Bone Reports 20 (2024) 101737

Contents lists available at ScienceDirect

Bone Reports

journal homepage: www.elsevier.com/locate/bonr

Total serum pentosidine quantification using liquid chromatography-tandem mass spectrometry

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ARTICLE INFO

Keywords:
Pentosidine
Liquid chromatography-tandem mass spectrometry
Advanced glycation end product
Osteoporosis

ABSTRACT

Pentosidine (PEN) is an Advanced Glycation End-product (AGE) that is known to accumulate in bone collagen with aging and contribute to fracture risk. The PEN content in bone is correlated with serum PEN, making it an attractive, potential osteoporosis biomarker. We sought to develop a method for quantifying PEN in stored serum. After conducting a systematic narrative review of PEN quantification methodologies, we developed a liquid chromatography tandem mass spectrometry (LC-MS/MS) method for quantifying total serum PEN. Our method is both sensitive and precise (LOD 2 nM, LOQ 5 nM, %CV < 6.5 % and recovery 91.2–100.7 %). Our method is also equivalent or better than other methods identified in our review. Additionally, LC-MS/MS avoids the pitfalls and limitations of using fluorescence as a means of detection and could be adapted to investigate a broad range of AGE compounds.

1. Introduction

Advanced Glycation End-products (AGEs) occur when proteins become glycated with sugar. Glycation is a spontaneous, non-enzymatic, multi-step reaction by which sugar metabolites become covalently linked to amino acid residues in protein. Approximately 20 different AGEs are known to occur, with varying chemical structures and properties (Stinghen et al., 2016). We can ingest them from food or they can form within the body. AGEs play a significant role in human health and disease. In some tissues, AGE formation is a normal physiologic process whereby AGEs facilitate the lysosomal degradation of proteins, normal tissue remodelling and the turnover of senescent extracellular matrix components (Stinghen et al., 2016; Miyata et al., 1997; Brownlee et al., 1988). AGEs are also known to be pathological and accumulate with aging and in a variety of disease states. AGEs contribute to the complications associated with diabetes, atherosclerosis, Alzheimer's disease, chronic kidney disease and osteoporosis, to name a few (Vistoli et al., 2013; Singh et al., 2014; Seeman, 2006). AGE accumulation contributes to disease progression in 3 ways: 1) The glycation of intra- and extracellular proteins can affect their function and adversely affect physiology at both the cellular and tissue level (Kerkeni et al., 2014). 2) AGEs form cross-links within collagen. Collagen fibrils make up the scaffold or extracellular matrix for many bodily tissues. The accumulation of AGE cross-links in collagen affects its microarchitectural structure and can negatively affect overall tissue composition and function (Miyata et al., 1996). 3) Many cells in the body express the protein the Receptor for AGE (RAGE) and increased RAGE activation exacerbates inflammation and oxidative stress (Kerkeni et al., 2014; Miyata et al., 1996; Prasad et al., 2012; Di Marco et al., 2013).

Pentosidine (PEN), is an AGE which is formed from ribose sugar and arginine and lysine amino acid residues (Sell et al., 1991). In collagen, PEN creates non-enzymatic crosslinks between exposed arginine and lysine residues on collagen fibers. This is particularly relevant in bone, which is comprised of a collagen matrix. Bone turnover slows down as we age (Demontiero et al., 2012). Thus, the collagen in bone is not turned over as quickly, leading to increased levels of AGEs in bone (Vashishth, 2009). Due to its fluorescent nature and ease of detection, PEN is the most intensely studied AGE in bone (Willett et al., 2022).

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https://doi.org/10.1016/j.bonr.2024.101737
Received 30 August 2023; Received in revised form 9 January 2024; Accepted 11 January 2024
Available online 17 January 2024

Increased bone PEN has been shown to affect the microarchitecture of bone tissues, making it more brittle and likely to fracture (Vashishth et al., 2001; Yamamoto and Sugimoto, 2016). Vaculik et al. 2016 have previously shown that serum PEN levels are correlated with the PEN content of bone (Vaculik et al., 2016). We sought to develop a method to assess total PEN in stored serum samples, with the intention of using this methodology in future studies, exploring PEN's utility as a serum biomarker of osteoporosis and fracture.

A systematic narrative review of the literature identified multiple enzyme-linked immunosorbent assay (ELISA), high-performance liquid chromatography (HPLC) and liquid chromatography tandem mass spectrometry (LC-MS/MS) methods for quantifying PEN in serum or plasma. We chose to explore the utility of LC-MS/MS for the quantification of PEN in stored serum samples, due to its superior sensitivity and specificity in comparison to ELISA and HPLC. Some of the information that we can get with this hybrid analytical technique are: specific fragmentation patterns, constant ratio of the fragments being monitored during the LC-MS/MS run, and specific time at which the measurand (PEN) is detected (retention time). These parameters are available for each analyzed sample (calibrator, quality control, unknown specimen) and help confidently identify and quantify PEN.

Four LC-MS/MS methodologies are currently reported in the literature for the assessment of PEN in blood. Thornalley et al. 2003 describe a methodology for the assessment of PEN in stored plasma (Thornalley et al., 2003). Kerkeni et al., 2014 describe the assessment of PEN in human serum (both fresh and frozen) in patients with and without coronary artery disease via LC-MS/MS but do not report detailed precision data for the methodology (Kerkeni et al., 2014). O'Grady et al., 2020 report a methodology for quantification of PEN in frozen serum from individuals with either chronic renal insufficiency or diabetes (O'Grady et al., 2020). Precision data is reported but the limit of quantification is quite high in comparison to other methods. And finally, Monnier et al., 2022 assess PEN in frozen plasma samples from individuals with Type 1 diabetes, but supply minimal precision data in the manuscript supplement (Monnier et al., 2022). Based on these reported methods, we have developed a method for PEN quantification in archived human serum via LC-MS/MS. Herein, we describe this methodology in detail as well as its precision data. We also discuss the results of our systematic narrative review and provide a detailed comparison of multiple PEN quantification methods, their benefits and limitations.

2. Materials and methods

2.1. Systematic narrative review

Ovid Medline was searched in October 2019 and updated in February 2022 and January 2023 for any research reporting on the quantification of total PEN in blood (serum or plasma) that also reported precision data. A detailed overview of our search strategy and review flow diagram are included as supplementary materials.

2.2. Chemicals and materials

Two vials of PEN (1.05 mg; $\geq$ 98 % purity) were purchased from Cedarlane, 0416662–49 and 0473950–8, respectively. Isotopically labelled pentosidine-d3 TFA salt (0.25 mg, 97 % purity for the salt and 99.2 % isotopic purity) was obtained from Toronto Research Chemicals. Hydrochloric acid was of American Chemical Society (ACS) purity, methanol was Optima grade and both were supplied by Fisher Scientific. In-house ultrapure water with a high resistivity (18.2 MΩ.cm) was used. Ammonium formate was obtained from Sigma-Aldrich. Anhydrous ethyl alcohol was supplied by Commercial alcohols, while formic acid (Honeywell Fluka), for mass spectrometry, was obtained from Fisher Scientific. Ammonium hydroxide, ACS reagent with 28–30 % NH_3 basis was from Sigma-Aldrich.

2.3. Standard solutions, calibration and quality control samples

A primary stock solution of 2.77 mmol/L (solution A) of PEN was prepared in ethanol. Standard solutions with PEN concentrations of 277 μmol/L (solution B), 27.7 μmol/L (solution C) and 2.77 μmol/L (solution D) were prepared by serial dilution of the primary stock standard in water. All solutions were stored at −20 °C. Calibrators were prepared by spiking ultrapure water with the appropriate amount of PEN standard solutions to obtain final concentrations of 1, 10, 25, 50, 100, 250, 500, and 1000 nM. Internal standard stock solution was also prepared in ethanol and contained PEN at the concentration of 0.3 mM. Semi-stock solution and working internal standard solution were prepared by diluting the stock solution to obtain 1 μM and 100 nM concentrations, respectively. Semi-stock solution was made in water, while working solution was ethanol-based.

Quality control (QC) samples were prepared from remnant serum specimens (gold top tubes). Three specimen pools were prepared by pooling serum into 20 mL scintillation vials. Two pools were used without alteration. Pool 1 for QC low (~ 70 nM) and pool 2 for QC medium (~170 nM), while the third pool of serum specimens was spiked with PEN to create a quality control material with higher (~500 nM) PEN concentration.

2.4. Sample preparation

Specimens for this study were surplus, unlinked material. The Research Ethics Board at the University Health Network in Toronto, Ontario, Canada deemed the study exempt from review. Extraction of total (free and protein-bound) PEN was achieved by taking 50 μL of serum and transferring to a Pyrex screw cap culture tube with Polytetrafluoroethylene (PTFE) lined phenolic cap. 50 μL of working internal standard (100 nM in ethanol) was then added. To this, 500 μL of 6 M hydrochloric acid was added and the mixture was subjected to acid hydrolysis that was carried out at 110 °C for 18 h. Following hydrolysis, hydrolysates were evaporated to dryness under nitrogen stream. Dried hydrolysates were then re-suspended in 0.5 mL of ultrapure water. 0.5 mL of 1.0 M potassium phosphate dibasic solution was added and the mixture was filtered using Basix™ Syringe Filters (0.2 μm, 13 mm, PTFE, Thermo Scientific).

Filtered sample was applied to a solid phase extraction (SPE) cartridge (Strata X-C, Phenomenex) for further purification and concentration. Cation exchange mechanism of the Strata X-C SPE cartridge results in strong retention for basic compounds such as PEN. The SPE is useful as it eliminates matrix contaminants resulting in a better limit of detection (LOD) and limit of quantification (LOQ).

SPE protocol was as follows: cartridge was conditioned with 1 mL of methanol. Equilibration was achieved with 1 mL of ultrapure water. Sample was then loaded and allowed to flow by gravity through sorbent material. The cartridge was subsequently washed with water and methanol (1 mL each). The cartridge was dried under vacuum for 5 min. PEN was eluted with 1 mL of 5 % ammonium hydroxide in methanol and collected in clean borosilicate tubes. The solvent was evaporated under stream of nitrogen gas at 80 °C. The dried sample was reconstituted in 100 μL of mobile phase and transferred to a 2 mL autosampler vial with insert or 96-well plate. 15 μL of the sample extract was analyzed by LC-MS/MS.

2.5. LC-MS/MS – instrumentation and chromatographic conditions

Liquid chromatography (LC) separation was performed using an Agilent 1200 series system (Agilent Technologies, US) consisting of a degasser, solvent binary pump, autosampler, and a column oven. Sample extracts were analyzed at 30 °C using reversed-phase Kinetex 2.6 μm F5 100 Å liquid chromatography column, 100 × 2.1 mm (Phenomenex). The mobile phase consisted of a single solvent composed of 5 mmol/L ammonium formate, 0.02 % formic acid, 2 % methanol in ultrapure

Table 1
Review of pentosidine quantification methodologies which report precision metrics.

Author/Reference	Sample Type	Method	LOD	LOQ	CV%	% Recovery	Protein Normalization
Odetti et al., 1992	Plasma	HPLC	–	–	Inter-assay 7.1 %	70 %	Total Protein
Takahashi et al., 1996	Serum	HPLC			Intra-assay 5.7 % Inter-assay 5.8 %	97.7–99.9 %	None
Miyata et al., 1996	Plasma	HPLC	4 nM	–	–	–	Albumin
Floridi et al., 1999	Plasma	HPLC	150fmol	–	–	90 ± 3.8 %	Total Protein
Slowik-Zylka et al., 2004	Plasma	HPLC	0.75 pmol/mg	–	Inter-day 8 %	80 ± 5 – 88 ± 3 %	Total Protein
Spacek and Adam, 2006	Serum	HPLC	1.76 nM	–	Inter-assay 4.44 % (RSD)	77 ± 3.5 %	None
Scheijen et al., 2009	Plasma	HPLC	LOD 2.2 nM or 0.02 pmol/mg protein	–	Intra-assay 2.0–6.5 % Inter-assay 1.6–3.1 %	102 ± 10 %	Total Protein
Lee et al., 2017a	Plasma	HPLC	–	1 nM	Intra-day 6.8 % (4.96–8.78 %) Inter-day 4.27 %	52.1 % (41.6–57.3 %)	None
Palma-Duran et al., 2018	Serum	HPLC	–	0.005 μmol/L	Intra-assay & Inter-assay <13 %	79–115 %	Unknown
Yamamoto et al., 2008	Serum	ELISA	–	–	Intra-assay 8.0 % Inter-assay 6.6 %	–	Total Protein†
Uchiyama et al., 2015	Serum	ELISA	–	–	6.4 %	–	Total Protein†
Kurt et al., 2016	Plasma	ELISA			Intra-assay 5.1 % Inter-assay 9.3 %		Total Protein†
Haddad et al., 2016	Plasma	ELISA	31–2000 nM	–	Intra-assay <8 % Inter-assay <10 %	–	Total Protein†
Thornalley et al., 2003	Plasma	LC-MS/MS	0.23 pmol	–	Inter-batch 2.3 %	101 %	Lysine
Kerkeni et al., 2014	Serum	LC-MS/MS	–	–	Inter-assay 11 %	–	Lysine
O'Grady et al., 2020	Serum	LC-MS/MS	–	5 ng/mL	Intra-assay 3.66–5.91 % Inter-assay 3.8–10.3 %	104 % (97–116 %)	None
Monnier et al., 2022	Plasma	LC-MS/MS	–	–	Intra-assay 26.5 % Inter-assay 37.0 %	Not Assessed	Total Protein
Blencowe & Božović et al., 2024	Serum	LC-MS/MS	2 nM (0.8 ng/mL)	5 nM (1.9 ng/mL)	Intra-day 3.0–5.5 % Inter-day 4.4–6.5 %	91.2–100.7 %	None

RSD – Relative Standard Deviation, †normalization inherent in methodology.

water. Wash solvent was water:methanol 1:1 (*v*/v). Chromatographic separation of PEN was achieved using isocratic elution at the flow rate of 0.3 mL/min. The injection volume was 15 μL and the total run time was 4 min.

The LC system was coupled to an API 5000 triple quadrupole mass spectrometer (Applied Biosystems/MDS Sciex, Concord, ON, Canada) equipped with an electrospray ionization (ESI) probe and was used in positive ion mode by multiple reaction monitoring (MRM). Protonated PEN (for both labelled and unlabelled forms) was the major ion produced by ESI and was chosen as a precursor ion. Instrument operating parameters were optimized for PEN. The optimal working parameters for the mass spectrometer were as follows: curtain gas setting 15, gases GS1 and GS2 on settings 50 and 20, respectively. Ion source temperature 650 °C, collisionally activated dissociation (CAD) gas setting 4, ion spray voltage 5500 V, declustering potential 120 V, entrance potential 7 V. Using tandem mass spectrometry, we can select specific fragment ions to monitor using our method. These are called selective reaction monitoring (SRM) scans that help us get specific information about the molecule (i.e., PEN) being measured. Ratio of the fragment1/fragment 2 is constant and is being checked for each processed sample prior to accepting the result. The ion-transitions of m/z 379.1 > 187.1 (quantifier) and m/z 379.1 > 135.1 (qualifier) were monitored to identify and quantitate PEN, while m/z 382.1 > 190.1 was monitored for the internal standard. Use of an isotopically labelled analogue of PEN, D3-pentosidine as internal standard helps us identify the measurand, and calculate the results.

The LC system and mass spectrometer were controlled by the Analyst software (version 1.6.2). Data acquisition and analysis were performed with the same software. After data acquisition was completed, peaks were integrated. PEN concentrations were corrected based on the internal standard and quantified using the calibration curves that were included in each batch.

2.6. Method validation

Method validation included assessments of: precision, linearity, analyte recovery, LOD and LOQ. Accuracy of a new diagnostic test is a crucial parameter. It is often assessed by comparing a newly developed assay with a reference standard/test, but in the absence of a certified reference material for PEN (which would help standardize tests using different platforms), we needed to rely on other ways of assessing trueness of our assay.

When developing an LC-MS/MS method, according to The Clinical & Laboratory Standards Institute's (CLSI) guidelines, we can assess accuracy of analyte concentration measurement using spike/recovery experiments. This is what we performed. We spiked the sample with a known concentration of PEN standard (different lot from the lot used to create calibration standards as this is best practice) and analyzed the sample using the method being validated. Percent recovery calculated in this type of experiment is equivalent to the degree of agreement of test result to the true value, i.e., accuracy. Inter-day precision was determined by analyzing the three levels of QC in triplicates for 7 days. The intra-day precision was assessed by analyzing the same QCs in triplicate within a single batch. The criteria for acceptability of the precision data was a coefficient of variation (CV) < 10 % for all 3 QC levels. Linearity was evaluated by assessing three sets of calibration curves analyzed in separate batches. The ratio of analyte peak area to internal standard peak area was plotted against PEN concentration in the Analyst software. Linear regression using a 1/x weighted least squares regression algorithm was used. Next, analyte recovery experiments were performed by spiking synthetic PEN at concentrations of 50, 90, 130, and 170 nM into two serum samples each containing endogenous PEN concentration of about 29 nM. Recovery was determined by comparing the concentrations in the spiked samples with expected concentrations. Finally, the LOD was defined as the concentration of PEN that produced a peak with

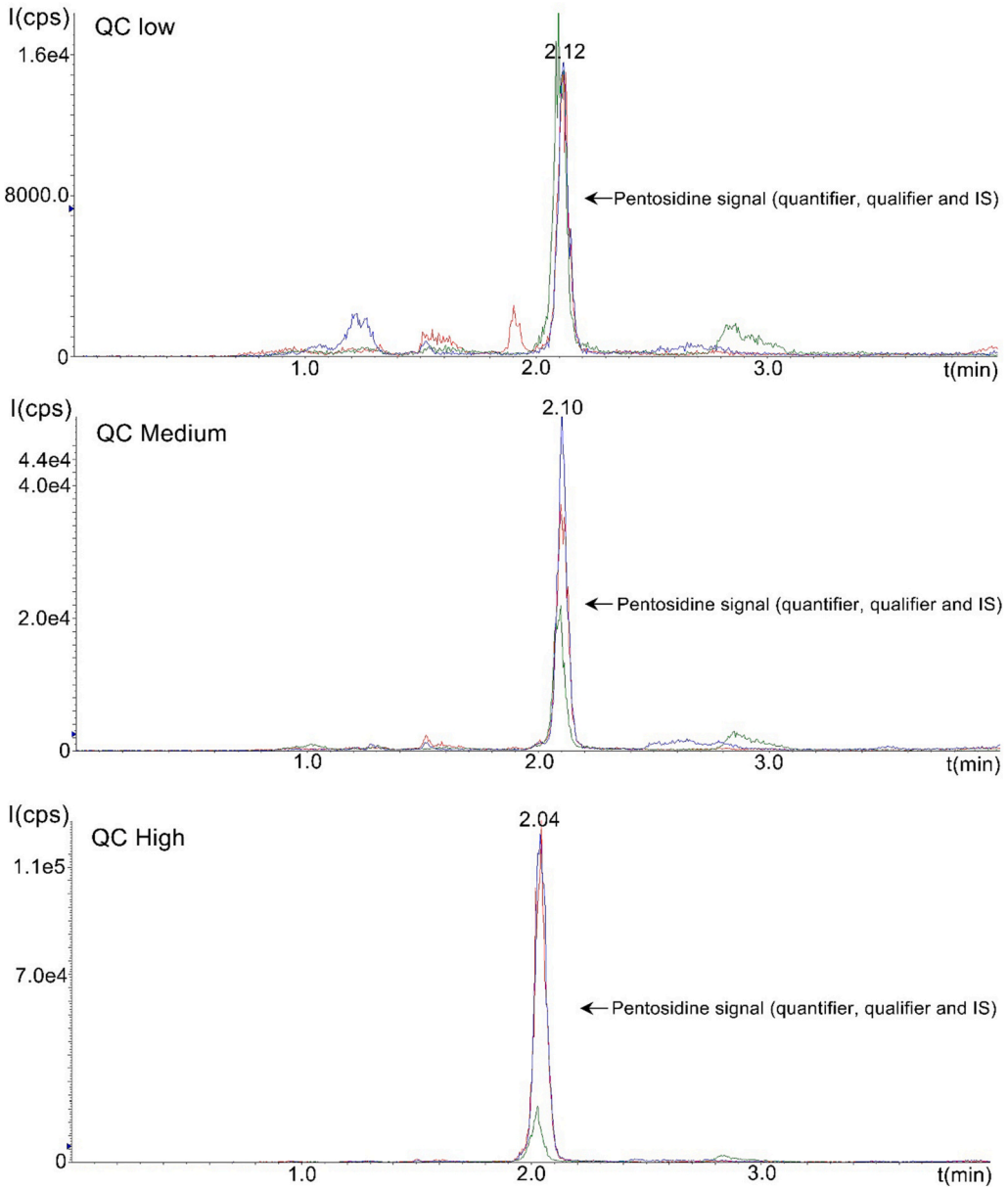

Fig. 1. LC-MS/MS chromatograms representing Quality Control (QC) specimens. The most abundant peaks (at retention time, t_R = 2.12 min) visible in the chromatograms belong to ion-transitions *m/z* 379.1 > 187.1 (pentosidine quantifier), *m/z* 379.1 > 135.1 (pentosidine qualifier), and *m/z* 382.1 > 190.1 (d3-pentosidine). Satisfactory resolution from other components of the matrix was achieved. I (cps) – Intensity (counts per second), IS - Internal Standard.

a signal-to-noise ratio of 3:1. Serum sample with PEN measurement of 29 nM was diluted 10-fold and assayed in five replicates for LOD assessment. The LOQ was defined as the concentration of PEN that produced signal-to-noise ratio of at least 10:1 and precision deviation of <20 %. For LOQ experiment, a different serum specimen with previously measured PEN concentration of 22 nM was diluted 4× and assayed five times.

3. Results

3.1. Systematic narrative review

Our search yielded 17 unique PEN quantification methods which had reported at least one precision metric; LOD, LOQ, variation and/or recovery. These results are shown in Table 1. It should be noted that some

Table 2
Within- and between-day precision data for pentosidine in human serum (n = 21).

	Within-day			Between-day		
	Mean concentration (nM)	SD	CV (%)	Mean concentration (nM)	SD	CV (%)
QC Low	64	1.9	3.0	64	4.2	6.5
QC Medium	162	3.0	1.8	165	7.8	4.7
QC High	509	27.8	5.5	534	23.2	4.4

SD – Standard Deviation.

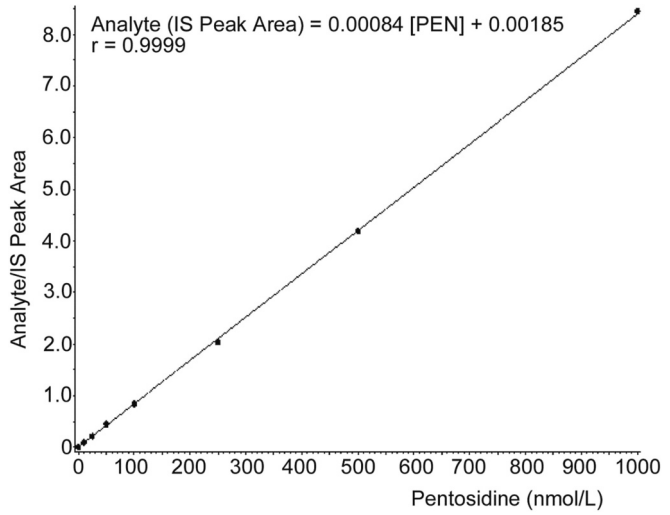

Fig. 2. Standard curve for the Pentosidine method. Assay shows a dynamic linear response up to at least 1000 nmol/L.

publications reported using commercially available PEN ELISA kits. We did not investigate the precision metrics for these methods beyond what was reported in the publication, though these metrics may be available from the manufacturer(s).

3.2. LC-MS/MS development and validation

Fig. 1 shows the LC-MS/MS chromatogram obtained for three QC samples containing 62, 165, and 521 nM of PEN. Extracted ion chromatogram (XIC) for both the quantifier and qualifier for the analyte, as well as the XIC for the IS are shown overlaid on the same plot, demonstrating sharp, symmetrical peaks with high selectivity. The most abundant peaks (at retention time, t_R = 2.12 min) visible in the chromatograms belong to PEN and its internal standard. Satisfactory resolution from other components of the matrix was achieved. Triplicate injections of QC pools yielded within-day imprecision of 3.0, 1.8, and 5.5 % at concentrations of 64, 162, and 509 nM, respectively. The between-day imprecision for the same QC pools were 6.5, 4.7, and 4.4 % (Table 2). These results indicate that the developed method has excellent precision for the measurement of PEN in serum samples.

In addition, the developed assay exhibited excellent linearity, as shown in Fig. 2, where the measured PEN values correlated well across the measuring range to the expected output; a linear response up to at least 1000 nM. The linear regression line fitted the data with a correlation coefficient R^2 of 0.997. Next, we observed the efficiency with which our analytes were recovered from their native serum matrices (Table 3). PEN was added at four different concentrations to two different serum specimens to assess analyte recovery. Spike recovery was calculated as a ratio of the recovered and added PEN amounts and was expressed as percentage. Average calculated recoveries were 100.7 % (% CV = 10.6) and 91.2 % (% CV = 7.3) for the two assessed samples.

LOD and LOQ were determined by analyzing diluted serum specimens with endogenous PEN concentrations of 29 (VS-1) and 22 (VS-2) nM. 400 μL of each specimen was made by diluting serum with 6 M HCl. VS-1 was diluted 10-fold, while VS-2 was diluted 4-fold. Each sample was assayed 5 times in the same batch. PEN concentrations of 2.8 nM (mean value for VS-1) produced an average signal-to-noise ratio of 10:1, with % CV of 31.2. Mean PEN concentration for diluted sample VS-2 was 5.4 nM (% CV = 9.2) with S/N ratios above 30 for all replicates. Extrapolating from this data, we assigned 2 nM and 5 nM, for LOD and LOQ, respectively. The above results confirm that the developed LC-MS/MS method for serum PEN is precise (CV < 10 %), linear (5–1000 nM) and accurate, as demonstrated by the excellent recovery.

4. Discussion

Our LC-MS/MS protocol yields rigorous results for LOD, LOQ, coefficients of variation and recovery of PEN. In comparison to other reported methodologies (Table 1), our precision and validation metrics are equivalent or better (Kerkeni et al., 2014; Miyata et al., 1996; Thornalley et al., 2003; O'Grady et al., 2020; Monnier et al., 2022; Odetti et al., 1992; Takahashi et al., 1996; Floridi et al., 1999; Slowik-Zylka et al., 2004; Spacek and Adam, 2006; Scheijen et al., 2009; Lee et al., 2017a; Palma-Duran et al., 2018; Yamamoto et al., 2008; Uchiyama et al., 2015; Kurt et al., 2016; Haddad et al., 2016). While there are HPLC and LC-MS/MS methodologies for PEN utilized for various research purposes in the literature and there are many established PEN ELISA kits available on the market, validation and precision data are only sporadically reported in the literature (Supplement Fig. 3). This is also evident in Table 1, which includes only publications which reported details on either LOD, LOQ, variation and/or recovery. Without reported precision data, it is difficult to evaluate the credibility of study results and to compare PEN quantification between studies and patient populations. This is particularly relevant, given that AGEs are fast emerging as valuable biomarkers in many disease states (diabetes, atherosclerosis, Alzheimer's, kidney disease, and osteoporosis) (Vistoli et al., 2013; Singh et al., 2014; Seeman, 2006).

ELISA and HPLC have been most commonly used to quantify PEN because PEN's fluorescence can be easily measured with a fluorescence detector (λ_{ex} 328–335 nm and λ_{em} 378-385 nm) (Nogajczyk et al., 2015). Unfortunately, ELISA and HPLC have limited specificity and reproducibility (Slowik-Zylka et al., 2004; Lee et al., 2017b). ELISA may both over-detect and under-detect PEN. False elevations in PEN may be detected if the proteins used to block non-specific binding in the immunoassay contain PEN epitopes. Conversely, under-detection of PEN may occur if the antibody does not bind all PEN-protein epitopes due to

Table 3
Pentosidine recovery by spiking 2 patient samples with 4 levels of standard.

Sample 1	Endogenous PEN (nM)	Added PEN amount [a] (nM)	Expected PEN amount (nM)	Measured PEN amount (nM)	Recovered PEN amount [b] (nM)	% Recovery (b/a) *100
neat		10.0	38.7	40.6	11.9	104.9
spike 1		50.0	78.7	77.6	48.9	98.6
spike 2	28.7	90.0	118.7	116.8	88.1	98.4
spike 3		130.0	158.7	144.4	115.7	91.0
spike 4		170.0	198.7	191.8	163.1	96.5
Sample 2	Endogenous PEN (nM)	Added PEN amount [a] (nM)	Expected PEN amount (nM)	Measured PEN amount (nM)	Recovered PEN amount [b] (nM)	% Recovery (b/a) *100
Neat		10.0	40.6	39.2	8.6	86.0
spike 1		50.0	80.6	78.5	47.9	97.4
spike 2	30.6	90.0	120.6	100.9	70.3	83.7
spike 3		130.0	160.6	145.5	114.9	90.6
spike 4		170.0	200.6	197.6	167.0	98.5

steric restrictions. Additionally, it has been shown that the 100 °C incubation (without the addition of an acid), required when using ELISA to detect PEN in serum or plasma, can result in de novo PEN formation, further affecting accurate quantitation (Nakano et al., 2013). Antibody specificity is difficult to define and ELISA is considered semi-quantitative (Nogajczyk et al., 2015; Teerlink et al., 2004).

With regards to HPLC, acid hydrolysis is required to extract PEN from protein prior to detection. This step creates artefacts that can interfere with PEN and reduces the sensitivity of HPLC (Odetti et al., 1992; Slowik-Zylka et al., 2004). Additionally, hydrolysis must be performed under a stream of nitrogen to prevent the reaction of sample components with oxygen (Odetti et al., 1992). Exposure to oxygen can create fluorescent products which interfere with the UV detection of PEN (O'Grady et al., 2020; Odetti et al., 1992).

Mass spectrometry (MS) is more precise than UV fluorescence detection. MS is not affected by contaminating fluorescent compounds or hydrolysis artefacts and does not require the use of antibodies. LC-MS/MS avoids the pitfalls of ELISA and HPLC while being both sensitive and specific. There are numerous publications in the literature that utilize LC-MS/MS to quantify AGEs in various tissues types and food products (Monnier et al., 2022; Nur et al., 2010; Odani et al., 2001; Rabbani and Thornalley, 2014; Soboleva et al., 2017). Carboxy-methyl-lysine (CML) and carboxy-ethyl-lysine (CEL) are two AGEs which have been frequently quantified using LC-MS/MS. Despite the advantages of using LC-MS/MS and its documented utility in studying other AGEs, the method has only recently been applied to PEN. For many decades, PEN has been the most intensely studied AGE because its fluorescent nature allowed for easy detection. While fluorescence detection is certainly an important tool in molecular biology, a shift towards newer, more sensitive detection methods, like LC-MS/MS, has begun.

Table 1 also compares the Protein Normalization methodologies commonly employed when quantifying PEN. PEN is typically protein-bound (hence the need for sample hydrolysis) and variations in sample protein content could lead to spurious results. For this reason, it is common practice to normalize quantification of PEN to sample protein content (Kerkeni et al., 2014; Thornalley et al., 2003; Thornalley and Rabbani, 2014). Protein quantification is an inherent step in ELISA optimization and equal amounts of protein per sample are assessed, so a true normalization calculation is not required. Current HPLC and LC-MS/MS methods typically normalize to collagen, albumin, total protein or lysine content of the sample. Similar to the heterogeneity of PEN quantification methods, there are many protein quantification methods utilized in the literature (O'Rourke et al., 2019). Protein quantification methods also vary depending on their appropriateness for the tissue or sample type being assessed. For the sake of simplicity, we have chosen to describe and report the methodology for the detection of PEN alone. True quantification would require further evaluation of sample protein content as well (O'Rourke et al., 2019).

5. Conclusion

Our intention in developing an LC-MS/MS method for PEN was to assess total PEN in frozen serum samples. We plan to employ this methodology in the future to examine the utility of PEN as an osteoporosis and fracture biomarker. It is encouraging to see LC-MS/MS emerging as a frequently used tool in AGE research. Our ability to more accurately quantify AGEs will only further expand our understanding of the pathological effects of these compounds and the role they play in osteoporosis.

Funding

L. Blencowe was supported by the Osteoporosis Canada PhD Studentship and the Canadian Federation of University Women Dr. Alice E. Wilson Award.

AM. Cheung is supported by a Tier 1 Canada Research Chair in Musculoskeletal and Postmenopausal Health.

CRediT authorship contribution statement

Lindsie A. Blencowe: Writing – review & editing, Writing – original draft, Investigation, Conceptualization. **Andrea Božović:** Writing – review & editing, Writing – original draft, Validation, Resources, Methodology, Investigation, Formal analysis. **Evelyn Wong:** Writing – review & editing, Conceptualization. **Vathany Kulasingam:** Writing – review & editing, Project administration, Conceptualization. **Angela M. Cheung:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

All authors declare no relevant conflicts of interest.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bonr.2024.101737.

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