ijlpr 2023; doi 10.22376/ijlpr.2023.13.6.P287-P298

International Journal of Life science and Pharma Research

ISSN 2250-0480

Research Article

Chemistry for New drug discovery

Stability Indicating RP-HPLC Method for Quantification of Lefamulin and Its Impurities in Lefamulin Injection

P. Venkata Prabhakara Rao[1], P. Bharath[1], Syed Mastan Ali[1] and D. Ramachandran*[1]

1Department of Chemistry, Acharya Nagarjuna University, Nagarjuna Nagar, Guntur, Andhra Pradesh, India.

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 Lefamulin and its impurities in the Lefamulin parenteral dosage form. The separation was achieved using a stationary phase waters X-Bridge shield RP18 (250 x 4.6 mm, 5µ). The mobile phase consists of ammonium acetate buffer and acetonitrile in the proportion of gradient elution. The flow rate was 1.0 mL/min. Lefamulin was detected using a UV detector at the wavelength of 210 nm. The column temperature was 25°C, the sample cooler temperature was 5°C, the injection volume was 10µL, and the run time was 35 minutes. The developed method was validated for parameters per ICH guidelines like accuracy, precision, linearity, specificity, and solution stability. The developed HPLC method was validated with respect to specificity, the chromatograms were recorded for blank, placebo, standard, and sample solutions of Lefamulin. Specificity studies reveal that the peaks are well separated from each other. Results were found to be within the acceptance limits for system precision and method precision. The linearity results for Lefamulin in the specified concentration range (50.0240-150.0720 µg/mL) are satisfactory, with a correlation coefficient of 0.9999.

Keywords: Lefamulin, Liquid chromatography, Related substances, Assay, Forced degradation and Validation.

***Corresponding Author**

D.Ramachandran , Department of Chemistry, Acharya Nagarjuna University, Nagarjuna Nagar, Guntur, Andhra Pradesh, India.

Received On 9 June, 2023

Revised On 27 September, 2023

Accepted On 10 October, 2023

Published On 1 November, 2023

Funding This research did not receive any specific grant from any funding agencies in the public, commercial or not for profit sectors.

Citation P. Venkata Prabhakara Rao, P. Bharath, Syed Mastan Ali and D.Ramachandran , Stability Indicating RP-HPLC Method for Quantification of Lefamulin and Its Impurities in Lefamulin Injection.(2023).Int. J. Life Sci. Pharma Res.13(6), P287-P298 http://dx.doi.org/10.22376/ijlpr.2023.13.6.P287-P298

1. INTRODUCTION

The chemical name of Lefamulin is (1S,2R,3S,4S,6R,7R,8R,14R)-3-Hydroxy-2,4,7,14-tetramethyl-9-oxo-4-vinyltricyclo [5.4.3.01,8] tetradec-6-yl{[(1R,2R,4R)-4-amino-2-hydroxycyclohexyl] sulfanyl} acetate corresponding to the molecular formula $C_{28}H_{45}NO_5S$. It has a relative molecular mass of 507.74 (free base) g/mol. Lefamulin is available as an acetic acid salt (acetate) with a molecular weight of 567.79 g/mol and a molecular formula of $C_{30}H_{49}NO_7S$[1-10]. The chemical structure of Lefamulin is shown in Fig. 1. Lefamulin acetate is a single stereoisomer semi-synthetically derived from pleuromutilin, a homochiral, natural fermentation product of known absolute stereochemistry. Lefamulin acetate is a diastereomer with R-configuration at carbons of the cyclohexane moiety. The absolute configuration of the active substance has been confirmed by single-crystal structure determination. Lefamulin is indicated to treat adults diagnosed with community-acquired bacterial pneumonia (CABP) caused by susceptible bacteria. Its use should be reserved for confirmed susceptible organisms or a high probability of infection with susceptible organisms. The list of susceptible bacteria includes Streptococcus pneumoniae, Staphylococcus aureus (methicillin-susceptible), Legionella pneumophila, Haemophilus influenzae, Chlamydophila pneumoniae, and Mycoplasma pneumoniae. Lefamulin demonstrates strong antibacterial activity against several microbes that are found to be common in both acute bacterial skin and skin structure infections, as well as community-acquired bacterial pneumonia.1,3 It shows antibacterial activity against gram-positive and atypical microbes (for example, Streptococcus pneumoniae, Legionella pneumophila, Mycoplasma pneumoniae, Haemophilus influenzae, and Chlamydophila pneumoniae). Lefamulin also exerts activity against Staphylococcus aureus, methicillin-resistant Staphylococcus aureus, and vancomycin-resistant Enterococcus faecium. It does not treat Pseudomonas aeruginosa infections. During in vitro studies, the drug has also demonstrated activity against Neisseria gonorrhoeae and Mycoplasma genitalium. Lefamulin acetate is a white to off-white solid. It is a stable compound (requiring no special storage conditions) that is highly soluble in water and 0.9% sodium chloride solution (>300mg/ml). The hydration level of Lefamulin acetate at ambient conditions is typically below 1% w/w. Two polymorphic forms are observed (Form A and Form B), both crystalline. The manufacturing process yields the thermodynamically more stable Form B. FT-IR is used to distinguish between Form A and B. The finished product (Xenleta concentrate 150 mg/15 mL) comprises a sterile, pyrogen-free solution of the active substance in 0.9 % sodium chloride solution. As the finished product is a concentrate solution for infusion, the solubility of the active substance is the most critical physicochemical parameter. Lefamulin acetate is a BCS class III compound, highly soluble in water and 0.9% sodium chloride solution (> 300 mg/mL). Lefamulin inhibits prokaryotic ribosomal protein synthesis by binding to the peptidyl transferase center (PTC) of the ribosomal bacterial 50S subunit. It inhibits protein translation by binding to both the A and P sites of the PTC via four hydrogen bonds, interrupting peptide bond formation.[6] Lefamulin's tricyclic mutilin core is the common moiety for binding all members of its drug class, the pleuromutilins. Although the tricyclic motilin core doesn't form any hydrogen bonds with the PTC nucleotides, it is stabilized or anchored by hydrophobic and Van der Waals interactions.[10] Lefamulin exerts a selective inhibition of protein translation in eukaryotes; however, it does not affect the ribosomal translation of eukaryotes. Lefamulin demonstrates a unique induced-fit action that closes the binding pocket within a ribosome, conferring close contact with the drug to its target improving therapeutic efficacy.[3] Cross-resistance to other antibiotic classes is less likely because of its mechanism of action that differs from other antimicrobials.[2] In a pharmacokinetic study of healthy subjects, lefamulin was rapidly absorbed after oral administration. The median Tmax was measured at 1.00 h for the intravenous preparation and 1.76 h for the tablet preparation. 7 At steady-state doses, the Cmax of oral Lefamulin is 37.1 mcg/mL.[11] The AUC at steady-state concentrations of this drug is 49.2 mcg·h/mL. The estimated bioavailability of the oral tablets is 25%. Clinical studies have found that the AUC of Lefamulin is decreased by about 10-28% in the fed state.[3] To optimize absorption, this drug should be administered a minimum of 1 hour before a meal or, at minimum, 2 hours after a meal with water.[11] CYP3A4 is the main enzyme responsible for the metabolism of Lefamulin. [3,11]

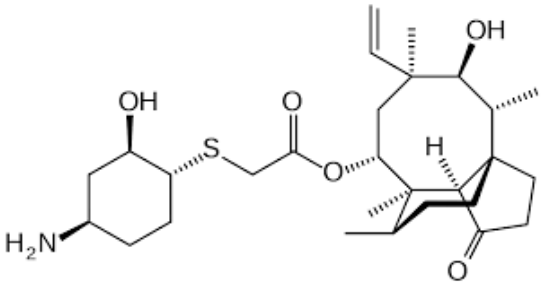

Fig. 1: Chemical structure of Lefamulin

The literature survey reveals that no HPLC methods were reported in major pharmacopeias like USP, EP, JP, and BP. Only a few methods reported to date for estimating Lefamulin in biological fluid were carried out by LC-MS/MS[11]. Hence, we tried to develop stability indicating the HPLC method for quantification of Lefamulin and its impurities in Lefamulin parenteral dosage form according to ICH guidelines[12,13].

2. MATERIALS AND METHODS

2.1. 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.

2.2. *Chemicals and Reagents*

Ammonium acetate (AR grade), acetitrile (HPLC grade), Hydrochloric acid, Sodium hydroxide, Hydrogen peroxide, water, reagents, and chemicals were procured from Merck chemicals. Mumbai, India.

2.3. *Preparation of mobile Phase-A*

Accurately weighed and transferred 0.7754 g of ammonium acetate into 1000 mL of milli-Q water and mixed well. Filtered the solution with 0.45 µm membrane filter and sonicate to degas.

2.4. *Preparation of mobile Phase-B*

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

2.5. Preparation of diluent

Prepared a mixture of 500 mL of water and 500 mL of acetonitrile in a ratio of 50:50 (%volume/volume). Filtered the solution with 0.45 µm membrane filter and sonicate to degas.

2.6. *Preparation of standard solution*

Weighed 20.38 mg of Lefamulin working standard into a 100 mL volumetric flask, added 70 mL diluent, sonicated 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. (Standard concentration contains about 2µg/mL of Lefamulin).

2.7. *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 Lefamulin).

2.8. *Preparation of placebo solution*

Transferred 5.0 mL of placebo solution into 50 mL volumetric flask, added 35 mL diluent, and shaken for 10 minutes to dissolve and dilute to volume with diluent and mixed well.

2.9. *Preparation of test solution*

Transferred 5.0 mL of sample solution into a 50 mL volumetric flask, added 35 mL diluent, and shaked for 10 minutes to dissolve and diluted to volume with diluent and mixed well.

2.10. Preparation of standard solution

Weighed accurately 50.27 mg of Lefamulin working standard into a 50 mL volumetric flask, added 25 mL diluent, sonicated for 2 minutes to dissolve, diluted to volume with diluent and mixed well. Further diluted 5.0 mL of this solution into a 50 mL volumetric flask, made up to volume with diluent and mixed well. (Standard concentration contains about 0.1mg / mL of Lefamulin).

2.11. Preparation of placebo solution

Transferred 5.0 mL of placebo solution into 50 mL volumetric flask, added 35 mL diluent, and shaked for 10 minutes to dissolve and diluted to volume with diluent and mixed well. Further diluted 5.0 mL of this solution into 50 mL volumetric flask diluted to volume with diluent and mixed well.

2.12. Preparation of test solution

Transferred 5.0 mL of sample solution into a 50 mL volumetric flask, added 35 mL diluent, and shaked for 10 minutes to dissolve and diluted to volume with diluent and mixed well. Further diluted 5.0 mL of this solution into 50 mL volumetric flask diluted to volume with diluent and mixed well.

2.13. Method development

UV-spectroscopic analysis of the Lefamulin drug substance showed maximum UV absorbance (λmax) at 210 nm, respectively. To develop a suitable and robust HPLC method for the quantification of Lefamulin and its impurities in Lefamulin parenteral dosage form, different mobile phases were employed to achieve an efficient quantification of Lefamulin and separation of impurities from blank, placebo, and Lefamulin analyte peak.

2.14. Effect of Column Selectivity

The method development started with waters x-bridge shield RP-18 (250 x 4.6 mm, 5µ) with the following mobile phase compositions: mobile phase-A 0.1% orthophosphoric acid buffer and mobile phase-B acetonitrile in gradient mode. There was no proper resolution of impurities, the analyte peak and efficiency of the peak were also not achieved, and peak interferences were present. For the next trial, the mobile phase consisted of pH 2.8 phosphate buffer and acetonitrile in gradient mode, respectively, with a flow rate of 1.0 mL/min, a column temperature of 25°C, and a sampler cooler of 5°C. UV detection was performed at 210nm. There was no proper resolution of impurities and analyte peaks. For the next attempt, the mobile phase consisted of ammonium acetate buffer 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. The resolution of both drug and impurities was achieved. These chromatographic conditions were selected for validation studies.

2.15. Optimized chromatographic conditions

Chromatographic analysis was performed on the water's 2695 HPLC system. The chromatograms are recorded and analyzed by Empower3 software. The separation was performed on waters x-bridge shield RP-18 (250 x 4.6 mm, 5µ) mobile phase consisting of mobile phase-A, ammonium acetate buffer, and mobile phase-B, acetonitrile, and water in gradient mode. The HPLC gradient program was time (min)/B% v/v: 0/15, 10/50, 20/900, 25/90, 30/15, 35/15. The flow rate was 1.0 mL/min, the column oven temperature was 25°C, 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).

3. RESULTS (Related substances and Assay)

The developed RP-HPLC method was extensively validated to quantify Lefamulin and its impurities in Lefamulin parenteral dosage form using the following parameters.

3.1. Specificity (Blank and placebo interference)

Specificity[14,15] was demonstrated by injecting the blank, placebo, standard, and sample solutions and analyzed as per the optimized method. The observations are tabulated below in Table 1 and Fig. 2-5.

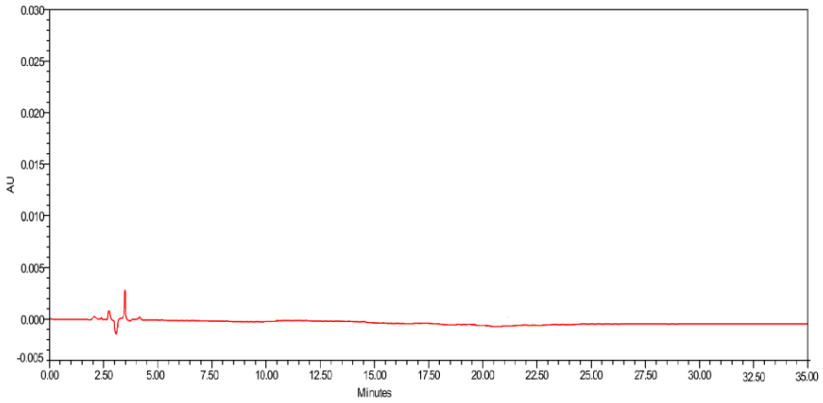

Fig.2. Typical chromatogram blank

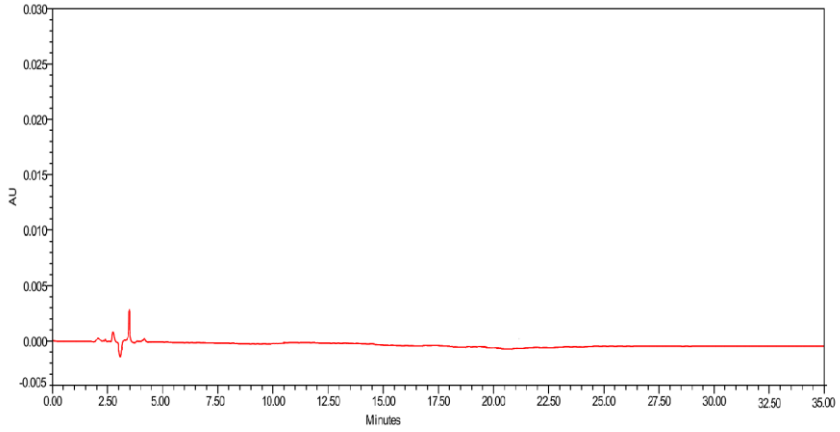

Fig.3. Typical chromatogram placebo

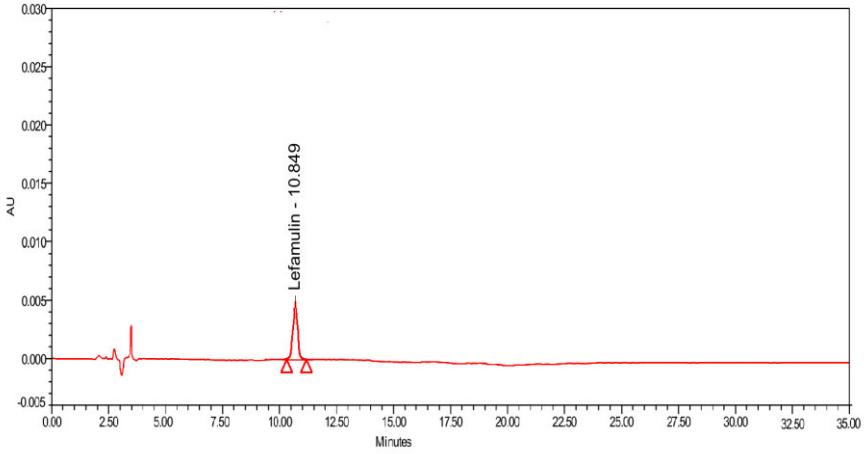

Fig. 4. Typical chromatogram standard

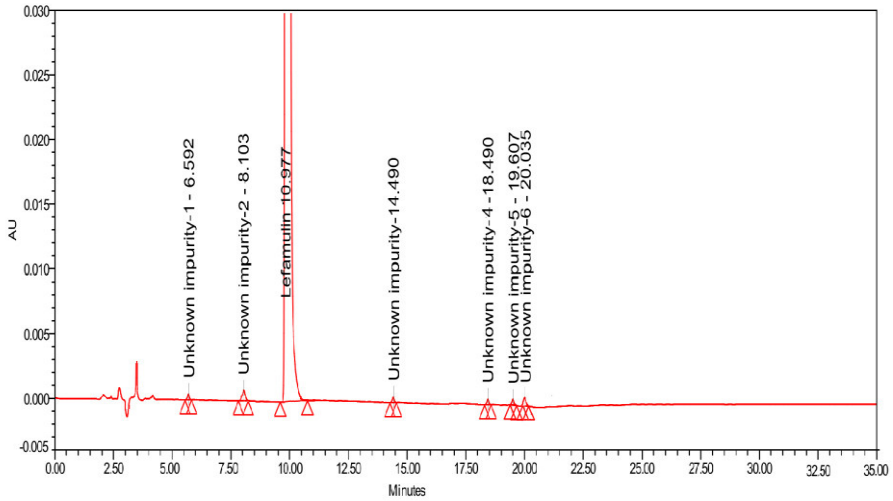

Fig. 5. Typical chromatogram sample

Figures 2 to 5 illustrate that the specificity of the chromatograms was recorded for blank, placebo, standard, and sample solutions of Lefamulin. Specificity studies reveal that the peaks are well separated from each other.

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	10.849	No	No
4	Sample solution	10.977	No	No

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

3.2. System suitability

Table 2: System suitability results

S.No.	Name	Retention Time (min)	Theoretical plates	Tailing factor
1	Standard solution	10.849	7410	1.1

3.3. Force degradation studies

A study was conducted to demonstrate the effective separation of degradants/impurities from the Lefamulin 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 are presented in Table 3 and Table 4.

3.4. Necessity and importance of stability-indicating method

The stabilization studies aim to track potential improvements to a substance or material over time and under various storage conditions. The factors and parameters that affect the stability are production timeframe, batch factors, process parameters, excipient efficiency, and environmental conditions like temperature and humidity. Access to appropriate deteriorated samples for method production assistance is a major challenge when designing a stability indicator method (SIM). Such deteriorated samples in a perfect environment must be real-time stability samples containing all applicable degradants and those developed during ordinary storage conditions. For this cause, pharmacists must use forced degradation samples to create SIMs. Many experiments have explored the potential of forced deterioration studies to predict real-time degradation. The precision of the stability methods showing potential impurities of the drug material and components is demonstrated by forced degradation (FD). Stress experiments help to generate impurities in a much shorter period. The formulations scientist will then generate consistent formulations in less time. FD studies now include completing the file and comprehending the drug production mechanism for globally controlled markets.

Table 3: Forced degradation results				
Stress condition	Impurity at RRT about 0.60 (%)	Impurity at RRT about 0.70 (%)	Any single impurity (%)	Total impurities (%)
As such	0.02	0.05	0.06	0.25
Acid	0.02	0.05	0.05	0.32
Alkali	0.02	0.05	0.06	0.28
Oxidative	0.02	0.05	0.05	0.51
Photolytic	0.28	0.31	0.11	10.3
Humidity	0.02	0.04	0.05	0.27
Thermal	0.27	0.38	0.12	4.5

Based on the above-forced degradation results, major degradation impurities are observed at RRT about 0.60 and 0.70 in the photolytic and thermal stress conditions.

Table 4: Mass balance results				
Stress condition	Degradation condition	% Assay	% Degradation	Mass Balance
As such	Control sample	100.4	0.25	NA
Acid	1.0 N HCl/60°C/6 hrs	99.7	0.32	99.4
Alkali	1.0 N NaOH/60°C/6 hrs	100.1	0.28	99.7
Oxidative	30% H_2O_2/BT/24 hrs	99.9	0.51	99.8
Photolytic	(200 watt hours/m2 & 1.2 million Lux hours)	89.7	10.3	99.4
Humidity	90%RH exposed for 7 days	100	0.27	99.6
Thermal	105°C/7 days	95.2	4.5	99.1

Table 3 and Table 4 illustrate the degradation study results that significant degradation was observed in photolytic and thermal stress conditions. Hence, Lefamulin is sensitive to photolytic and thermal. The results proved that the developed method has good selectivity and specificity.

3.5. System precision

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

Table 5: System precision results		
S.No.	No.of injections	Peak area
1	Inj-1	20320
2	Inj-2	20343
3	Inj-3	20417
4	Inj-4	20811
5	Inj-5	20683
6	Inj-6	20842
	Avg.	20569
	Std.Dev.	237.6002
	%RSD	1.2

Table 5 illustrates that the %RSD of peak area for the Lefamulin standard was 1.20%, below 5.0%, indicating that the system gives precise results.

3.6. Method Precision

Method precision was demonstrated by preparing six samples of Lefamulin 150 mg/15 ml concentrate for solution for infusion as per method and injected into the chromatographic system. The method's precision was evaluated by calculating the impurities found and the % relative standard deviation for impurities found for each set of samples. The results of the precision study are tabulated below in Table 6.

Table 6: Method precision results

Preparations	Impurity at RRT about 0.60 (%)	Impurity at RRT about 0.70 (%)	Individual maximum unknown impurity (%)	Total impurities (%)
Prep-1	0.024	0.051	0.061	0.25
Prep-2	0.025	0.052	0.058	0.24
Prep-3	0.026	0.048	0.055	0.24
Prep-4	0.022	0.054	0.062	0.25
Prep-5	0.025	0.048	0.059	0.24
Prep-6	0.024	0.047	0.057	0.24
Average	0.024	0.05	0.059	0.243
STDEV	0.001	0.003	0.003	0.005
% RSD	5.6	5.5	4.4	2.1

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

3.7. Specificity (Blank and placebo interference)

Specificity[14-15] was demonstrated by injecting a blank, placebo, standard, and sample solution and analyzed as per the optimized method. The observations are tabulated below in Table 7 and Fig. 6-9.

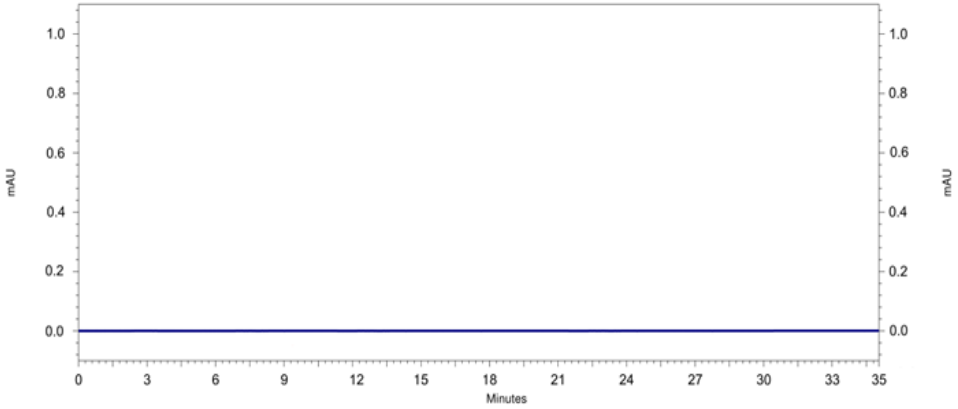

Fig. 6: Typical chromatogram blank

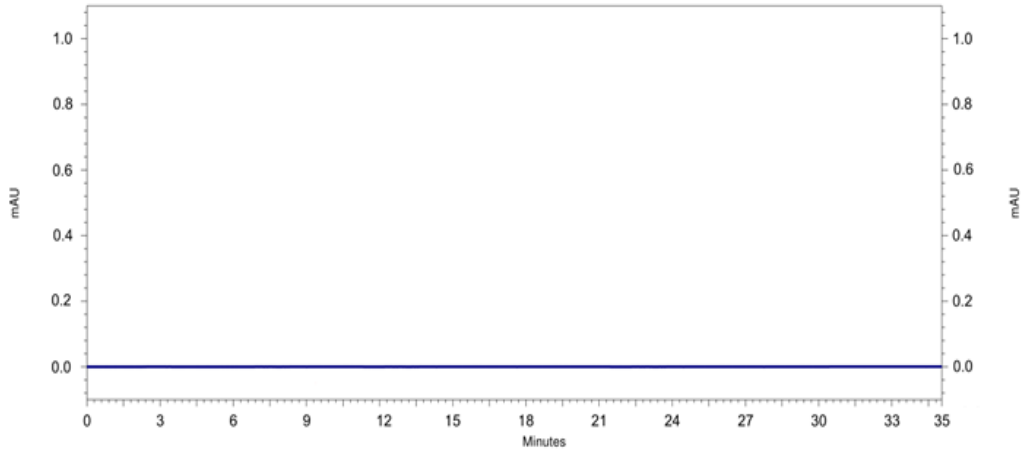

Fig. 7: Typical chromatogram placebo

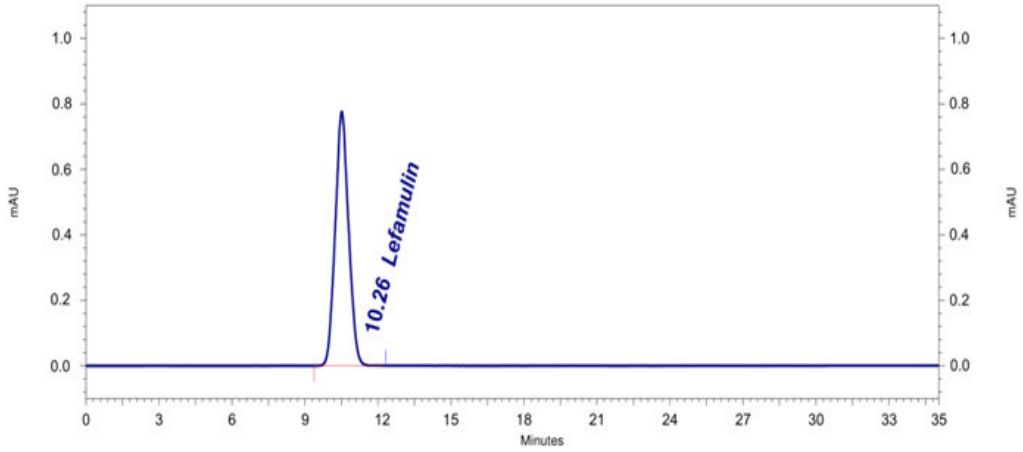

Fig. 8: Typical chromatogram standard

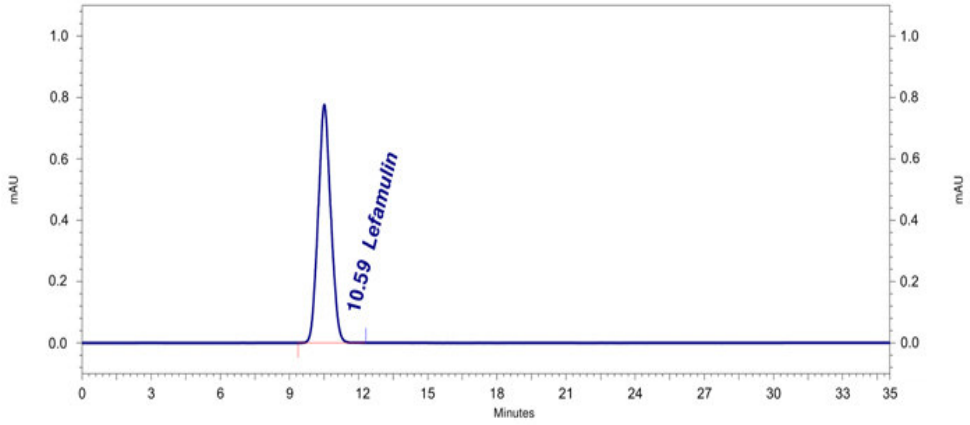

Fig. 9: Typical chromatogram sample

Table 7 and Figures 6 to 9 illustrate that the specificity of the chromatograms was recorded for blank, placebo, standard, and sample solutions of Lefamulin. Specificity studies reveal no interference between diluent and placebo at the Lefamulin analyte peak. Therefore, the method is selective for the Quantification of Lefamulin in Lefamulin 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	10.849	No	No
4	Sample solution	10.977	No	No

3.8. System precision

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

Table 8: System precision results

S.No.	No.of injections	Peak area
1	Inj-1	2057745
2	Inj-2	2014315
3	Inj-3	2029224
4	Inj-4	2063686
5	Inj-5	2029142
6	Inj-6	2036758
Average		2038478
STDEV		18795.0362
% RSD		0.9

Table 8 illustrates that the relative standard deviation of six replicates standard solution consequences was established to be within the specification limit, i.e.0.9%.

3.9. Method Precision

The method precision of the test method was estimated by doing an assay for six samples of Lefamulin 150 mg/15 ml concentrate for solution for infusion as per the optimized technique. The % assay for Lefamulin for each of the test preparations was calculated. The middling content of the six arrangements and % RSD for the six observations were determined. The statistics are revealed in Table 9.

Table 9: Method precision results

S.No	No. of Preparations	% Assay
1	Preparation 1	100.3
2	Preparation 2	100.3
3	Preparation 3	100.3
4	Preparation 4	100.2
5	Preparation 5	100.1
6	Preparation 6	100.2
	Average	100.2
	SD	0.0816
	%RSD	0.1

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

3.10. Linearity

The linearity[18-23] of an analytical method is its ability to obtain test results, which have a definite mathematical relation to the concentration of the analyte. The linearity of response for Lefamulin was determined in the 50% to 150 % (50.0240-150.0720 µg/mL for Lefamulin). The statistics are revealed in Fig.10 and Table 10.

Table 10: Linearity studies for Lefamulin

S.No	Linearity Level	Concentration (ppm)	Area response
1	50	50.0240	1004935
2	80	80.0384	1599244
3	100	100.0480	2005707
4	120	120.0576	2410222
5	150	150.0720	3000042
	Correlation coefficient (r^2)		1.0000
	Slope		19986.0808
	Intercept		4462.5862
	% Y-intercept		0.22

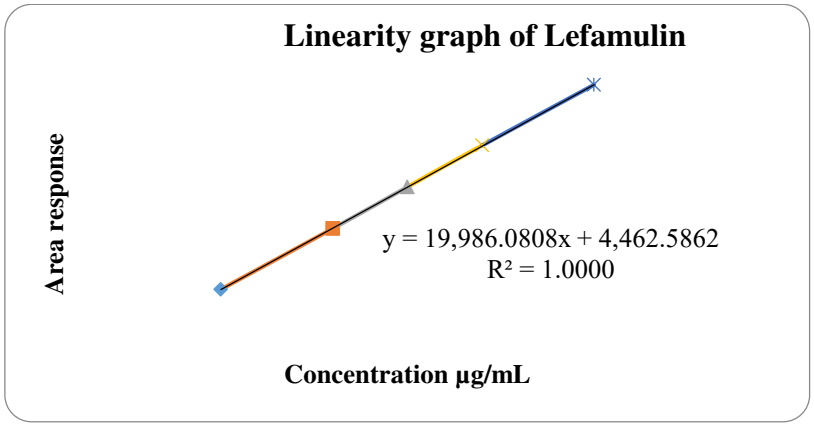

Fig. 10: Linearity graph of Lefamulin

Table 10 and Figure 10 illustrate that the linearity results for Lefamulin in the specified concentration range are satisfactory. The linearity results for Lefamulin in the specified concentration range are satisfactory, with a correlation coefficient greater than 0.9999.

3.11. Accuracy

The accuracy[24] 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 sample results. The data obtained was given in Table 11, and the method was accurate.

Table 11: Recovery studies for Lefamulin

Level	Added (µg)	Found (µg)	% Recovery	Mean % Recovery	%RSD
Accuracy at 50% Level-1	49.8767	49.9785	100.2	99.9	0.3
Accuracy at 50% Level-2	49.8677	49.7355	99.7		
Accuracy at 50% Level-3	49.8199	49.6908	99.7		
Accuracy at 100% Level-1	100.3968	100.3732	100.0	100.1	0.2
Accuracy at 100% Level-2	100.1976	100.3457	100.1		
Accuracy at 100% Level-3	99.9984	100.2939	100.3		
Accuracy at 150% Level-1	149.6988	150.3575	100.4	100.5	0.1
Accuracy at 150% Level-2	149.8980	150.4476	100.4		
Accuracy at 150% Level-3	149.4000	150.2670	100.6		

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

3.12. Solution stability of analytical solutions

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

Table 12: Solution stability of standard

Time Interval	Similarity factor	
	Room temperature	Refrigerator
Initial	NA	NA
24hrs	1.05	1.04
48hrs	1.05	1.05

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 a 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	1.02	1.01

Table 16: Solution stability of Assay sample at room temperature		
Time Interval	%Assay	%Assay difference
Initial	100.3	NA
24hrs	100.2	0.1
48hrs	100.0	0.3

Table 17: Solution stability of Assay sample in a refrigerator		
Time Interval	**%Assay**	**%Assay difference**
Initial	100.3	NA
24hrs	100.3	0.0
48hrs	100.2	0.1

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

4. DISCUSSION

A simple, economical, accurate, and precise HPLC method was successfully developed. This method was carried out using waters X-Bridge shield RP18 (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. Lefamulin was detected using a UV detector at the wavelength of 210 nm. The column temperature was 25°C, the sample cooler temperature was 5°C, the injection volume was 10µL, and the run time was 35 minutes. The results obtained were accurate and reproducible. The method developed was statistically validated regarding the solution's selectivity, accuracy, linearity, precision, and stability. The developed HPLC method was validated concerning specificity[14,15], and the chromatograms were recorded for blank, placebo, standard sample solutions of Lefamulin. Specificity studies reveal that the peaks are well separated from each other. For system precision and method precision[16-17] results were within the acceptance limits. The linearity [18-23] results for Lefamulin in the specified concentration range (50.0240-150.0720 µg/mL) are satisfactory, with a correlation coefficient of 0.9999. The accuracy studies were shown as % recovery [24] for Lefamulin at the specification level, and the results obtained were within the limits. Solution stability[25] parameter was established Assay and RS, standard, sample solutions are stable up to 48 hrs on bench top at refrigerator. Degradation studies [26-28] showed significant degradation in photolytic and thermal stress conditions. Hence, it can be concluded that Lefamulin is sensitive to photolytic and thermal. The results proved that the developed method has good selectivity and specificity.

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, the developed method is simple, precise, cost-effective, eco-friendly, and safe and can be successfully employed for the routine analysis of Lefamulin in bulk and pharmaceutical dosage forms.

6. ACKNOWLEDGMENT

The authors thank the Department of Chemistry, Acharya Nagarjuna University, Nagarjuna Nagar, Guntur. Andhra Pradesh, India, for providing facilities for this research work.

7. AUTHORS CONTRIBUTION STATEMENT

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

8. CONFLICT OF INTEREST

Conflict of interest declared none.

9. REFERENCES

1. Dillon C, Guarascio AJ, Covvey JR. Lefamulin: a promising new pleuromutilin antibiotic in the pipeline. Expert Rev Anti-Infect Ther. 2019;17(1):5-15. doi: 10.1080/14787210.2019.1554431.
2. Rodvold KA. Introduction: lefamulin and pharmacokinetic/pharmacodynamic rationale to support the dose selection of Lefamulin. J Antimicrob Chemother. 2019;74;Suppl 3:iii2-4. doi: 10.1093/jac/dkz084, PMID 30949709.
3. Veve MP, Wagner JL. Lefamulin: review of a promising novel pleuromutilin antibiotic. Pharmacotherapy. 2018;38(9):935-46. doi: 10.1002/phar.2166. PMID 30019769.
4. Bhavnani SM, Zhang L, Hammel JP, Rubino CM, Bader JC, Sader HS et al. Pharmacokinetic/pharmacodynamic target attainment analyses to support intravenous and oral Lefamulin dose selection for the treatment of patients with community-acquired bacterial pneumonia. J Antimicrob Chemother. 2019;74;Suppl 3:iii35-41. doi: 10.1093/jac/dkz089, PMID 30949705.
5. Crowther GS, Wilcox MH. Antibiotic therapy and Clostridium difficile infection - primum non nocere - first do no harm. Infect Drug Resist. 2015;8(8):333-7. doi: 10.2147/IDR.S87224, PMID 26396535.
6. Paukner S, Riedl R. Pleuromutilins: potent drugs for resistant Bugs-Mode of action and resistance. Cold Spring Harb Perspect Med. 2017;3(1):7(1).doi:

10.1101/cshperspect.a027110. doi: 10.1101/cshperspect.a027110, PMID 27742734.
7. Wicha WW, Prince WT, Lell C, Heilmayer W, Gelone SP. Pharmacokinetics and tolerability of Lefamulin following intravenous and oral dosing. J Antimicrob Chemother. 2019;74;Suppl 3:iii19-26. doi: 10.1093/jac/dkz087, PMID 30949704.
8. File TM Jr, Goldberg L, Das A, Sweeney C, Saviski J, Gelone SP et al. Efficacy and safety of IV-to-oral lefamulin, a pleuromutilin antibiotic, for treatment of community-acquired bacterial pneumonia: the Phase 3 LEAP 1 Trial. Clin Infect Dis. 2019 February 4;69(11):1856-67. doi: 10.1093/cid/ciz090, PMID 30722059.
9. Amalakuhan B, Echevarria KL, Restrepo MI. Managing community-acquired pneumonia in the elderly - the next generation of pharmacotherapy on the horizon. Expert Opin Pharmacother. 2017;18(11):1039-48. doi: 10.1080/14656566.2017.1340937. PMID 28598693.
10. Eyal Z, Matzov D, Krupkin M, Paukner S, Riedl R, Rozenberg H et al. A novel pleuromutilin antibacterial compound, its binding mode and selectivity mechanism. Sci Rep. 2016;6(6):39004. doi: 10.1038/srep39004, PMID 27958389.
11. Strickmanna DB, JörgFaberb TK. Development, validation, and application of an LC–MS/MS method forthe quantification of the novel antibiotic drug Lefamulin (Xenleta®)and its main metabolite 2R-hydroxy Lefamulin in human plasma. J Pharm Biomed Anal. 2021;205:114293. doi: 10.1016/j.jpba.2021.114293.
12. ICH guidelines for stability testing of new drug substances and products. 2004;Q1A:(R2).
13. ICH guidelines for validation of analytical procedures: text and methodology. 2005;Q2:(R1).
14. IUPAC. Harmonized guidelines for single-laboratory validation of methods of analysis [IUPAC technical report]. Pure and Applied Chemistry. Vol. 74(5); 2002.
15. Shah BP, Jain S, Prajapati KK, Mansuri NY. Stability indicating HPLC method development: a review. Int J Pharm Res Sci. 2012;3(9):2978-88.
16. Precision of test methods-Repeatability and reproducibility. International Standard ISO 5725; 1986.
17. Snyder LR, Kirkland JJ, Glajch JL. Completing the method: validation and transfer. In: Practical HPLC method validation. 1st ed. NJ: John Wiley & Sons; 2007.
18. Linear calibration using reference material. ISO standard 11095; 1996.
19. Dong MW. Regulatory aspects of HPLC analysis: HPLC system and method validation, modern HPLC for practicing scientists. 1st ed. NJ: John Wiley & Sons; 2006.
20. Araujo P. Key aspects of analytical method validation and linearity evaluation. J Chromatogr B Analyt Technol Biomed Life Sci. 2009;877(23):2224-34. doi: 10.1016/j.jchromb.2008.09.030, PMID 18929516.
21. Van Loco J, Elskens M, Croux C, Beernaert H. Linearity of calibration curves: use and misuse of the correlation coefficient. Accred Qual Assur. 2002;7(7):281-5. doi: 10.1007/s00769-002-0487-6.
22. Hayashi Y, Matsuda R, Ito K, Nishimura W, Imai K, Maeda M. Detection limit estimated from slope of calibration curve: an application to competitive ELISA. Anal Sci. 2005;21(2):167-9. doi: 10.2116/analsci.21.167, PMID 15732478.
23. Zhao Y, Liu G, Shen JX, Aubry AF. Reasons for calibration standard curve slope variation in LC-MS assays and how to address it. Bioanalysis. 2014;6(11):1439-43. doi: 10.4155/bio.14.71, PMID 25046045.
24. Gonzalez AG, Herrador MA. A practical guide to analytical method validation, including measurement uncertainty and accuracy profiles. Trends Anal Chem. 2007;26(3):11.
25. Soni NR. Stability indicating analytical methods (SIAMS); 2018.
26. Blessy M, Patel RD, Prajapati PN, Agrawal YK. Development of forced degradation and stability indicating studies of drugs-A review. J Pharm Anal. 2014;4(3):159-65. doi: 10.1016/j.jpha.2013.09.003, PMID 29403878.
27. Reynolds DW, Facchine KL, Mullaney JF. Available guidance and best practices for conducting forced degradation studies. Pharm Technol. 2002;26:48-56.
28. Maheswaran R. FDA perspectives: scientific considerations of forced degradation studies in ANDA submissions. Pharm Technol. 2012;36:73-80.
29. Kochanek KD, Murphy SL, Xu J, Tejada-Vera B. Deaths: final data for 2014 Rep 65. Natl Vital Stat Rep. 2016;65(4):1-122. PMID 27378572.
30. World Health Organization. Pneumonia: key facts; 2021, June 10. Available from: http://www.who.int/mediacentre/factsheets/fs331/en/ed.
31. Centers for Disease Control and Prevention. Antibiotic Resistance Threats in the United States [accessed Jun 10, 2021]. Vol. 2019; 2019.
32. Paukner S, Riedl R. Pleuromutilins: potent drugs for resistant bugs – mode of action and resistance. Cold Spring Harb Perspect Med, Antibiotics and Antibiotic Resistance. 2017;7 (Jan. (1.
33. US Food and Drug Administration. FDA NEWS! RELEASE: FDA approves new antibiotic to treat community-acquired bacterial pneumonia; 2019.
34. Sunovion Pharmaceutical Canada Inc, Xenleta (Lefamulin) [package Insert].Canadian Drug and Health Product Register Website, 2020.
35. European Medicines Agency, XenletaLefamulin, 2020.
36. Zeitlinger M, Schwameis R, Burian A, Burian B, Matzneller P, Müller M et al. Simultaneous assessment of the pharmacokinetics of a pleuromutilin, Lefamulin, in plasma, soft tissues and pulmonary epithelial lining fluid. J Antimicrob Chemother. 2016;71(4):1022-6. doi: 10.1093/jac/dkv442, PMID 26747098.
37. European Medicines Agency. Xenleta: EPAR – product information[accessed Jun 22 2021]; 2020.
38. Zhanel GG, Deng C, Zelenitsky S, Lawrence CK, Adam HJ, Golden A, Berry L, Schweizer F, Zhanel MA, Irfan N, Bay D. Lefamulin: a novel oral and intravenous pleuromutilin for the treatment of community-acquired bacterial pneumonia. Drugs. 2021 Feb;81:233-56.