Biosimilar Characterization
- Teriparatide

Teriparatide is the only currently available anabolic agent in the osteoporosis market that provides remediation against glucocorticoid-induced osteoporosis. The patent expiry of the originator product, Eli Lilly's Forteo/Forsteo, in August 2019 had piqued the interest of biosimilar developers worldwide. Number of Teriparatide biosimilars are already in various stages of development. To be demonstrated as 'biosimilar', regulatory approval of these biosimilars will depend heavily on the quality and extensiveness of the physicochemical and functional characterization studies. Solution providers having in-depth understanding of biopharma characterization and focusing solely on providing analytical characterization packages, that comprehensively compare innovator and biosimilar product, offers an added advantage to the biosimilar industry. In this study we discuss some of the tests that were utilized to characterize Teriparatide.

Osteoporosis is a disease characterized by low bone mass and deterioration of bone tissue, which leads to an increased risk of fracture. Some of the currently available medications for the treatment of osteoporosis includes Bisphosphonates, Prolia (Denosumab), Forteo (Teriparatide), Estrogen-Like Drugs and Fortical (Calcitonin).

Osteoporosis market currently is dominated by generic bisphosphates and is likely to undergo significant changes over the next 10 years due to the launch of biosimilar anabolic (bone-forming) agents. The Global osteoporosis market is expected to reach a sale of $11.2 bn by 2027, 33% market share of which will be occupied by the biosimilars sales. *(Reference: https://www.pharmaceutical-technology.com/comment/global-osteoporosis-market-reach-11-2bn-2027)* One such effective anabolic agent is Teriparatide which is a recombinant form of parathyroid hormone (PTH).

In terms of the primary structure the first 34 amino acid of Teriparatide are identical to the bioactive portion of PTH. Teriparatide is used in the treatment of osteoporosis in postmenopausal women and men at high risk for fracture. Intermittent use of Teriparatide activates osteoblasts (bone building cells) more than osteoclasts, which stimulates new bone growth and leads to an overall increase in bone density.

The innovator product, Eli Lilly's Forteo/Forsteo (Teriparatide), was approved by the USFDA in November 2002 and by the EMA in June 2003. The patents for Forteo/Forsteo will expire in August 2019.

Some of the Teriparatide Biosimilars in various stages of regulatory approvals are listed in Table-1. Other leading biosimilar developers are also gearing up to take advantage of the patent's expiry.

The regulatory approval for these biosimilars will depend on the array of data generated through the comparative analytical, *in-vivo* and clinical studies. Most crucial factor in establishing the bio-similarity will be the detailed analytical (structural and functional) characterization and comparison of the biosimilar products with the innovator's product. A full-blown characterization study, a key component of the CMC has therefore been the prime focus of analytical companies like Shantani, which has the in-depth understanding and extensive experience of providing bio-analytical solutions.

Shantani utilizes well established workflows to generate high quality analytical data, as per the ICH-Q6B guidelines. Our aim is to support the regulatory requirements of biopharma developers, by providing

Saba Naaz Siddique
Director-Sales
saba@shantani.com

Chaitanya Saxena
CEO
csaxena@shantani.com

Shantani Proteome Analytics Pvt., Ltd.
Pune

comprehensive comparability analysis based on the totality of the analytical evidences.

In this segment, we will showcase a few examples of primary and secondary structure characterization and comparability studies between the Biosimilar and Reference Teriparatide samples; which were performed at Shantani using well optimized Standard Operating Procedures (SOPs).

Intact Mass-Analysis using ESI-MS

Methodology

Proteins from the samples were precipitated using Trichloroacetic Acid based protein precipitation method. 10 µg of protein sample was passed through a C4 resin HPLC-CHIP and eluates were infused into a Q-TOF mass-spectrometer over a period of 10 minutes. The mass over charge spectra of the sample was obtained and the mass of the protein sample was calculated by deconvoluting the charges using vendor provided software which uses Peak Modelling Charge Deconvolution method (pMod), an enhanced deconvolution algorithm based on Maximum Entropy deconvolution.

Analysis

Based on the chemical composition of Teriparatide ($C_{181}H_{291}N_{55}O_{51}S_2$), its theoretical molecular mass is 4117.72Da. Intact mass of the samples were analyzed using Electrospray Ionization-Mass Spectrometry (ESI-MS) based method. Mass of the observed +4, +5 and +6 charged species (Figure 1 and 2) was deconvoluted.

Results

The mass of the biosimilar and reference sample was found to be 4117.17 Da which is very close to the theoretical predicted mass of the samples. The deconvoluted molecular mass of the samples are tabulated in Table-2.

Peptide Mapping using LC-MS/MS

Methodology

Step-1: Protein Reduction, Alkylation and Digestion with Trypsin: 20 µg of protein was taken from the samples and was precipitated by adding four volumes of chilled acetone. The mixture was kept for 1 hour at -20°C. and then centrifuged at 10000 RPM for 10 minutes at 4^0C. Supernatant was removed and the protein pellets were air-dried briefly. The pellets obtained were then dissolved in 20 µl of the reduction buffer (8 M Urea in 20 mM NH_4HCO_3 and 10 mM Dithiothreitol).

Company, Country	Product Name	Approval Status
Intas Pharmaceuticals, India	Teriparatide (Terifrac)	'Similar biologic' approved in India in November 2010
Cadilla Healthcare, India	Teriparatide (Bonmax)	'Similar biologic' approved in India in August 2012
USV Private Limited, India	Teriparatide	'Similar biologic' approved in India in August 2012
Gedeon Richter/Mochida Pharmaceutical, Hungary/Japan	Terrosa	Approved by EMA in November 2016. In-licensed by Mochida for Japanese market
StadaArzneimittel, Germany	Movymia	Approved by EMA in November 2016.
Pfenex, USA	PF708	Expects to submit NDA to the USFDA in the third quarter of 2018. Entered in agreement with Alvogen for commercialization in USA. Out licensed to China NT Pharma Group to market in Asia.

Table 1. *Teriparatide Biosimilar and Stage of Marketing Approval*

(Reference: http://www.gabionline.net/Biosimilars/General/Biosimilars-of-teriparatide)

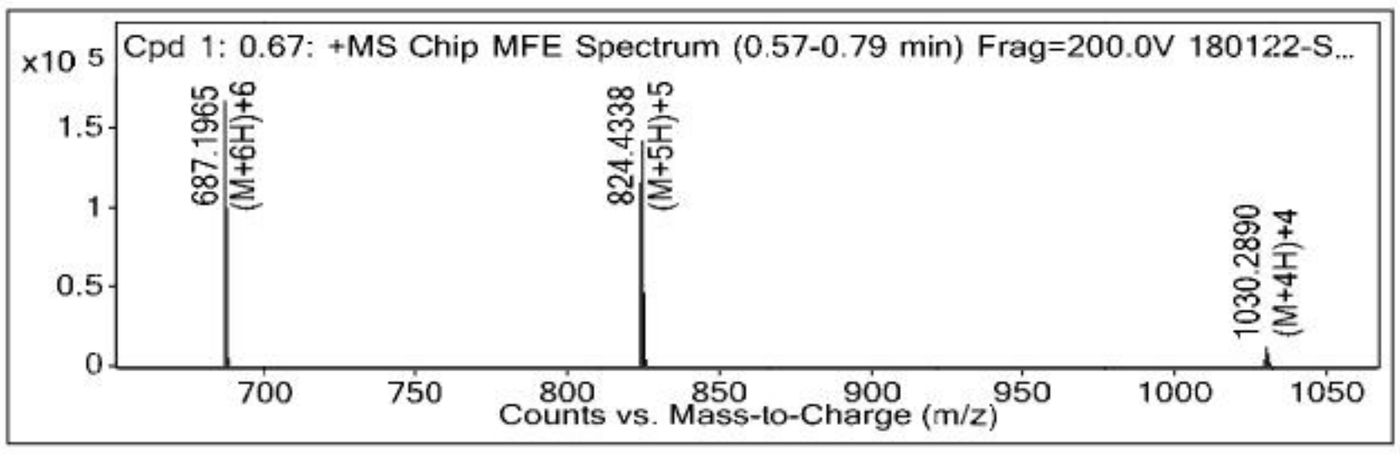

Fig. 1: Intact Mass of Biosimilar Teriparatide Sample.

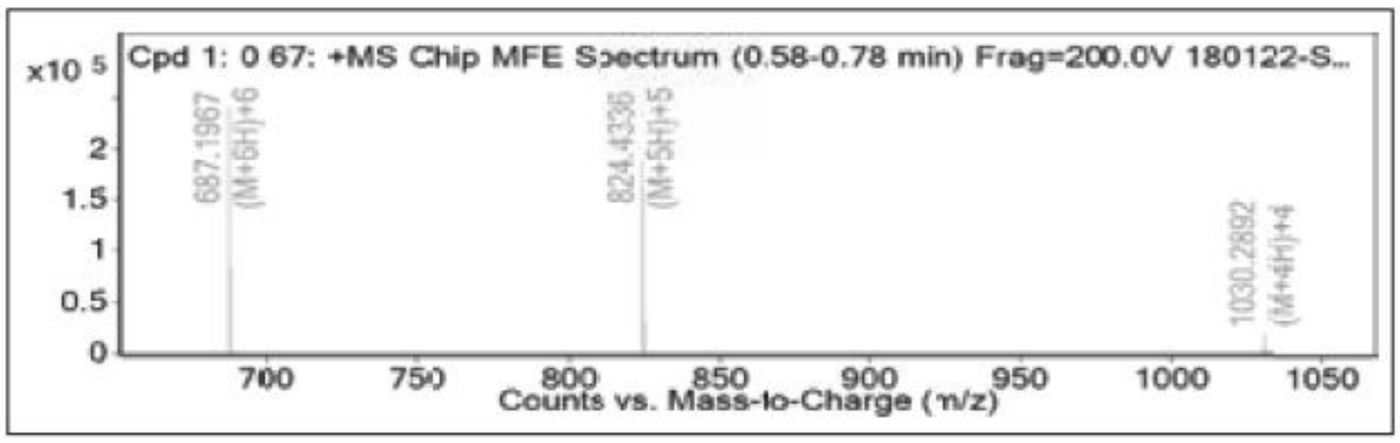

Fig. 2: Intact Mass of Reference Forteo Sample.

Samples were further denatured and reduced by heating at 70^{0}C for 5 minutes. Post denaturation, sample solution was diluted to 100 µl with 20 mM ammonium bi-carbonate. Protease trypsin was added in the ratio of 1:20 (Protein:Substrate) and proteolysis was carried out using an optimized, ultrafast protein digestion method, for 5 minutes at 37^{0}C. After 5 minutes, the digestion was quenched by adding 10% Trifluoroacetic Acid (1:10 ratio). Peptides were concentrated close to dryness and were dissolved in 10 µl of 0.1% Trifluoroacetic Acid. 5 µl of this sample was injected onto mass-spectrometer for peptide identification purposes.

Step-2: Peptide Separation and Mass-Spectrometry based Analysis: Obtained peptides were separated using reverse-phase liquid chromatography using 1-hour gradient and eluate was directly infused into ESI-Q-TOF mass-spectrometer. Peptides MS and MS/MS spectra were acquired, and the compiled mass list was searched against the protein sequence and theoretical trypsin protein digest of Teriparatide.

Analysis

Peptide search was carried out using morpheus software tools using the amino acid sequence of parathyroid hormone precursor. Protein Identification was performed with the following criteria: (a) Trypsin digested peptides with 2 missed cleavages allowed, (b) Peptide tolerance < 10 ppm, (c) FDR < 1% and (d) Free N-terminus Tryptic cleavage.

Results

A schematic of peptides obtained from theoretical trypsin digestion is shown the given Figure 3. The peptide search resulted in the amino acid coverage of 100% of the known sequence of Teriparatide, confirming the identity of the protein to be Teriparatide. T-9 peptide was not identified in biosimilar sample, however, covering similar sequence as of T-9, T-8 and T-10 peptides were identified that confirms that complete amino acid sequence of peptide is present in biosimilar sample. Mirror map of extracted ion chromatogram of the two samples (Figure 4) show significant similarity. The list of different peptides identified in both the samples are provided in Table-3.

S. No.	Sample	Theoretical Mass (dalton)	Deconvoluted Mass (dalton)
1.	Biosimilar Replicate-1	4117.72	4117.17
2.	Biosimilar Replicate-2	4117.72	4117.17
3.	Reference Replicate-1	4117.72	4117.17
4.	Reference Replicate-2	4117.72	4117.17

Table 2. *Deconvoluted Mass of Teriparatide Reference and Biosimilar*

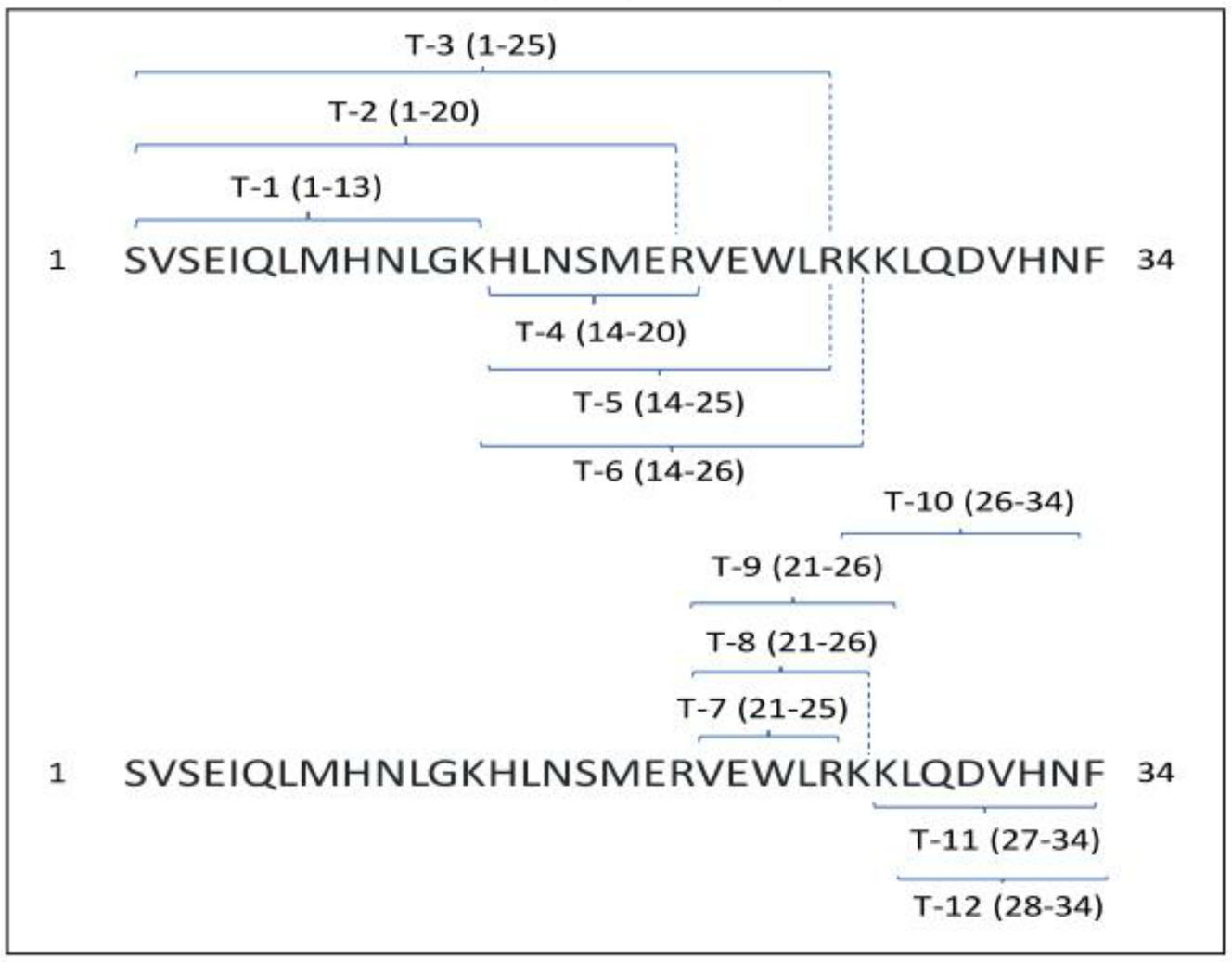

Fig. 3: Probable Tryptic Peptides that can be obtained from the sample.

Isoelectric focusing using AEC (Anion Exchange Chromatography)

Methodology

To generate a linear, descending pH gradient through AEC, a mixture of buffering components with 9.6 mM Tris base, 6 mM Piperazine and 11 mM Imidazole was prepared. The mixture was divided into two equal half's and pH was adjusted with 37% HCL, one with pH 6 and other to pH 9.5. Buffer A (pH 9.5) and Buffer B (pH 6) was used for generating the linear pH gradient. The samples were then precipitated using acetone precipitation method and precipitated protein samples were reconstituted in water. Protein concentration was estimated using Bradford method and equal amount of protein was used for the experiment. The HPLC Run Conditions are shown in Table 4.

Analysis

Ion exchange chromatography, that allows charged based retention of biomolecules on a chromatographic surface (Yamamoto *et al*. 1999), was utilized in measuring and comparing the isoelectric point of biosimilar and reference sample. The reconstituted samples were diluted in high pH (pH 9.5) buffer before HPLC analysis. In this condition it is expected that sample will have a net negative charge. Samples were then injected on a cation exchange HPLC column equilibrated at pH 9.5 and later eluted from the column using a linear pH gradient.

Results

The chromatographic profile of the samples is shown in the Figure 5. From the chromatographic profile it is evident that > 99.8% of the biosimilar and reference sample eluted at the same retention time of 29.1±0.1 minutes. Based on the pH gradient, at this retention time, pH in the system was in the range of 7.4±0.2. Theoretical isoelectric point of Teriparatide is in the range of 8.2 to 8.4, however, precipitation and de-formulation of peptide may partially alter its charge profile. Considering that both the reference sample and the biosimilar were treated and de-formulated using identical protocol, the analytical procedure adopted should affect both the product similarly. It is evident from the Figure-5 that the chromatographic profiles of the biosimilar and the reference samples are similar, and it confirms that the net charge on two samples are similar.

Far-UV CD Spectroscopy

Methodology

For acquiring CD spectra in Far-UV region, buffer of the samples was exchanged with HPLC water using Gel-filtration chromatography. De-formulated samples were diluted to 40 µg / ml in 40% Methanol: HPLC Water system. The CD spectra of the protein was collected using with following instrument settings:

Measure Range Wavelength	:250-190 nm
D.I.T.	:1 sec.
Slit Width	:1 mm
CD detector	:PMT (voltage set to Auto)
Accumulations	:5

Tryptic Peptide Sequence	Tryptic Peptide Number	Start position of Amino Acid	End position of Amino Acid	Peptide De-convoluted Mass (amu)	Reference Sample (Forteo)	Biosimilar Sample (Teriparatide)
SVSEIQLMHNLGK	T-1	1	13	1454.76	Identified	Identified
SVSEIQLMHNLG-KHLNSMER	T-2	1	20	2322.16	Identified	Identified
SVSEIQLMHNLG-KHLNSMERVEWLR	T-3	1	25	3005.53	Identified	Identified
HLNSMER	T-4	14	20	885.41	Identified	Identified
HLNSMERVEWLR	T-5	14	25	1568.79	Identified	Identified
HLNSMERVEWLRK	T-6	14	26	1696.88	Identified	Identified
VEWLR	T-7	21	25	701.39	Identified	Identified
VEWLRK	T-8	21	26	829.48	Identified	Identified
VEWLRKK	T-9	21	27	957.58	Identified	Not Identified
KKLQDVHNF	T-10	26	34	1127.61	Identified	Identified
KLQDVHNF	T-11	27	34	999.51	Identified	Identified
LQDVHNF	T-12	28	34	871.42	Identified	Identified

Table 3. *List of Identified Peptides.*

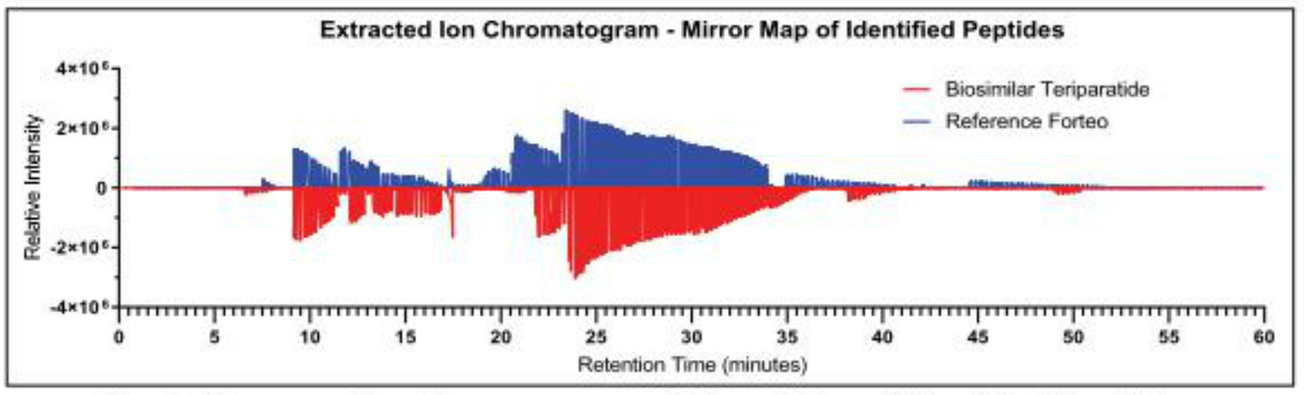

Fig. 4: Extracted Ion Chromatogram - Mirror Map of Identified Peptides.

Analysis

Baseline was acquired using 40% Methanol: HPLC water and a sample spectrum was acquired in baseline-subtracted mode. Ellipticity values were corrected for the concentration of protein and the path length of cuvette. Dichroport software was used to analyze the CD data, and curves were fitted with constrained least square self-consistent method (Sreerama *et al*. 1994) to obtain the value of alpha-helical, beta-pleated sheet and random coil conformations of protein.

Results

When the samples were analyzed in aqueous solution, CD spectrum showed only extended and disordered pattern suggesting absence of defined secondary structure (date not shown). This observation is consistent with the literature (Strickland *et al*. 1993). Yet, to probe the similarity at secondary structure level, CD spectra of the de-formulated samples were acquired in 40% Methanol (Figure 6).

In methanol, although, no major increase in ellipticity due to parallel n -> Π* transition (negative maximum at ~222 nm) and Π -> Π* parallel (negative maximum at ~208 nm) was observed, however, a clear increase in Π -> Π* perpendicular transition (positive maxima at ~192 nm) was recorded in both, the Biosimilar and the reference sample. Spectrum was analyzed with the Dichroport software following self-consistent method. In Biosimilar and reference samples 6 ± 2% and 8 ± 2% alpha helix components were recorded respectively. Based on the observation that in aqueous solution both the samples show no defined secondary structure and in 40% methanol, both the samples equally respond to a forbidden transition (Π -> Π* perpendicular transition) suggest that both may have similar secondary structure.

Chromatographic Column:	Anion Exchange Column (250 X 4.1 mm), Particle Size– 10 µ, Stationary phase – PRP X 100, Pore size 100 Å		
Mobile Phase A (Solvent A):	9.6 mM Tris base, 6 mM Piperazine and 11 mM Imidazole in HPLC Grade water (pH = 9.5)		
Mobile Phase B (Solvent B):	9.6 mM Tris base, 6 mM Piperazine and 11 mM Imidazole in HPLC Grade water (pH = 6)		
Flow Rate:	1 ml / min		
Injection Volume:	5 µl		
Detection (Wavelength):	280 nm		
Gradient	Time	Pump/Controller	Concentration
	0.01 Min	Controller	Start
	10.00 Min	Solvent B	0%
	10.01 Min	Solvent B	0%
	40.00 Min	Solvent B	100%
	40.01 Min	Solvent B	0%
	50.00 Min	Controller	Stop

Table 4. *HPLC Conditions for generating linear pH Gradient and Analysis of IE point of Teriparatide*

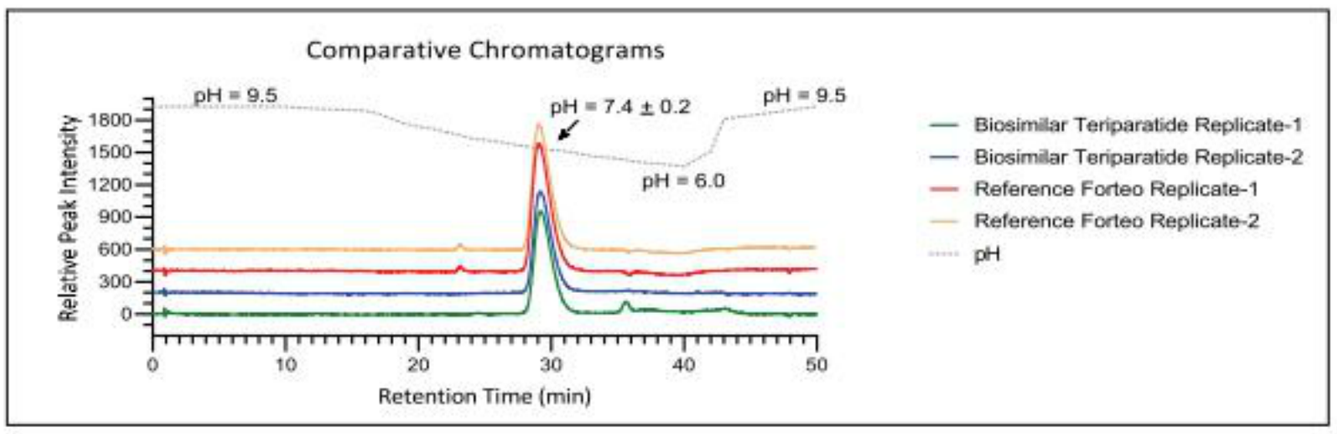

Fig. 5: Determination of Iso-Electric Points using Anion Exchange Chromatography (linear pH gradient)

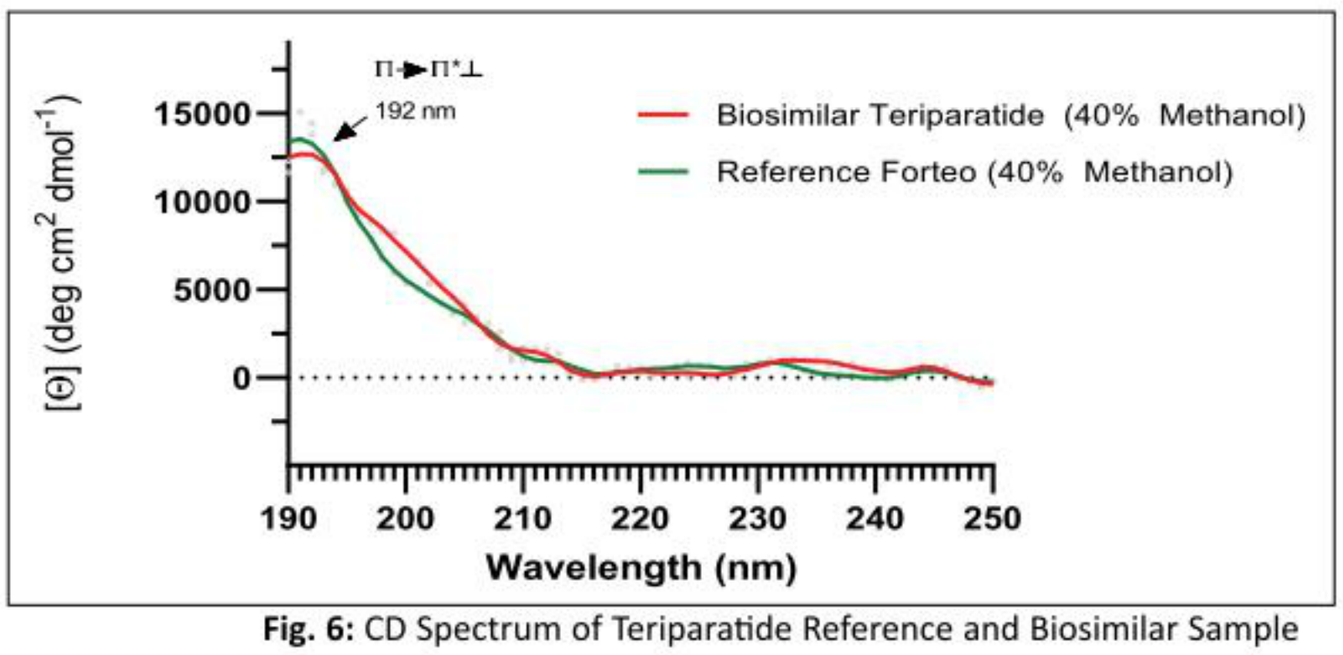

Fig. 6: CD Spectrum of Teriparatide Reference and Biosimilar Sample

Study Conclusion

Based on the studies carried out using LC-MS and LC-MS/MS, HPLC-CEX-UV, and Circular-Dichroism Spectroscopy based methods, physicochemical properties of Teriparatide Injection (Biosimilar Sample) and Forteo Teriparatide Injection (Reference Sample) was compared. It was concluded that the physicochemical properties of both the samples are highly similar.

Shantani's Strength

Shantani apply a 'totality of analytical evidences' approach to understand and establish the biosimilarity of the biopharma product. Shantani carry out experiments following the ICH-Q6B guidelines under a Quality Management System that confers to ISO 17025:2017. A team of dedicated, specialized protein scientists utilize their decades old expertise in analyzing data obtained from different techniques in making appropriate conclusions. Further, an extra effort is made to simplify and present the data in a manner that (a) does not compromise the integrity of detailed analysis but, yet (b) provide an impression that can be easily comprehended by biosimilar manufacturer and regulators alike. Moreover, Shantani's expertise in regulatory affairs and its consultative approach helps in developing product characterization dossier that can be directly utilized by biopharma manufacturer for the regulatory filings.

Acknowledgement

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References

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