.5
	LiHep	102 (11)	102 (11)	100 (11)	102 (11)	

whereas it was stable for 3 h only in LiHep plasma (Fig. 1a). In contrast, CTX was stable in EDTA plasma for at least 72 h (Fig. 1a). After centrifugation, the stability was likewise affected in serum or LiHep plasma (Fig. 1b). In EDTA plasma, the CTX level was stable for 72 h of storage after centrifugation (Fig. 1b). The differences between LiHep plasma and serum were already evident at the first analysis time point (half hour), as the CTX level was 6% lower in LiHep and 13% lower in serum, compared to EDTA plasma (Fig. 1a and Table 1). There was a significant difference between the initial level in EDTA plasma and LiHep plasma and serum, but also between Lihep and serum levels (two-tailed Student's t test $p < 0.05$). Calculating the acceptable change limit, a change up until 14.7% is within the limit of analysis uncertainty. Thus, the initial changes between EDTA plasma and serum are in the outer range, but do not exceed acceptable change limits. Initial values measured in different sample materials did not differ for the remaining three markers.

The levels of P1NP were stable in all sample materials both before (Table 1) and after centrifugation.

Osteocalcin was stable for 3 h before centrifugation and analysis in all three sample materials. Longer storage, whether samples were centrifuged or not, reduced osteocalcin stability in all sample types (Fig. 1c and d).

BAP was stable in both serum and LiHep plasma. No change in analyte levels was observed for up to 72 h of storage at room temperature before centrifugation (Table 1). After centrifugation, there was statistical significance in analyte levels after 72 h of storage (6% in LiHep and 4% in serum). These changes were minor compared to the acceptable change limit of the analysis.

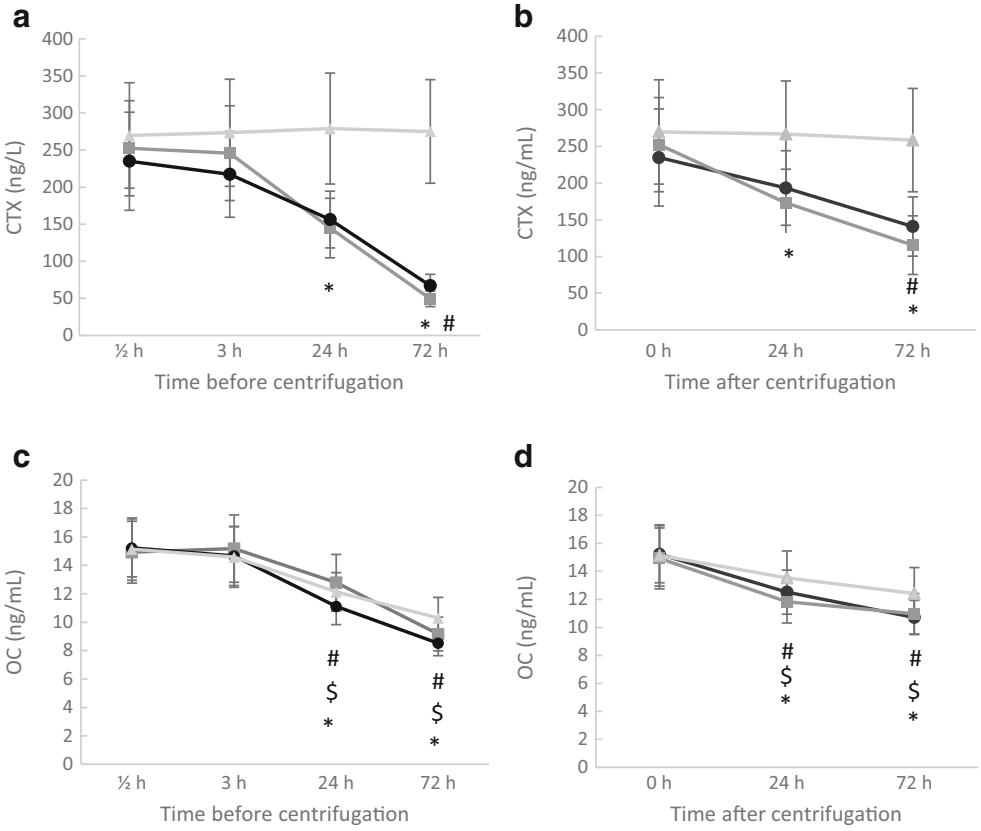

Fig. 1 Stability of CTX and osteocalcin at room temperature before and after centrifugation for isolation of serum, lithium heparin plasma, and EDTA plasma. Samples from ten individuals were incubated for the indicated timepoints before centrifugation and analysis (**a** and **c**) or centrifuged after 30 min and incubated for the indicated timepoints before analysis (**b** and **d**). Light-gray triangles, EDTA. Dark-gray squares, LiHep. Black circles, serum. p values < 0.05 indicate significant difference from a half-hour sample with one-way repeated measures ANOVA with Dunnett's post hoc test. * = LiHep # = serum $ = EDTA

Discussion

In this study, P1NP was equal in all three sample materials and can accordingly be analyzed in any of these. It is not essential to analyze samples for P1NP immediately, as stability is not affected upon storage for up to 72 h. This is in agreement with previous observations [9, 10]. Despite high stability of P1NP, it is relevant to evaluate the degree of hemolysis, which significantly affects the P1NP measurement [10].

BAP levels were similar in LiHep plasma and serum and were stable in both sample types, before separation. After centrifugation, incubation slightly affected BAP levels after 24 h. Given that BAP concentration in average was reduced by 6% in serum and 4% in LiHep plasma, this was not higher than the average intermediary precision for the assay (6.4%) and definitely within the acceptable change limits (23.5%). BAP was not analyzed in EDTA plasma as it is stated by the manufacturer that EDTA and sodium citrate plasma cannot be used with the IDS-iSYS BAP assay (IDS-iSYS Ostase® BAP data sheet). It has previously been reported that BAP is stable at 25 °C for up to 14 days in serum [11]. Stokes and colleagues reported no effect on storage for up to 48 h before centrifugation or 7 days after centrifugation in either serum or EDTA plasma in another immunoassay (Metra biosystems). Using the iSYS assay, it can be recommended to analyze BAP in either serum or LiHep plasma, preferably directly after centrifugation, but concentrations are not markedly affected by several days of storage.

Storage of samples for CTX measurement has been reported previously to be more critical. The levels of CTX found in LiHep plasma and serum were significantly lower than those in EDTA plasma already in the half-hour sample and throughout the study. The differences in initial values were close to but did not exceed acceptable change limit for the analysis. Given the low stability in LiHep plasma and serum, these initial differences may reflect fast degradation in serum and LiHep plasma. In two previous studies, no difference between initial levels in serum and EDTA plasma was observed with the Cobas E170/Elecsys platform [9, 12]; however, lower levels in LiHep plasma compared to EDTA plasma were observed [12]. It should be kept in mind that CTX assays are not harmonized and commercial assays do not measure the same level of analytes [6, 7, 13]. Therefore, the observed difference may also reflect differences in specificity of the antibodies used in the Cobas and IDS-iSYS assays for degraded CTX peptides. Importantly, the stability of CTX can be prolonged to at least 72 h before and after centrifugation by the use of EDTA plasma as sample material. This is also true for the more concentrated samples, which tend to show faster degradation in serum and LiHep samples. These observations support the recommendation for sampling of CTX analysis by National Bone Health Alliance (NBHA), who recommend that EDTA plasma should be used if samples cannot be processed and analyzed or frozen immediately [8]. Given the differences observed already after half an hour, we would recommend always to use EDTA plasma for CTX analysis.

OC is known to be very unstable. We find osteocalcin to be partially degraded in all three types of sample material 24 h after sampling. There were no differences in initial levels in the half-hour samples, and we did not observe differences in stability between the three sample materials tested. Three hours of storage does not significantly affect the levels of OC. Previously, it has been reported that OC samples can be stored at room temperature for 8 h in LiHep or EDTA plasma without affecting the analyte level [9]. Earlier reports describe the highest stability of OC in serum. Hemolysis has been reported to affect the measurement of OC in several assays [14, 15]. It has been speculated that even low degrees of hemolysis results in the release of proteases, which degrade the peptide [14]. However, for some assays, it is an assay interference with hemoglobin which affects the measurement [15]. The reason for differing reports on the stability and interference with hemoglobin on OC measurement is presumably a result of specificity of antibodies and assay type. Preservation of OC samples after centrifugation can be achieved at 2–8 °C for up to 48 h in all three sample materials [9].

The current study underscores the importance of correct handling of samples prior to analysis of bone turnover markers. As these analyses are currently not performed in all laboratories and samples are often shipped to central laboratories, it is important to use the optimal sample material and storage condition for improving preservation of the individual marker the most.

Limitations of the study

This is a minor study evaluating the stability of three sample types each in ten subjects. The starting levels of analytes varied between individuals and were therefore also differentially affected by time until analysis. Therefore, minor changes did not reach statistical significance, although there may be effect on more concentrated samples. We evaluated timepoints of interest in everyday clinical evaluation, but did not include storage at 4 °C.

In conclusion, P1NP and BAP have high stability in both plasma and serum at room temperature for up to 72 h, and all sample materials can be used. OC shows high tendency to degrade in all sample types and samples should be refrigerated until analysis.

For CTX analysis, the use of EDTA plasma is recommended, as lower concentrations are measured already after 30 min in LiHep plasma or serum. The recommendations by the National Bone Health Alliance state that serum can be used for CTX analysis, if samples are processed promptly [8]. Given the time it takes for serum to coagulate, we consider EDTA plasma to be the optimal choice. On the other hand, we

do not find it necessary to freeze CTX samples in EDTA plasma and samples for P1NP and BAP within 2 h as recommended by the National Bone Health Alliance. These samples show good stability at room temperature for up to 72 h in all the tested sample materials.

Compliance with ethical standards

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. For this type of study, formal consent is not required.

Conflict of interest None.

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