Stability indicating RP-HPLC method for quantification of Eravacycline and its impurities in Eravacycline for injection

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ABSTRACT

The main aim and objective of the research work is to develop an effective, sensitive, economical and simple reverse phase HPLC method for quantification of Eravacycline and its impurities in Eravacycline parenteral dosage form. The separation was achieved by using a stationary phase waters Primesil C18 (250 x 4.6 mm, 5μ) and the mobile phase consists of ammonium acetate buffer and acetonitrile in the proportion of gradient elution. The flow rate was 1.0 mL/min. Eravacycline was detected by using UV detector at the wavelength of 210 nm. The column temperature was maintained at 40°C and sample cooler temperature was maintained at 5°C, injection volume 10 μL, run time was 60 minutes. The developed method was validated for various parameters as per ICH guidelines like accuracy, precision, linearity, specificity, solution stability.

1. Introduction

Eravacycline (TP-434, Xerava) is a synthetic halogenated tetracycline class antibiotic by Tetra phase Pharmaceuticals. It is closely related to tigecycline. It has a broad spectrum of activity including many multi-drug resistant strains of bacteria. Phase III studies in complicated intra-abdominal infections (cIAI) [1] and complicated urinary tract infections (cUTI) [2] were recently completed with mixed results. Eravacycline was granted fast track designation by the FDA [3] and is currently available in USA.

Eravacycline has shown broad spectrum of activity against a variety of Gram-positive and Gram-negative bacteria, including multi-drug resistant strains, such as methicillin-resistant Staphylococcus aureus (MRSA) and carbapenem-resistant

Enterobacteriaceae [4]. It is currently being formulated as for intravenous and oral administration.

The chemical name of Eravacycline dihydrochloride is [(4S,4aS,5aR,12aS)-4-(dimethylamino)-7-fluoro-3,10,12,12a-tetrahydroxy-1,11-dioxo-9-[2-(pyrrolidin-1-yl) acetamido]-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide] dihydrochloride corresponding to the molecular formula $C_{27}H_{31}FN_4O_8.2HCl$. It has a relative molecular mass of 631.48 g/mol and the following structure:
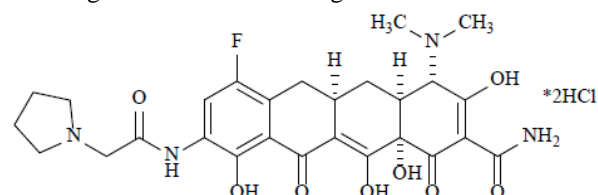

Figure 1. Chemical structure of Eravacycline

Drug product is a sterile, preservative-free, yellow to orange, lyophilized powder in a glass single dose vial for intravenous infusion after reconstitution and dilution. Each vial of Drug product (XERAVA) contains 50 mg of Eravacycline (equivalent to 63.5 mg of Eravacycline dihydrochloride) and the excipient, mannitol (150 mg). Sodium hydroxide and hydrochloric acid are used as needed for pH adjustment to 5.5 to 7.0. The literature survey reveals that there are no HPLC methods were reported in major pharmacopoeias like USP, EP, JP and BP. Only a few methods were reported to date for the estimation of Eravacycline in biological fluid were carried out by LC-MS/MS [5] method.

Hence we tried to develop stability indicating the HPLC method for quantification of Eravacycline and its impurities in Eravacycline parenteral dosage form according to ICH guidelines [6-7].

2. Materials and Methods

Chemicals and Reagents

Eravacycline dihydrochloride (EC) (Clearsynth, Mumbai, India), Acetonitrile (J.T Baker, USA), Ammonium acetate (Merck, Mumbai, India), Ultra pure water (Milli-Q system, Millipore, Bedford, MA, USA) Hydrochloric acid (Merck, Mumbai, India), Sodium hydroxide (Merck, Mumbai, India), Hydrogen peroxide (Merck, Mumbai, India). The chemicals and solvents were used in this study analytical and HPLC grade.

Instrumentation

Waters HPLC model: e2695 with DAD, Bandelin ultrasonic bath, pH Meter (Thermo Orion Model) and Analytical Balance (Metller Toledo Model) were used in the present study.

Methods

Chromatographic conditions

The chromatographic analysis was performed on waters 2695 HPLC system. The chromatograms are recorded and analysed Empower[3] software. The separation was performed on Primesil C18 (250 x 4.6 mm, 5μ) mobile phase consisting of mobile phase-A is ammonium acetate buffer and mobile phase-B is acetonitrile and water in gradient mode. The HPLC gradient program was time (min)/B% v/v: 0/10, 10/45, 30/85, 50/85, 55/10, 60/10. The flow rate was 1.0 mL/min, the column oven temperature was 40°C and the sampler cooler temperature was 5°C, the injection volume was 10μL, and detection was performed at 210 nm using a photodiode array detector (PDA).

Preparation of mobile phase-A

Accurately weighed and transferred 0.7715 g of ammonium acetate into a 1000 mL of milli-Q water and mixed well. Filtered the solution with 0.45 µm membrane filter and sonicate to degas.

Preparation of mobile phase-B

Prepared a mixture of 900 mL of Acetonitrile and 100 mL of water in the ratio of 90:10 (%v/v). Filtered the solution with 0.45 µm membrane filter and sonicate to degas.

Preparation of diluent

Prepared a mixture of 800 mL of water and 200 mL of acetonitrile in the ratio of 50:50 (%volume/volume). Filtered the solution with 0.45 µm membrane filter and sonicate to degas.

Preparation of standard solution

Weighed accurately 20.21 mg of Eravacycline working standard into a 100 mL volumetric flask, added 70 mL diluent, sonicate for 2 minutes to dissolve, diluted to volume with diluent and mixed well. Further diluted 1.0 mL of this solution into a 100 mL volumetric flask, made up to volume with diluent and mixed well. (Concentration of the standard contains about 2µg/mL of Eravacycline).

Preparation of sensitivity solution

Transferred 5 mL of the standard solution into 20 mL volumetric flask, diluted to volume with diluent and mixed well. (Concentration of the standard solution contains about 0.5µg/mL of Eravacycline).

Preparation of placebo solution

Reconstituted 2 vials (placebo) with 5 mL of diluent and transferred the entire contents to 50 mL volumetric flask with suitable hypodermic needle and syringe. Rinsed the each vial with 5 mL diluent for 2 times and transferred the entire contents to same 50 mL volumetric flask with suitable hypodermic needle and syringe. Diluted to volume with diluent and mixed well.

Preparation of sample solution

Reconstituted 2 vials (sample) with 5 mL of diluent and transferred the entire contents to 50 mL volumetric flask with suitable hypodermic needle and syringe. Rinsed the each vial with 5 mL diluent for 2 times and transfer the entire contents to same 50 mL volumetric flask with suitable hypodermic needle and syringe. Diluted to volume with diluent and mixed well.

3.0 Method development

UV-spectroscopic analysis of Eravacycline drug substance showed maximum UV absorbance (λmax) at 210 nm respectively.

To develop a suitable and robust HPLC method for the quantification of Eravacycline and its impurities in Eravacycline parenteral dosage form, different mobile phases were employed to achieve a efficiently quantification of Eravacycline and separation of impurities from blank, placebo and Eravacycline analyte peak.

The method development was started with waters x-bridge shield RP-18 (250 x 4.6 mm, 5µ) with the following different mobile phase compositions like mobile phase-A 0.1% orthophosphoric acid buffer and mobile phase-B acetonitrile in gradient mode. There was no proper resolution of impurities and analyte peak and efficiency of the peak is also not achieved and peak interferences are present.

For the next trial the mobile phase consisted of pH 2.8 phosphate buffer and acetonitrile in gradient mode respectively, flow rate 1.0 mL/min, column temperature 25°C and sampler cooler maintained 5°C. UV detection was performed at 210nm. There was no proper resolution of impurities and analyte peak.

For the next attempt the mobile phase consisted of ammonium acetate buffer and acetonitrile and water in the ratio of (90:10 v/v) in gradient mode respectively, flow rate 1.0 mL/min, column temperature 25°C and sampler cooler maintained 5°C. UV detection was performed at 210nm. There was no proper resolution of impurities and analyte peak.

For the next attempt column was changed from waters x-bridge shield RP-18 (250 x 4.6 mm, 5µ) to Primesil C18 (250 x 4.6mm, 5µm) and the mobile phase consisted of ammonium acetate buffer and acetonitrile and water in the ratio of (90:10 v/v) in gradient mode respectively, flow rate 1.0 mL/min, column temperature 40°C and sampler cooler maintained 5°C. UV detection was performed at 210nm. The resolution of both drug and impurities was achieved. These chromatographic conditions were selected for validation studies.

4. Results and Discussion (Related substances)

The developed RP-HPLC method was extensively validated for quantification of Eravacycline and its impurities in Eravacycline parenteral dosage form using the following parameters.

4.1.1 *Specificity* (Blank and placebo interference) [8-9]

Specificity was demonstrated by injected blank solution, placebo solution, standard solution, sample solution and analyzed as per the optimised method. The observations are tabulated below Table 1and Figure 2 to Figure 5.
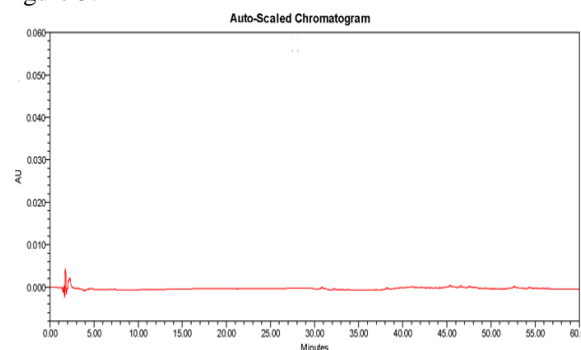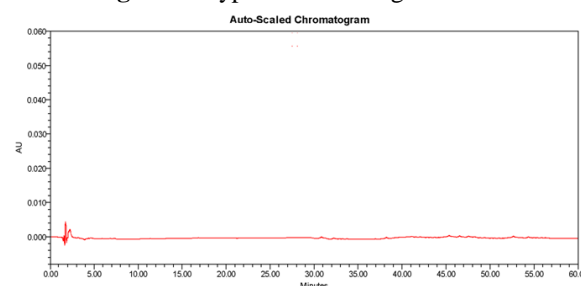

Figure 2. Typical chromatogram blank

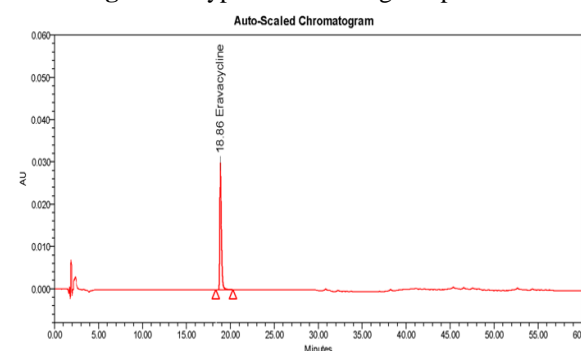**Figure 3.** Typical chromatogram placebo
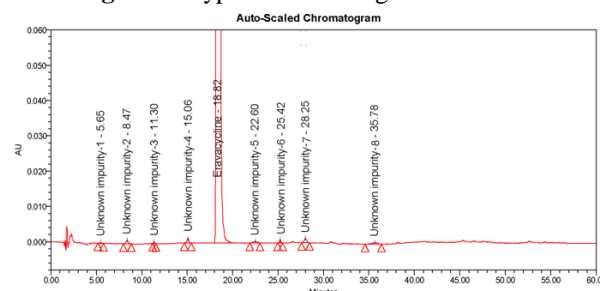

Figure 4. Typical chromatogram standard

Figure 5. Typical chromatogram sample

Table 1 and Figures 2 to 5 illustrates that the specificity the chromatograms were recorded for blank, placebo, standard and sample solutions of Eravacycline. Specificity studies reveal that the peaks are well separated from each other. Therefore the method is selective for the quantification of Eravacycline and related substances in Eravacycline parenteral dosage formulations. There is no interference of diluent and placebo at Eravacycline analyte peak.

Table 1. Specificity results

S.No.	Name	Retention Time (min)	Blank	Placebo
1	Blank	ND	NA	NA
2	Placebo solution	ND	NA	NA
3	Standard solution	18.86	No	No
4	Sample solution	18.82	No	No

4.1.2 *System suitability*

Table 2. System suitability results

S.No.	Name	Retention Time (min)	Theoretical plates	Tailing factor
1	Standard solution	18.86	7895	1.1

4.1.3 *Force degradation studies*

A study was conducted to demonstrate the effective separation of degradants/impurities from Eravacycline analyte peak. Separate portions of sample and placebo solutions were exposed to the following stress conditions to induce degradation. Stressed and unstressed samples were injected into the HPLC system with a PDA detector. The degradation study results were presented in Table 3 and Table 4.

Table 3. Forced degradation results

Stress condition	Impurity at RRT about 0.45 (%)	Impurity at RRT about 1.35 (%)	Any single impurity (%)	Total impurities (%)
As such	0.02	0.05	0.06	0.17
Acid	0.02	0.05	0.05	0.19
Alkali	0.02	0.05	0.06	0.21
Oxidative	0.56	1.89	0.05	2.73
Photolytic	1.12	0.11	0.11	1.51
Humidity	0.02	0.04	0.05	0.15
Thermal	0.03	0.05	0.06	0.22

Based on the above forced degradation results major degradation impurities are observed at RRT about 0.45 and 1.35 in the oxidation and photolytic stress conditions.

Table 4. Mass balance results

Stress condition	Degradation condition	% Assay	% Degradation	Mass Balance
As such	Control sample	100.1	0.11	NA

Acid	1.0 N HCl/60°C/12 hrs	99.9	0.19	99.9
Alkali	1.0 N NaOH/60°C/12 hrs	100.3	0.21	100.3
Oxidative	30% H_2O_2/BT/24 hrs	97.11	2.73	99.6
Photolytic	(200 watt hours/m2 & 1.2 million Lux hours)	98.52	1.51	99.8
Humidity	90%RH exposed for 7 days	100.3	0.15	100.2
Thermal	105°C/7 days	99.8	0.22	99.8

Table 3 and Table 4 illustrates that the degradation study results were shown significant degradation was observed in oxidation and photolytic stress conditions. Hence it can be concluded that Eravacycline is sensitive to photolytic and thermal. The results proved that the developed method has good selectivity and specificity.

4.1.4 System precision [10-11]

The standard solution was prepared as per the optimised method, injected into the HPLC system six times, and evaluated the % RSD for the area responses. The data were shown in Table 5.

Table 5. System precision results

S.No.	No.of injections	Peak area
1	Injection-1	63145
2	Injection-2	62896
3	Injection-3	62884
4	Injection-4	61997
5	Injection-5	61355
6	Injection-6	63001
Avg.		62546
Std.Dev.		710.2748
%RSD		1.14

Table 5 illustrates that the %RSD of peak area for Eravacycline standard was found to be 1.14% which is below 5.0% indicates that the system gives precise result.

4.1.5 *Method Precision*

Method precision was demonstrated by preparing six samples of Eravacycline 50 mg/vial concentrate for solution for infusion as per method and injected in to the chromatographic system. The precision of the method was evaluated by calculating the impurities found and % relative standard deviation for impurities found for each set of samples. The results of the precision study are tabulated below Table 6.

Table 6. Method precision results

Preparations	Impurity at RRT about 0.45 (%)	Impurity at RRT about 1.35 (%)	Individual maximum unknown impurity (%)	Total impurities (%)
Prep-1	0.023	0.027	0.041	0.17

Prep-2	0.025	0.022	0.048	0.19
Prep-3	0.026	0.028	0.045	0.18
Prep-4	0.022	0.024	0.042	0.17
Prep-5	0.025	0.028	0.049	0.15
Prep-6	0.024	0.027	0.047	0.16
Average	0.024	0.026	0.045	0.170
STDEV	0.00147	0.00245	0.00327	0.01414
% RSD	6.09	9.42	7.20	8.32

Table 6 illustrates that the method precision was demonstrated by prepared six control samples and analyzed as per the method. The results control samples results were within the limits. From the above results, it is concluded that method is precise.

4.2 Results and Discussion (Assay)

Preparation of standard solution

Weighed accurately 25.18 mg of Eravacycline working standard into a 25 mL volumetric flask, added 10 mL diluent, sonicate for 2 minutes to dissolve, diluted to volume with diluent and mixed well. Further diluted 5.0 mL of this solution into a 25 mL volumetric flask, made up to volume with diluent and mixed well. (Concentration of the standard contains about 0.2mg / mL of Eravacycline).

Preparation of placebo solution

Reconstituted 2 vials (placebo) with 5 mL of diluent and transferred the entire contents to 50 mL volumetric flask with suitable hypodermic needle and syringe. Rinsed the each vial with 5 mL diluent for 2 times and transferred the entire contents to same 50 mL volumetric flask with suitable hypodermic needle and syringe. Diluted to volume with diluent and mixed well. Further diluted this solution 5 mL in to 50 mL volumetric flask and made up the volume with diluent and mixed well.

Preparation of sample solution

Reconstituted 2 vials (sample) with 5 mL of diluent and transferred the entire contents to 50 mL volumetric flask with suitable hypodermic needle and syringe. Rinsed the each vial with 5 mL diluent for 2 times and transfer the entire contents to same 50 mL volumetric flask with suitable hypodermic needle and syringe. Diluted to volume with diluent and mixed well. Further diluted this solution 5 mL in to 50 mL volumetric flask and made up the volume with diluent and mixed well.

4.2.1 Specificity (Blank and placebo interference)

Specificity was demonstrated by injected blank solution, placebo solution, standard solution, sample solution and analyzed as per the optimised method. The observations are tabulated below Table 7 and Fig. 6-9.
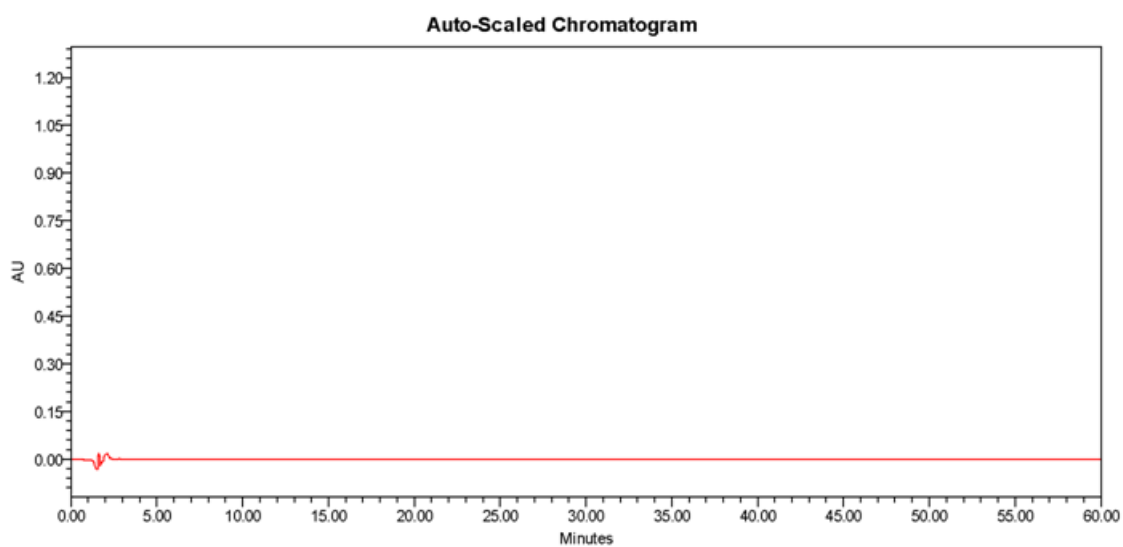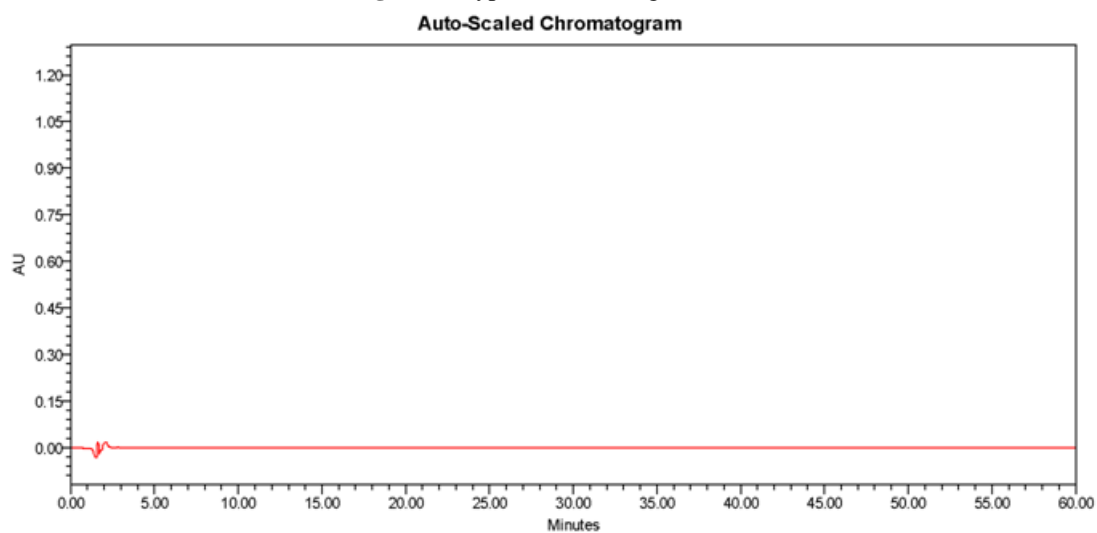
Figure 6. Typical chromatogram blank

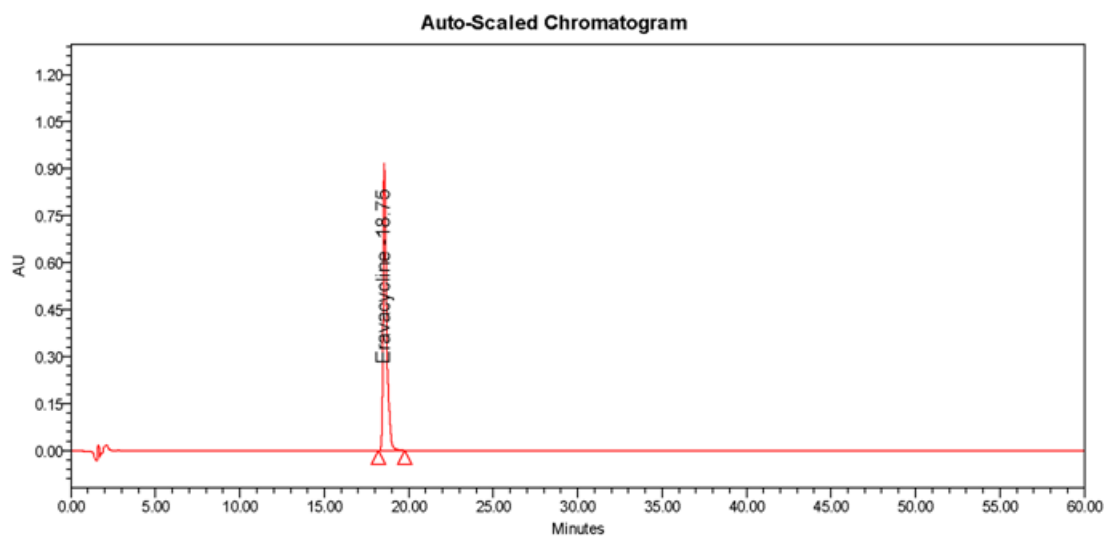**Figure 7.** Typical chromatogram placebo
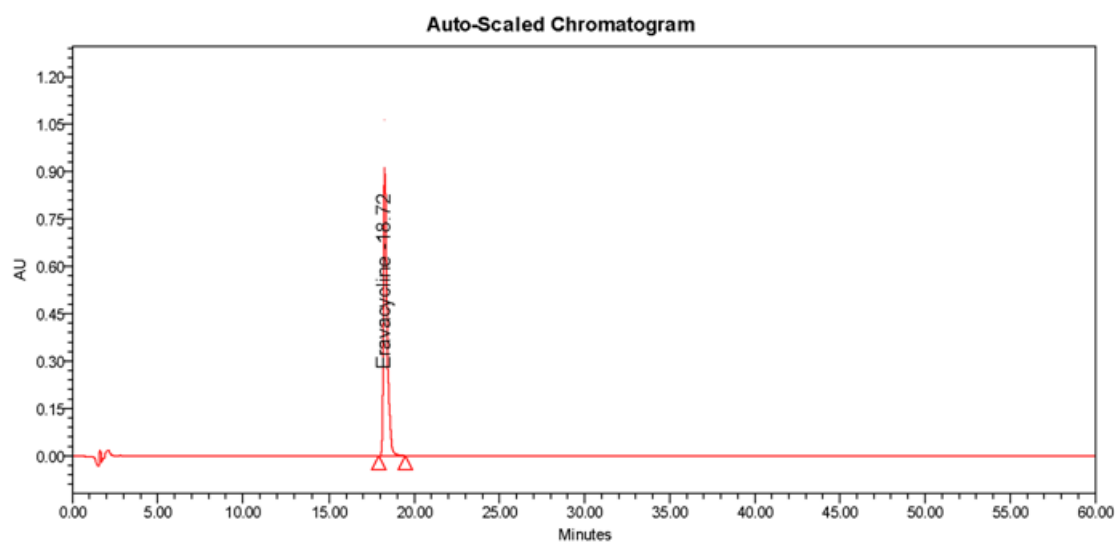
Figure 8. Typical chromatogram standard

Figure 9. Typical chromatogram sample

Table 7 and Figures 6 to 9 illustrates that the specificity the chromatograms were recorded for blank, placebo, standard and sample solutions of Eravacycline. Specificity studies reveal that there is no interference of diluent and placebo at Eravacycline analyte peak. Therefore the method is selective for the Quantification of Eravacycline in Eravacycline parenteral dosage form.

Table 7. Specificity results

S.No.	Name	Retention Time (min)	Blank	Placebo
1	Blank	ND	NA	NA
2	Placebo solution	ND	NA	NA
3	Standard solution	18.75	No	No
4	Sample solution	18.72	No	No

4.2.2 *System precision*

The standard solution was arranged as per the test technique, infused keen on the HPLC system six times, and calculated the % RSD for the vicinity responses. The statistics were revealed in Table 8.

Table 8. System precision results

S.No.	No.of injections	Peak area
1	Injection-1	31563090
2	Injection-2	31484178
3	Injection-3	31498978
4	Injection-4	31568845
5	Injection-5	31458952
6	Injection-6	31504574
Average		31513103
STDEV		43925.2
% RSD		0.14

Table 8 illustrates that the relative standard deviation of six replicates standard solution consequences were establish to be within the specification limit i.e.0.14%.

4.2.3 *Method Precision*

The method precision of the test method was estimated by doing an assay for six samples of Eravacycline 50 mg/vial concentrate for solution for infusion as per the optimised technique. The % assay for Eravacycline for each of the test preparation was calculated. The middling content of the six arrangements and % RSD for the six observations were determined. The statistics were revealed in Table 9.

Table 9. Method precision results

S.No.	No. of Preparations	% Assay
1	Preparation 1	100.1
2	Preparation 2	100.4
3	Preparation 3	100.2
4	Preparation 4	99.9
5	Preparation 5	100.0
6	Preparation 6	100.3
Average		100.2
SD		0.18708
%RSD		0.19

Table 9 illustrates that the method precision was demonstrated by prepared six control samples at specification level and analyzed as per the method. The results control samples results were well within the limits. From the above results, it is concluded that method is precise.

4.2.4 Linearity [12-17]

The linearity of an analytical method is its ability to obtain test results which has a definite mathematical relation to the concentration of the analyte. The linearity of response for Eravacycline was determined in the range of 25% to 150 % (50.36-302.16 µg/mL for Eravacycline). The statistics were revealed in Fig.10 and Table 10.

Table 10. Linearity studies for Eravacycline

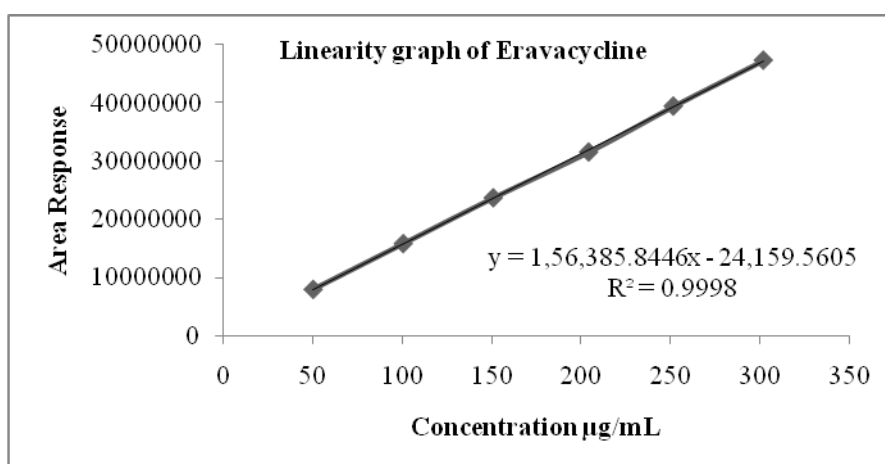| S.No	Linearity Level	Concentration (ppm)	Area response
1	25	50.36	7900254
2	50	100.72	15780351
3	75	151.08	23670957
4	100	204.44	31554565
5	125	251.8	39453537
6	150	302.16	47351950
Correlation coefficient (r^2)			0.9998
Slope			156385.8446
Intercept			-24159.5605
% Y-intercept			-0.08

Figure 10. Linearity graph of Eravacycline

Table 10 and Figure 10 illustrates that the linearity results for Eravacycline in the specified concentration range are found satisfactory. The linearity results for Eravacycline in the specified concentration range are found satisfactory, with a correlation coefficient greater than 0.9998.

4.2.5 *Accuracy* [18]

The accuracy of the test method was demonstrated by preparing recovery samples at 50%, 100 % and 150 %of the target concentration level. The recovery samples were prepared in triplicate for each concentration level. The above samples were injected and the percentage recovery of each sample was calculated for the amount added. Evaluated the precision of the recovery at each level by computing the % Relative Standard Deviation of triplicate recovery samples results. The data obtained which given in Table 11. and the method was found to be accurate.

Table 11. Recovery studies for Eravacycline

Level	Added (µg)	Found (µg)	% Recovery	Mean % Recovery	%RSD
Accuracy at 50% Level-1	50.1767	49.9985	99.64		
Accuracy at 50% Level-2	50.8677	50.7355	99.74	99.7	0.06
Accuracy at 50% Level-3	50.8199	50.6908	99.75		

Accuracy at 100% Level-1	200.3468	200.3132	99.98		
Accuracy at 100% Level-2	200.1914	200.3412	100.07	99.9	0.22
Accuracy at 100% Level-3	200.9984	200.2939	99.65		
Accuracy at 150% Level-1	250.6988	250.3575	99.86		
Accuracy at 150% Level-2	250.8981	250.4476	99.82	99.9	0.06
Accuracy at 150% Level-3	250.4145	250.267	99.94		

Table 11 illustrates that the accuracy at 50% level, 100% level and 150% level for Eravacycline is meeting the acceptance criteria. From the above results, it is concluded that method is accurate.

4.2.6 *Solution stability of analytical solutions*

Solution stability of standard, sample solutions were established at various conditions such as bench top on room temperature and at refrigerator 2-8°C. The stability of standard, sample solutions was determined by comparison of initially prepared standard, sample solutions with freshly prepared standard solution. The data obtained which given in Table 12 to Table 17.

Table 12. Solution stability of standard

Time Interval	Similarity factor	
	Room temperature	Refrigerator
Initial	NA	NA
24hrs	1.03	1.02
48hrs	1.04	1.02

Table 13. Solution stability of RS sample at room temperature

Component	Initial	After 24Hrs	% Difference	After 48Hrs	% Difference
Impurity at RRT about 0.60 (%)	0.02	0.02	0.00	0.03	0.01
Impurity at RRT about 0.70 (%)	0.05	0.06	0.01	0.07	0.02
Any single impurity (%)	0.06	0.06	0.00	0.07	0.01
Total impurities	0.25	0.26	0.01	0.29	0.04

Table 14. Solution stability of RS sample in refrigerator

Component	Initial	After 24Hrs	% Difference	After 48Hrs	% Difference
Impurity at RRT about 0.60 (%)	0.02	0.02	0.00	0.03	0.01
Impurity at RRT about 0.70 (%)	0.05	0.05	0.00	0.06	0.01
Any single impurity (%)	0.06	0.06	0.00	0.06	0.00
Total impurities	0.25	0.26	0.25	0.27	0.02

Table 15. Solution stability of Assay standard

Time Interval	Similarity factor	
	Room temperature	Refrigerator
Initial	NA	NA
24hrs	1.01	1.00
48hrs	1.01	1.00

Table 16. Solution stability of Assay sample at room temperature

Time Interval	%Assay	%Assay difference
Initial	100.1	NA
24hrs	100.2	0.1
48hrs	100.3	0.2

Table 17. Solution stability of Assay sample in refrigerator

Time Interval	%Assay	%Assay difference
Initial	100.1	NA
24hrs	100.1	0.0
48hrs	100.2	0.1

Table 12 to Table 17 illustrates that the solution stability of standard, sample at different time intervals studied, from the above results, it is concluded that standard, sample solutions are stable up to 48 hours in both the conditions (bench top and refrigerator).

5. CONCLUSION

The developed method was validated for various parameters as per ICH guidelines like accuracy, precision, linearity, specificity and solution stability. The results obtained were within the acceptance criteria. So, it can be concluded that the developed method is simple, precise, cost-effective, eco-friendly, and safe and can be successfully employed for the routine analysis of Eravacycline in bulk and pharmaceutical dosage forms.

AUTHORS CONTRIBUTION STATEMENT

We have assured that "all authors have read and approved the manuscript." All the authors have equal contribution and participation in this research work. VB has analyzed all samples on HPLC instrument and completed the experimental work and was a major contributor in writing the manuscript. He had completed his work under the supervision of DRC a who help him to elaborate the methodology as well as theoretical approach.

CONFLICT OF INTERESTS

The authors claim that there is no conflict of interest.

REFERENCES

1. Solomkin, Joseph; Evans, David; Slepavicius, Algirdas; Lee, Patrick; Marsh, Andrew; Tsai, Larry; Sutcliffe, Joyce A.; Horn, Patrick (2016-11-16). "Assessing the Efficacy and Safety of Eravacycline vs Ertapenem in Complicated Intra-

abdominal Infections in the Investigating Gram-Negative Infections Treated With Eravacycline (IGNITE 1) Trial: A Randomized Clinical Trial". JAMA Surgery. **152** (3): 224–232. doi:10.1001/jamasurg.2016.4237.

2. "Tetraphase Announces Top-Line Results From IGNITE2 Phase 3 Clinical Trial of Eravacycline in cUTI (NASDAQ:TTPH)". ir.tphase.com. Archived from the original on 2016-11-21. Retrieved 2016-11-20.

3. "FDA Grants QIDP Designation to Eravacycline, Tetraphase's Lead Antibiotic Product Candidate | Business Wire". businesswire.com. 2013-07-15. Retrieved 2016-11-20.

4. Zhanel, George G.; Cheung, Doris; Adam, Heather; Zelenitsky, Sheryl; Golden, Alyssa; Schweizer, Frank; Gorityala, Bala; Lagacé-Wiens, Philippe R. S.; Walkty, Andrew (2016-04-01). "Review of Eravacycline, a Novel Fluorocycline Antibacterial Agent". Drugs. 76 (5): 567–588. doi:10.1007/s40265-016-0545-8.

5. Satya Prasad, B.; Jaya Kumari, S.; Estimation of Eravacycline Dihydrochloride in Biological Matrices by LC-MS/MS. Pharm Methods, 2019;10(2):47-52. DOI : 10.5530/phm.2019.2.9.

6. ICH guidelines, for stability testing of new drug substances and products Q1A (R2), 2004.

7. ICH guidelines for validation of analytical procedures: text and methodology Q2 (R1) 2005.

8. IUPAC, Harmonized Guidelines for Single-Laboratory Validation of Methods of Analysis, IUPAC Technical Report. Pure and Applied Chemistry. 2002; 74(5).

9. Shah BP, Jain S, Prajapati KK and Mansuri NY. Stability Indicating HPLC Method Development: A Review. International Journal of Pharmaceutical Research Science. 2012; 3(9):2978-2988.

10. Precision of test methods-Repeatability and reproducibility. International Standard ISO 5725. 1986.

11. Snyder LR, Kirkland JJ and Glajch JL. Completing the Method: Validation and Transfer, in Practical HPLC Method Validation, 1st ed. John Wiley and Sons. New Jersey. 2007.

12. Linear calibration using reference material. ISO standard 11095. 1996.

13. Dong MW, Regulatory Aspects of HPLC Analysis: HPLC System and Method Validation, Modern HPLC for Practicing Scientists. 1st ed. John Wiley and Sons. New Jersey. 2006.

14. Araujo P. Key aspects of analytical method validation and linearity evaluation. Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences. 2009; 877(23): 2224-2234.

15. Van Loco J, Elskens M, Croux C, Beernaert H. Linearity of calibration curves: Use and misuse of the correlation coefficient. Accreditation and Quality Assurance. 2002;7: 281-285.

16. Hayashi Y et al. Detection limit estimated from slope of calibration curve: An application to competitive ELISA. Analytical Sciences. 2005; 21(2):167-169.

17. Zhao Y et al. Reasons for calibration standard curve slope variation in LC-MS assays and how to address it. Bioanalysis. 2014; 6(11): 1439-1443.

18. Gonzalez AG, Herrador MA. A practical guide to analytical method validation including measurement uncertainty and accuracy profiles. Trends in Analytical Chemistry. 2007;26(3):11.