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RESEARCH ARTICLE

Characterization of Trivalently Crosslinked C-Terminal Telopeptide of Type I Collagen (CTX) Species in Human Plasma and Serum Using High-Resolution Mass Spectrometry

Justine Demeuse[1] | William Determe[1] | Elodie Grifnée[2] | Philippe Massonnet[2] | Matthieu Schoumacher[1] | Loreen Huyghebeart[2] | Thomas Dubrowski[2] | Stéphanie Peeters[2] | Caroline Le Goff[1,2] | Etienne Cavalier[1,2]

[1]Clinical Chemistry Laboratory, CIRM, University of Liège, Liege, Belgium | [2]Clinical Chemistry Department, University Hospital of Liège, Liege, Belgium

Correspondence: Justine Demeuse (Justine.demeuse@uliege.be)

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ABSTRACT

With an aging population, the increased interest in the monitoring of skeletal diseases such as osteoporosis led to significant progress in the discovery and measurement of bone turnover biomarkers since the 2000s. 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, which is rich in collagen, does not represent the true structure of the peptides in the biofluids as it was cleaved. 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 LC, affinity chromatography, and HR-MS. Subsequently, we successfully identified numerous CTX species, providing valuable insights into the characterization of these crucial biomarkers.

1 | Introduction

Osteoporosis is a skeletal disease characterized by low bone mass and loss of bone microarchitecture, which represents a major global health issue [1–4]. The disease is caused by an increased rate of bone resorption, which involves osteoclasts acidifying the medium and releasing 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

Abbreviations: ACN, acetonitrile; CID, collision-induced fragmentation; CTX, C-terminal telopeptide of type I collagen; DIA, data-independent acquisition; DMSO, dimethyl sulfoxide; FA, formic acid; FDR, false discovery rate; HR-MS, high-resolution mass spectrometry; IDS, Immunodiagnostic Systems; ICTP, trivalently crosslinked C-telopeptide of the α1-chain of type I collagen; LC, liquid chromatography; LC-MS, liquid chromatography coupled to mass spectrometry; LC-MS/MS, liquid chromatography coupled to tandem mass spectrometry; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; NHS, N-hydroxysuccinimide; NTX, N-terminal telopeptide of type I collagen; PBS, phosphate buffer saline; PINP, N-terminal propeptide of type I procollagen; PTM, posttranslational modifications; Rt, retention time; UPLC, ultra-performance liquid chromatography.

Summary

- In this study, we devised a proteomics workflow for the identification and characterization of trivalently crosslinked peptides/proteins directly extracted from plasma and serum.
- Presently, proteomics software, such as Merox, is limited to the identification of divalently crosslinked molecules, and the identification and characterization of trivalently crosslinked proteins remain challenging.
- Our method involves preparative liquid chromatography (LC), affinity chromatography for protein/peptide extraction from biofluids, and high-resolution mass spectrometry for analysis, with data processed using standard tools such as Skyline and Peaks.
- Our primary contribution lies in extending the applicability of this workflow beyond CTX (trivalently crosslinked type I collagen fragment) to diverse proteins, facilitating the characterization of trivalently crosslinked peptides/proteins.
- Secondly, this approach significantly advanced medical reasearch by characterizing over 40 CTX species, a key bone resorption marker in osteoporosis follow-up.
- Considering the variability in current CTX immunoassays, our work paved the way for a reference LC-MS/MS method development, crucial for standardizing these assays.
- Standardization will enhance assay consistency, making them interchangeable and streamlining patient follow-up.

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]. This investigation led to the identification of the trivalently crosslinked C-terminal telopeptide (CTX).

While CTX can presently be quantified through immunoassays, its comprehensive characterization remains incomplete. Only specific portions of the molecule are recognized and employed as epitopes in immunoassays [15–17]. CTX is known to be made up of three peptides trivalently crosslinked together by a pyridinoline crosslink [1]. CTX originates from the same type I collagen region and is released through the activity of cathepsin K during physiological bone resorption [18].

To this day, CTX serves as the recommended marker for bone resorption, 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 [19, 20]. 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 LC coupled to tandem mass spectrometry (LC-MS/MS), for accurate quantitation. 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 mass spectrometry-based methods rely on the measurement of both the mass and charge of the molecule, demanding a comprehensive understanding of its primary structure. Therefore, we conducted an analysis of the various CTX species present in human plasma and serum using a workflow involving preparative chromatography, affinity chromatography, and high-resolution mass spectrometry (HR-MS) analysis.

2 | Materials and Methods

2.1 | Proteins Precipitation of Plasma and Serum Pools

One pool of plasma and one serum were created by combining leftover plasma and serum samples from dialyzed patients, without considering distinctions based on gender, health, or age. No ethical committee was necessary for this condition according to the Belgian Law of December 19, 2008, on remnant human corporal samples. Dialyzed patient samples were chosen to maximize peptide concentration. Individuals with severe chronic kidney diseases, such as those undergoing dialysis, display decreased renal clearance of protein and peptides leading to increased blood levels of these molecules.

To precipitate the proteins in these pools, 250 µL of H_2O and 10% $ZnSO_4$ (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 min at 10°C and subsequently centrifuged at high speed for 10 min. The resulting supernatant was transferred to 2 mL LoBind Eppendorf tubes and then evaporated to dryness overnight under vacuum conditions at 35°C. Following evaporation, the pools were reconstituted with 1 mL 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, USA).

2.2 | Preparative Chromatography

A total of 50 µL of the concentrated pool was introduced into a Shimadzu Nexera X2 ultra-performance liquid chromatography (UPLC) system (Shimadzu Corporation, Kyoto, Japan). Chromatographic separation was achieved using an XSelect PRM PST HSS T3 column (Waters) with a particle size of 2.5 µm and dimensions of 2.1 mm × 150 mm, 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 min, followed by an increase to 10% of phase B over 9 min, then a 1-min period at 100% of phase B, and finally a 5-min step at 0% of phase B.

Postcolumn flow splitting was conducted every 15 s. Fractions obtained were subsequently evaporated and reconstituted with 30 µL of H_2O containing 5% DMSO and 0.4% FA. The retention time (Rt) of CTX was determined by quantifying CTX in fractions collected through postcolumn 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).

2.3 | Two-Step Preparative LC

We employed a two-step preparative LC technique to gain deeper insights into the nature of the peaks observed in the elution profile obtained through preparative LC. 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 high concentration of CTX, urine was chosen as the primary human matrix for two-step preparative 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 LC method as previously explained. The CTX content of each resulting fraction was assessed using the IDS-iSYS CTX-I (CrossLaps) kit. The two-step preparative LC separation workflow is represented in Figure 1.

2.4 | Affinity Chromatography

Antibodies specific to β-isomerized CTX were 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 β-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 phosphate buffer saline (PBS). Subsequently, 12 mg of 1M0122 antibodies, prediluted 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 preactivated N-hydroxysuccinimide (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 0.5 M, NaCl at 0.5 M, and pH 8.3 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 0.1 M, NaCl at 0.5 M, and pH 4; H_2O, NaCl at 1 M, glycerol at 5%, KI at 0.1 mM, Triton X-100 at 0.1%, and NH_4OH at 0.05%; H_2O, ethanolamine at 0.5 M, NaCl at 0.5 M, and pH 8.3. 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 0.1 M and pH 3.

Subsequently, the samples were evaporated and reconstituted in H_2O containing 0.4% FA before injection. The remaining of each sample was subsequently diluted five times and then quantified using the IDS-iSYS CTX-I (CrossLaps) kit to confirm the presence of CTX after purification.

2.5 | Micro-LC-Data-Independent Acquisition (DIA) Analysis

Samples obtained following preparative chromatography and affinity chromatography were introduced into the microACQUITY UPLC-system Micro-LC (Waters), which was coupled to the Synapt-XS mass spectrometer (Waters). The column employed for chromatographic separation was the microEase m/z HSS T3 column with a pore size of 100 Å, particle size of 1.8 µm, and dimensions of 300 µm × 150 mm (Waters). The mobile phases consisted of a mixture of H_2O and ACN, supplemented with 0.1% FA. The gradient began by holding at 0% of ACN for 2 min and subsequently increased to 90% over a span of 43 min, with a total flow rate of 5 µL/min.

Data acquisition was conducted according to the parameters described in our previous work [21]. DIA was chosen as the acquisition mode to maximize peptide identification. The MS data generated in this study have not been uploaded to a public platform due to the use of proprietary antibodies provided by IDS, which are subject to patent restrictions. IDS has requested that these data not be shared publicly to protect industrial confidentiality. However, researchers who are interested in accessing the MS data may contact the corresponding author.

2.6 | Data Processing

Type I collagen-derived linear peptides and their posttranslational modifications (PTMs) were identified using PEAKS X software (Bioinformatics Solutions Inc., Waterloo, CA, USA). 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. Tolerance of 0.1 and 0.2 Da was respectively allowed on the precursor and the fragment masses. A threshold of 15 for -10Log$p$ (p value = 1%) was defined as advised by the suppliers for a small dataset.

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

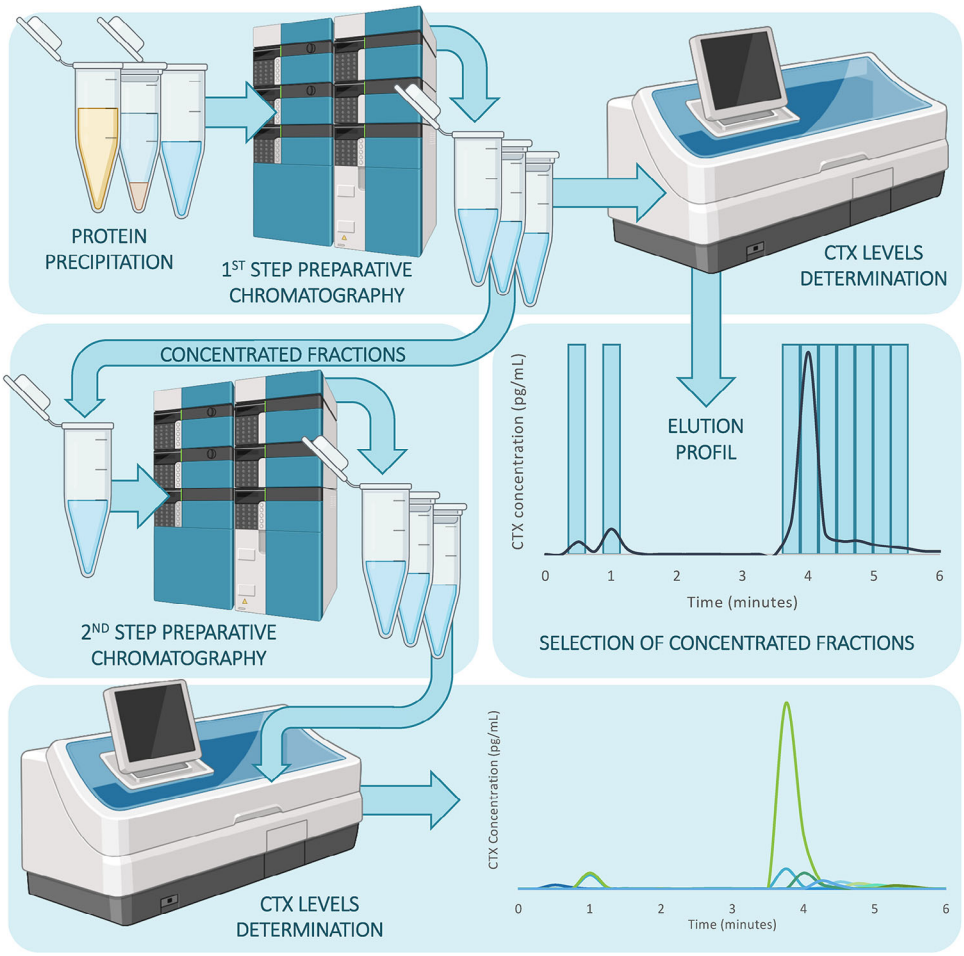

FIGURE 1 | Two-step preparative LC separation protocol. A concentrated urine sample was prepared and concentrated using protein precipitation. This prepared sample was then subjected to separation through preparative LC. The fractions obtained from this sepa separation were subsequently assessed for 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. LC, liquid chromatography.

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 software, as described by Götze et al. [22], and accessible at http://stavrox.com/Download.htm. Regarding settings, the divalent crosslinks researched were the hydroxylysino-ketonorleucine and the lysino-ketonorleucine divalent crosslinks. It was specified that the crosslinks only occur on lysine residues. A false-rate discovery (FDR) of 1% and a mass precision of MS1 and MS2 of respectively 5.0 and 10.0 ppm were tolerated.

2.7 | Analysis of Trivalently Crosslinked CTX-Like Peptides in High-Resolution-MS Spectra

2.7.1 | Design of CTX-Like Peptides

All the type I collagen-derived peptides identified by the PEAKS X software displaying a pyridinoline crosslink site were used to design trivalently crosslinked CTX-like peptides. The molecular formula and thus, theoretical precursor mass of each CTX-like peptide was then calculated and uploaded into Skyline Software (available at https://skyline.ms/project/home/begin.view) for subsequent visualization. The MS1 data obtained during the UDMS^{E} acquisition were extracted and also uploaded into Skyline software. Each theoretical mass of each CTX-like peptide was then matched in the MS1 spectra.

2.7.2 | Identification of CTX-Like Peptides and Acceptance Criteria

Chromatograms obtained from samples purified by affinity chromatography were uploaded to Skyline along with the molecular formulas of all CTX-like peptides, a mass tolerance of 10 ppm was allowed. To enhance the confidence in identifying CTX species in our samples, three strategies were employed.

Firstly, we generated a comprehensive list of all potential linear fragment ions (b and y) that could result from collision-induced dissociation (CID) fragmentation of each theoretical CTX species.

These ions were then matched against the MS/MS spectra extracted from our data using the Skyline software. If 80% of the expected ions were detected at a specific Rt, that Rt was designated as a potential CTX species Rt, while those eluting at other times were discarded. This step aimed to pinpoint potential Rts of CTX species.

Secondly, Isotope Dot Product (IDotP) values for each theoretical CTX species were calculated in Skyline. This analysis involved comparing the experimental isotopic distribution with the expected isotope distribution based on the peptide's molecular formula. Peaks for each CTX species were required to exhibit an IDotP value above 0.9 to minimize the risk of false positives. Each CTX-like peptides eluting at one of the Rt pinpointed by the CID fragments with an IDotP higher or equal to 0.9 were noted as likely present in the sample.

Thirdly, a database of random molecular formula was generated using InfoChem (available at https://www.cheminfo.org/Spectra/Mass/Generate_list_of_MF/index.html). The list of molecular formulas was then uploaded to skyline along with the MS1 spectra of the sample purified by affinity chromatography. Molecular formulas were then matched in the spectra and the ones with IDotP higher or equal to 0.9 were kept as potentially present while the others were discarded. The false positive rate was calculated by dividing the number of kept molecular formulas by the total number of molecular formulas for each sample. The entire workflow is depicted in Figure 2.

3 | Results

3.1 | Separation of CTX Species by Two-Step Preparative LC Separation

Concentrated pools of serum and plasma, separated by preparative chromatography, exhibited similar elution profiles regardless of the kits used for quantitating the presence of CTX in 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 is likely that the various immunoassays are affected differently. In light of these findings, it was determined that the use of the IDS-iSYS CTX-I (CrossLaps) kit for further antibody-based quantitation is appropriate.

Regarding Rt, 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 may undergo, such as glucuronidation, sulfonation, methylation, and acetylation, in order to be excreted [23]. Consequently, different CTX metabolites may be present in higher concentrations in urine.

Concerning the two-step preparative 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 and 3.75 min, while the remaining fractions yielded elution profiles with only one peak at different Rt. Given the similarity in the elution profiles of fractions 5 and 17, it is conceivable that the species present in these fractions may be monomers and polymers that aggregated due to the high concentration. In the initial chromatography, monomers and polymers of CTX are separated into distinct fractions. It is plausible that following postcolumn flow splitting, the isolated monomers aggregated to form polymers, and the isolated polymers disaggregated into monomers.

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 min or the presence of different molecules than those responsible for the peak at 3.75 min. 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 min but rather at 4, 4.25, 4.5, 4.75, and 5 min.

3.2 | Quantitation of CTX by IDS-iSYS CTX-I (CrossLaps) Kit in Sample Obtained From Affinity Chromatography

After purifying 50 mL of plasma and serum via affinity chromatography with 1M0161, the resulting samples displayed CTX concentrations of 525.2 and 495.7 pg/mL, respectively, following a 5-fold dilution. Regarding plasma and serum purified using the antibodies 1M0122, calculated concentrations were respectively 971.5 and 817.1 pg/mL. The differences between the samples purified by 1M0122 and 1M0161 may be explained by different factors such as the matrix effect of the immunoassays and the difference between 1M0161 and 1M0122 protocols. However, this confirms the effectiveness of affinity chromatography in capturing CTX species.

3.3 | 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 [24–27]. Additional investigations are required

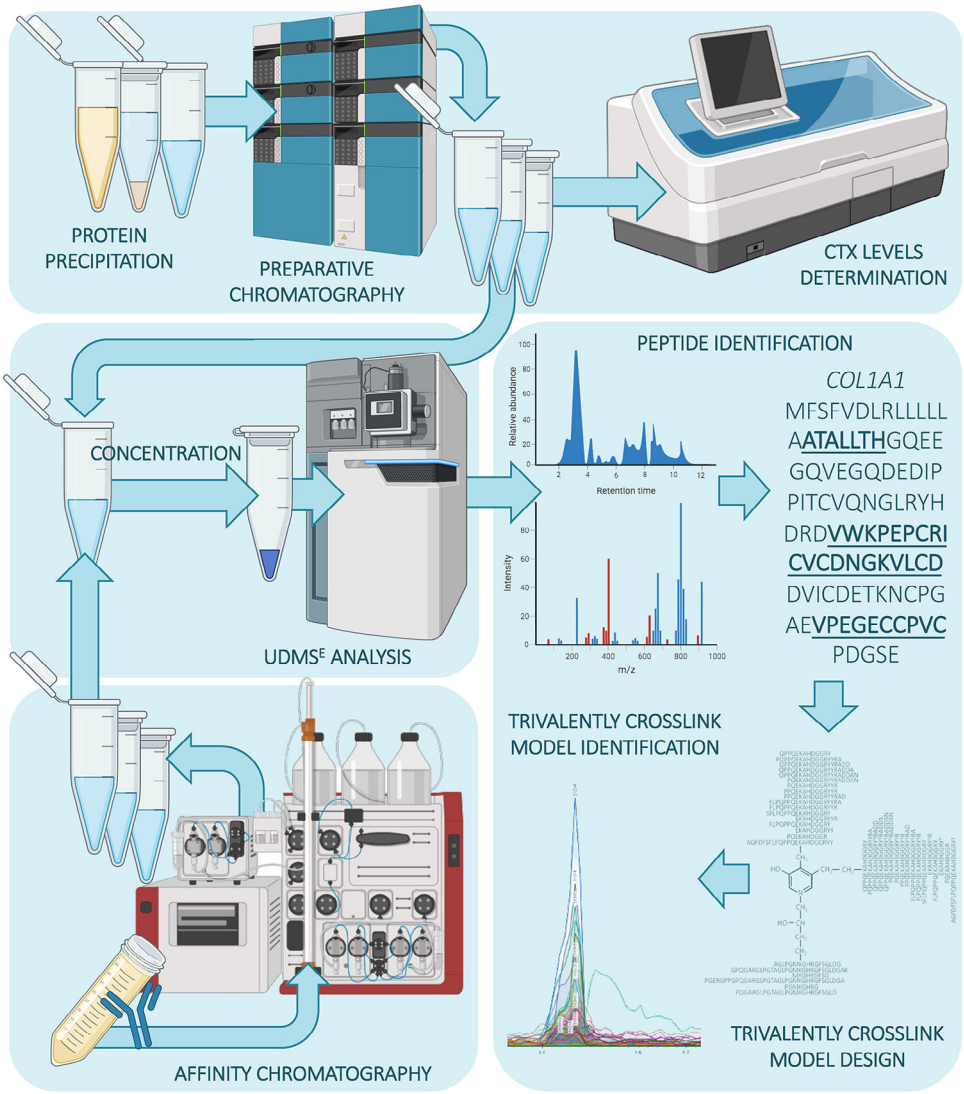

FIGURE 2 | Developed workflow. Plasma and serum samples, prepared through protein precipitation, underwent separation via LC preparative. The fractions obtained were then subjected to CTX concentration analysis. Fractions containing CTX concentrations exceeding 1 ng/mL were subsequently analyzed using HR-MS. The acquired spectra were processed using PEAKS software for the identification of noncrosslinked 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 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 noncrosslinked peptides, which were used to construct additional trivalently crosslinked CTX-like peptides. Once all the CTX-like peptides were designed, Skyline software was employed to identify trivalently crosslinked CTX species. HR-MS, high-resolution mass spectrometry; LC, liquid chromatography.

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 nine 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. All peptides containing a lysine or hydroxylysine residue involved in pyridinoline crosslinks are detailed in Table 1.

3.4 | Analysis of Theoretical Trivalently Crosslinked Models

3.4.1 | Design of CTX-Like Peptides

To achieve a comprehensive identification of CTX species, particularly acknowledging that trivalently crosslinked peptides

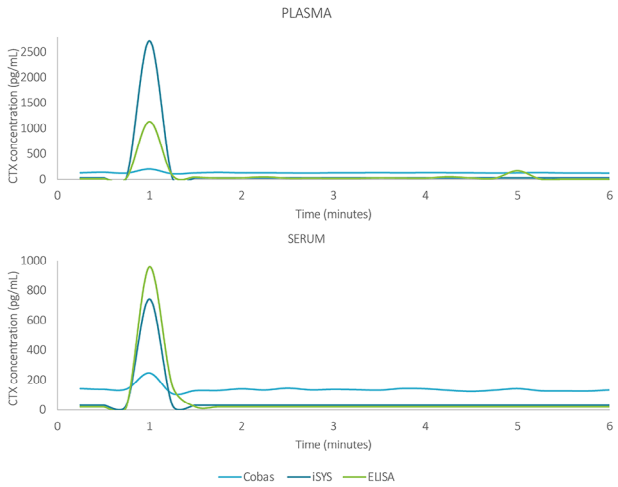

FIGURE 3 | Elution profile obtained after preparative chromatography separation of human plasma and serum. Fractions levels of CTX were assessed using the iSYS CTX-I (CrossLaps) and CrossLaps ELISA kits from IDS Diagnostics, as well as the B-Crosslaps ECLIA kit by Cobas (Roche).

cannot be directly identified using standard peptide/protein identification software, we developed CTX-like peptides. These CTX-like peptides consisted of three peptides selected from the previously identified list of linear peptides, crosslinked together by a pyridinoline or deoxypyridinoline. Subsequently, these CTX-like peptides were employed in the chromatogram search using specialized software.

A total of 3230 CTX-like peptides, outlined in Figure S1, were generated by combining the various peptides accordingly. The molecular formula for each CTX-like peptide was calculated as part of this process.

3.4.2 | Identification of Retention Times via Post-CID Fragments

According to our analysis of linear y and b fragments originating from each CTX-like peptide model, we were able to pinpoint two potential Rt for CTX species at 1.5 and 3.0 min in the chromatogram corresponding to the plasma purified with 1M0161 and 1.4 and 3.0 min in the chromatogram corresponding to the plasma purified with 1M0122. Regarding the chromatograms obtained for the serum purified with 1M0122 and 1M0161, one Rt was found at 1.4 min. Post-CID fragments and their Rt are reported in Table S2. All CTX-like peptides eluting at other times were discarded as potential CTX species.

3.4.3 | Identification of CTX-Like Peptides in Chromatograms

Skyline was utilized to visualize traces corresponding to the trivalently crosslinked CTX-like peptides based on their molecular formulas. Only CTX-like peptides eluting at predefined Rt with an IDotP value above 0.9 were selected. Multiple trivalently crosslinked CTX-like peptides were identified by our analysis, these crosslinked peptides may thus be considered as potential CTX species.

Analysis of the spectra obtained from the plasma sample purified with antibody 1M0161 led to the identification of 26 CTX-like peptides, while the analysis of the spectra from the plasma purified with antibody 1M0122 led to the identification of 24 CTX-like peptides. In terms of serum samples purified by antibodies 1M0161 and 1M0122, skyline analysis identified 28 and 27 CTX-like peptides, respectively. Among these peptides, 11 were common to all 4 samples, 6 were identified in 3 samples, 14 were found in 2 samples, and only 10 were unique to 1 sample. Chromatograms are provided in Figure 5 and Figure S2–S9.

No significant differences in properties or structures were observed between the group of CTX-like peptides recognized in plasma or serum by antibody 1M0161 and the group recognized by antibody 1M0122. However, it is noteworthy to mention that none of the larger CTX-like peptides were identified using our method. Identified peptides were actually part of the smallest CTX-like peptides.

3.4.4 | Assessment of False Discovery Rate

None of the calculated FDR was above 10%. The FDR was assessed as 8.6%, 5.7%, 1.3%, and 4.6% for plasma sample purified with 1M0161, plasma sample purified with 1M0122, serum sample purified with 1M0161 and serum sample purified with 1M0122, respectively.

4 | Discussion

In this work, we employed a multi-step approach in order to fully characterize CTX. First, uncrosslinked and linear peptides derived from type I collagen from plasma and serum were extracted using preparative LC before being analyzed by HR-MS. These peptides were subsequently identified using protein/peptide identification software. The peptides involving a C-terminal pyridinoline crosslink previously identified were then used to build trivalently crosslinked CTX-like peptides. Traces corresponding to these CTX-like peptides were then sought in the different chromatograms obtained from plasma and serum samples purified using affinity chromatography. Identification of CTX-like peptides was done according to strict acceptance criteria including Rt, IDotP values, and FDR. More than 40 trivalently crosslinked CTX-like peptides were found for plasma and serum purified with antibodies 1M0122 and 1M0161. This marks a significant step toward enhancing our understanding of these critical markers in the context of osteoporosis management.

The significant number of CTX species identified in our study may be explained by 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 nonreproducible [21]. 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,

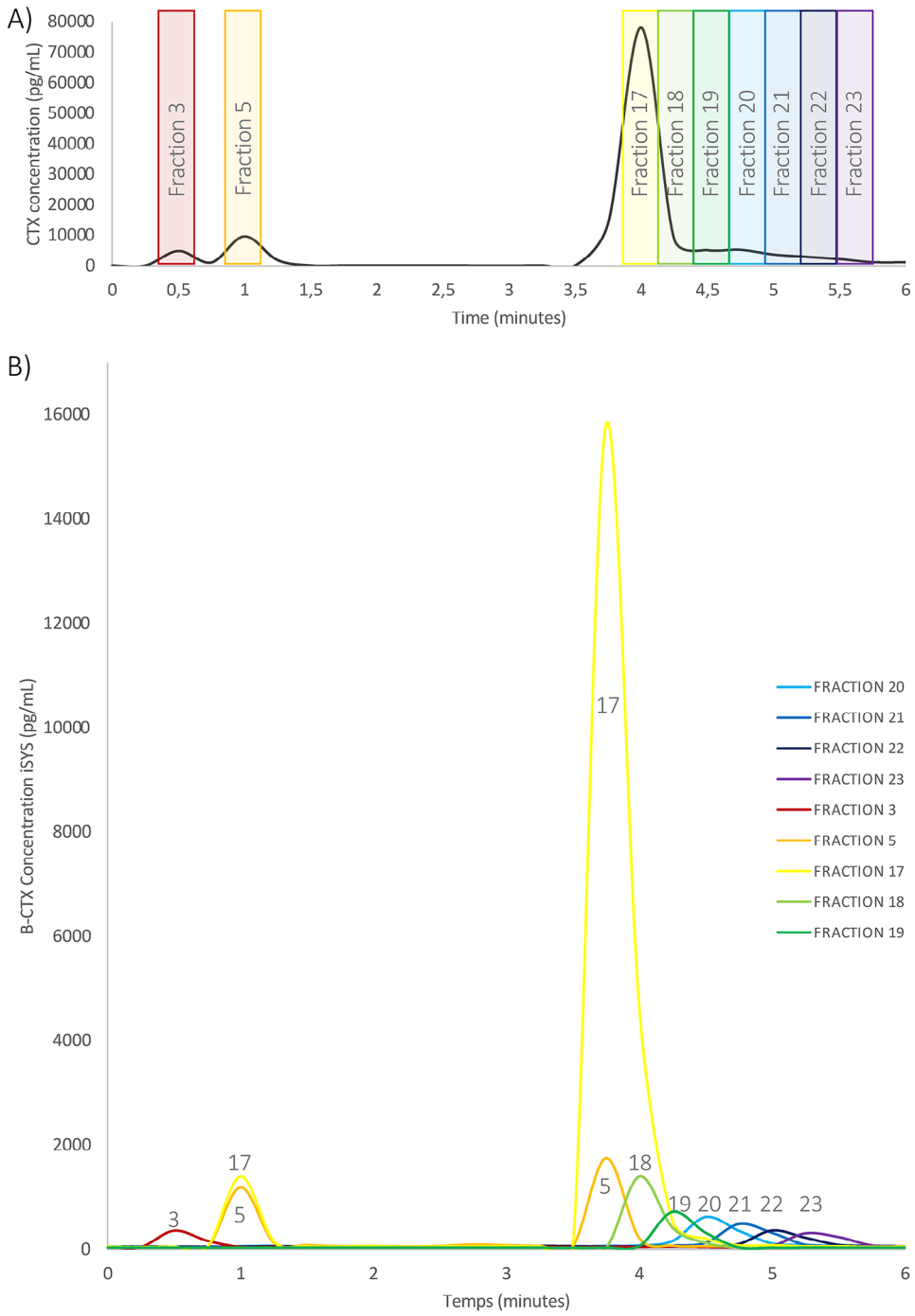

FIGURE 4 | Elution profile obtained by the two-step preparative LC separation of a concentrated urine sample. (A) First run of preparative LC (B) second run of preparative LC. Each color represents a fraction. LC, liquid chromatography.

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. Secondly, 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. Thirdly, 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 species 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-isomerized 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 but also potentially due to the fact that smaller peptides ionize more effectively in

TABLE 1 | List of the identified peptides containing a lysine known to be involved in the pyridinoline crosslinks.

Amino acid sequence	Origin	−10Log*p*	Separative technique
AGFDFSFLPQHyPHyPQEKAHDGGRYY	Type I collagen α1 chain	20.12	Affinity LC
AGFDFSFLPQHyPPQEHyLAHDGGRYY	Type I collagen α1 chain	16.68	Affinity LC
EKAHDGGRYY	Type I collagen α1 chain	19.36	Affinity LC
EKAHDGGRYYR	Type I collagen α1 chain	20.52	Affinity LC
FLPQHyPPQEKAHDGGRY	Type I collagen α1 chain	23.82	Affinity LC
FLPQPHyPQEKAHDGGRYYR	Type I collagen α1 chain	28.01	Affinity LC
FLPQPPQEKAHDGGRYYRA	Type I collagen α1 chain	19.21	Affinity LC
FSFLPQPPQEKAHDGG	Type I collagen α1 chain	16.57	Affinity LC
KAHDGGRYYRAD	Type I collagen α1 chain	26.34	Affinity LC
PPQEKAHDGGRYYRAD	Type I collagen α1 chain	31.90	Affinity LC
PQEKAHDGGRYYR	Type I collagen α1 chain	22.32	Affinity LC
PQEKAHDGGRYYRADDAN	Type I collagen α1 chain	15.87	Affinity LC
PQPHyPQEKAHDGGRYYRA	Type I collagen α1 chain	22.99	Affinity LC
QPHyPQEKAHDGGRYYRADD	Type I collagen α1 chain	28.82	Affinity LC
QPPQEKAHDGGRY	Type I collagen α1 chain	35.44	Affinity LC
QPPQEKAHDGGRYYRADDA	Type I collagen α1 chain	16.59	Affinity LC
QPPQEKAHDGGRYYRADDAN	Type I collagen α1 chain	21.57	Affinity LC
QPPQEKAHDGGRYYRADDANVVRDRDLEVDTTLHyLSLSQQ IENIRSHyPEGSRHyLNPARTCRDL	Type I collagen α1 chain	17.69	Affinity LC
SFLPQPPQEHyLAHDGGRY	Type I collagen α1 chain	17.03	Affinity LC
AGFDFSFLHyPQPPQEKAHDGG	Type I collagen α1 chain	26.69	Preparative LC
AGFDFSFLPQPPQEKA	Type I collagen α1 chain	26.15	Preparative LC
AGLPGMKGHRGFSGLDG	Type I collagen α1 chain	31.86	Preparative LC
DFSFLPQPPQEKAHDG	Type I collagen α1 chain	21.70	Preparative LC
FDFSFLPQPPQEKAHD	Type I collagen α1 chain	26.59	Preparative LC
FKGIRGHNG	Type I collagen α2 chain	23.02	Preparative LC
FKGIRGHNGLDGLKGQ	Type I collagen α2 chain	16.88	Preparative LC
FLPQPPQEKAH	Type I collagen α1 chain	19.33	Preparative LC
FSFLPQPPQEKAH	Type I collagen α1 chain	18.32	Preparative LC
GPQGARGLPGTAGLPGMKGHRGFSGLDGAK	Type I collagen α1 chain	17.90	Preparative LC
MKGHRGFSG	Type I collagen α1 chain	15.21	Preparative LC
PGERGPPGPQGARGLPGTAGLPGMKGHRGFSGLDGA	Type I collagen α1 chain	21.12	Preparative LC
PGFKGIRGHNG	Type I collagen α2 chain	19.72	Preparative LC
PGMKGHRG	Type I collagen α1 chain	20.84	Preparative LC
PPGPPSAGFDFSFLQPPQEKAH	Type I collagen α1 chain	18.85	Preparative LC
PQEKAHDGGR	Type I collagen α1 chain	26.46	Preparative LC
PQGARGLPGTAGLPGMKGHRGFSGLD	Type I collagen α1 chain	15.27	Preparative LC
SAGFDFSFLPQPPQEKAHD	Type I collagen α1 chain	16.44	Preparative LC
SFLHyPQPPQEKAHDG	Type I collagen α1 chain	20.41	Preparative LC
PPQEKAHDGGRYYR	Type I collagen α1 chain	16.33/21.09	Preparative LC/Affinity LC
SFLPQPPQEKAHDG	Type I collagen α1 chain	15.70/19.34	Preparative LC/Affinity LC

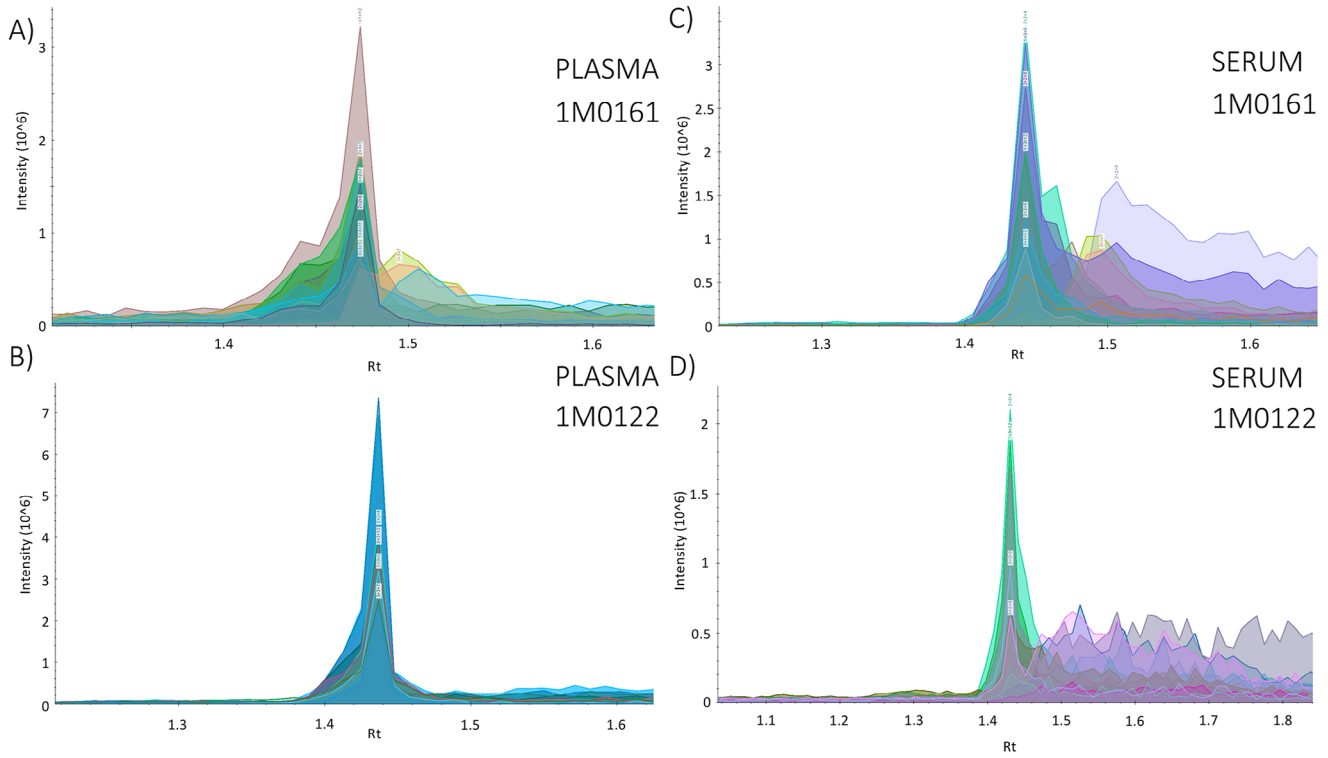

FIGURE 5 | Chromatograms obtained from (A) Plasma sample captured by antibody 1M0161, (B) Plasma sample captured by antibody 1M0122, (C) Serum sample captured by antibody 1M0161, and (D) Serum samples captured by antibody 1M0122.

the mass spectrometer source than bigger ones. Additionally, larger peptides may have partially precipitated during the sample preparation process.

Regarding type I collagen divalently crosslinked fragments, it was not surprising that they were not found, as divalent crosslinks are typically associated with newly synthesized bone tissue, serving as precursors to trivalent crosslinks found in older bone tissue. The newly synthesized bone tissue rarely undergoes bone resorption compared to older bone tissue except in some metabolic bone disorders, such as Paget's disease. Therefore, finding no divalently crosslinked peptides in the plasma and serum is in line with the expectations.

A notable constraint in our study is the challenge of doubly confirming the presence of our CTX-like peptides through MS/MS spectra, owing to the unknown and therefore unpredictable fragmentation patterns of these molecules. LC-MS/MS methods were already developed for the quantitation of free pyridinoline and deoxypyridinoline [28] and described a CID fragment with an m/z of 84 and 82, respectively. However, there is currently no description of the CID fragmentation pattern for bound pyridinoline.

In this work, Skyline was used as a visualization tool. Unfortunately, it lacks the advanced identification features found in dedicated identification software, which led to the need to find stringent inclusion and exclusion criteria and filters. The analysis of y and b ions originating from each CTX-like peptide, as well as the analysis of IDotP values, should reduce the risks of having false positive risks to an acceptable level, which has been calculated as under 10% according to our results.

A minor limitation of this study is the absence of negative controls. It would have been beneficial to conduct the same workflow on samples devoid of CTX, such as *Escherichia coli* samples. The inclusion of negative control samples would have allowed us to verify if any CTX was present in these samples, thereby enhancing the confidence in our analysis.

Another minor limitation of our work is the use of plasma from patients with chronic kidney disease. While this choice was made to capitalize on the higher concentrations of peptides and proteins in these samples due to impaired renal filtration, it may have inadvertently led to a bias in the representation of species found in osteoporotic patients. Specifically, certain species that are more representative of osteoporotic patients may not have been sufficiently concentrated in our samples from nonosteoporotic patients.

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 [29–31]. 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 identified a significant 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 an 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 toward 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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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The MS data generated in this study have not been uploaded to a public platform due to the use of proprietary antibodies provided by IDS, which are subject to patent restrictions. IDS has requested that these data not be shared publicly to protect industrial confidentiality. However, researchers who are interested in accessing the MS data may contact the corresponding author. Data can potentially be shared upon request, provided that the interested parties sign a confidentiality agreement to ensure the protection of the proprietary information.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.