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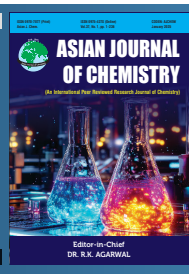

RP-HPLC Method for the Quantification of Impurities in Etelcalcetide Injection and a Comparative Characterization Study of In-House Samples of Etelcalcetide Injection with Reference-Listed Drug Samples

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In present study, an accurate and simple reversed phase-high performance liquid chromatography (RP-HPLC) method for the determination of potential impurities resulting from etelcalcetide injection was validated. An Ace Excel 3 C18 amide column with 100% 0.1 M sodium perchlorate (pH 2.0) buffer (100%) was used as mobile phase A and a 60:10 (%v/v) ratio of acetonitrile and 0.1 M sodium perchlorate (pH 2.0) was used as mobile phase B. The wavelength detection performed at 210 nm. Etelcalcetide injection was subjected to thermal, photolytic, acid, base, and peroxide degradation conditions, which were studied using the current methodology. The method was validated as per the International Council for Harmonization guideline (ICH). Consistent recoveries were obtained for all the impurities of etelcalcetide between 85% and 105%. Furthermore, comparative characterization studies and impurity profiling were successfully performed for in-house samples of etelcalcetide with reference-listed drug (RLD) samples through circular dichroism (CD), Fourier transform infrared (FTIR), size exclusion chromatography-high performance liquid chromatography (SEC-HPLC) and liquid chromatography-high resolution mass spectrometry (LC-HRMS), results indicates that found similar in both in-house and RLD samples.

Keywords: Etelcalcetide injection, Circular dichroism, SEC-HPLC.

INTRODUCTION

Secondary hyperparathyroidism (SHPT) is a problem encountered in the care of hemodialysis patients [1-3]. The conditions of hypophosphatemia, hypocalcaemia and a reduction in vitamin D production that accompany renal failure lead to increased synthesis and secretion of parathyroid hormone (PTH). While this increase may be viewed as a compensatory mechanism for the impaired metabolism of minerals and vitamin D, the increase in SHPT may occur independently of PTH. The expression levels of calcium-sensing receptors (CaSR) and vitamin-D receptors progressively decrease during parathyroid hyperplasia [4,5], potentially explaining the resistance to vitamin D receptor activators observed in SHPT.

Etelcalcetide is a newly developed calcimimetic agent used to treat SHPT in elderly dialysis patients [6-8]. Etelcalcetide is an octapeptide consisting of seven D-amino acids arranged in a row. Significantly, whereas certain peptides, such as protamine and poly-L-arginine, are recognized as agonists of calcium-sensing receptors (CaSRs), etelcalcetide functions as an allosteric modulator with a distinct signaling effect. It establishes a covalent disulfide connection with Cys482 in the extracellular domain of CaSR [9]. This bond is a covalent interaction that allows the exchange of thiols with other proteins and small molecules without sulfhydryl groups. Furthermore, CaSR persists in its development as additional receptors are introduced to the cell surface, indicating that the covalent modifications are temporary rather than permanent.

There is a growing interest in peptide drugs within the pharmaceutical sector, fuelled by advancements in their design and manufacturing processes. Peptides constitute a unique class biopharmaceutical compounds that harness the benefits of both

small molecules and protein therapeutics. They combine the advantageous size of small molecules, which facilitates cell membrane penetration, with the high specificity of proteins, enabling them to effectively interfere with protein–protein interactions (PPIs) and target or inhibit intracellular molecules. The increasing complexity of peptides is becoming crucial in targeting various diseases [10]. High-performance liquid chromatography is widely recognized as the standard approach for analyzing peptide drugs, employing techniques such as reverse-phase chromatography [11-13], ion-exchange chromatography [14] and size exclusion chromatography [15].

The primary sequence of etelcalcetide is Ac-D-Cys-D-Ala-D-Arg-D-Arg-D-Arg-D-Ala-D-Arg-NH_2-H-Cys-OH. The characteristics of peptide molecules necessitate particular considerations in the development of relevant analytical methodologies. The challenge for small molecules lies in their ability to blind to larger sites, whereas proteins and antibodies are restricted from crossing the cell membrane to reach these targets. Moreover, the formulation of peptides for therapeutic purposes encounters specific scientific hurdles that stem from the distinct physio-chemical traits of peptide molecules [16]. Identifying suitable characterization tools to develop peptide formulations with the right properties for safe and effective therapeutic applications is vital. Nonetheless, the high complexity inherent in their chemical structures poses substantial challenges for effective quantification and characterization. A thorough understanding of the structure and physio-chemical properties is essential for maintaining quality, stability and consistency across various batches.

To date, no methodologies have been established for the determination of potential impurities of etelcalcetide injection and characterization, including within the United States Pharmacopoeia (USP) and European Pharmacopoeia (EP). Thus, in this work, a RP-HPLC method is described for the sepearation of the potential impurities of etelcalcetide injection. The proposed method has been successfully validated in accordance with the International Conference on Harmonization (ICH) guidelines [17]. Moreover, in this investigation, a comparative characterization studies of in-house samples of etelcalcetide injection with RLD samples were.

EXPERIMENTAL

Etelcalcetide samples (Batch nos. EEC2001, EEC2007 and EEC2008) were obtained from Eugia Pharma Research Centre, Hyderabad, India. Etelcalcetide API (Active Pharma Ingredient) was purchased from Eugia Pharma Specialities Limited. Parsabiv® (RLD) (Batch No. 1105656) was manufactured by Amgen Thousand Oaks, USA. Sodium perchlorate monohydrate (Merck), perchloric acid (70%, HPLC grade) and acetonitrile (gradient grade) were used in this study. Water for injection was used.

Instrumentation: The experiments were conducted using the Waters-PDA e 2695 series in conjunction with Empower-3 software. The stationary phase employed was an ACE Excel 3 C18 amide (150 mm × 4.6 mm, 3 µm) connected to a ghost guard column with dimensions of 30 mm × 4.6 mm. A 0.1 M sodium perchlorate monohydrate solution was prepared by dissolving 14 g of sodium perchlorate monohydrate in 1000 mL of water, the pH (Eutech pH 2700 model) adjusted to 2.5 and the mixture as diluted with per chloric acid. Mobile phase A consisted of a mixture of 100% 0.1 M sodium perchlorate monohydrate (pH 2.5) and mobile phase B consists of a mixture of 60% 0.1 M sodium perchlorate monohydrate (pH 2.5) and 40% acetonitrile. A degassed mixture of 90% 0.1 M sodium perchlorate monohydrate (pH 2.5) and 10% acetonitrile used as a diluent. The column oven temperature and injection volume were 30 ºC and 10 µL, respectively. The detector wavelength was 210 nm and the flow rate was 0.5 mL/min. The gradient programme for the RS-I methodology (time (min)/%B) is 0.01/10, 5/10, 10/18, 28/30, 35/33, 55/55, 70/90, 75/90, 75.1/10, 95/10.

LC-HRMS consists of a Waters-ACQUITY UPLC H-Class PLUS with PDA coupled with a Waters-HRMS (Vion IMS QTof) with Unifi software and used for the characterization of peptide impurities. The generation of peptide ions was achieved through an electrospray ionization (ESI) source integrated with a mass spectrometer. For the Tandem mass spectrometry analysis, collision-induced dissociation (CID) was executed in the linear quadrupole ion trap, alongside high-energy collision dissociation in a trap.

Preparation of etelcalcetide standard solution: Accurately weighed 60 mg of etelcalcetide standard in a dehumidifier room due to its hygroscopic nature in to a 100 mL VF, added 3mL of diluent, mixed well and make up with diluent up to the mark. Then, 2mL of above solution was transferred into a 50 mL VF and make up with diluent up to the mark. The standard solution was prepared 2% level with respect to the sample concentration (1000 µg/mL).

Sample preparation: A 1000 µg/mL stock solution of etelcalcetide sample was prepared in the diluent for the analysis of related impurities. A 300 µg/mL stock solution mixture of impurities was also prepared in the diluent. Working solutions of impurities were prepared by diluting the impurity stock solution.

RESULTS AND DISCUSSION

The potential impurities of etelcalcetide were determined by using ACE Excel 3 C18 amide (150 mm × 4.6 mm, 3 µm) connected to a ghost guard column with dimensions of 30 mm × 4.6 mm in the related substances (RS)-I methodology. An unknown impurity peak was observed at the relative retention time (RRT) of 0.08, which is close to the tartaric acid peak and is disregarded in the RS-I method monitored *via* the RS-II method. The gradient methodology adopted for RS-II (time (min)/%B) is 0.01/0, 5/100, 15/90, 20/90, 20.1/0 and 40/0.

Method validation

System precision and method precision: Approximately 0.0240 mg/mL etelcalcetide was prepared in the diluent as a standard solution and six replicates were injected into the chromatograph to determine the system precision. %RSD for the peak areas of etelcalcetide obtained from six replicated injections of standard solution was 0.3.

Six sample solutions were prepared separately using a single batch of etelcalcetide injection, which was spiked with known

related impurities at a specification level of 0.5% and each level was injected accordingly. The spiked sample was prepared by adding impurities at the 0.5 % level according to the test method and injected each sample into the HPLC system according to the per methodology used to determine the method precision. The % RSD values for the results of etelcalcetide and its related impurities in the six spiked samples were not more than 2% (Table-1).

Limit of detection and limit of quantification (LOD and LOQ): The linearity study, slope and standard deviation measurements were analyzed to determine the LOD and LOQ for etelcalcetide and its impurities. The predicted concentrations for LOD and LOQ were prepared and injected six times for precision verification. The LOD and LOQ concentrations of ECT and its impurities are shown in Table-1.

Linearity: A series of solutions were prepared using etelcalcetide standard and its related impurities at concentration ranging from 1% to 150% of the specification level. The assessment of linearity was conducted *via* standard curves ranging from 0.812 to 30.46 μg mL^{-1} for etelcalcetide, 2.017 to 15.12 μg mL^{-1} for deacetyl-etelcalcetide, 1.506 to 30.110 μg mL^{-1} for Arg7-etelcalcetide impurity, 2.006 to 12.034 μg mL^{-1} for des-cys etelcalcetide, 2.001 to 22.654 μg mL^{-1} for dimer etelcalcetide impurity, 2.068 to 12.389 μg mL^{-1} for L-cys etelcalcetide. The correlation coefficient is more than 0.990 for etelcalcetide and its impurities. Hence, the response of etelcalcetide and its impurities is linear from the LOQ level to the 150% specification level. The results are summarized in Table-1.

Accuracy: Sample solutions were thoroughly prepared in triplicate through ECT injection, incorporating all impurities at levels of LOQ, 50%, 100%, and 150% of the specification level. Each of these levels was subsequently injected using HPLC. The percentage of recovery was between 85% and 125% and the %RSD values were between 0.2 and 1.8 for the impurities of etelcalcetide (Table-2).

Specificity: Solutions of the etelcalcetide standard, etelcalcetide sample, sample spiked with impurities at specification level, sodium chloride and L-tartaric acid were injected. The peak purity was established by using water Empower 3 software. No peak was observed in the blank, placebo and excipients at the retention times of etelcalcetide and its related impurities. The specificity data are summarized in Table-3.

Forced degradation study: A forced degradation study was performed to determine the stress conditions that can accelerate the degradation process of the drug product. Hence different stress conditions such as acid, base, peroxide, photolytic degradation were employed to degrade the etelcalcetide and its related impurities. The different stress conditions were (i)

TABLE-1
PRECISION, LOD, LOQ, RF DATA AND REGRESSION DATA FOR ECT AND ITS IMPURITIES

Parameter	De acetyl etelcalcetide	Arg^7– etelcalcetide	Des-Cys-etelcalcetide	Dimer etelcalcetide	Etelcalcetide	L-Cys etelcalcetide
% RSD (Mean of six spiked samples)	1.0	0.5	1.0	1.5	0.8	1.7
LOD (%)	0.067	0.050	0.066	0.066	0.027	0.068
LOQ (%)	0.202	0.151	0.201	0.200	0.081	0.207
Slope (m)	6208	12410	10971	12179	14383	7589
Intercept	-827	2127-1210	-2518	-4229	-559	-2973
Residual sum of squares	263	450	186	1957	559	854
Correlation coefficient	0.9999	0.9999	0.9999	0.9998	0.9999	0.9996
Response factor	2.32	1.16	1.31	1.18	–	1.13

TABLE-2
ACCURACY DATA OF RELATED SUBSTANCES IN ECT INJECTION

Amount spiked*	De acetyl etelcalcetide	Arg^7–etelcalcetide	Des-Cys-etelcalcetide	Dimer etelcalcetide	L-Cys etelcalcetide
LOQ level	100.9 ± 3.0	99.6 ± 1.1	86.1 ± 1.2	83.7 ± 3.6	123.3 ± 0.5
50%	103.7 ± 0.6	101.0 ± 0.4	97.6 ± 0.6	107.9 ± 0.8	114.4 ± 0.7
100%	103.9 ± 0.4	100.3 ± 0.3	97.4 ± 0.2	105.1 ± 1.8	105.8 ± 0.5
150%	104.0 ± 0.8	100.2 ± 1.1	96.6 ± 1.1	105.7 ± 0.9	103.9 ± 0.4

TABLE-3
SPECIFICITY DATA FOR ECT SPIKED SAMPLE AND ITS IMPURITIES

Name	Retention time (min)	Relative retention time	Peak purity	
			Purity angle	Purity threshold
De acetyl-etecalcetide	35.557	0.92	1.259	1.896
Etelcalcetide	39.219	1.00	0.142	0.549
Arg^7(acid)-etelcalcetide	42.656	1.09	0.435	0.652
Des-Cys etelcalcetide	49.871	1.27	0.808	1.323
Dimer-etelcalcetide	67.202	1.70	0.191	0.451
L-Cys etelcalcetide#	30.39	1.07	0.076	0.342
Sodium chloride	2.920	–	–	–
L-Tartaric acid	3.501	–	–	–

#RS-II methodology

for acid degradation (1 M HCl at 85 ºC for 5 min); (ii) for base degradation (1 M NaOH at room temperature for 5 min); (iii) for thermal degradation at 85 ºC for 6 h; (iv) for oxidation degradation (0.05% 3-chloro perbenzoic acid at 85 ºC for 5 min); and (v) humidity degradation (90% RH at 25 ºC for 72 h). The percentage degradation obtained for the samples stressed under various conditions was not less than 95% for all degradation conditions. Furthermore, the evaluation of the peak purity of etelcalcetide from the analysis of every degradation sample indicated that the etelcalcetide peak was homogenous and had no co-eluting peaks. Hence, the present proposed method is specific and stability indicating method for the determination of related impurities of etelcalcetide.

Robustness: Robustness assessments were performed by adjusting the flow rate by ± 10%, the wavelength by ± 5 nm, the column oven temperature by ± 5 ºC, organic composition in mobile phase-B by ± 1% absolute, the pH of buffer by ± 0.1 units and gradient composition by ± 1 % absolute from prescribed methodology values. In this study, various system suitability parameters, such as the signal-to-noise ratio, tailing factor and USP plate count, were analyzed. The findings indicate that there is no notable influence on the system suitability results of etelcalcetide and its impurities, thus affirming the robustness of the method.

Stability of solution and mobile phase: An investigation was conducted on etelcalcetide and its impurities standard and etelcalcetide and its impurities spiked samples at different intervals of time up to 72 h. The spiked sample exhibited a change of less than 1% from initial over a 48 h storage period in a refrigerator condition (~8 ºC). Moreover, there were no visible particles or haziness in the mobile phase after 70 h, thus indicating that the mobile phase is stable for a minimum of 70 h.

Peptide characterization studies of etelcalcetide injection

Primary sequence and physical properties: The primary sequence and physical properties of the active pharmaceutical gradient (API) of etelcalcetide were previously established by Eugia Pharma specialities Ltd. The structural elucidation data generated by Eugia Pharma specialities Limited were used to qualify the drug substance (API) as the same analytical facility needs to be used for qualification of the drug product (ECT injection) also.

Secondary structure analysis of etelcalcetide injection: The secondary structure of etelcalcetide injection was assessed by circular dichroism (CD) and Fourier-transform infrared spectroscopy (FTIR) orthogonal methods.

Circular dichroism (CD): In-house samples (obtained from Eugia Research Centre) of etelcalcetide injection and RLD product samples were subjected to circular dichroism (CD) analysis at 0.1 mg/mL concentration. Analysis was carried out with 0.1 mg/mL etelcalcetide (10 mg/2 mL, 5 mg/mL and 2.5 mg/0.5 mL) diluted with water. At actual sample concentrations, HT voltage was above 700, hence, to bring the HT voltage within limits, the drug product was diluted to 0.1 mg/mL. In-house and RLD were analyzed under similar conditions and comparable secondary structures were found. The quantitative secondary structure results obtained from JASCO's multivariant program are tabulated in Table-4.

The CD spectra of the in-house and RLD samples show a minimum in between 210 and 220 and a maximum in between 190-200, which is a characteristic value for β sheets. A low α-helical content was also observed in the etelcalcetide peptide in liquid and structural interpretation data revealed 0.0 to 7.7 % α-Helix content in in-house samples and 1.9% in RLD samples, where the differences were negligible. However, the CD spectrum found to be comparable between in-house samples (B.No-EEC2001, EEC2007 and EEC2008) at 2-8 ºC for 12 months and RLD samples aged near expiry. On the basis of tabulated data and spectral overlay demonstrate that the secondary structures of in-house ECT injection (2-8 ºC 12 M) and RLD (near expiry) are comparable. The overlaid CD spectra of the in-house etelcalcetide with RLD samples are shown in Fig. 1.

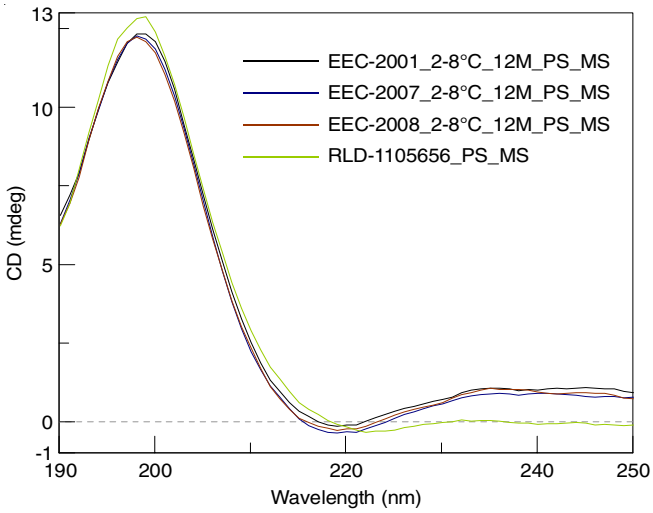

Fig 1. Overlaid CD spectra of in-house ECT and RLD samples

FTIR spectral sutides: In-house samples of etelcalcetide injection and RLD product samples were analyzed. Etelcalcetide at a concentration of 0.1 mg/mL was subjected to FTIR analysis and the secondary structure was evaluated with the built-in secondary structure evaluation algorithm. Interference was observed when the direct drug product sample was subjected to analysis, hence the samples were diluted to 0.1 mg/mL with water.

TABLE-4
SECONDARY STRUCTURE ANALYSIS RESULTS OF ECT INJECTION OBTAINED FROM CD

Sample name	Strength	α-Helix (%)	β-Sheet (%)	β-Turn (%)	Others (%)
EEC2001_2-8 ºC_12M	10 mg/2 mL	0.0	65.2	9.8	25.0
EEC2007_2-8 ºC_12M	5 mg/mL	7.7	55.7	9.1	27.5
EEC2008_2-8 ºC_12M	2.5 mg/0.5 mL	5.2	58.9	8.3	27.6
1105656_Near expiry	2.5 mg/0.5 mL	1.9	60.7	10.1	27.3

TABLE-5
SECONDARY STRUCTURE ANALYSIS RESULTS OF ECT INJECTION OBTAINED FROM FTIR

Sample name	Strength	α-Helix (%)	β-Sheet (%)	β-Turn (%)	Others (%)
EEC2001_2-8 ºC_12M	10 mg/2 mL	6	44	24	26
EEC2007_2-8 ºC_12M	5 mg/mL	6	44	24	26
EEC2008_2-8 ºC_12M	2.5 mg/0.5 mL	7	43	24	26
1105656_Near expiry	2.5 mg/0.5 mL	6	43	24	27

A lower α-helical content was observed in the etelcalcetide liquid sample and the structural interpretation results revealed a 6 to 7% α-helix content in the in-house and RLD samples (Table-5). The % RSD of the overall samples was within 5% for the remaining structures, thus, the secondary structures of the in-house ECT injection and RLD are comparable.

Aggregation profile study of etelcalcetide injection: The peptide aggregation profile was analyzed using size exclusion chromatography (SEC-HPLC) coupled with UV and multi-angle light scattering (MALS) detector. The chromatographic profile of SEC-HPLC-MALS suggested that etelcalcetide contain dimer only, no other aggregated species are exist. The LC-HRMS technique employed for peptide impurity profiling separates three dimer compounds through chromatography, allowing for the extraction of aggregate profiles of the dimer species, which were regarded as the orthogonal method for SEC-HPLC.

Size exclusion chromatography (SEC-HPLC): Size exclusion chromatography (SEC-HPLC) was performed on Shimadzu HPLC system coupled with UV and Wyatt multiangle light scattering systems (MALS). The etelcalcetide samples at a concentration of 1.0 mg/mL were injected in to Shodex protein kw 802.5 column for separation. At the actual drug product concentration, the response of etelcalcetide reaches the UV saturation limit. Hence, the sample was diluted to 1.0 mg/mL and to minimize the baseline issues with the actual sample, it was diluted with the mobile phase. The overlaid auto scale UV chromatograms (SEC-HPLC) of In-house and RLD samples are shown Fig. 2.

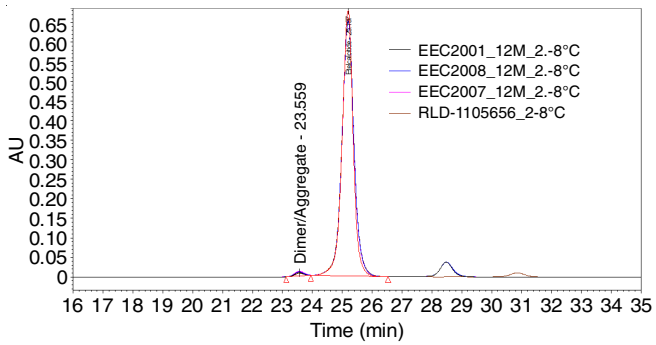

Fig 2. Overlaid auto scaled UV chromatograms (SEC-HPLC) of in-house ECT and RLD samples

Sodium phosphate (50 mM, pH 3.0) was used as the mobile phase and analysis was carried out for 40 min at a 0.4 mL/min flow rate. Etelcalcetide spiked with dimer was injected into chromatography to establish the retention time (RT) of the dimer and to establish resolution between the etelcalcetide monomer and dimer peaks. The resolution between etelcalcetide monomer and dimer found to be 2.55. In-house samples of etelcalcetide injection and reference listed drug samples were evaluated using a UV detector at 225 nm to determine aggregates and their percentage by size exclusion chromatography. Dimers were detected in etelcalcetide injections, while larger aggregates like trimers and tetramers were not identified in the MALS detector (Fig. 3).

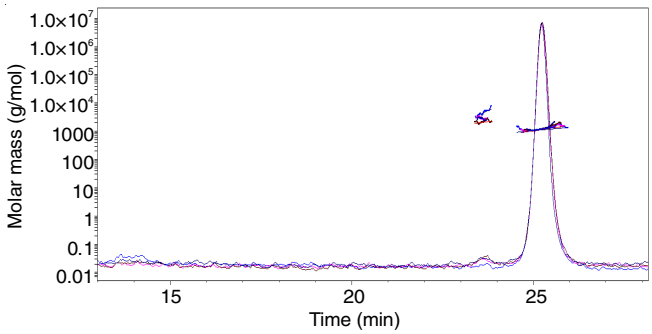

Fig 3. Overlaid auto scaled MALS chromatograms of in-house ECT and RLD samples

The aggregation tendency of in-house samples stored at condition (2-8 ºC) for 12 M shows between 1.06 and 1.48, whereas the RLD sample (aged near expiry) shows it as 0.76 (Table-6). Furthermore, it can be concluded that the aggregation tendency of in-house samples under long-term storage condition (*i.e.* 2-8 ºC) and RLD samples (aged near expiry) are well-controlled.

TABLE-6
AGGREGATE PROFILE STUDY ANALYSIS OF
ECT INJECTION OBTAINED FROM SEC-HPLC

Sample name	Strength	Condition	Aggregates (%)
EEC2001	10 mg/2 mL	2-8 ºC_12M	1.06
EEC2007	5 mg/mL	2-8 ºC_12M	1.48
EEC2008	2.5 mg/0.5 mL	2-8 ºC_12M	1.21
1105656	2.5 mg/0.5 mL	Near expiry	0.76

Liquid chromatography-high resolution mass spectrometry (LC-HRMS): Data on aggregation profiling were obtained from the impurity profiling carried out using the LC-HRMS technique. Three dimer impurities were observed in etelcalcetide injection at 22.51 min, 27.98 min and 28.98 min. Dimers and their respective trifluoroacetic acid (TFA) adducts were analyzed and relative response was calculated. The aggregation tendency of the in-house samples under long term storage conditions (*i.e.*, 2-8 ºC for 12 months) and RLD samples (aged near expiry) found comparable (Table-7). The dimer response observed through LC-HRMS was found to be higher than that obtained from the SEC-MALS method, attributed to the impact

TABLE-7
AGGREGATE PROFILE ANALYSIS OF ECT INJECTION OBTAINED FROM UPLC-HRMS

Sample name	Dosage	Condition	RT (min)			Total % aggregates
			21.51	27.98	28.98	
EEC2001	10 mg/2 mL	2-8 ºC_12M_UP	1.08	2.85	0.00	3.93
EEC2007	5 mg/mL	2-8 ºC_12M_UP	1.23	4.22	0.01	5.46
EEC2008	2.5 mg/0.5 mL	2-8 ºC_12M_UP	1.26	3.42	0.01	4.69
1105656	2.5 mg/0.5 mL	Near expiry	1.51	1.97	0.08	3.56

of various factors, including the mobile phase and solvent composition, on ionization. However, the obtained aggregation profile data were comparable between the in-house and RLD products.

Impurity profiling: Analysis of impurity profiling was conducted using the HPLC-UV method as well as he UPLC-HRMS method. For detecting and relative quantification of peptide impurities UPLC-HRMS method was used as orthogonal technique. The HPLC-UV results are tabulated in Table-8. Impurity levels are slightly higher in RLD samples (near expiry) except the etelcalcetide dimer impurity, this may be due to ageing (near expiry) of the RLD sample. However, all the impurity levels of the in-house samples under storage condition (2-8 ºC) for 12 months were well controlled. The second orthogonal technique for impurity profiling is the LC-HRMS method, which uses a Waters Vion HRMS to characterize the impurity

TABLE-8
IMPURITY PROFILE OF IN-HOUSE SAMPLES (STORAGE 2-8 ºC FOR 12M) AND RLD (2-8 ºC-NEAR EXPIRY) USING HPLC-UV METHOD

Sample details	EEC2001	EEC2007	EEC2008	1105656
Condition		In-House 2-8 ºC_ 12 Months		RLD_2_8 ºC_Near expiry
Dosage	10 mg/2 mL	5 mg/mL	2.5 mg/0.5 mL	2.5 mg/0.5 mL
Age of sample	12M	12M	12M	Near Expiry
De acetyl etelcalcetide	0.351	Below LOQ	Below LOQ	0.746
Arg 7 Acid etelcalcetide	0.349	0.303	0.307	1.63
Des cys etelcalcetide	Below LOD	Below LOD	Below LOD	Not detected
Etelcalcetide dimer	1.224	1.809	1.774	0.851
Unknown impurity	0.083	0.109	0.106	0.917

TABLE-9
% AREA RESPONSE COMPARISON OF IMPURITY PROFILE OF IN-HOUSE (STORAGE 2-8 ºC FOR 12Months) AND RLD (2-8 ºC-NEAR EXPIRY) USING LC-HRMS METHOD

Sample details			EEC2001	EEC2007	EEC2008	1105656
Condition			In-House 2-8 ºC_ 12 Months			2-8 ºC_Near expiry (RLD)
Dosage			10 mg/2 mL	5 mg/mL	2.5 mg/0.5 mL	2.5 mg/0.5 mL
Age of Sample			12M	12M	12M	Near expiry
Impurity name	RT	Observed mass	Area (%)			
Etelcalcitide main peptide	22.63	1048.5364	85.32	83.11	83.58	84.30
Arg7 acid etelcalcitide peptide	23.45	1049.5235	0.40	0.37	0.37	1.59
De-acetyl etelcalcitide peptide	20.20	1006.5268	0.05	0.04	0.00	0.26
Des Cys etelcalcitide peptide	25.02	929.5318	0.02	0.00	0.05	0.00
Unknown impurity 1	22.51	895.5448	5.84	5.79	5.96	5.70
Unknown impurity 2	22.51	927.5167	1.76	1.87	1.81	1.77
Unknown impurity 3	22.51	1031.5093	1.23	1.55	1.67	1.16
Unknown impurity 4	22.48	1050.3986	0.30	0.29	0.29	0.29
Unknown impurity 5	22.50	1162.5268	0.49	0.69	0.67	0.50
Unknown impurity 6	22.50	1276.5189	0.64	0.83	0.90	0.86
Unknown impurity 7	22.51	2552.0320	0.00	0.44	0.45	0.43
Unknown impurity 8	22.52	2324.0464	0.20	0.00	0.00	0.19
Unknown impurity 9	22.51	2438.0387	0.88	0.79	0.81	0.88
Etelcalcitide dimer 2_1	27.98	1856.0378	0.80	1.31	1.05	0.56
Etelcalcitide dimer 2_2	27.98	1970.0296	0.65	0.94	0.76	0.44
Etelcalcitide dimer 2_3	27.98	2084.0214	0.74	1.04	0.85	0.52
Etelcalcitide dimer 2_4	27.98	2198.0140	0.39	0.54	0.45	0.28
Etelcalcitide dimer 2_5	27.98	2312.0064	0.09	0.13	0.11	0.05
Etelcalcitide dimer 2_6	27.98	2425.9999	0.09	0.14	0.11	0.06
Etelcalcitide dimer 2_7	27.98	2539.9917	0.08	0.12	0.09	0.06
Etelcalcitide dimer 2_8	28.98	2002.0023	0.00	0.00	0.00	0.03
Etelcalcitide dimer 2_9	28.99	2115.9931	0.00	0.01	0.01	0.04
Etelcalcitide dimer 2_10	28.99	2229.9884	0.00	0.00	0.00	0.02

profiling. The impurities found above 0.10% were identified and characterized. The relative abundance was calculated and is presented in Table-9.

All the peptide-associated impurities, encompassing those related to the process and degradation, were thoroughly characterized using LC-HRMS. All the peptide-related impurities were observed in more than 0.10% in drug products were identified. All impurities exhibiting area responses greater than 0.1% were identified and characterized through MS/MS analysis. Other masses found below the main peak of etelcalcetide are generated through source ionization, hence these masses are considered to contribute to the main peptide. The Arg7-etelcalcitide (1049.5), deacetyl etelcalcitide (1006.5) and des-cys etelcalcitide (929.5) impurities are found within the specification limit and are much lower than the RLD impurity level. Similar to the UV-HPLC method, Arg7-etelcalcitide peptide impurity found higher compared to in-house and RLD products.

Conclusion

In this work, the proposed RP-HPLC method herein is a novel and efficient analytical technique for the quantification of related impurities of etelcalcetide injection in both bulk and finished formulations. The method has been rigorously validated according to established guidelines, emphasizing critical parameters such as specificity, precision, accuracy, linearity and robustness. The secondary structure profile of the in-house etelcalcitide injection samples was similar to that of the RLD samples according to the FTIR and CD analysis. The aggregation tendency of in-house samples under long term storage conditions (*i.e.*, 2-8 ºC) were found well controlled. Based on the results, the proposed method is crucial for pharmaceutical quality control laboratories facilitating the precise evaluation of etelcalcitide injection in various formulations.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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