

응용 자료

Development of a Quantitative SPE-LC-MS/MS Assay for Teriparatide in Human Plasma

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Abstract

This study provides a single, simple method for the quantification of teriparatide in human plasma. The method uses UPLC and fast, selective sample prep in 96-well format to achieve a limit of detection (LOD) of 15 pg/mL, a dynamic range of 15 to 500 pg/mL, and an average QC accuracy of 97.5% (across six sources of matrix) from 200 µL of human plasma.

Benefits

- Selective, fast extraction without time-consuming affinity purification
- More accurate and precise quantification than traditional LBA methods
- High sensitivity nearly equivalent to more time-consuming and laborious LBA methods
- Rapid analysis time increases productivity
- Specificity
- Excellent asset utilization allows the use of current LC/MS instrumentation

Introduction

Teriparatide (FORTEO) is a recombinant form of a fragment of human parathyroid hormone, used in the treatment of osteoporosis. As a major global health problem, osteoporosis is responsible for 1.5 million bone fractures each year. Teriparatide is the first treatment for osteoporosis that actually stimulates new bone formation. It is an anabolic drug that acts to strengthen bones with the potential to improve skeletal micro architecture, thereby increasing bone density. In contrast to bisphosphonate drugs that treat osteoporosis by reducing or preventing bone loss, teriparatide promotes bone production.

Teriparatide is comprised of the first 34 amino acids (the biologically active region) of the 84-amino acid human parathyroid hormone (PTH), also referred to as [rhPTH (1-34)].

Although biologics like teriparatide have historically been quantified using ligand binding assays (LBAs), recently there has been a trend toward the analysis of large molecules by LC-MS/MS. This is, in part, driven by the fact that LBAs can suffer from significant cross-reactivity issues and lack of standardization. LC-MS/MS offers the following benefits: shorter development times, greater accuracy and precision, the ability

to multiplex, and readily distinguish between closely related analogues, metabolites, or endogenous interferences. Large peptides, such as teriparatide, are particularly difficult to analyze by LC-MS/MS, as MS sensitivity is low due to poor transfer into the gas phase and poor fragmentation. In addition, teriparatide suffers from significant non-specific binding and poor solubility, making LC and sample preparation method development challenging. The pharmacokinetics of teriparatide are characterized by rapid absorption within 30 minutes and rapid elimination with a half-life of one hour, resulting in a total duration of exposure to the peptide of approximately four hours. At the practical clinical dose of 20 µg, typical teriparatide levels are ~50 pg/mL, making detection by LC-MS/MS even more difficult. Traditional LC-MS/MS assays for teriparatide have involved time-consuming and laborious immunoaffinity purification and/or multidimensional or nano-flow LC. This study provides a single, simple method for the quantification of teriparatide in human plasma, as shown in Figure 1. The method uses UPLC and fast, selective sample prep in 96-well format to achieve a limit of detection (LOD) of 15 pg/mL, a dynamic range of 15 to 500 pg/mL, and an average QC accuracy of 97.5% (across six sources of matrix) from 200 µL of human plasma.

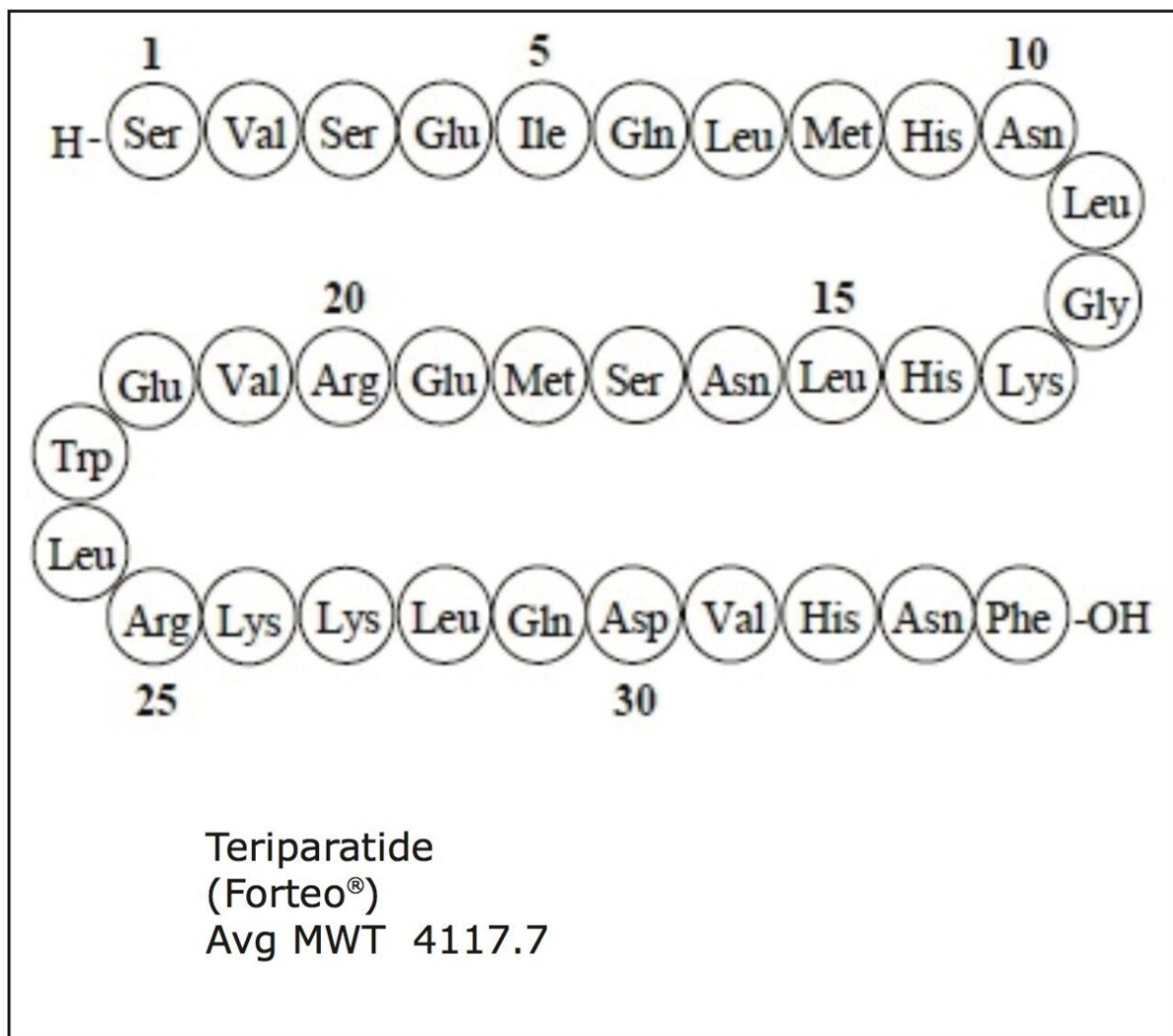


Figure 1. Representative structure and molecular weight of teriparatide.

Experimental

Sample Description

Step 1: Protein Precipitation (PPT): 20 μ L of human parathyroid (1–38), which was used as an internal standard (2 ng/mL), was added to 200 μ L of human plasma and mixed.

Samples were precipitated in a 1-mL 96-well plate with 200 μ L acetonitrile containing 5% ammonium

hydroxide, and centrifuged for 15 min at 4000 rpm.

The supernatant was transferred to a 2-mL 96-well plate containing 1 mL of water then mixed.

Step 2:	Solid-phase extraction (SPE) using an Oasis HLB μ Elution 96-well plate (p/n 186001828BA)
Condition:	200 μ L methanol
Equilibrate:	200 μ L water
Load sample:	Entire diluted PPT supernatant (~1.4 mL) was loaded onto the extraction plate in two steps
Wash:	200 μ L 5% methanol
Elute:	2 x 25 μ L 60:34:5:1 acetonitrile/water/trifluoroethanol/ trifluoroacetic acid
Dilute:	50 μ L water
Inject:	30 μ L

UPLC conditions

System:	ACQUITY UPLC
Column:	ACQUITY UPLC CSH C ₁₈ , 2.1 x 50 mm, 1.7 μ m (p/n 186005296)
Mobile phase A:	0.1% HCOOH in water
Mobile phase B:	0.1% HCOOH in acetonitrile
Gradient:	Start at 15% B and hold for 0.2 min, linear ramp

	to 50% B at 3.8 min, flush column at 98% B for 0.6 min and return to initial
Flow rate:	0.4 mL/min
Column temp.:	60 °C
Sample temp.:	15 °C
Injection volume:	30 µL
Run time:	5.5 min
Collection plates:	Waters 1-mL ACQUITY collection plates

MS conditions

Mass spectrometer:	Xevo TQ-S
Ionization mode:	ESI positive
Capillary voltage:	3.0 kV
Desolvation temp.:	600 °C
Cone gas flow:	150 L/h
Desolvation gas flow:	1000 L/h
Collision cell pressure:	3.8×10^{-3} mbar
Collision energy:	Optimized by component, see Table 1
Cone voltage:	Optimized by component, see Table 1

Data management

Chromatography software: MassLynx

Quantification software: TargetLynx

Peptide	MRM Transition	Cone Voltage (V)	Collision Energy (eV)
Teriparatide	687.05 > 787.26	45	18
	824.25 > 983.79	45	25
Human Parathyroid 1–38 (ISTD)	637.58 > 712.61	45	10
	892.22 > 854.80	45	22

Table 1. MS conditions for teriparatide and internal standard.

Results and Discussion

Several multiple-charged precursors were observed for teriparatide and human parathyroid hormone 1–38 (ISTD), with spectra shown in Figure 2. MS/MS spectra for the three most abundant precursors for teriparatide, obtained at their optimal collision energies, are shown in Figure 3. The 6+ charge state of teriparatide at m/z 687 was determined to be the most intense and yielded a selective fragment at m/z 787 for quantitative analysis. The 7+ precursor at m/z 589 was also intense, but did not yield any usable fragments. CID of the 5+ precursor at m/z 824 produced fragment ions of sufficient intensity to be used for confirmatory purposes. Although many peptides produce intense fragments below m/z 200, these ions (often immonium ions) result in high background in extracted samples due to their lack of specificity. In this assay, the use of highly specific y ion fragments above m/z 700 yielded significantly improved specificity, facilitating the use of simple LC and SPE methodologies.

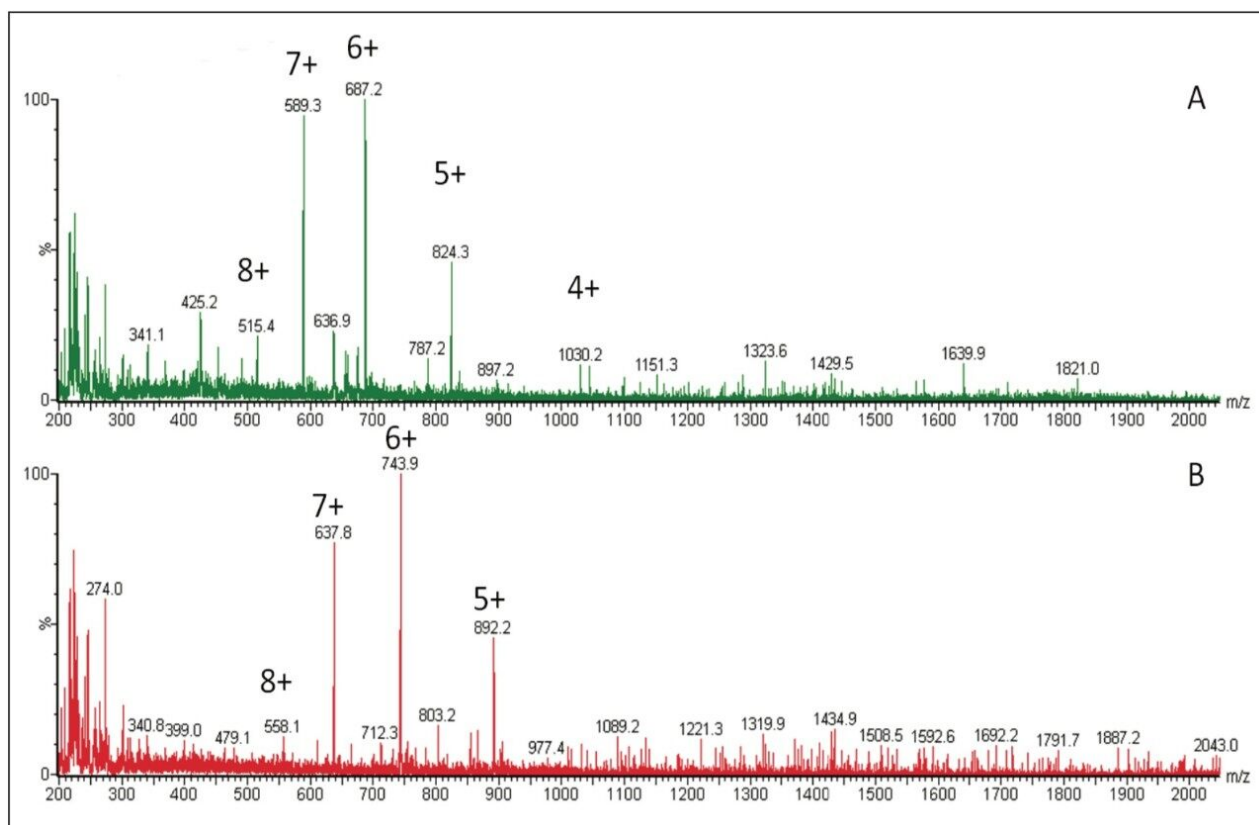


Figure 2. MS spectra of (A) teriparatide and (B) human parathyroid 1-38.

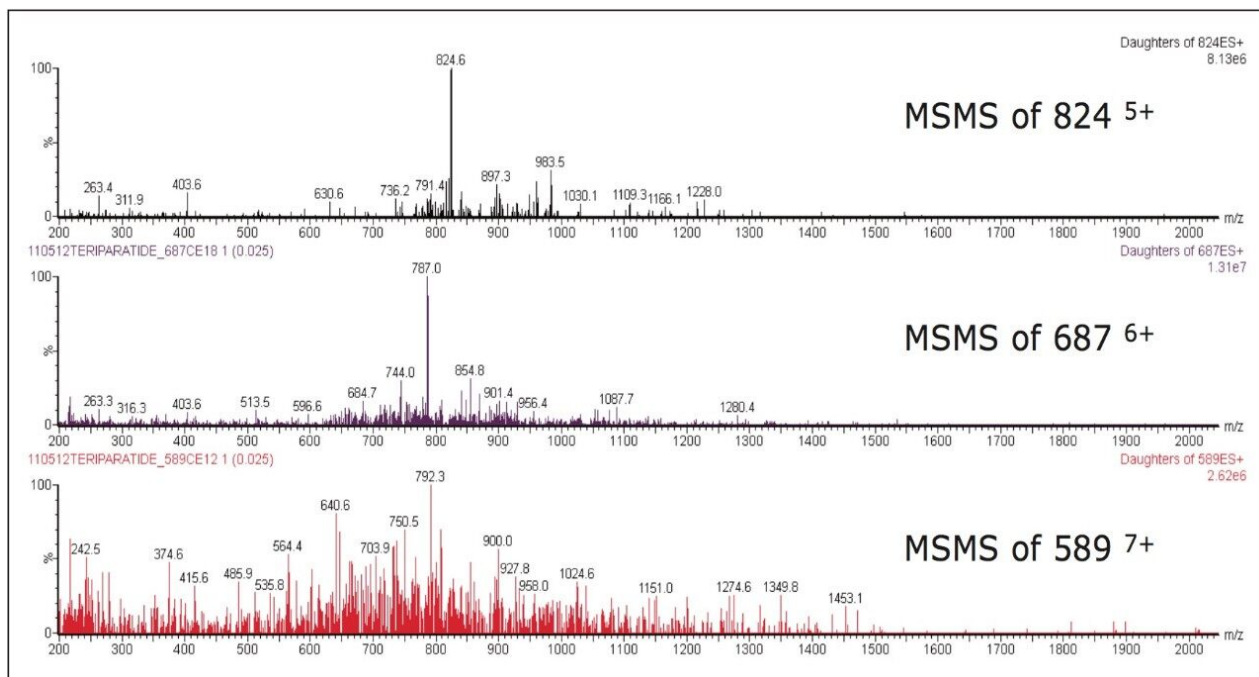


Figure 3. MS/MS spectra of teriparatide.

Narrow peak widths were obtained for teriparatide and the ISTD, using a novel charged surface hybrid (CSH) column. The resulting separation of a 125 pg/mL QC sample is shown in Figure 4. Peak widths at base are <6 s wide.

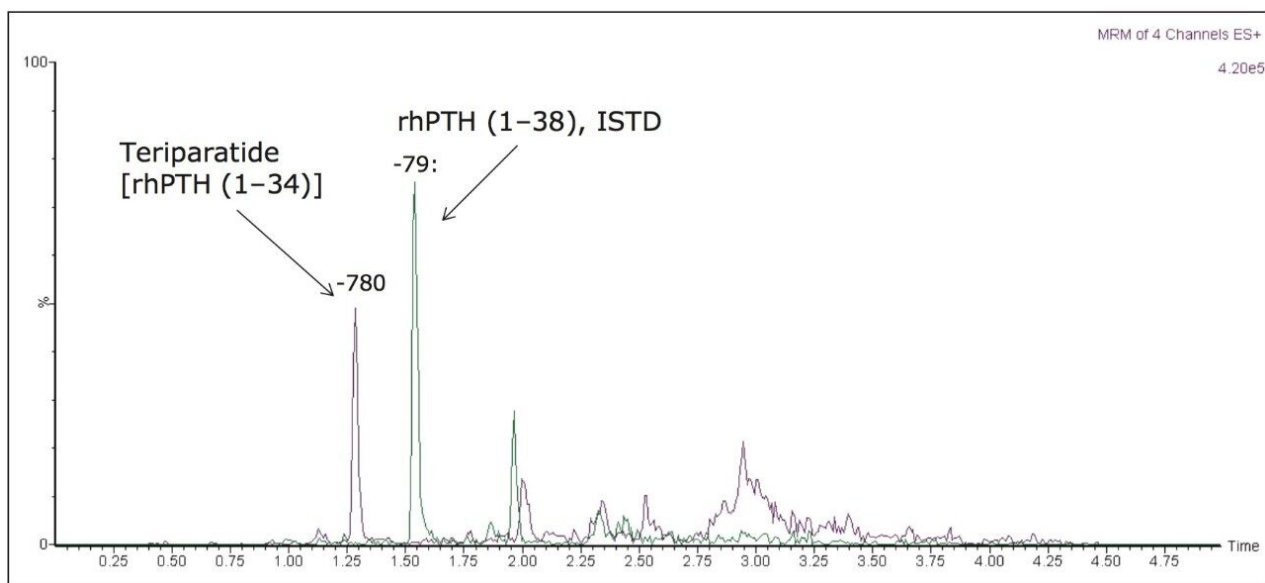


Figure 4. UPLC separation of teriparatide and internal standard, from a 125 pg/mL extracted plasma sample, using a 2.1 x 50 mm ACQUITY UPLC CSH column.

Development of this assay was challenging due to a high degree of non-specific binding (NSB) and difficulty maintaining peptide solubility throughout the SPE extraction and elution processes. Sample pre-treatment prior to SPE proved to be critical to improving recovery and specificity. Protein precipitation (1:1) with 5% NH₄OH acetonitrile resulted in 80% to 100% recovery without precipitating the peptide itself. Protein precipitation with higher organic ratios resulted in peptide loss due to undesirable precipitation of teriparatide. The PPT pre-treatment minimized protein binding and eliminated endogenous interferences from large proteins, such as albumin. The supernatant was then applied to conditioned SPE plates, and analytes were well retained on the SPE sorbent during the basic pH load step, with no breakthrough occurring. Optimization of the elution solution was critical to fully elute teriparatide, maintain its solubility, and minimize interferences from the plasma matrix. The optimum elution solution was 60% organic, with 1% trifluoroacetic acid and 5% trifluoroethanol (TFE), the latter being added to maintain solubility of the compound. The addition of TFE also aided in sensitivity and increased the MS signal by 50%. The combination of proper MS fragment choice, selective SPE cleanup, and optimal LC column enabled detection and quantification limits of 15 and 30 pg/mL, respectively, in all six lots of control plasma tested.

Figure 5 contains a representative chromatogram for low level QC's samples containing teriparatide at 20, 35, and 75 pg/ml extracted from human plasma as compared to blank extracted plasma. Figure 6 is a representative extracted standard curve for teriparatide, from 15 to 500 pg/mL, in human plasma. Finally, the standard curve and QC statistics for teriparatide are summarized in Tables 2 and 3.

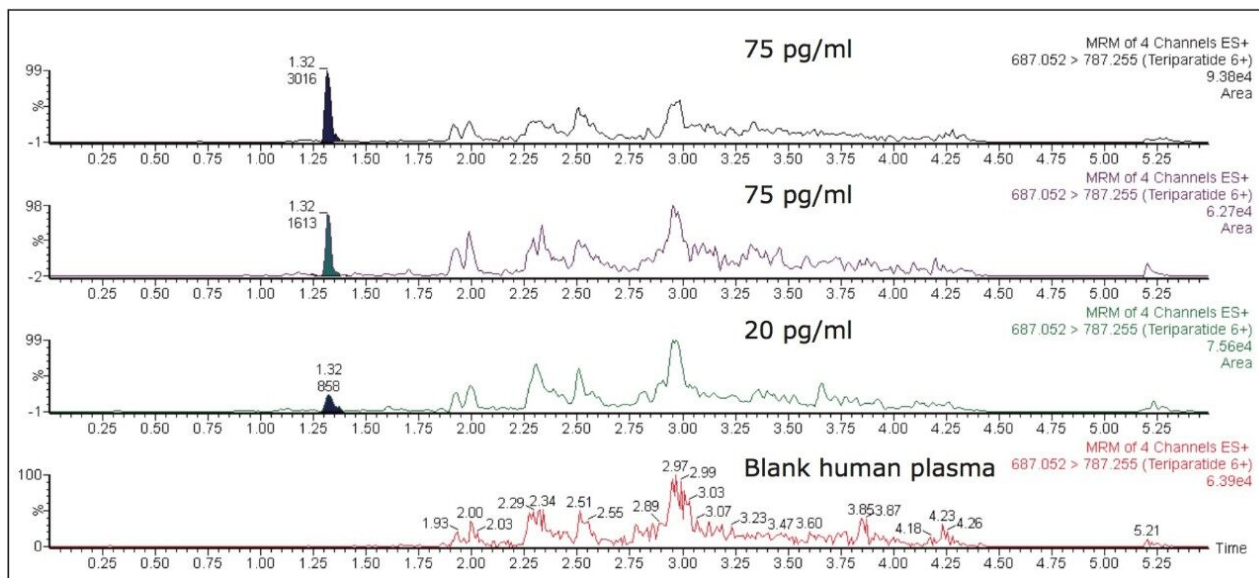


Figure 5. Representative chromatograms of teriparatide extracted from human plasma at 20, 35, and 75 pg/mL compared to extracted blank plasma.

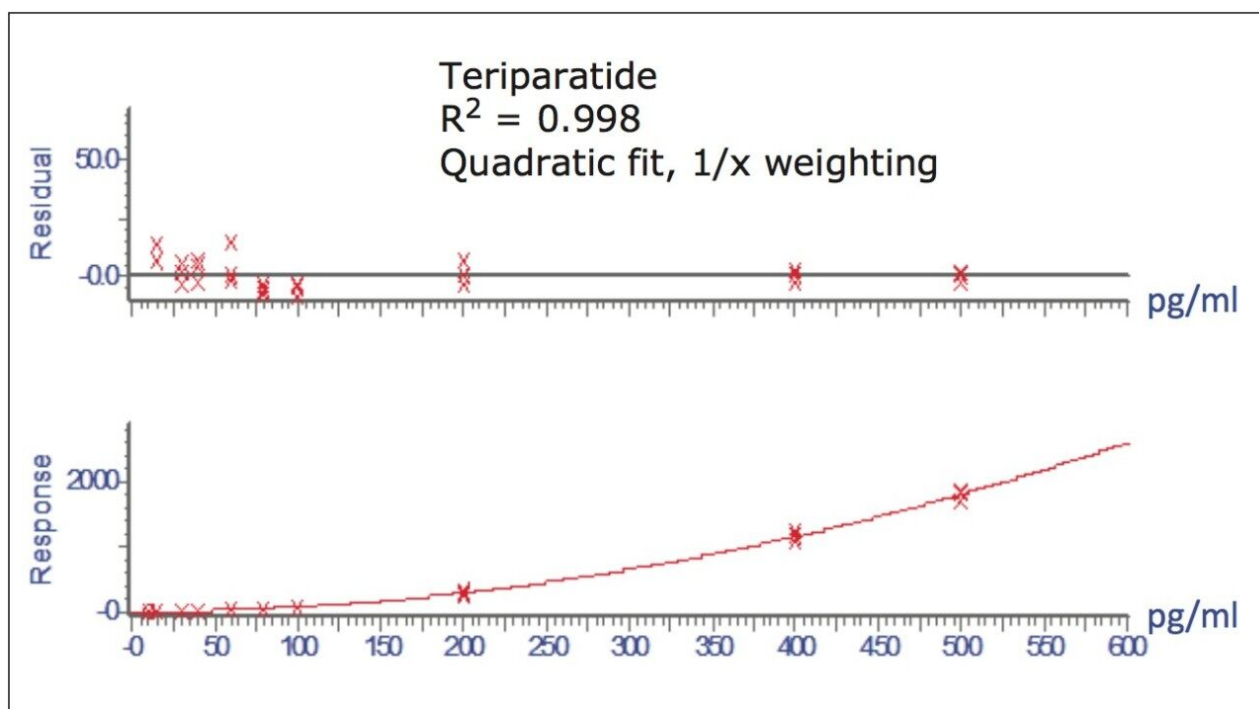


Figure 6. Representative standard curve of teriparatide extracted from human plasma, ranging from 15 to 500 pg/mL.

Human Plasma Lot# X1793C (Biological Specialty Corp.)	Std. Conc. (pg/mL)	Mean Calculated Conc. (pg/mL)	Std. Deviation	%CV	Number of Replicates Passed	Mean Accuracy
15 pg/ml	15	16.65	0.64	3.87	3/3	111.03
30 pg/ml	30	30.48	1.39	4.56	3/3	101.60
40 pg/ml	40	41.09	1.95	4.75	3/3	102.70
60 pg/ml	60	62.78	5.20	8.28	3/3	104.63
80 pg/ml	80	75.38	1.56	2.07	3/3	94.23
100 pg/ml	100	94.39	2.78	2.94	3/3	94.37
200 pg/ml	200	202.60	10.63	5.24	3/3	101.27
400 pg/ml	400	401.90	10.86	2.70	3/3	100.50
500 pg/ml	500	498.47	10.36	2.08	3/3	99.67

Table 2. Representative standard curve of teriparatide extracted from human plasma.

Human Plasma Lot# (Biological Specialty Corp.)	Gender	QC Conc. (pg/mL)	Mean Calculated Conc. (pg/mL)	Std. Deviation	%CV	Number of Replicates Passed	Mean Accuracy
X1793C	Mixed	20	20.25	1.50	7.3	3/3	102.2
82111	Female	20	33.38	1.75	8.6	3/3	102.2
57298	Male	20	18.30	0.67	3.7	3/3	91.5
82740	Female	20	20.74	1.59	7.7	3/3	103.7
57901	Male	20	19.67	0.60	3.0	3/3	98.3
X1803C	Mixed	20	21.27	0.96	4.5	2/3	106.4

Human Plasma Lot# (Biological Specialty Corp.)	Gender	QC Conc. (pg/mL)	Mean Calculated Conc. (pg/mL)	Std. Deviation	%CV	Number of Replicates Passed	Mean Accuracy
X1793C	Mixed	35	33.35	1.99	6.0	2/3	95.3
82111	Female	35	34.48	1.84	5.3	3/3	98.5
57298	Male	35	31.64	1.81	5.7	3/3	90.4
82740	Female	35	33.29	1.73	5.2	3/3	95.1
57901	Male	35	34.71	0.30	0.9	3/3	99.2
X1803C	Mixed	35	34.24	2.66	7.8	3/3	97.8

Human Plasma Lot# (Biological Specialty Corp.)	Gender	QC Conc. (pg/mL)	Mean Calculated Conc. (pg/mL)	Std. Deviation	%CV	Number of Replicates Passed	Mean Accuracy
X1793C	Mixed	75	75.07	1.17	1.6	3/3	100.1
82111	Female	75	73.29	4.23	5.8	3/3	97.7
57298	Male	75	71.92	3.50	4.9	3/3	95.9
82740	Female	75	70.34	5.14	7.3	2/3	93.8
57901	Male	75	72.30	5.28	7.3	3/3	96.4
X1803C	Mixed	75	69.63	3.25	4.7	3/3	92.8

Human Plasma Lot# (Biological Specialty Corp.)	Gender	QC Conc. (pg/mL)	Mean Calculated Conc. (pg/mL)	Std. Deviation	%CV	Number of Replicates Passed	Mean Accuracy
X1793C	Mixed	125	131.21	2.73	2.1	3/3	105.0
82111	Female	125	130.01	1.67	1.3	3/3	104.0
57298	Male	125	125.82	1.42	1.1	3/3	100.7
82740	Female	125	117.34	6.94	5.9	3/3	93.9
57901	Male	125	115.21	5.42	4.7	3/3	92.2
X1803C	Mixed	125	113.70	4.92	4.3	3/3	91.0

Human Plasma Lot# (Biological Specialty Corp.)	Gender	QC Conc. (pg/mL)	Mean Calculated Conc. (pg/mL)	Std. Deviation	%CV	Number of Replicates Passed	Mean Accuracy
X1793C	Mixed	250	262.26	5.14	2.0	3/3	104.9
82111	Female	250	265.24	1.77	0.7	3/3	106.1
57298	Male	250	244.54	15.75	6.4	3/3	97.8
82740	Female	250	225.71	11.42	5.1	3/3	90.3
57901	Male	250	232.51	12.58	5.4	3/3	93.0
X1803C	Mixed	250	186.76	67.42	36.1	3/3	89.3

Table 3. QC statistics from teriparatide extracted from six lots of human plasma.

Conclusion

- An SPE extraction method was developed for teriparatide from human plasma. The μ Elution format SPE plate eliminates the need for evaporation, reducing teriparatide losses due to adsorption and non-specific binding.
- A fast, simple, analytical-scale LC method was developed for separation of teriparatide with a total LC cycle time of 5.5 minutes.
- Detection and quantification limits of 15 and 30 pg/mL were achieved for teriparatide extracted from only 200 μ L of human plasma.
- Standard curves were accurate and precise from 15 to 500 pg/mL.
- QC samples at all levels, from six individual sources of human plasma easily passed FDA regulatory criteria, with mean accuracies ranging from 90% to 106%.
- This study demonstrates the importance of column chemistry, sample pre-treatment, addressing non-specific binding, concentration without evaporation, and proper fragment choice.

- The method shows promise for high sensitivity quantification of patient samples from PK studies or clinical trials using LC-MS/MS.
- Average CV of all points on three independent standard curves was 4%, average accuracy was 101% indicating a very reproducible and accurate method.

References

1. Eli Lilly and Company (2009) Teriparatide [rhPTH(1-34)] (FORTEO) United States package insert.
2. Satterwhite J, Heathman M, Miller PD, Marín F, Glass EV, Dobnig H. Pharmacokinetics of teriparatide (rhPTH[1-34]) and calcium pharmacodynamics in postmenopausal women with osteoporosis. *Calcif Tissue Int* 2010 Dec;87(6):485-92.

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