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Research Article

**DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD
FOR THE ESTIMATION OF ETELCA LCETIDE IN BULK AND
MARKETED PHARMACEUTICAL FORMULATION****Prashanthi Gali ^{1*}, B.V.N.S. Jaya Lahari ², Ratnam Sarayu², G. Usha Laxmi ², P. Venu²**¹Assistant professor, Department of Pharmaceutical Chemistry, St. Mary's College of Pharmacy,
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9/1/248, St. Francis Street, Hyderabad, Telangana - 500025, India.**Abstract:**

A simple, rapid, specific and accurate reverse phase high performance liquid chromatographic method has been developed for the validated of Etelcalcetide in bulk as well as in marketed pharmaceutical dosage form. This separation was performed on a Symmetry ODS C18 (4.6mm×250mm, 5µm) column with Methanol: Phosphate Buffer (35:65%) v/v as mobile phase at a flow rate of 1.0 mL min⁻¹ with UV detection at 235 nm; the constant column temperature was Ambient. The run time under these chromatographic conditions was less than 8 min. The retention time of Etelcalcetide was found to be 2.293min. The calibration plot was linear over the concentration range of 6–14 µg mL⁻¹ with limits of detection and quantification values of 1.2 and 3.6 ng mL⁻¹ respectively. The mean % assay of marketed formulation was found to be 99.86%, and % recovery was observed in the range of 98–102%. Relative standard deviation for the precision study was found <2%. The developed method is simple, precise, specific, accurate and rapid, making it suitable for estimation of Etelcalcetide in bulk and marketed pharmaceutical dosage form.

Keywords: Etelcalcetide, RP-HPLC, Method Development, Validation, ICH Guidelines.**Corresponding author:****Prashanthi Gali,**Assistant Professor, Department of Pharmaceutical Chemistry,
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INTRODUCTION:

Etelcalcetide is a calcium sensing receptor agonist used to treat secondary hyperparathyroidism in patients with chronic kidney disease who require hemodialysis. Etelcalcetide is a Calcimimetic drug for the treatment of secondary hyperparathyroidism in patients undergoing hemodialysis¹. Etelcalcetide was approved (trade name Parsabiv) for the treatment of secondary hyperparathyroidism (HPT) in adult patients with chronic kidney disease (CKD) on hemodialysis in February, 2017. Following a single intravenous bolus administration of Etelcalcetide, PTH levels decreased within 30 minutes post dose. In the single-dose study, the extent and duration of the

reduction in PTH increased with increasing dose². Reduction in PTH levels correlated with plasma Etelcalcetide concentrations in hemodialysis patients. The reduction in PTH resulted in reductions in calcium and attenuation of post-dialytic phosphate elevation. The effect of reducing PTH levels was maintained throughout the 6-month dosing period when Etelcalcetide was administered by intravenous bolus three times a week. Etelcalcetide (brand name Parsabiv) is a Calcimimetic medication primarily prescribed to treat secondary hyperparathyroidism (SHPT) in adults with chronic kidney disease (CKD) who are on hemodialysis³. The Chemical Structure of Etelcalcetide is shown in follows

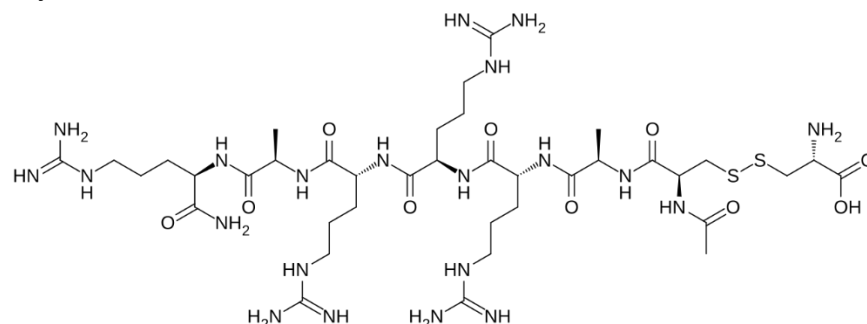


Fig-1: Chemical Structure of Etelcalcetide

The considerable literature survey disclose that the analytical methods reported for estimation of Etelcalcetide alone and in combination with other drug, but no analytical methods was reported for estimation of Etelcalcetide. So, it was thought of interest to develop simple, accurate, precise and reproducible RP-HPLC method for estimation of Etelcalcetide in their bulk form and Marketed Pharmaceutical Dosage forms²⁸⁻³⁰. Methods were validated as per ICH guidelines²⁷ [Q2 (R1)].

MATERIALS AND METHODS:

The Instruments and Materials

The instruments and glassware used in the present study included a WATERS Alliance 2695 separation module equipped with Empower 2 software and a 996 PDA detector for chromatographic analysis. A Lab India pH meter was used for pH measurements, while a Sartorius weighing machine was employed for accurate weighing of the materials. Borosil volumetric flasks, pipettes, burettes, and beakers were used for preparation, measurement, and handling of solutions. A Labman digital ultrasonicator was used for sonication and degassing of the prepared solutions.

Etelcalcetide was procured from the local market and used as the working drug substance. HPLC-grade water and methanol were obtained from Lichrosolv

(Merck), while HPLC-grade acetonitrile was procured from Merck. All chemicals and solvents were of appropriate analytical/HPLC grade and were used as received for the development and validation of the analytical method.

HPLC Method Development:

Preparation of Standard Solution:

Accurately weigh and transfer 10 mg of Etelcalcetide working standard into a 10ml of clean dry volumetric flasks add about 7ml of Methanol and sonicate to dissolve and removal of air completely and make volume up to the mark with the same Methanol. Further pipette 0.1ml of the above Etelcalcetide stock solutions into a 10ml volumetric flask and dilute up to the mark with Methanol.

Procedure:

Inject the samples by changing the chromatographic conditions and record the chromatograms, note the conditions of proper peak elution for performing validation parameters as per ICH guidelines²⁷.

Mobile Phase Optimization:

Initially the mobile phase tried was Methanol and Methanol: Water with varying proportions. Finally, the mobile phase was optimized to Methanol: Phosphate Buffer in proportion 35:65% v/v.

Optimization of Column:

The method was performed with various C18 columns like, X- bridge column, Xterra, and C18 column. Symmetry ODS C18 (4.6mm x 250mm, 5µm) was found to be ideal as it gave good peak shape and resolution at 1.0ml/min flow.

Preparation of Potassium dihydrogen Phosphate (KH₂PO₄) buffer (pH-3.6):

Dissolve 6.8043 of potassium dihydrogen phosphate in 1000 ml HPLC water and adjust the pH 3.6 with diluted orthophosphoric acid. Filter and sonicate the solution by vacuum filtration and ultra-sonication⁴.

Preparation of Mobile Phase:

Accurately measured 350 ml (35%) of Methanol, 650 ml of Phosphate buffer (65%) were mixed and degassed in digital ultra sonicator for 15 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Diluent Preparation:

The Mobile phase was used as the diluent⁵.

Method Validation Parameters**System Suitability**

Accurately weigh and transfer 10 mg of Etelcalcetide working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.1ml of the above Etelcalcetide stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

Procedure:

The standard solution was injected for five times and measured the area for all five injections in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits.

Specificity:**Preparation of Standard Solution:**

Accurately weigh and transfer 10 mg of Etelcalcetide working standard into a 10ml of clean dry volumetric flasks add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)
Further pipette 0.1ml of the above Etelcalcetide stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

Preparation of Sample Solution:

Weight 10 mg equivalent weight of Etelcalcetide sample into a 10mL clean dry volumetric flask and add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

Further pipette 0.1ml of Etelcalcetide above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Procedure:

Inject the three replicate injections of standard and sample solutions and calculate the assay by using formula:

$$\% \text{ASSAY} = \frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Weight of standard}}{\text{Dilution of standard}} \times \frac{\text{Dilution of sample}}{\text{Weight of sample}} \times \frac{\text{Purity}}{100} \times \frac{\text{Weight of tablet}}{\text{Label claim}} \times 100$$

Linearity and Range:

Accurately weigh and transfer 10 mg of Etelcalcetide working standard into a 10ml of clean dry volumetric flasks add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Preparation of Level – I (6ppm of Etelcalcetide):

Take 0.6ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluents and sonicate the solution for bubble entrapment using ultrasonicator.

Preparation of Level – II (8ppm of Etelcalcetide):

Take 0.8ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluents and sonicate the solution for bubble entrapment using ultrasonicator.

Preparation of Level – III (10ppm of Etelcalcetide):

Take 0.1ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluents and sonicate the solution for bubble entrapment using ultrasonicator.

Preparation of Level – IV (12ppm of Etelcalcetide):

Take 0.12ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluents and sonicate the solution for bubble entrapment using ultrasonicator.

Preparation of Level – V (14ppm of Etelcalcetide):

Take 0.14ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark

with diluents and sonicate the solution for bubble entrapment using ultrasonicator.

Procedure:

Inject each level into the chromatographic system and measure the peak area.

Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient⁶.

Precision**Repeatability****Preparation of Etelcalcetide Product Solution for Precision:**

Accurately weigh and transfer 10 mg of Etelcalcetide working standard into a 10ml of clean dry volumetric flasks add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.1ml of the above Etelcalcetide stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

Procedure:

The standard solution was injected for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

Intermediate Precision:

To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different days by maintaining same conditions.

Procedure:**Analyst 1:**

The standard solution was injected for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

Analyst 2:

The standard solution was injected for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

Accuracy:**For preparation of 50% Standard Stock Solution:**

Accurately weigh and transfer 10 mg of Etelcalcetide working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.05ml of the above Etelcalcetide stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

For preparation of 100% Standard Stock**Solution:**

Accurately weigh and transfer 10 mg of Etelcalcetide working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.1ml of the above Etelcalcetide stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

For preparation of 150% Standard Stock**Solution:**

Accurately weigh and transfer 10 mg of Etelcalcetide working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.15ml of the above Etelcalcetide stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

Procedure:

Inject the Three replicate injections of individual concentrations (50%, 100%, 150%) were made under the optimized conditions. Recorded the chromatograms and measured the peak responses. Calculate the Amount found and Amount added for Etelcalcetide and calculate the individual recovery and mean recovery values.

Limit of Detection and Limit of Quantification (LOD & LOQ):**Preparation of 0.95µg/ml Solution (For LOD):**

Accurately weigh and transfer 10 mg of Etelcalcetide working standard into a 10ml of clean dry volumetric flasks add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.0095ml of the above Etelcalcetide stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

Preparation of 2.9µg/ml Solution (For LOQ):

Accurately weigh and transfer 10 mg of Etelcalcetide working standard into a 10ml of clean dry volumetric flasks add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.029ml of the above Etelcalcetide stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

Robustness:

The analysis was performed in different conditions to find the variability of test results. The following conditions are checked for variation of results. .

For preparation of Standard solution:

Accurately weigh and transfer 10 mg of Etelcalcetide working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.1ml of the above Etelcalcetide stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

Effect of Variation of Flow Conditions:

The sample was analyzed at 0.9 ml/min and 1.1 ml/min instead of 1ml/min, remaining conditions are same. 10 μ l of the above sample was injected and chromatograms were recorded.

Effect of Variation of Mobile Phase Organic Composition:

The sample was analyzed by variation of mobile phase i.e. Methanol: Phosphate Buffer was taken in the ratio and 40:60, 30:70 instead (35:65), remaining conditions are same. 10 μ l of the above sample was injected and chromatograms were recorded.

RESULTS AND DISCUSSION:**Analytical Method Development:****Sample & Standard Preparation for the UV-Spectrophotometer Analysis:**

25 mg of Etelcalcetide standard was transferred into 25 ml volumetric flask, dissolved & make up to volume with mobile phase. Further dilution was done by transferring 0.1 ml of the above solution into a 10ml volumetric flask and make up to volume with mobile phase.

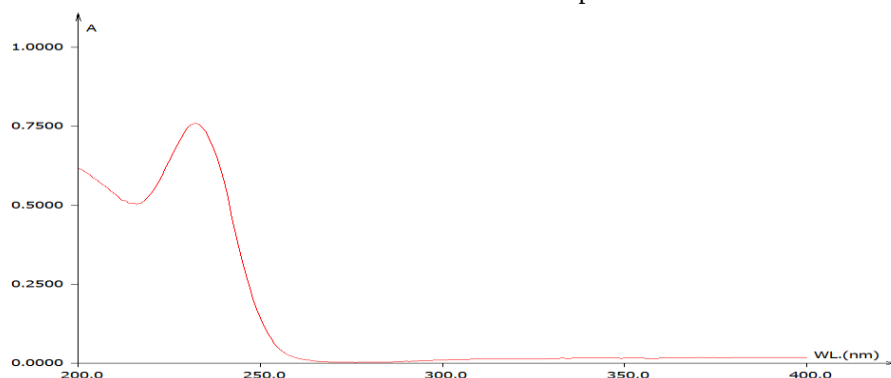


Fig-2: UV Spectrum for Etelcalcetide (235nm)

Observation: While scanning the Etelcalcetide solution we observed the maxima at 235nm. The UV spectrum has been recorded on T60-LAB INDIA make UV – Vis spectrophotometer model UV-2450.

Optimized Chromatographic Conditions:

Mobile phase ratio : Methanol: Phosphate Buffer (35:65%) v/v
 Column : Symmetry ODS C₁₈ (4.6mm×250mm, 5 μ m)
 Column temperature : Ambient
 Wavelength : 235 nm
 Flow rate : 1.0 mL/min
 Injection volume : 10.0 μ l
 Run time : 8.0 min

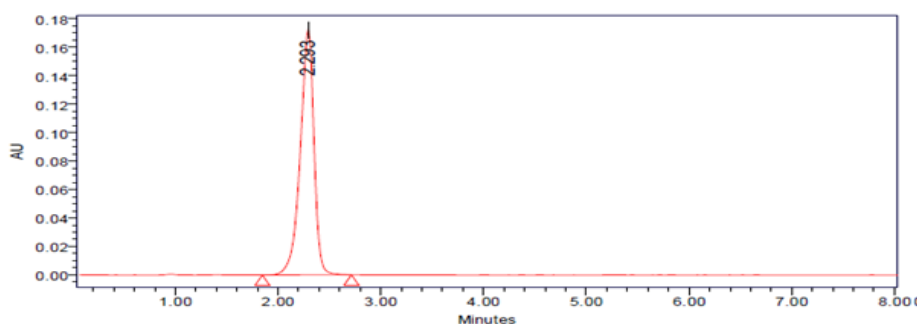


Fig-3: Optimized Chromatographic Condition

Validation of Analytical Method**System Suitability:**

The test was carried out by making five replicate injections of a standard solution containing 10µg/ml

of Etelcalcetide and analyzing each solute for their peak area, theoretical plates (N), tailing factor (T) and asymmetric factors (As). The results of System Suitability test⁷ were shown in table-1.

Table-1: Results of System Suitability for Etelcalcetide

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Etelcalcetide	2.277	1652847	185647	6589	1.24
2	Etelcalcetide	2.277	1653658	186254	6587	1.26
3	Etelcalcetide	2.267	1654521	185475	6584	1.28
4	Etelcalcetide	2.265	1653564	186594	6582	1.29
5	Etelcalcetide	2.277	1658745	187884	6895	1.24
Mean			1654667			
Std. Dev.			2355.764			
% RSD			0.142371			

Specificity

The ICH documents define specificity as the ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such

as impurities, degradation products, and matrix components⁸. Analytical method was tested for specificity to measure accurately quantitates Etelcalcetide in drug product.

%ASSAY =

$$\frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Weight of standard}}{\text{Dilution of standard}} \times \frac{\text{Dilution of sample}}{\text{Weight of sample}} \times \frac{\text{Purity}}{100} \times \frac{\text{Weight of tablet}}{\text{Label claim}} \times 100$$

= 99.40%

Conclusion: The % purity of Etelcalcetide in pharmaceutical dosage form was found to be 99.40%⁹.

Etelcalcetide over the concentration of 6 to 14µg/mL. The linearity plots were acquired by plotting peak response (on X-axis) vs. concentration (on Y-axis)¹⁰⁻¹¹. The results of the linearity and calibration curve were represented in Table 2, and Fig-4.

Linearity

The linearity of the developed method was evaluated by processing five different concentration levels of

Table-2: Data for Linearity of Etelcalcetide

Concentration (µg/ml)	Average Peak Area
6	1078475
8	1461129
10	1808358
12	2211573
14	2593778

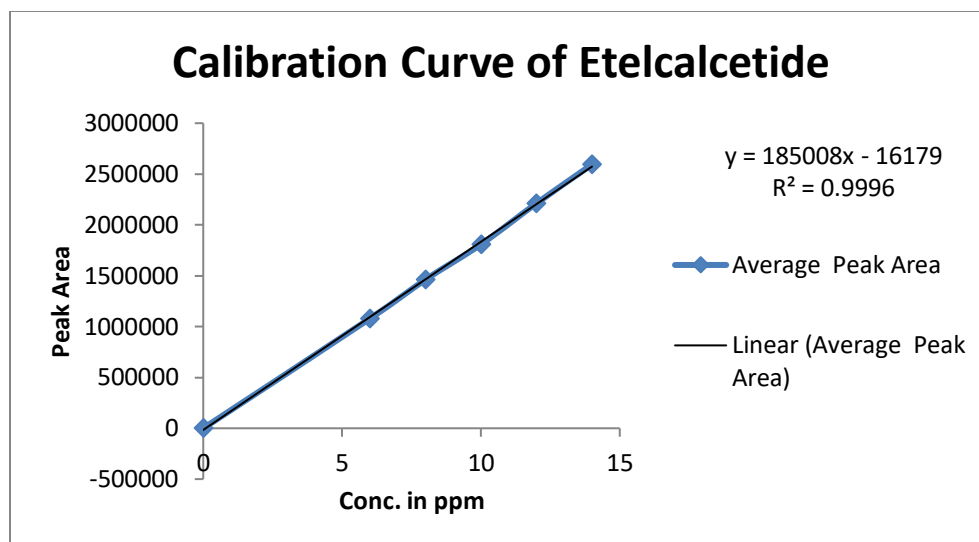


Fig-4: Linearity Curve of Etelcalcetide

Linearity Plot: The plot of Concentration (x) versus the Average Peak Area (y) data of Etelcalcetide is a straight line.

$$Y = mx + c$$

$$\text{Slope (m)} = 18500$$

$$\text{Intercept (c)} = 16179$$

$$\text{Correlation Coefficient (r)} = 0.999$$

Validation Criteria: The response linearity is verified if the Correlation Coefficient is 0.99 or greater.

Conclusion: Correlation Coefficient (r) is 0.99, and the intercept is 0.16179. These values meet the validation criteria.

Precision:

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions¹².

Repeatability: Obtained Six (6) replicates of 100% accuracy solution as per experimental conditions¹³. Recorded the peak areas and calculated % RSD and results were shown in table-3.

Table-3: Results of Repeatability for Etelcalcetide:

S. No.	Peak name	Retention time	Area($\mu\text{V}\cdot\text{sec}$)	Height (μV)	USP Plate Count	USP Tailing
1	Etelcalcetide	2.293	1658954	186958	1.26	6785
2	Etelcalcetide	2.276	1658745	187548	1.27	6854
3	Etelcalcetide	2.286	1659865	189854	1.26	6852
4	Etelcalcetide	2.277	1653254	186985	1.25	6784
5	Etelcalcetide	2.280	1654781	189542	1.24	6895
6	Etelcalcetide	2.293	1661324	187586	1.28	6965
Mean			1657821			
Std. Dev			3120.433			
%RSD			0.188225			

Intermediate Precision¹⁴:**Analyst1:****Table-4: Results of Intermediate Precision Analyst 1 for Etelcalcetide**

S.No.	Peak Name	RT	Area (μV*sec)	Height (μV)	USP Plate Count	USP Tailing
1	Etelcalcetide	2.274	1678541	186589	6587	1.26
2	Etelcalcetide	2.258	1685985	186598	6321	1.26
3	Etelcalcetide	2.267	1685745	186985	6385	1.25
4	Etelcalcetide	2.270	1685987	187854	6580	1.26
5	Etelcalcetide	2.264	1698526	187549	6721	1.27
6	Etelcalcetide	2.265	1685943	186598	6637	1.26
Mean			1686788			
Std. Dev			6463.466			
% RSD			0.383182			

Analyst 2:**Table-5: Results of Intermediate Precision Analyst 2 for Etelcalcetide**

S.No.	Peak Name	RT	Area (μV*sec)	Height (μV)	USP Plate count	USP Tailing
1	Etelcalcetide	2.277	1665847	167481	6854	1.25
2	Etelcalcetide	2.255	1658989	167854	6785	1.26
3	Etelcalcetide	2.265	1659845	167895	6854	1.24
4	Etelcalcetide	2.255	1665964	167854	6895	1.26
5	Etelcalcetide	2.253	1659863	168585	6459	1.25
6	Etelcalcetide	2.252	1665986	167859	6456	1.26
Mean			1662749			
Std. Dev			3501.766			
% RSD			0.210601			

Accuracy

Method accuracy¹⁵ was estimated at three variable concentrations of 50%, 100%, and 150% levels by spiking the known amount of the drug analytes. The % recovery¹⁶⁻¹⁸ at each level was calculated, and the findings were represented in Table 6.

Table-6: The Accuracy Results for Etelcalcetide

% Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	109068.3	5	5.021	100.420%	100.72%
100%	202187	10	10.054	100.540%	
150%	297032.3	15	15.181	101.206%	

Limit of Detection

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value¹⁹.

$$LOD = 3.3 \times \sigma / s$$

Where

σ = Standard deviation²⁰ of the response

S = Slope of the calibration curve

Result: = 0.95 µg/ml

Quantitation Limit

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined²¹.

$$LOQ = 10 \times \sigma / S$$

Where

σ = Standard deviation of the response

S = Slope of the calibration curve²²

Result: = 2.9 µg/ml

Robustness:

The robustness was performed for the flow rate variations from 0.9 ml/min to 1.1 ml/min and mobile phase ratio variation from more organic phase to less organic phase ratio for Etelcalcetide²³. The method is robust only in less flow condition. The standard of Etelcalcetide was injected by changing the conditions of chromatography. There was no significant change in the parameters like resolution, tailing factor, asymmetric factor, and plate count²⁴⁻²⁶.

Table-7: Results for Robustness of Etelcalcetide

Parameter Used for Sample Analysis	Peak Area	Retention Time	Theoretical Plates	Tailing Factor
Actual Flow rate of 1.0 mL/min	1658242	2.312	6569	1.24
Less Flow rate of 0.9 mL/min	1854215	2.458	6865	1.35
More Flow rate of 1.1 mL/min	1758468	2.032	6254	1.32

SUMMARY AND CONCLUSION:

The analytical method was developed by studying different parameters. First of all, maximum

absorbance was found to be at 235nm and the peak purity was excellent. Injection volume was selected to be 10 µl which gave a good peak area. The column

used for study was Symmetry ODS C₁₈ (4.6mm×250mm, 5µm) because it was giving good peak. Ambient temperature was found to be suitable for the nature of drug solution. The flow rate was fixed at 1.0ml/min because of good peak area and satisfactory retention time. Mobile phase is Methanol: Phosphate Buffer pH-3.6 in the ratio of 35:65% v/v was fixed due to good symmetrical peak. So this mobile phase was used for the proposed study. Methanol was selected because of maximum extraction sonication time was fixed to be 10.0min at which all the drug particles were completely soluble and showed good recovery. Run time was selected to be 8min because analyze gave peak around 2.293min and also to reduce the total run time. The percent recovery was found to be 98.0-102% was linear and precise over the same range. Both system and method precision was found to be accurate and well within range. The analytical method was found linearity over the range of 6-14ppm of the Etelcalcetide target concentration. The analytical passed both robustness and ruggedness tests. On both cases, relative standard deviation was well satisfactory.

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