Characterization of trivalently crosslinked C-terminal telopeptide of type I collagen (CTX) species in human plasma and serum using high resolution mass spectrometry

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Abstract

With an aging population, there has been significant progress in the discovery and measurement of bone turnover biomarkers since the 2000s, especially for monitoring skeletal diseases like osteoporosis. Multiple markers derived from type I collagen, such as CTX, NTX, PINP, and ICTP, have been developed. Extensive efforts have been devoted to characterizing these molecules; however, their complex crosslinked structures have posed significant analytical challenges, and to date, these biomarkers remain poorly characterized. Previous attempts at characterization involved gel-based separation methods and MALDI-TOF analysis on collagen peptides directly extracted from bone. However, using bone powder, while rich in collagen, does not represent the true structure of the peptides in the biofluids. In this study, our goal was to characterize plasma and serum CTX for subsequent LC-MS/MS method development. We extracted and characterized type I collagen peptides directly from human plasma and serum using a proteomics workflow that integrates preparative liquid chromatography, affinity chromatography, and high-resolution mass spectrometry. Subsequently, we successfully identified numerous CTX species, providing valuable insights into the characterization of these crucial biomarkers.

Introduction

Osteoporosis is a skeletal disease characterized by low bone mass and loss of bone microarchitecture, which represents a major global health issue [1–3]. It accounts for more than 4% of the annual EU health expenditure[2–4]. The disease is caused by an increased rate of bone resorption, which involves osteoclasts releasing acids and proteases like cathepsin K to digest type I collagen.[5] This process results in the release of collagen-derived peptides into the bloodstream. Due to the importance of finding biomarkers for osteoporosis and other bone metabolism disorders, these peptides have been extensively studied and investigated as potential biomarkers[6–11]. During the course of these investigations, the primary emphasis was put on the analysis of pyridinoline/deoxypyridinoline crosslinks, highly specific to collagen molecules, and peptides bound to these crosslinks. This particular focus was intentional, as the preference for these markers over linear peptides stemmed from the fact that pyridinoline crosslinks present in biofluids are primarily derived from bones due to bone resorption [12]. This specificity makes them indicative of bone metabolism, whereas linear peptides could originate from various tissues rich in type I collagen. Furthermore, the inclusion of deoxypyridinoline crosslinks in the study was driven by their specificity to bones and dentin.[13,14] The outcomes of these investigations led to the identification and characterization of trivalently crosslinked

C-telopeptide of the α1-chain of type I collagen (ICTP) and free pyridinoline[15–24]. These characterizations relied on preparative chromatography coupled with UV detection, MALDI TOF spectrometry, and N-sequencing analysis[25–27]. Subsequently, immunoassays were developed for the quantitative analysis of ICTP[28,29]. While the trivalently crosslinked C-Terminal telopeptide (CTX), trivalently crosslinked N-Terminal telopeptide (NTX), and amino-terminal propeptide of type I procollagen (PINP) can presently be quantified through immunoassays, their comprehensive characterization remains incomplete. Only specific portions of these molecules, such as the sequence EKAHDGGR in CTX, are recognized and employed as epitopes in immunoassays[28,30,31]. CTX and NTX are known to be made up of three peptides trivalently crosslinked together by a pyridinoline crosslink[32]. CTX and ICTP both originate from the same type I collagen region and share a common origin. However, they are released under different conditions and are cleaved by distinct enzymes. Specifically, CTX is released through the activity of cathepsin K during physiological bone resorption, while ICTP is likely cleaved by matrix metalloproteases during pathological bone resorption, as observed in bone metastasis[33]. To this day, CTX and PINP serve as the recommended markers for bone resorption and bone formation, respectively, according to the guidelines set forth by the International Osteoporosis Foundation and the International Federation of Clinical Chemistry and Laboratory Medicine for monitoring the therapeutic progress of osteoporotic patients[34,35]. Despite CTX endorsement, the absence of a reference method and variability in immunoassay results have diminished clinician confidence, leading to underutilization. The lack of complete marker characterization hampers the development of a definitive reference method, like liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), for precise quantitation. HR-MS analysis. Consequently, our research is dedicated to the comprehensive characterization of CTX found in human plasma and serum. Developing a LC-MS/MS method necessitates precise knowledge of the exact structure of the targeted molecule. This is crucial to achieve the highest level of specificity attainable by triple quadrupole mass spectrometry. In contrast to the development of immunoassays, where specific regions of a molecule can be targeted, LC-MS/MS methods require the comprehensive targeting of the entire molecule. This is because the method relies on the measurement of both the mass and charge of the molecule, demanding a comprehensive understanding of its structure. Therefore, we conducted an analysis of the various CTX species present in plasma and serum using a comprehensive workflow involving preparative chromatography, affinity chromatography, and HR-MS analysis.

Materials and methods

Proteins precipitation

One pool of plasma and one of serum were created by combining leftover plasma and serum samples from hemodialyzed patients, without considering distinctions based on gender, health, or age. Such samples were chosen to maximize peptide concentration since the peptides are cleared by the kidney. To precipitate the proteins in these pools, 250 µL of H2O and 10% ZnSO4 (w/v) per milliliter of the matrix were employed. All solutions were prepared using LC-MS grade solvents. The pools were subjected to agitation on a reciprocating shaker for 20 minutes at 10°C and subsequently centrifuged at high speed for 10 minutes. The resulting supernatant was transferred to 2mL LoBind Eppendorf tubes and then evaporated to dryness overnight under vacuum conditions at 35°C. Following evaporation, the pools were reconstituted with 1mL of a solution containing H_2O, 5% dimethyl sulfoxide (DMSO), and 0.4% formic acid (FA). The FA and DMSO were procured from Sigma-Aldrich (Saint-Louis, MO, US).

Preparative chromatography

A total of 50 µL of the concentrated pool was introduced into a Shimadzu Nexera X2 UPLC system (Shimadzu Corporation, Kyoto, Japan). Chromatographic separation was achieved using a XSelect PRM PST HSS T3 column (Waters) with a particle size of 2.5µm and dimensions of 2.1x150mm, maintained at 35°C. The mobile

phases consisted of H_2O and acetonitrile (ACN) with the addition of 5% DMSO and 0.4% FA. A gradient method was employed at a flow rate of 0.5 mL/min, as follows: initiation and maintenance at 0% of phase B (ACN, 5% DMSO, 0.4% FA) for 0.5 minute, followed by an increase to 10% of phase B over 9 minutes, then a 1-minute period at 100% of phase B, and finally a 5-minute step at 0% of phase B. Post-column flow splitting was conducted every 15 seconds. Fractions obtained were subsequently evaporated and reconstituted with 30 µL of H_2O containing 5% DMSO and 0.4% FA. The retention times of CTX were determined by quantifying CTX in fractions collected through post-column flow splitting during the entire run, utilizing the IDS-iSYS CTX-I (CrossLaps®) and CrossLaps® ELISA kits from Immunodiagnostic System (IDS), as well as the B-Crosslaps ECLIA kit by Cobas (Roche).

Off-line 2D LC/LC

We employed an off-line 2D LC/LC technique to gain deeper insights into the nature of the peaks observed in the elution profile obtained through preparative liquid chromatography. Given the presence of multiple peaks, it was imperative to determine whether these peaks resulted from suboptimal LC parameter optimization or if they represented distinct species. Due to its elevated CTX concentration, urine was selected as the primary human matrix for 2D LC/LC analysis. A total of 50 µL of urine concentrated through evaporation was subjected to separation utilizing the previously described method. Fractions obtained were assessed for their CTX content using the IDS-iSYS CTX-I (CrossLaps®) kits by IDS. Fractions exhibiting a high CTX concentration were subsequently evaporated to dryness overnight under vacuum conditions at 35°C and reconstituted with 100 µL of the injection solvent. Each reconstituted fraction was then subjected to separation once more using the same preparative liquid chromatography method as previously explained. The CTX content of each resulting fraction was assessed using the IDS-iSYS CTX-I (CrossLaps®) kit. Off-line 2D preparative LC/LC separation workflow is represented in figure 1.

Affinity chromatography

Antibodies specific to β-CTX were generously provided by IDS (Boldon, UK). These antibodies, named 1M0161 and 1M0122, target a well-established β-CTX sequence: EKAHDGGR. Both 1M0161 and 1M0122 antibodies are integral components of the commercially available kits for B-CTX quantitation. Regarding the columns used for affinity chromatography, the packing process was conducted in-house. For the 1M0122 column, a rProtein A Sepharose Fast Flow column (Merck) was initially equilibrated with PBS. Subsequently, 12 mg of 1M0122 antibodies, pre-diluted in PBS, were introduced into the column. Any excess antibodies were removed by washing with PBS before injecting 35 mL of pooled plasma/serum. The flow-through was collected, and immune complexes were subsequently eluted using a solution of H_2O and citrate at pH 3. For the 1M0161 column, a pre-activated NHS column was packed and stored in 7 mL of isopropanol. After equilibrating the column with H_2O containing 1 mM HCl, 11.3 mg of 1M0161 antibodies, diluted in a solution of H_2O and $NaHCO_3$at 100 mM, were injected twice. Unbound antibodies were washed with a carbonate buffer. To saturate any remaining free NHS groups, a solution of H_2O, Ethanolamine at 0.5M, NaCl at 0.5M, and pH 8.30 was injected into the column (70 mL). The column was subsequently washed and conditioned twice using the following solutions in the specified order: H_2O, citrate at 0.1M, NaCl at 0.5M, and pH 4; H_2O, NaCl at 1M, glycerol at 5%, KI at 0.1 mM, Triton X 100 at 0.1%, and NH_4OH at 0.05%; H_2O, ethanolamine at 0.5M, NaCl at 0.5M, and pH 8.30. Finally, the column was equilibrated in PBS before injecting 35 mL of pooled plasma/serum. The flow-through was collected, and the antigens were eluted using a solution of H_2O and glycine at 0.1M and pH 3. Subsequently, the samples were evaporated and reconstituted in H_2O containing 0.4% FA before injection.

Nano-LC-DIA analysis

Samples obtained following preparative chromatography and affinity chromatography were introduced into the nanoAQUITY UPLC-system Nano-LC (Waters), which was coupled to the SYNAPT XS mass spec-

trometer (Waters). The column employed for chromatographic separation was the nanoEaseTM M/Z HSS T3 column with a pore size of 100Å, particle size of 1.8µm, and dimensions of 300µm x 150mm (Waters). The mobile phases consisted of a mixture of H_2O and ACN, supplemented with 0.1% formic acid (FA). The gradient began by holding at 0% of ACN for 2 minutes and subsequently increased to 90% over a span of 43 minutes, with a total flow rate of 5 µL/min. Data acquisition was conducted according the parameters described previously in our previous work[36]. Data-independent analysis (DIA) was chosen as acquisition mode in order to maximize peptide identification.

Data processing

Type I collagen-derived linear peptides and their post-translational modifications (PTMs) were identified using PEAKS X software (Bioinformatics Solutions Inc., Waterloo, CA). Sequences of type I collagen α1 and type I collagen α2 were obtained from the UniProt database (https://www.uniprot.org/) and utilized for data-based research. Variable PTMs, including hydroxylation of lysine and proline, oxidation of methionine, glycation, and glycosylation, were considered. Hydroxylation is a common occurrence in type I collagen, and oxidation can potentially occur during sample preparation. Glycation and glycosylation are also known to be present in type I collagen. For in-silico digestion performed by PEAKS X Software, the settings were configured as "unspecific" since various enzymes, such as matrix metalloproteinases (MMPs), may cleave CTX, especially in the bloodstream, subsequent to its initial cleavage by cathepsin K. The identification of divalently crosslinked peptides was carried out using Stavrox/Merox software, as described by Götze *et al* . in 2012[37], and accessible at http://stavrox.com/Download.htm . To identify trivalently crosslinked peptides, their chemical formulas were determined and visualization of chromatogram was done using Skyline software, available at https://skyline.ms/project/home/begin.view.

Confirmation of the presence of model molecules in high resolution-MS spectra

The MS1 data obtained during $UDMS^E$ analysis were extracted and subjected to analysis using Skyline to detect the presence of model molecules. These model molecules were designed by appropriately combining the identified peptides that contained lysines and hydroxylysines involved in the C-terminal pyridinoline crosslink. The entire workflow is depicted in Figure 2.

Results

Separation of CTX species by preparative chromatography and off-line 2D LC/LC

Concentrated pools of serum and plasma, separated by preparative chromatography, exhibited similar elution profiles regardless of the kits used for quantitating the fractions, as illustrated in Figure 3. However, there were significant variations in the concentrations calculated by the different kits for highly concentrated fractions. This discrepancy can potentially be attributed to the matrix effect, which often has a substantial impact on immunoassays. Since our fractions differ significantly from a biological matrix, it's likely that the various immunoassays are affected differently. In light of these findings, it was determined that reliance on the IDS-iSYS CTX-I (CrossLaps®) kit for further antibody-based quantitation would be more appropriate. Regarding retention time, CTX molecules appear to be highly hydrophilic, given their elution at a very low percentage of ACN. The differences observed in the elution profiles between urine and plasma/serum could be attributed to modifications that CTX molecules undergo, such as conjugation, in order to be excreted in urine. Consequently, different CTX metabolites may be present in higher concentrations in urine. Concerning the off-line 2D LC/LC separation, out of all the fractions obtained during the initial step of preparative chromatography (figure 4A), nine (fractions 3, 5, and 17-23) displayed concentrations above 1 ng/mL, the arbitrarily chosen threshold, and were retained for the second round of preparative chromatography (figure

4B). Two types of elution profiles were observed for fractions 3, 5, 17-23. Fractions 5 and 17 displayed very similar profiles, both with two peaks at 1 minute and 3.75 minutes, while the remaining fractions yielded elution profiles with only one peak at different retention time. Given the similarity in the elution profiles of fractions 5 and 17, it's conceivable that the species present in these fractions may be isomers. In the initial chromatography, both isomers are separated into distinct fractions. It is plausible that following post-column flow splitting, the isolated isomer undergoes isomerization to transform into the other isomer, reaching equilibrium in solution. The fact that fractions 19 to 23 exhibited concentrations exceeding 1 ng/mL could be attributed to either significant tailing of the peak at 3.75 minutes or the presence of different molecules than those responsible for the peak at 3.75 minutes. In light of these results, the hypothesis that different CTX molecules coexist and are recognized by the immunoassays gains credence, as the elution profiles obtained from fractions 19 to 23 do not exhibit a peak at 3.75 minutes but rather at 4, 4.25, 4.5, 4.75, and 5 minutes.

Peptide identification

From the samples obtained after protein precipitation coupled with preparative chromatography, a total of 502 linear peptides derived from type I collagen were successfully identified. Among these, 22 peptides containing the C-terminal pyridinoline crosslink site were identified. Pyridinoline crosslinks involve the fusion of two telopeptide hydroxyallysine and one helix lysine. While only hydroxylysine residues, and by extension, hydroxyallysine, are directly implicated in pyridinoline crosslinks at the telopeptide level, our focus also encompasses peptides containing a lysine residue at the crosslink site of the telopeptide. This broader consideration aims to gather information about the cleavage sites around the crosslink site, rather than identifying precursors of the crosslink. Peptides featuring hydroxylation of proline residues (n=7) in the telopeptidic regions were excluded, as this PTM is reported to be absent in this region of collagen molecules [24,38–40]. Additional investigations are required to validate the presence of hydroxyproline in the telopeptidic peptides. For the samples purified by affinity chromatography, 23 peptides containing the epitope EKAHDGGR or EhylHDGGR were identified. These peptides varied in length, ranging from 7 to 60 residues. Hydroxyprolines were identified at various sites in 9 of these peptides. However, no sugar-mediated PTMs were detected. It is worth noting that the absence of glycosylation detection is not unexpected, as glycosylation can be challenging to analyze using electrospray ionization, the method employed in this study. Additionally, it is crucial to acknowledge that certain PTMs, such as the oxidation of hydroxylysine residues by lysyl oxidase—an essential step preceding crosslinking—were not considered in this analysis. Consequently, more linear peptides may have been present but not identified. Significantly, no divalently crosslinked peptides were found in the analysis. Divalent crosslinks are typically associated with newly-synthetized bone tissue, serving as precursors to trivalent crosslinks found in older bone tissue. The newly-synthetized bone tissue rarely undergoes bone resorption compared to older bone tissue except in some metabolic bone disorders, such as Paget's disease. Therefore, founding no divalently crosslinked peptides in the plasma and serum is in line with the expectations. All peptides containing a lysine or hydroxylysine residue involved in pyridinoline crosslinks are detailed in Table 1.

Analysis of theorical trivalently crosslinked models

Given the multitude of peaks observed in the chromatogram resulting from affinity chromatography coupled to nano-LC-HR-MS, we postulated that the number of crosslinked species was substantial. To achieve a comprehensive identification of these species, particularly acknowledging that trivalently crosslinked peptides cannot be directly identified using standard peptide/protein identification software, we developed model molecules. These model molecules consisted of three peptides selected from the previously identified list of linear peptides, crosslinked together by a pyridinoline or deoxypyridinoline. Subsequently, these model molecules were employed in the chromatogram search using specialized software. A total of 3,230 model molecules, outlined in Supplemental Info 1, were generated by combining the various peptides. The chemical formula for each model molecule was calculated as part of this process. Skyline was employed to identify traces corresponding to our model molecules based on their chemical formulas. All model molecules were

successfully identified in samples obtained through preparative chromatography (fractions 3, 5, 17-23) and samples obtained via affinity chromatography in both plasma and serum. For samples purified by preparative chromatography, the majority of the molecules coeluted within the first 3 minutes of liquid chromatography. Unfortunately, the peak shapes were poorly defined, characterized by low intensity. This phenomenon could be attributed to the inherent complexity of the sample, even after separation via preparative chromatography. The important competition for ionization in the ionization source becomes apparent when numerous molecules coelute. Additionally, molecules like phospholipids and albumin, which significantly compete for the signal, were not eliminated, further contributing to this challenge. In contrast, samples purified by affinity chromatography exhibited chromatograms (figure 5) with fewer interferences and better peak shapes, which can be attributed to the higher level of sample purity. The peak intensities in these chromatograms were more than 50 times higher. Following Skyline analysis, similar chromatograms were obtained for both plasma and serum using the same antibodies. However, commonalities were identified among the four chromatograms, including the coelution of numerous CTX species at 1.5 minutes and a prominent peak corresponding to the "3+3+4" species. While C-terminal pyridinoline and deoxypyridinoline predominantly involve the α1 chain helix of type I collagen and less the α2 chain helix, the "3+3+4" species, composed of two peptides, PQEKAHDGGR (α1 chain of type I collagen) crosslinked to the peptide FKGIRGHNG (α2 chain of type I collagen) by a deoxypyridinoline, was designated as the primary CTX species due to its consistent presence in all chromatograms. It is important to note, however, that this designation does not necessarily imply it is the most concentrated species in the blood. The prominence of the "3+3+4" species may stem from its superior ionization, possibly due to better isolation from other molecules or its specific structure and size[41,42]. Nonetheless, it is evident that different species were present in plasma and serum, and distinct species were also captured by the antibodies. In terms of differences between plasma and serum, it is apparent that plasma contains larger species than serum. This distinction can be explained by the fact that proteolysis occurs to a lesser extent in plasma after collection, as plasma proteases are inhibited by the chelation of divalent ions by EDTA. Moreover, it is evident that degradation products were simultaneously captured with the initial species they originated from, as very similar species were identified within the same sample. Regarding the differences between the species captured by the antibodies, the most prevalent species differed for both antibodies. There were no significant differences in terms of properties or structures between the group of species recognized by antibody 1M0161 and the group of species recognized by antibody 1M0122, except that the species captured by 1M0161 tended to have a lower retention time and were thus more polar than the species captured by 1M0122. However, a big number of the identified species were captured by the same antibody in both matrices. Therefore, it can be hypothesized that the capture of different species is reproducible.

Discussion

Osteoporosis, characterized by excessive bone resorption over bone formation, diminishes bone mass, heightening fracture vulnerability. Currently endorsed by the International Osteoporosis Foundation and the International Federation of Clinical Chemistry and Laboratory Medicine for the assessment of bone resorption in osteoporotic patients, CTX faces challenges due to variable immunoassay outcomes, eroding clinician confidence and limiting clinical use. Addressing this, the development of a reliable reference method is imperative, but the incomplete understanding of CTX structure hinders progress. In this work, we employed a multi-step approach in order to fully characterize CTX. We extracted CTX directly from human biological matrices using preparative liquid chromatography (LC) and affinity chromatography techniques. Preparative LC served as a straightforward preparation step for the subsequent identification of uncrosslinked linear peptides derived from type I collagen via high-resolution mass spectrometry (HR-MS). The objective of the preparative chromatography-based separation was to identify a comprehensive set of peptides originating from type I collagen present in the blood, independent of antibodies, in order to use them to build CTX models. Affinity chromatography was then used for the analysis of CTX species, as it provided a significantly more concentrated and purified sample thanks to its high selectivity for CTX species. We then applied our previously described proteomics workflow[36] to characterize all the distinct CTX species present in both

plasma and serum samples. This marks a significant step towards enhancing our understanding of these critical markers in the context of osteoporosis management. The substantial number of CTX species identified in our study can be attributed to the complex nature of CTX proteolysis, occurring at multiple stages. Firstly, our prior research on cathepsin K cleavage sites has demonstrated that the digestion of type I collagen by cathepsin K remains non-reproducible[36]. Consequently, during bone resorption, multiple CTX species are produced. Moreover, once released into the bloodstream, these species are susceptible to cleavage by circulating metalloproteases and undergo further proteolysis in the liver by Kupffer cells, leading to an increased number of coexisting species in the bloodstream. Depending on the type of blood collection tube used, proteolysis by circulating proteases may still occur after blood collection. In addition to cleavages, the presence of PTMs that are not uniform further increases the variety of species observed in the chromatograms. These PTMs primarily involve the hydroxylation of lysine and proline residues, which are highly prevalent in type I collagen. Given that an aspartic acid residue is located near the crosslink site, many of the species may undergo isomerization resulting in the manifestation of multiple species. However, β-isomerization of type I collagen is associated with older bone tissue, suggesting that the majority of resorbed bone tissue should exhibit β-isomerization. Consequently, CTX should also predominantly exist in the β-isomeric form, as it is released during bone resorption. Nevertheless, in specific bone diseases such as Paget's disease, α-CTX may be released during bone resorption. It is worth noting that smaller species were more prominent in the chromatograms, possibly due to their higher abundance in the blood as a result of in situ proteolysis. Additionally, larger molecules may ionize less efficiently in the mass spectrometer source, and some of them may have partially precipitated during the sample preparation process. The observation that more large species were found in plasma aligns with this theory. A significant limitation in our study is the inability to doubly confirm the presence of our model molecules through MS/MS spectra, given the unknown and thus, unpredictable fragmentation pattern of such molecules. Nevertheless, the superposition of traces from multiple states of charge at the same retention time serves as an intriguing indicator of our target, making it less likely to be an interference. The diversity of CTX species identified and the differences in the species recognized by the antibodies in the three commercially available kits may contribute to the variations in results obtained by these assays[43–45]. Each kit may use a different combination of antibodies, leading to differential recognition of CTX species. However, it's worth noting that despite these differences, the overall results from the kits do not show significant discrepancies. This suggests that a significant portion of CTX species is still effectively captured by the antibodies used in these assays, leading to relatively consistent measurements across the kits. In conclusion, this study successfully identified a substantial number of CTX species extracted from human plasma and serum. Our findings highlight the complexity of CTX proteolysis, and the diverse array of species present in these biological matrices. In light of our findings, it becomes evident that, prior to the development of a LC-MS/MS method for the quantitation of all CTX species, the development of a digestion step aimed at yielding a single, standardized CTX species is crucial. Once this digestion step is optimized within the biological matrix, we will be well-positioned to progress towards the development and subsequent validation of an LC-MS/MS method for the quantitation of the total CTX. This method is anticipated to play a crucial role in advancing the precision and standardization of CTX immunoassays, thereby improving osteoporosis monitoring. Following standardization, these immunoassays are expected to exhibit reduced variability, becoming fully interchangeable. This, in turn, is projected to instill greater confidence among clinicians, fostering enhanced utilization of the marker in patient follow-up. With improved assessment of medication compliance, the risk of fractures is anticipated to decrease in osteoporotic patients.

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their financial support. [1] Szulc, P., Bone turnover: Biology and assessment tools.*Best Pract. Res. Clin. Endocrinol. Metab.* 2018, 32, 725–738.[2] Lane, N.E., Epidemiology, etiology, and diagnosis of osteoporosis. *Am. J. Obstet. Gynecol.* 2006, 194.[3] Burge, R., Dawson-Hughes, B., Solomon, D.H., Wong, J.B., et al., Incidence and economic burden of osteoporosis-related fractures in the United States, 2005-2025. *J. Bone Miner. Res.* 2007, 22, 465–475.[4] Hernlund, E., Svedbom, A., Ivergård, M., Compston, J., et al., Osteoporosis in the European Union: Medical management, epidemiology and economic burden: A report prepared in collaboration with the International Osteoporosis Foundation (IOF) and the European Federation of Pharmaceutical Industry Associations (EFPIA). *Arch. Osteoporos.* 2013, 8.[5] Bossard, M.J., Tomaszek, T.A., Thompson, S.K., Amegadzie, B.Y., et al., Proteolytic activity of human osteoclast cathepsin K: Expression, purification, activation, and substrate identification. *J. Biol. Chem.* 1996, 271, 12517–12524.[6] Chapurlat, R.D., Garnero, P., Brárt, G., Meunier, P.J., Delmas, P.D., Serum type I collagen breakdown product (serum CTX) predicts hip fracture risk in elderly women: The EPIDOS study. *Bone* 2000, 27, 283–286.[7] Garnero, P., Hausherr, E., Chapuy, M.-C., Marcelli, C., et al., Markers of Bone Resorption Predict Hip Fracture in Elderly Women: The EPIDOS Prospective Study 1994, 620, 2104–2107.[8] Garnero, P., Sornay-Rendu, E., Claustrat, B., Delmas, P.D., Biochemical Markers of Bone Turnover, Endogenous Hormones and the Risk of Fractures in Postmenopausal Women: The OFELY Study. *J. Bone Miner. Res.* 2000, 15, 1526–1536.[9] Vasikaran, S., Eastell, R., Bruyère, O., Foldes, A.J., et al., Markers of bone turnover for the prediction of fracture risk and monitoring of osteoporosis treatment: A need for international reference standards. *Osteoporos. Int.* 2011, 22, 391–420.[10] Bruyere, O., Collette, J., Delmas, P., Rouillon, A., et al., Interest of biochemical markers of bone turnover for long-term prediction of new vertebral fracture in postmenopausal osteoporotic women. *Maturitas* 2003, 44, 259–265.[11] Li, M., Li, Y., Deng, W., Zhang, Z., et al., Chinese Bone Turnover Marker Study: Reference ranges for C-terminal telopeptide of type i collagen and procollagen I N-terminal peptide by age and gender. *PLoS One* 2014, 9, 5–11.[12] Chapurlat, R.D., Genant, H.K., in:,*Endocrinol. Adult Pediatr.* , vol. 1–2, Elsevier, 2015, pp. 1184–1213.[13] Kline, G., Orton, D., Sadrzadeh, H., *Bone metabolism* , Elsevier Inc., 2017.[14] Medio, L. Di, in:, *Adv. Clin. Chem.* , 2021, pp. 101–140.[15] Knott, L., Bailey, A.J., Collagen cross-links in mineralizing tissues: A review of their chemistry, function, and clinical relevance. *Bone* 1998, 22, 181–187.[16] Yamauchi, M., Taga, Y., Hattori, S., Shiiba, M., Terajima, M., *Analysis of collagen and elastin cross-links* , vol. 143, Elsevier Inc., 2018.[17] Eyre, D., Collagen Cross-Linking Amino Acids 1987, 144.[18] Sorushanova, A., Delgado, L.M., Wu, Z., Shologu, N., et al., The Collagen Suprafamily: From Biosynthesis to Advanced Biomaterial Development. *Adv. Mater.* 2019, 31, 1–39.[19] Gamsjaeger, S., Robins, S.P., Tatakis, D.N., Klaushofer, K., Paschalis, E.P., Identification of Pyridinoline Trivalent Collagen Cross-Links by Raman Microspectroscopy. *Calcif. Tissue Int.* 2017, 100, 565–574.[20] Eyre, D.R., Paz, M.A., Gallop, P.M., Cross-Linking in Collagen and Elastin. *Annu. Rev. Biochem.* 1984, 53, 717–748.[21] Smith-Mungo, L.I., Kagan, H.M., Lysyl oxidase: Properties, regulation and multiple functions in biology. *Matrix Biol.* 1998, 16, 387–398.[22] McCudden, C.R., Kraus, V.B., Biochemistry of amino acid racemization and clinical application to musculoskeletal disease. *Clin. Biochem.* 2006, 39, 1112–1130.[23] Robins, S.P., Bailey, A.J., The chemistry of the collagen cross links. The mechanism of stabilization of the reducible intermediate cross links. *Biochem. J.* 1975, 149, 381–385.[24] Hanson, D.A., Eyre, D.R., Molecular site specificity of pyridinoline and pyrrole cross-links in type I collagen of human bone. *J. Biol. Chem.* 1996, 271, 26508–26516.[25] Tanzer, M., Cross-Linking of Collagen. *Science (80-.).* 1973, 180, 561–566.[26] Fujimoto, D., Moriguchi, T., Pyridinoline, a Non-Reducible Crosslink of Collagen 1978, 83, 863–867.[27] Eriksen, H.A., Sharp, C.A., Robins, S.P., Sassi, M.L., et al., Differently cross-linked and uncross-linked carboxy-terminal telopeptides of type I collagen in human mineralised bone. *Bone* 2004, 34, 720–727.[28] Sassi, M.L., Eriksen, H., Risteli, L., Niemi, S., et al., Immunochemical characterization of assay for carboxyterminal telopeptide of human type I collagen: loss of antigenicity by treatment with cathepsin K. *Bone* 2000, 26, 367–373.[29] Risteli, J., Elomaa, I., Niemi, S., Novamo, A., Risteli, L., Radioimmunoassay for the pyridinoline cross-linked carboxy-terminal telopeptide of type I collagen: A new serum marker of bone collagen degradation. *Clin. Chem.* 1993, 39, 635–640.[30] Chubb, S.A.P., Measurement of C-terminal telopeptide of type I collagen (CTX) in serum. *Clin. Biochem.* 2012, 45, 928–935.[31] Koivula, M.K., Risteli, L., Risteli, J., Measurement of aminoterminal propeptide of type I procollagen (PINP) in serum. *Clin. Biochem.* 2012, 45, 920–927.[32] Fledelius, C., Johnsen, A.H., Cloos, P.A.C., Bonde, M., Qvist, P., Characterization of Urinary Degradation Products Derived from Type I

Collagen. *J. Biol. Chem.* 1997, 272, 9755–9763.[33] Garnero, P., Ferreras, M., Karsdal, M.A., Nicamhlaoibh, R., et al., The Type I Collagen Fragments ICTP and CTX Reveal Distinct Enzymatic Pathways of Bone Collagen Degradation.*J. Bone Miner. Res.* 2003, 18, 859–867.[34] Szulc, P., Naylor, K., Hoyle, N.R., Eastell, R., Leary, E.T., Use of CTX-I and PINP as bone turnover markers: National Bone Health Alliance recommendations to standardize sample handling and patient preparation to reduce pre-analytical variability. *Osteoporos. Int.* 2017, 28, 2541–2556.[35] Vasikaran, S., Cooper, C., Eastell, R., Griesmacher, A., et al., International Osteoporosis Foundation and International Federation of Clinical Chemistry and Laboratory Medicine Position on bone marker standards in osteoporosis. *Clin. Chem. Lab. Med.* 2011, 49, 1271–1274.[36] Demeuse, J., Massonnet, P., Schoumacher, M., Grifnée, E., et al., Innovative workflow for the identification of cathepsin K cleavage sites in type I collagen. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* 2023, 1228.[37] Götze, M., Pettelkau, J., Schaks, S., Bosse, K., et al., StavroX-A software for analyzing crosslinked products in protein interaction studies. *J. Am. Soc. Mass Spectrom.* 2012, 23, 76–87.[38] Gjaltema, R.A.F., Bank, R.A., Molecular insights into prolyl and lysyl hydroxylation of fibrillar collagens in health and disease. *Crit. Rev. Biochem. Mol. Biol.* 2017, 52, 74–95.[39] Rappu, P., Salo, A.M., Myllyharju, J., Heino, J., Role of prolyl hydroxylation in the molecular interactions of collagens. *Essays Biochem.* 2019, 63, 325–335.[40] Gistelinck, C., Weis, M.A., Rai, J., Schwarze, U., et al., Abnormal Bone Collagen Cross-Linking in Osteogenesis Imperfecta/Bruck Syndrome Caused by Compound Heterozygous PLOD2 Mutations. *JBMR Plus* 2021, 5, 1–15.[41] Pace, C.N., Grimsley, G.R., Scholtz, J.M., Protein ionizable groups: pK values and their contribution to protein stability and solubility. *J. Biol. Chem.* 2009, 284, 13285–13289.[42] Liigand, P., Kaupmees, K., Kruve, A., Influence of the amino acid composition on the ionization efficiencies of small peptides. *J. Mass Spectrom.* 2019, 54, 481–487.[43] Bhattoa, H.P., Cavalier, E., Eastell, R., Heijboer, A.C., et al., Analytical considerations and plans to standardize or harmonize assays for the reference bone turnover markers PINP and β-CTX in blood. *Clin. Chim. Acta* 2021, 515, 16–20.[44] Cavalier, E., Eastell, R., Jørgensen, N.R., Makris, K., et al., A Multicenter Study to Evaluate Harmonization of Assays for C-Terminal Telopeptides of Type I Collagen (ß-CTX): A Report from the IFCC-IOF Committee for Bone Metabolism (C-BM). *Calcif. Tissue Int.* 2021, 108, 785–797.[45] Vasikaran, S.D., Miura, M., Pikner, R., Bhattoa, H.P., Cavalier, E., Practical Considerations for the Clinical Application of Bone Turnover Markers in Osteoporosis. *Calcif. Tissue Int.* 2023, 112, 148–157. Table 1. List of the identified peptides containing a lysine known to be involved in the pyridinoline crosslinks.

Amino acid sequence	Origin
EKAHDGGRYY	Type I collagen
EKAHDGGRYYR	Type I collagen
FLPQPPQEKAHDGGRYYRA	Type I collagen
FSFLPQPPQEKAHDGG	Type I collagen
KAHDGGRYYRAD	Type I collagen
PPQEKAHDGGRYYRAD	Type I collagen
PQEKAHDGGRYYR	Type I collagen
PQEKAHDGGRYYRADDAN	Type I collagen
QPPQEKAHDGGRY	Type I collagen
QPPQEKAHDGGRYYRADDA	Type I collagen
QPPQEKAHDGGRYYRADDAN	Type I collagen
QPPQEKAHDGGRYYRADDANVVRDRDLEVDTTLHyLSLSQQIENIRSHyPEGSRHyLNPARTCRDL	Type I collagen
SFLPQPPQEHyLAHDGGRY	Type I collagen
AGFDFSFLPQPPQEKA	Type I collagen
AGLPGMKGHRGFSGLDG	Type I collagen
DFSFLPQPPQEKAHDG	Type I collagen
FDFSFLPQPPQEKAHD	Type I collagen
FKGIRGHNG	Type I collagen
FKGIRGHNGLDGLKGQ	Type I collagen
FLPQPPQEKAH	Type I collagen

Amino acid sequence	Origin
FSFLPQPPQEKAH	Type I collagen
GPQGARGLPGTAGLPGMKGHRGFSGLDGAK	Type I collagen
MKGHRGFSG	Type I collagen
PGERGPPGPQGARGLPGTAGLPGMKGHRGFSGLDGA	Type I collagen
PGFKGIRGHNG	Type I collagen
PGMKGHRG	Type I collagen
PPGPPSAGFDFSFLQPPQEKAH	Type I collagen
PQEKAHDGGR	Type I collagen
PQGARGLPGTAGLPGMKGHRGFSGLD	Type I collagen
SAGFDFSFLPQPPQEKAHD	Type I collagen
SFLHyPQPPQEKAHDG	Type I collagen
PPQEKAHDGGRYYR	Type I collagen
SFLPQPPQEKAHDG	Type I collagen

Figure 1. Developed workflow. Plasma and serum samples, prepared through protein precipitation, underwent separation via LC preparative. The fractions obtained were then subjected to CTX concentration analysis. Fractions containing CTX concentrations exceeding 1 ng/mL were subsequently analyzed using HR-MS. The acquired spectra were processed using PEAKS software for the identification of linear peptides derived from type I collagen. Peptides that contained lysine residues involved in pyridinoline crosslinks were selected. These selected peptides were then employed in the construction of trivalently crosslinked model molecules. The primary purpose of these models was to comprehensively represent all potential CTX species. In parallel, plasma and serum samples were purified through affinity chromatography and likewise analyzed using HR-MS. The spectra obtained were also processed using PEAKS to identify linear peptides, which were used to construct additional trivalently crosslinked model molecules. Once all the model molecules were designed, Skyline software was employed to identify trivalently crosslinked CTX species.

Figure 2. Off-line 2D preparative LC/LC separation protocol. A concentrated urine sample was prepared and concentrated using protein precipitation. This prepared sample was then subjected to separation through preparative LC. The fractions obtained from this separation were subsequently assessed for their CTX concentration, and an elution profile was established.

Fractions with CTX concentrations exceeding 1 ng/mL were singled out for a second round of preparative LC. The fractions from each of these selected fractions, which exceeded 1 ng/mL, were subjected to a second run of preparative LC. The results obtained from this process were compiled into a comprehensive elution profile.

Figure 3. Elution profile obtained after preparative chromatography separation of human plasma and serum. Fractions levels of CTX were assessed using the iSYS CTX-I (CrossLaps®) and CrossLaps® ELISA kits from IDS Diagnostics, as well as the B-Crosslaps ECLIA kit by Cobas (Roche).

Figure 4. Elution profile obtained by off-line 2D preparative LC/LC separation of concentrated urine samples. A) First run of preparative LC B) second run of preparative LC.

Figure 5. Chromatograms obtained from a) Plasma sample captured by antibody 1M0161, b) Plasma sample captured by antibody 1M0122, c) Serum sample captured by antibody 1M0161 and d) Serum samples captured by antibody 1M0122

Supplemental info 1. Schematic representation of all model molecule.

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`FIGURE 1 PROTEOMICS.pptx` available at https://authorea.com/users/833898/articles/1226677-characterization-of-trivalently-crosslinked-c-terminal-telopeptide-of-type-i-collagen-

ctx-species-in-human-plasma-and-serum-using-high-resolution-mass-spectrometry

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