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ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF LEFAMULIN BY PHARMACEUTICAL DOSAGE FORM BY CHROMATOGRAPHIC RP-HPLC METHOD

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ABSTRACT:

A rapid and precise reverse phase high-performance liquid chromatographic method has been developed for the validated of Lefamulin, in its pure form as well as in tablet dosage form. Chromatography was carried out on a Symmetry C18 (4.6 x 150mm, 5µm) column using a mixture of Methanol and water (45:55% v/v) as the mobile phase at a flow rate of 0.8ml/min, the detection was carried out at 260nm. The retention time of the Lefamulin was 2.359 ±0.02min respectively. The method produces linear responses in the concentration range of 24-120mg/ml of Lefamulin. The method precision for the determination of assay was below 2.0%RSD. The method is useful in the quality control of bulk and pharmaceutical formulations. The method was validated for accuracy, precision, linearity, robustness, ruggedness and LOD & LOQ of standard solution. The developed RP-HPLC method was found to be accurate, precise, linear, and robust and was successful applied to a pharmaceutical tablet formulation for qualitative estimation of Lefamulin in Bulk form and Marketed Pharmaceutical Dosage forms.

Key Words: Lefamulin, RP-HPLC, Method Development, Validation, Accuracy.

INTRODUCTION

Lefamulin is a Pleuromutilin antibacterial used to treat community-acquired bacterial pneumonia (CABP). Lefamulin is a Pleuromutilin antibiotic used for the treatment of bacterial community-acquired pneumonia1. A Pleuromutilin is a more recently developed type of antibiotic that is derived from the fungus, Pleurotus mutilus. Lefamulin is available in intravenous and oral preparations and was granted FDA approval in August 2019. This drug is the first semi-synthetic Pleuromutilin that has been designed for systemic administration. Lefamulin features a novel mechanism of action that shows benefit against resistant bacteria that cause pneumonia2. The chemical structure of Lefamulin contains a tricyclic mutilin core that is necessary for some of its antimicrobial activity. Lefamulin (brand name Xenleta) is an FDA-approved, semi-synthetic Pleuromutilin antibiotic. Its primary medical use is for treating adults with Community-Acquired Bacterial Pneumonia (CABP). It works by inhibiting bacterial protein synthesis, making it highly effective against both typical and atypical respiratory pathogens3. The IUPAC Name of Lefamulin is [(1S, 2R, 3S, 4S, 6R, 7R, 8R)-4-ethenyl-3-hydroxy-2, 4, 7, 14-tetra methyl-9-oxo-6-tricyclo [5.4.3.01, 8] tetra decanyl] 2-[(1R, 2R, 4R)-4-amino-2-hydroxycy clohexyl] sulfanyl acetate. The Chemical Structure of Lefamulin is as following

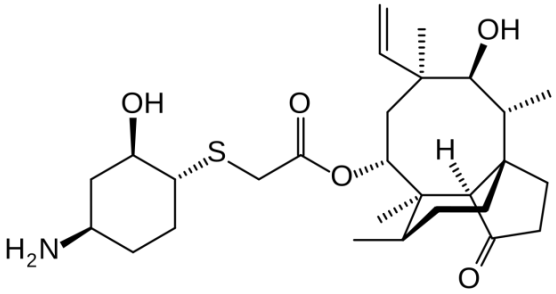

Fig-1: Chemical Structure of Lefamulin

A thorough literature survey25-26 of Lefamulin revealed that very few analytical methods had been reported for estimation of Lefamulin. Majority of methods for determination of Lefamulin in biological fluids and pharmaceutical dosage forms includes LC-MS/MS, LC-MS, and HPTLC-LC, HPTLC, UPLC-MS, HPLC-MS, RPHPLC and UV-Visible Spectrophotometric methods. This novel proposed method contributes quick estimation, correct peak shape, precise, simple, and quick, use of smaller sample volumes and utilizing Suitable Solvent System as a mobile phase which is economical when compared with other existing methods. So, it is necessary to develop a simple, precise, and rapid RP-HPLC method for the quantitative determination of Lefamulin. This work describes the validation parameters4 stated by the International Conference on Harmonization [ICH] guidelines23-24 Q2 (R1).

EXPERIMENTAL

Instruments Used

The instruments and glassware used for the present analytical method development and validation study included a WATERS Alliance 2695 Separation Module equipped with a PDA 996 Detector, with Empower 2 software used for chromatographic data acquisition and processing. A Lab India pH meter was used for pH measurements, while a Sartorius weighing machine was employed for accurate weighing of the drug and other materials. Borosil volumetric flasks, pipettes, burettes, and beakers were used for preparation, dilution, and handling of solutions. A Labman digital ultrasonicator was used for sonication and degassing of the prepared solutions.

Chemicals Used

The chemicals used in the present study included Lefamulin pure drug, obtained from Synpharma Research Lab, Hyderabad. HPLC-grade water and methanol were procured from LICHROSOLV (Merck), while HPLC-grade acetonitrile was obtained from Merck. All chemicals and solvents used in the study were of appropriate analytical/HPLC grade to ensure reliable and reproducible chromatographic results.

HPLC METHOD DEVELOPMENT:

Preparation of Standard Solution:

Accurately weigh and transfer 10 mg of Lefamulin 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 0.72ml of the above Lefamulin 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 guidelines.

Mobile Phase Optimization:

Initially the mobile phase tried was methanol: Water and Acetonitrile: Water with varying proportions. Finally, the mobile phase was optimized to Methanol and Water in proportion 45:55 v/v respectively.

Optimization of Column:

The method was performed with various C18 columns like ODS column, Xterra, and X Bridge C18 column. Symmetry C18 (4.6 x 150mm, 5µm) was found to be ideal as it gave good peak shape and resolution at 1ml/min flow.

Preparation of Mobile Phase:

Accurately measured 450 ml (45%) of HPLC Methanol and 550 ml of HPLC Water (55%) were mixed and degassed in a digital ultrasonicator for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Diluent Preparation:

The Mobile phase was used as the diluent.

METHOD VALIDATION PARAMETERS

System Suitability

Accurately weigh and transfer 10 mg of Lefamulin 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 0.72ml of the above Lefamulin 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 Lefamulin 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 0.72ml of the above Lefamulin stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

Preparation of Sample Solution:

Take average weight of the Tablet and crush in a mortar by using pestle and weight 10 mg equivalent weight of Lefamulin sample into a 10mL clean dry volumetric flask and add about 7mL of Diluent5 and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

Further pipette 0.72ml of Lefamulin 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 assay6 by using formula:

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

Linearity:

Accurately weigh and transfer 10 mg of Lefamulin 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 (24ppm of Lefamulin):

Pipette out 0.24ml of stock solution in to a 10ml volumetric flask and make up the volume up to mark by using diluent.

Preparation of Level – II (48ppm of Lefamulin):

Pipette out 0.48ml of stock solution in to a 10ml volumetric flask and make up the volume up to mark by using diluent.

Preparation of Level – III (72ppm of Lefamulin):

Pipette out 0.72ml of stock solution in to a 10ml volumetric flask and make up the volume up to mark by using diluent.

Preparation of Level – IV (96ppm of Lefamulin):

Pipette out 0.96ml of stock solution in to a 10ml volumetric flask and make up the volume up to mark by using diluent.

Preparation of Level – V (120ppm of Lefamulin):

Pipette out 1.2ml of stock solution in to a 10ml volumetric flask and make up the volume up to mark by using diluent.

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 coefficient.

Precision

Repeatability

Preparation of Lefamulin Product Solution for Precision:

Accurately weigh and transfer 10 mg of Lefamulin 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 0.72 ml of the above Lefamulin stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents. 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.

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:

Day 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.

Day 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 Lefamulin 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 0.36ml of the above Lefamulin 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 Lefamulin 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 0.72ml of the above Lefamulin 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 Lefamulin 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 1.08ml of the above Lefamulin 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 Lefamulin and calculate the individual recovery and mean recovery values.

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 Lefamulin 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 0.72ml of the above Lefamulin 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 0.7 ml/min and 0.9 ml/min instead of 0.8ml/min, remaining conditions are same. 10µl 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: Water was taken in the ratio and 40:60, 50:50 instead of 45:55, remaining conditions are same. 10µl of the above sample was injected and chromatograms were recorded.

RESULTS AND DISCUSSION

Development of Analytical Method:

The analytical method was developed and optimized by systematically evaluating the chromatographic conditions to obtain satisfactory separation and reproducible results. The optimized chromatographic conditions consisted of methanol and water in the ratio of 45:55 v/v as the mobile phase, using a Symmetry C18 column (4.6 × 150 mm, 5 µm) maintained at a column temperature of 40°C. The detection wavelength was set at 260 nm, with a flow rate of 0.8 mL/min and an injection volume of 10 µL. The total run time was 6 minutes, providing efficient chromatographic separation within a short analysis time.

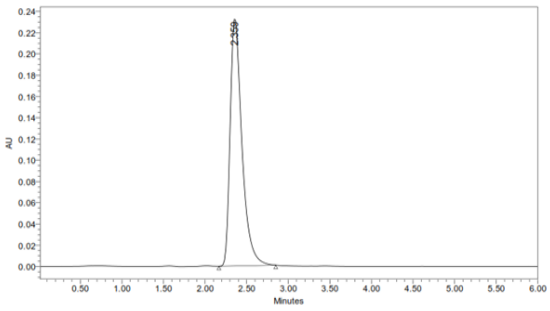

Fig-2: Optimized Chromatographic Condition of Lefamulin

Observation: In this trail it shows well peak shape and proper plate count and tailing under limit in the chromatogram. So, it's optimized chromatogram.

Method Validation

Once the chromatographic and the experimental conditions were established, the method was validated by the determination of the following parameters such as specificity, system suitability, linearity, precision, accuracy, robustness, limit of detection (LOD) and limit of quantitation (LOQ) as per ICH Q2 (R1) guidelines.

System Suitability:

The chromato-graphic systems used for analysis must pass system suitability7 before going to start the experiment. At first HPLC system is stabilized for forty minutes. Inject blank preparation (single injection) and standard preparation (five replicates) and record the chromatograms to evaluate the system suitability parameters such as tailing factor (NMT 1.5), theoretical plate count (NLT 3000) and retention time. The % RSD for the peak area of five replicate injections of Lefamulin standard NMT 2.0. The parameters, such as tailing factor, % RSD, and theoretical plates, were studied.

Table-3: Results of System Suitability for Lefamulin

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Lefamulin	2.317	2274631	239458	5728	1.2
2	Lefamulin	2.302	2284721	239582	5093	1.2
3	Lefamulin	2.323	2238127	236493	5391	1.2
4	Lefamulin	2.343	2259349	249482	6139	1.2
5	Lefamulin	2.321	2204850	239452	5281	1.2
Mean			2252336			
Std. Dev.			31827.08			
% RSD			1.41307			

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 components. Analytical method was tested for specificity8 to measure accurately quantitates Lefamulin in drug product.

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

The % purity of Lefamulin in pharmaceutical dosage form was found to be 99.7%.

Linearity: Standard stock solution of the Dasatinib (72 mg/ml) was prepared with the mobile phase. To study the linearity range9 of drugs, serial dilutions were made from a standard stock solution in the range of 24-120 µg/ml.

Table-4: Linearity Data of Lefamulin

Concentration Level (%)	Concentration µg/ml	Average Peak Area
33	24	791554
66	48	1647073
100	72	2283804
133	96	3058339
166	120	3839630

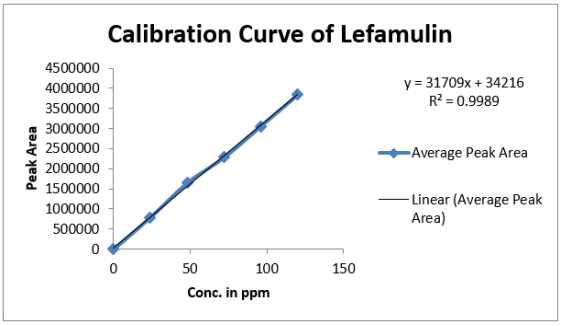

Fig-3: Calibration Curve of Lefamulin

Linearity Plot: The plot of Concentration10 (x) versus the Average Peak Area (y) data of Lefamulin is a straight line.

$Y = mx + c$

Slope (m) = 31709

Intercept (c) = 34216

Correlation Coefficient (r) = 0.998

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 34216. These values meet the validation criteria11.

Precision:

The precision12 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 conditions.

Repeatability: Obtained Five (5) replicates of 100% accuracy solution as per experimental conditions. Recorded the peak areas and calculated % RSD13. The results were shown in Table-5.

Table-5: Results of Repeatability for Lefamulin:

S. No.	Peak Name	Retention time	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Lefamulin	2.356	2259464	245362	5938	1.2
2	Lefamulin	2.356	2275915	248293	5827	1.2
3	Lefamulin	2.357	2282117	240795	5032	1.2
4	Lefamulin	2.358	2278675	230139	5978	1.2
5	Lefamulin	2.359	2282448	249605	6183	1.2
Mean			2275724			
Std. Dev			9476.485			
% RSD			0.416416			

Intermediate Precision:

Analyst1:

Table-6: Results of Intermediate Precision for Lefamulin

S. No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate count	USP Tailing
1	Lefamulin	2.380	2236184	202188	5472	1.2
2	Lefamulin	2.383	2238020	201837	6193	1.2
3	Lefamulin	2.385	2239352	201273	5980	1.2
4	Lefamulin	2.385	2242466	203923	7163	1.2
5	Lefamulin	2.389	2244692	202938	6182	1.2
6	Lefamulin	2.389	2247654	201982	7684	1.2
Mean			2241395			
Std. Dev.			4333.851			
% RSD			0.193355			

Analyst 2:

Table-7: Results of Intermediate Precision Analyst 2 for Lefamulin

S. No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate count	USP Tailing
1	Lefamulin	2.380	2236184	217363	5928	1.2
2	Lefamulin	2.383	2238020	218467	6183	1.2
3	Lefamulin	2.385	2239352	218346	5927	1.2
4	Lefamulin	2.385	2242466	221736	5163	1.2
5	Lefamulin	2.389	2244692	228361	4827	1.2
6	Lefamulin	2.346	2263431	217553	5019	1.2
Mean			2244024			
Std. Dev.			9988.458			
% RSD			0.445114			

Accuracy:

Accuracy at different concentrations (50%, 100%, and 150%) was prepared and the % recovery was calculated14-17.

Table-8: The accuracy Results for Lefamulin

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	1172485	36	35.8	99.4	99.5%
100%	2314753	72	71.6	99.4	
150%	3480210	108	107.9	99.9	

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 value18.

$LOD = 3.3 \times \sigma / s$

Where

σ = Standard deviation of the response

S = Slope of the calibration curve

Result:

=5.5µg/ml

Quantitation Limit

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined19.

$LOQ=10\times\sigma/S$

Where

σ = Standard deviation of the response

S = Slope of the calibration curve

Result:

=16.7µg/ml

Robustness

The robustness was performed for the flow rate variations from 0.7 ml/min to 0.9ml/min and mobile phase ratio variation from more organic phase to less organic phase ratio for Lefamulin. The method is robust only in less flow condition and the method is robust even by change in the Mobile phase ±5%. The standard and samples of Lefamulin were injected by changing the conditions of chromatography. There was no significant change in the parameters like resolution, tailing factor, asymmetric factor, and plate count20-24.

Table-9: Results for Robustness of Lefamulin

Parameter Used for Sample Analysis	Peak Area	Retention Time	Theoretical Plates	Tailing Factor
Actual Flow rate of 0.8mL/min	3119086	2.379	5837	1.2
Less Flow rate of 0.7mL/min	2640811	2.763	5361	1.2
More Flow rate of 0.9mL/min	2640354	2.234	5231	1.2
Less organic phase	2640758	2.765	4503	1.5
More organic phase	2640125	2.236	4491	1.5

SUMMARY

The analytical method was developed by studying different parameters. First of all, maximum absorbance was found to be at 260nm and the peak purity was excellent. Injection volume was selected to be 10µl which gave a good peak area. The column used for study was Symmetry C18 because it was giving good peak. 40 º C temperatures were found to be suitable for the nature of drug solution. The flow rate was fixed at 0.8ml/min because of good peak area and satisfactory retention time. Mobile phase is Methanol: water was fixed due to good symmetrical peak. So, this mobile phase was used for the proposed study. Methanol: water was selected because of maximum extraction sonication time was fixed to be 10min at which all the drug particles were completely soluble and showed good recovery. Run time was selected to be 6min because analyze gave peak around 2.3 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 24-120ppm of the Lefamulin target concentration. The analytical passed both robustness and ruggedness tests. On both cases, relative standard deviation was well satisfactory.

CONCLUSION

In the present investigation, a simple, sensitive, precise and accurate RP-HPLC method was developed for the quantitative estimation of Lefamulin in bulk drug and pharmaceutical dosage forms. This method was simple, since diluted samples are directly used without any preliminary chemical derivatization or purification steps. Methanol: water was chosen as the mobile phase. The solvent system used in this method was economical. The %RSD values were within 2 and the method was found to be precise. The results expressed in Tables for RP-HPLC method was promising. The RP-HPLC method is more sensitive, accurate and precise compared to the Spectrophotometric methods. This method can be used for the routine determination of Lefamulin in bulk drug and in pharmaceutical dosage forms.

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