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Development and validation of a stability-indicating RP-HPLC method for estimation of Lefamulin in pure and pharmaceutical drug products

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Abstract

Background Commonly occurring serious lung parenchymal infection is community-acquired bacterial pneumonia (CABP). Lefamulin acetate drug products are useful for the treatment of community-acquired bacterial pneumonia (CABP). The main objective of the study was the development and validation of a quick reverse-phase high performance liquid chromatographic analytical testing method for Lefamulin in pure and pharmaceutical dosage forms. The C18 column was the stationary phase for reverse-phase high performance liquid chromatographic separation, and the mobile phase consisted of 0.05 M phosphate buffer with a pH adjustment of 2.5 mixed with an equal volume of acetonitrile. A flow rate of 1.0 mL/min, a column temperature of 30 °C, an injection volume of 10 µL, and a detection wavelength of 210 nm were used as optimum conditions.

Results The developed method reported that Lefamulin peak's retention time was 3.1 min, the average theoretical plate count 5000, and the average peak asymmetry 1.12. In both pure and pharmaceutical dosage forms, the method was found to be accurate, precise, specific, linear calibration curve, and robust. Quantitative assay results from laboratory-made formulations showed similarity to existing commercial products of Lefamulin. Lefamulin is stable in acidic and photolytic environmental circumstances, while it degraded in oxidative, basic, and thermal humidity environment, according to the results of stress studies.

Conclusions The developed analytical method is fast, economic and stability indicating. It is useful for routine pharmaceutical analysis where the Lefamulin is synthesized in pure form, pharmaceutical tablet formulation, and parenteral dosage form, for their quality and safety.

Keywords Lefamulin acetate, Pharmaceutical formulations, RP-HPLC, Quantitative assay, Method validation

Background

Community-acquired bacterial pneumonia (CABP) is a frequent critical pulmonary parenchymal infection. It is the leading cause of death among the Asian adults [1]. Among the different infections that lead to sepsis, this is one of the most prevalent respiratory illnesses [2]. Lefamulin acetate chemically acetic acid;[(1S,2R,3S,4S,6R,7R,8R,14R)-4-ethenyl-3-hydroxy-2,4,7,14-tetramethyl-9-oxo-6-tricyclo[5.4.3.0^{1,8}]tetradecanyl]

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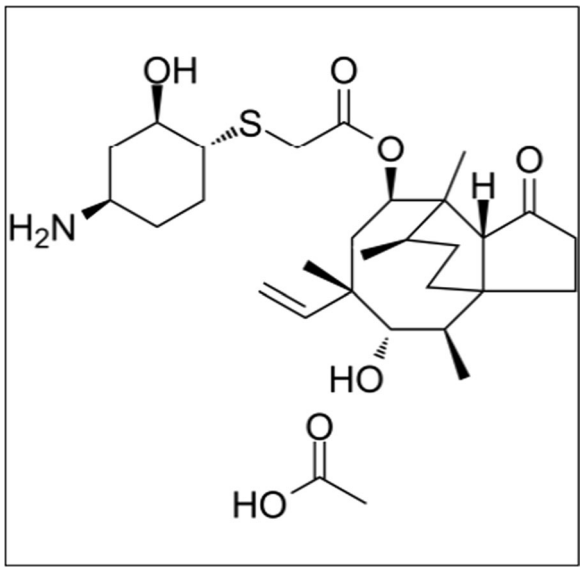

Fig. 1 Chemical structure of Lefamulin Acetate

2-[(1R,2R,4R)-4-amino-2-hydroxy-cyclohexyl]sulfanylacetate (The chemical structure is shown in Fig. 1), is a pleuromutilin derivative that is semi-synthetic [3, 4]. For the treatment of CABP in adults, it received its first approval from the FDA on 19th August 2019 [5], Health Canada on 10th July 2020 [6], and the EMA on 28th July 2020 [7]. Lefamulin (as acetate) is supplied in parenteral and oral dosage formulations.

Lefamulin is not listed in official Pharmacopoeial monographs as a drug substance or drug product, and the literature review indicated that only one LC–MS/MS analytical method was reported for the determination of Lefamulin in human plasma [8]. This method cannot be used for regular pharmaceutical analysis and is only relevant for the study of clinical trials. Mainly due to its complexity, cost, time requirements, and lack of reliability. So, based on our knowledge, there is a necessity for a low cost, simple, and quick analytical method for determining the purity and safety of Lefamulin. Therefore, our study's goal was to develop and validate a rapid analytical testing method for Lefamulin in both dosage forms while taking into account of ICH guidelines [9].

Methods

Chemicals

We used water and acetonitrile of Honeywell HPLC grade. Hydrogen peroxide (30% w/w), sodium hydroxide, potassium phosphate, orthophosphoric acid, hydrochloric acid of analytical-grade from Sigma-Aldrich were employed.

Standards and samples

Lefamulin acetate, which was procured from Kaifeng Pharmaceutical (Group) Company Limited China, was gratefully donated by Horizon Healthcare (Pvt.) Limited in Pakistan. The injection of 150 mg/15 mL and the 600 mg tablets of XENLETA® were bought from open market. Horizon Healthcare Pakistan granted all the commercial-grade excipients used throughout the validation study.

Instrumentation

Shimadzu Corporation of Japan quaternary gradient HPLC LC-10AD is outfitted with a degasser, column temperature oven, and a multi-channel photodiode array detector was used for the analytical method development and validation studies. The data curation was carried out by using Lab Solution software. To obtain a chromatographic separation, a Welchrom Ultisil® XB-C18 column with a 4.6 mm internal diameter, 25 cm length, and 5 µm particle size was employed. Analytical balance (ATX-224; Shimadzu Japan) was used to measure the weights of the standards and samples. The sonication process was carried out using an S15H ultra sonicator Elma Germany, and the pH of the mobile phase was maintained using a pH meter S210 from Mettler Toledo Switzerland. The Sartorius Germany filtration assembly was used to filter the Mobile phase. The glassware used during the whole study was calibrated. The climatic chambers HPP1060 Memmert Germany were used for the temperature and humidity stress experiments, while the xenon lamp 50W CEL-HXB F300 from China was used for the photolytic stress studies.

Chromatographic conditions

10 µL volumes of standards and samples were injected while the mobile phase flow rate was held at 1 mL/min, the column temperature was kept at 30 °C, and 210 nm was the detection wavelength. The HPLC needle rinsing solution was acetonitrile and water (50:50 v/v).

Preparation of mobile phase and diluting solution

The chromatographic separation used an isocratic mobile phase elution consisting of 0.05 M potassium phosphate buffer with a pH adjustment of 2.5 from diluted orthophosphoric acid. Then the buffer was combined with the acetonitrile as the organic modifier in equal volumes. The 45% v/v solution of acetonitrile in distilled water was used as a diluting solution.

Preparation of standard solution and calibration curves solutions

Lefamulin acetate, which is equivalent to 150 mg of Lefamulin base, was dissolved by diluent in a 100 mL volumetric flask to obtain the standard stock solution. Then, 3–7.5 mL were transferred to separate 50 mL volumetric flasks and diluted with a diluting solution to generate 90–225 ppm solutions in order to develop the calibration curve. The target concentration of standard solution was 150 ppm.

Preparation of tablet sample solution

The stock tablet sample solution was prepared by transferring the weight equivalent to one tablet (nominal amount of Lefamulin 600 mg) from powdered tablets to a 200 mL volumetric flask, diluted with diluent, and dissolved with the aid of ultra Sonicator for 15 min, finally made the volume to mark with diluent. The 5 mL of stock solution was diluted with diluent to 100 mL for preparation of the final sample solution.

Preparation of injection sample solution

The stock injection sample solution was prepared by diluting the 15 mL portion of injection (nominal amount of Lefamulin 150 mg) from cumulative volume of injections to 100 mL volumetric flask, diluted with diluent, and dissolved with the aid of ultra Sonicator for 15 min, finally made the volume to mark with diluent. The 5 mL of stock solution was diluted with diluent to 50 mL for preparation of the final sample solution.

Preparation of laboratory formulated tablet and injection sample solutions

The laboratory-formulated tablet sample solution was prepared by mixing the excipients (Croscarmellose sodium; 5 mg, colloidal silicon dioxide; 1 mg, magnesium stearate; 1 mg, mannitol; 45 mg, microcrystalline cellulose; 26 mg, polyethylene glycol; 1 mg, polyvinyl pyrrolidone K30; 5 mg, talc; 1 mg, and titanium dioxide; 2 mg) with 671 mg of Lefamulin acetate drug substance, and transferred in 200 mL volumetric flask, diluted with diluent, and dissolved with the aid of ultra Sonicator for 15 min, finally made the volume to mark with diluent. The 5 mL of stock solution was diluted with diluent to 100 mL for preparation of the final sample solution. Similarly, the Lefamulin laboratory injection sample solution was prepared by mixing the Lefamulin acetate with sodium chloride, citric acid, and trisodium citrate, diluted as per the above injection sample solution. Three replicates of these solutions were injected, measured in the % assay, and compared with commercial products.

Force degradation studies

Force degradation studies were employed to evaluate the stability characteristics of the Lefamulin analytical method [10]. Various stress conditions, oxidative, photolytic, thermal, and hydrolysis (acid and basic), were applied. The 150 ppm pure drug substance solution was exposed to acidic stress (10 mL of 0.1 N hydrochloric acid), basic stress (10 mL of 0.1 N sodium hydroxide solution), and oxidative stress (3% v/v hydrogen peroxide solution) and left in the dark for 5 days before injecting. The drug substance solution was subjected to thermal-humidity stress by being kept at 60 °C temperature and 75% relative humidity for 5 days. The standard solution was exposed to an illuminance of nearly 1500 lx for 5 days to apply the photolytic stress. The temperature control for acidic and basic stress samples along with their control blank solutions was 40 °C, while for oxidative stress along with its control blank was 25 °C.

Results

Analytical method development and optimization

Different modifications were made to the pH, organic solvent combination ratio, and potassium phosphate buffer concentration to obtain improved suitability conditions. The potassium dihydrogen phosphate buffer concentration was adjusted from 0.02 to 0.05 M, while the pH ranged from 6 to 2.5. Acetonitrile was utilized as an organic modifier, and different percentages of the buffer in combination with it were investigated. Thus, the suitable conditions were achieved by mixing acetonitrile in a 50% ratio with 0.05 potassium dihydrogen phosphate solution of pH 2.5. Although 20 µL of injection volume was attempted, the tailing factor was greater than 1.5. As a result, it was changed to 10 µL. The system suitability chromatogram is shown in Fig. 3 and results in Table 1.

Table 1 System Suitability results of the proposed method

System suitability parameters	Results[a]	Acceptance criteria
Retention time (t_R)	3.1 ± 0.1 min	± 5% of window
Tailing factor (T_f)	1.12 ± 0.05	0.8–2.0
No. of theoretical plates (*N*)	5000 ± 500	≥ 2000
Peak purity index	1.0000	≥ 0.999
%RSD of peak areas	0.108%	≤ 2.0%

[a] Mean of five replicates

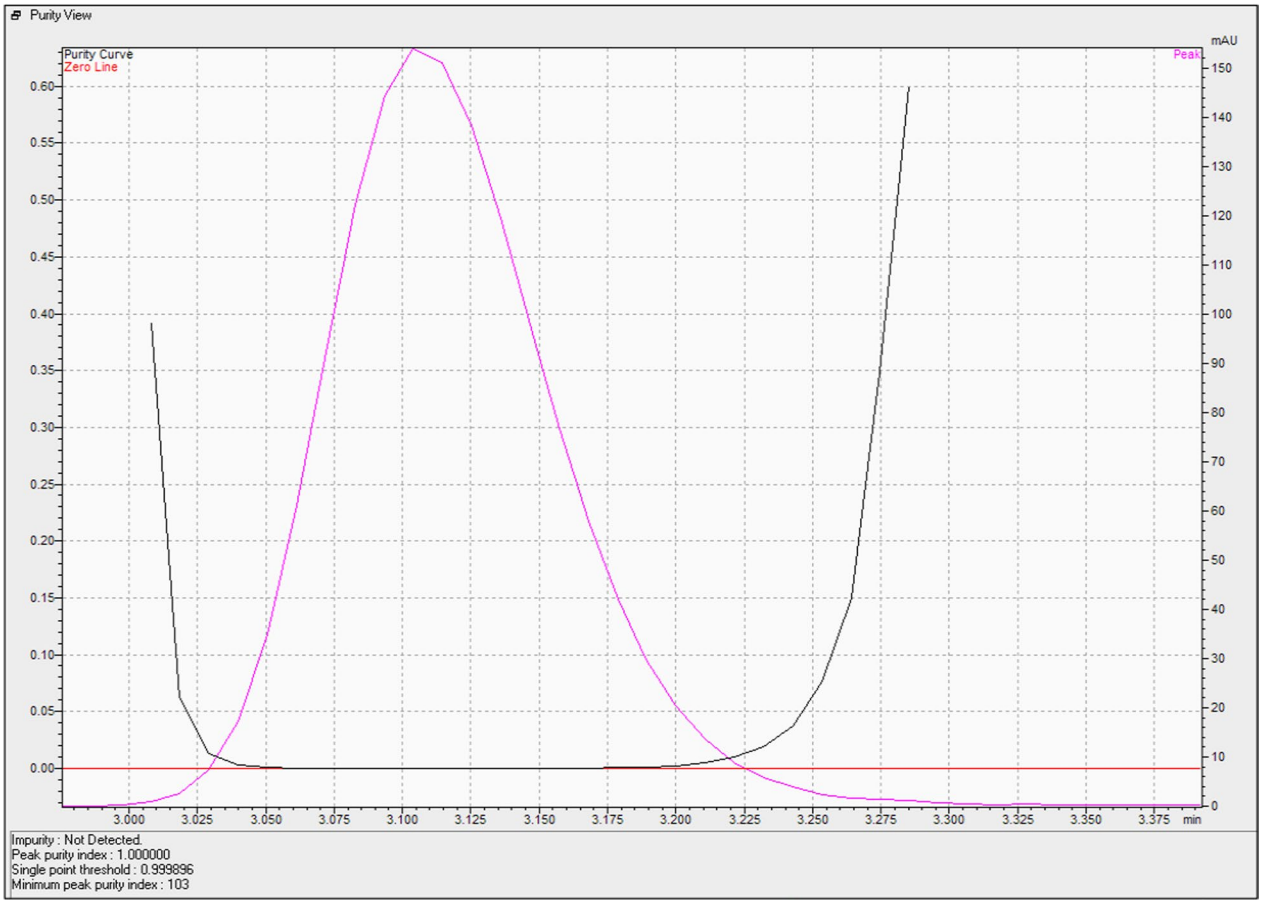

Fig. 2 Peak purity of Lefamulin

Analytical method validation

Specificity

By injecting target concentrations of standard, tablet sample, injection sample, blank, and placebos (excipients synthetic mixture of tablet and injection) solutions, the specificity was determined. Lefamulin sample measured had an average peak purity index of 1.0000 as shown in Fig. 2. Furthermore, no effect of diluent or placebo on analyte retention time was observed. The chromatograms are provided in Fig. 3.

Linearity, LOQ and LOD

The Linearity of analytical method was analyzed by preparing standard solutions 60 to 150% of target concentration having analyte concentration (90, 105, 120, 135, 150, 165, 180, 195, 210, and 225 ppm) solutions and injecting in triplicate. The quantitation and detection limits were calculated by using the standard mean error and slope from regression data as in Eq. (1) and (2);

$$\text{Limit of quantitation} = 10 \times \frac{\text{Standard error of slope}}{\text{Slope of clibration Curve}} \quad (1)$$

$$\text{Limit of quantitation} = 3.3 \times \frac{\text{Standard error of slope}}{\text{Slope of clibration Curve}} \quad (2)$$

The calibration curve was developed by plotting the analyte concentration along abscissa and peak areas along ordinate as shown in Fig. 4. The regression data are given in Table 2.

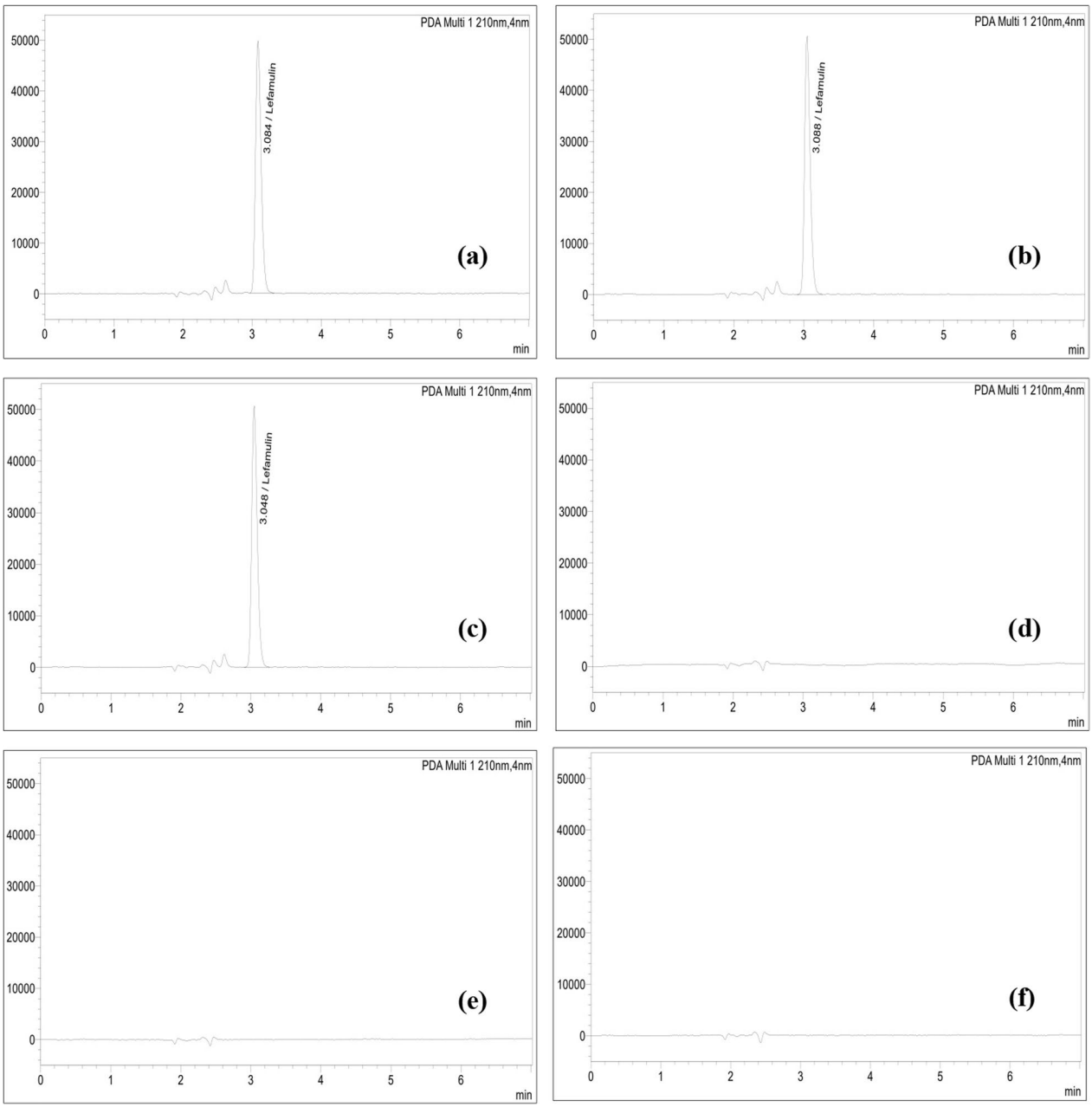

Fig. 3 Chromatograms representing **a** standard, **b** tablet sample, **c** injection sample, **d** blank, **e** placebo tablet, **f** placebo injection, **g** oxidative stress, **h** basic stress, **i** acidic stress, **j** photolytic stress, **k** thermal humidity stress

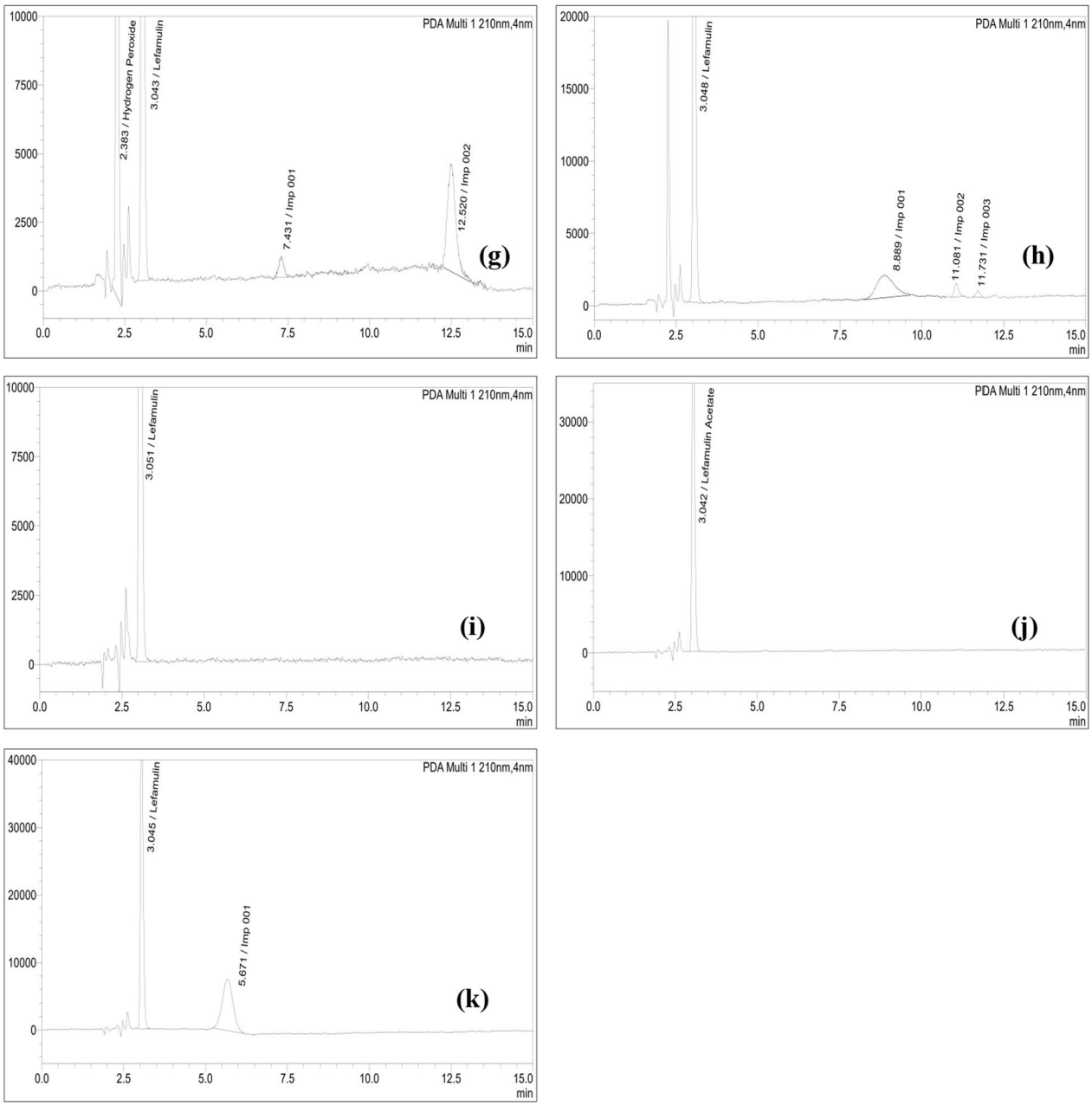

Fig. 3 continued

Accuracy and precision

The accuracy and precision were combinedly assessed by spiking the known amount of 80, 100, and 120% concentration of drug substance in separately placebo solutions of injection and Tablet. These solutions were prepared on three consecutive days and evaluated by noting the % recovery. The % recoveries between 98 and 102% and %RSD < 2% was observed. The recovery results are shown in Table 3.

Robustness

The chromatographic conditions for robustness [11] were deliberately altered by varying the flow rate ± 10%, the wavelength ± 2 nm, the column temperature ± 3 °C, and the acetonitrile ratio ± 3% in the mobile phase. Table 4 displays the variations in retention time, theoretical plate count, tailing factor, and % RSD of standard that were measured.

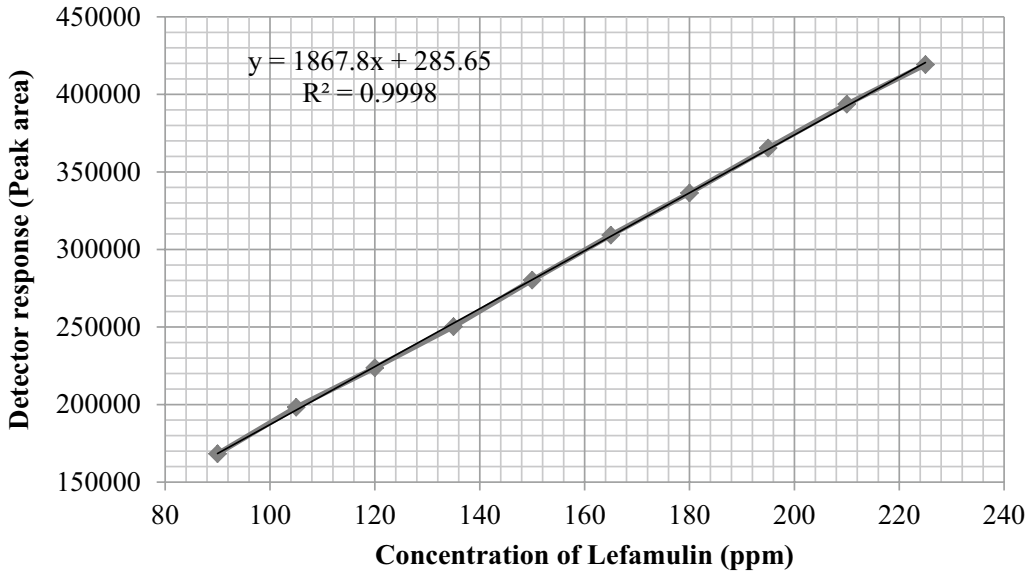

Fig. 4 Calibration curve of Lefamulin

Table 2 Regression data of the proposed analytical method

Parameters	Regression data
Multiple correlation (*R*)	0.99989
Correlation coefficient (R^2)	0.99979
Slope	1868
y-intercept	286
Standard mean error of slope	9.46
Standard mean error of intercept	1544.80
Limit of quantitation	0.051 ppm
Limit of detection	0.017 ppm

Stability of solutions

By storing the standards and samples in the dark for 48 h and injecting them at intervals of 6, 12, 24, 36, and 48 h, the stability of both was assessed. The results are given in Table 5.

Force degradation studies

By using several environmental conditions, such as acid, alkali, oxidative, photolytic, and thermal with humidity, force degradation or stress studies have been conducted. The preparations for degradation studies discussed above, the results in Table 6, and chromatograms provided in Fig. 3. The % degradation was calculated by subtracting the % recovery of samples influenced by environmental conditions from the % recovery of freshly prepared control sample.

Table 3 Recovery results of the proposed method

Time intervals	Spiked level (%)	Recovery of respective level		Recovery[a] (%)	
		Tablet	Injection	Tablet	Injection
Repeatability	80	80.52	80.47	100.65	100.59
	100	99.69	100.69	99.69	100.69
	120	120.02	119.79	100.02	99.83
Day 01	80	79.56	80.69	99.45	100.86
	100	100.64	99.45	100.64	99.45
	120	120.14	119.98	100.12	99.98
Day 02	80	81.02	79.09	101.28	98.86
	100	100.69	99.36	100.69	99.36
	120	120.51	120.04	100.43	100.03
Day 03	80	79.69	81.45	99.61	101.81
	100	99.98	101.41	99.98	101.41
	120	119.37	120.27	99.48	100.23
Average recovery (acceptance criteria 98% to 102%)				100.17%	100.26%
% RSD of recovery (should be not more than 2%)				0.572%	0.857%

[a] Mean of three measurements

Quantitative assay comparison

The quantitative assay of laboratory formulations of the tablet and injection was performed and compared with the quantitative assay of commercial brands (Xenleta tablet and Xenleta injection). The assay results are given in Table 7.

Table 4 Robustness results of the proposed method

Chromatographic conditions	Deliberate changes	Retention time	Tailing factor	Theoretical plates count	% RSD of peak area[a]
Flow rate (mL/min)	0.9	3.95	1.09	4705	0.35
	1.1	2.40	1.39	5450	0.21
Wavelength (nm)	208 nm	3.08	1.12	5204	0.19
	212 nm	3.09	1.13	5310	0.42
Column temperature (°C)	27	3.21	1.09	5023	1.04
	33	2.94	1.18	5125	0.49
Acetonitrile ratio	48.5%	3.47	1.14	4941	0.58
	51.5%	2.89	1.23	4532	0.79
Acceptance value		–	0.8–2.0	> 2000	< 2

[a] Mean of three measurements

Table 5 Results of stability of solution

Time intervals	% Recovery[a]	
	Tablet sample solution (%)	Injection sample solution (%)
Initial	101.21	99.68
After 6 h	100.59	100.12
After 12 h	101.25	99.29
After 24 h	100.11	99.21
After 36 h	99.89	98.49
After 48 h	99.31	97.89

[a] Mean of three measurements

Table 7 Quantitative assay results

Samples	Assay[a] (%)
Laboratory prepared tablet	99.79
Xenleta tablet	101.98
Laboratory prepared injection	100.69
Xenleta injection	100.98

[a] Mean of three measurements

Discussion

According to a review of the literature, there was only one LC–MS/MS analytical method that quantified Lefamulin in human plasma [6], and even then, it could only be used for a clinical study. Therefore, the objective of this work was to develop and validate a method for evaluating the safety and purity of Lefamulin in both its pure and pharmaceutical dosage forms. As the analytical method was being developed, it was observed that as the buffer concentration was decreased, the value of N decreased and t_R increased. Poor peak elution was noticed with an increase in pH of buffer. Poor N value and a higher t_R were seen when acetonitrile concentration dropped. The acquired results of validation showed that the analysis method had been stable for up to 48 h in the dark as shown in Table 5. Lefamulin's retention time was not impacted by excipients or diluent, according to the specificity data, as shown in chromatogram Fig. 3. The Linearity results indicated the analytical method followed Beer's law calibration curve shown in Fig. 4, providing a correlation coefficient of 0.99979. According to the regression data in Table 2, the LOQ concentration of the analyte was 0.051 ppm, and the LOD

Table 6 Force degradation studies results

Stress condition	Recovery (%)[a]			% Degradation	Extent of degradation
	1 day (%)	3 days (%)	5 days (%)		
Acidic	99.21	97.25	96.18	3	None
Basic	95.32	89.01	77.87	22	Substantial
Oxidative	94.23	87.25	79.52	20	Substantial
Thermal Humidity	96.01	90.32	87.69	10	Substantial
Photolytic	99.98	97.36	97.02	2	None

[a] Mean of three measurements

concentration was 0.017 ppm, demonstrating the sensitivity of the described analytical method. The recovery studies for three days provided in Table 3 demonstrated that the analyte recovered for tablet sample solutions ranged from 99.45 to 101.28% with %RSD 0.572%, and injection sample solutions ranged from 98.86 to 101.81% with %RSD 0.857%, demonstrating the accuracy and precision of the analytical method. The deliberate alteration in chromatographic conditions in Table 4 provided no change in %RSD of peak areas, whereas variations in wavelength led to changes in peak areas but had no effect on other system suitability parameters. The decrease in flow rate, column temperature, and acetonitrile ratio cause increasing the retention time of the analyte peak and decreasing the theoretical plates count while the tailing factor is unaffected while increasing in flow rate, column temperature, and acetonitrile ratio cause decreasing of retention time of the analyte peak and increasing of tailing factor while theoretical plates count unaffected. The force degradation studies were conducted under mild environmental conditions for 5 days, and the results indicate that Lefamulin degraded under oxidative, basic, and thermal humidity circumstances (Table 6) while maintaining stability under acidic and photolytic conditions. In oxidative conditions there were two unknown impurities observed at t_R 7.4 min and 12.5 min. In basic conditions there were three unknown impurities observed at t_R 8.8 min, 11.0 min, and 11.7 min while in thermal humidity circumstances only one unknown impurity observed at t_R 5.6 min. In acidic and photolytic conditions no impurities observed as shown in Fig. 3. Laboratory-made formulations quantitative assay results revealed similarities to commercial Lefamulin pharmaceutical dosage products.

Conclusion

Lefamulin estimation in pure, tablet, and injection dosage form was developed and validated using the current stability-indicating RP-HPLC method. The proposed method is simple, quick, affordable, accurate, precise, linear, specific, and stability-indicating. Regular pharmaceutical analysis of Lefamulin formulations can be successfully carried out with this RP-HPLC method.

Abbreviations

CABP	Community-acquired bacterial pneumonia
RP-HPLC	Reverse phase-high performance liquid chromatography
FDA	Food and drug administration
EMA	European Medicines Agency
LC–MS	Liquid chromatography-Mass spectrometry
ICH	International conference on harmonization
Pvt	Private
t_R	Retention time
T_f	Tailing factor
N	Number of theoretical plates
RSD	Relative standard deviation
R	Multiple correlation
R^2	Correlation coefficient

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Author contributions

TA conceptualized and collected the necessary data from literature. MU designed, developed the analytical methodology. MU and TA performed the validation; both are responsible for the data integrity. TA wrote the manuscript draft and MU proof read it. The authors declare that they have read and agreed to the published version of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All data and materials are available upon request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interest regarding this publication.

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