Novel LC-MS Method for Quantification of Lefamulin in Plasma Samples

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Abstract

The aim of present work is to develop cost effective, selective, precise and sensitive LC–MS method for the estimation of Lefamulin in rat plasma. The chromatographic separation of Lefamulin was successfully achieved with Zorbax Symmetry C18, (150mm x 4.6mm, 3.5µ) column and using mobile phase of 0.1%v/v formic acid: Acetonitrile (70:30 v/v) at a flow rate of 1 mL/min. The method was further validated to confirm the acceptability for pharmaceutical sector. The retention time of Lefamulin observed at 2.6min with the runtime of 4 min. The method was shown excellent linearity in the range of 1–20 ng/ml with R^2 of 0.999. The % CV of peak areas Lefamulin of different level sample solution were shown ≤ 15% for repeated injection in precision and acuuercy. Matrix effect on analyte was very negligible with % mean accuracy of ±15%.There was no significant carry over observed during this experiment. All the samples under investigation were found to be free from interferences by the other substance in plasma sample at the retention time of drug. The array of stability studies, specifically, bench-top, freeze-thaw, short term and long-term stability was done to different concentration levels of biological samples, which were showing the % CV for peak response of repeated injections should be ≤15%. The approach created was highly simple, accurate, dependable, sensitive, and sturdy. The retention time requires less time and has a good sensitivity, making the approach suitable for routine analysis and bioanalysis.

Keywords Lefamulin, LC-MS method, Sensitive, Matrix effect, stability studies

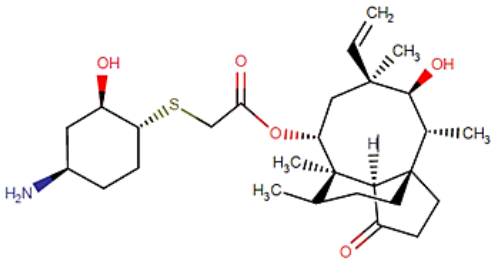
DOINumber:10.14704/nq.2022.20.8.NQ44746 **NeuroQuantology2022;20(8):7243-7254**

INTRODUCTION

Lefamulin is a pleuromutilin antibiotic that is used to treat community-acquired bacterial pneumonia. A pleuromotilin is a kind of antibiotic produced from the fungus Pleurotus mutilus that was created relatively recently [1-2]. The FDA granted clearance for intravenous and oral formulation of lefamulin in 2019 [2]. This medication is the first semi-synthetic pleuromutilin intended for systemic administration. Lefamulin's chemical structure has a tricyclic mutilin core, which is required for a portion of its antibacterial action [3-4] Lefamulin inhibits protein synthesis by binding to the peptidyl transferase core of the 50S bacterial ribosome, hence inhibiting the binding of transfer RNA for peptide transfer [1-4]. Lefamulin has a greater therapeutic impact in pneumonia due to its ability to reduce the resistance activity of several bacteria [3-

*e*ISSN1303-5150

4].The majority of doctors recommend Lefamulin as an alternative to various fluoroquinolones for the treatment of pneumonia [4]. The chemical structure, molecular weight and IUPAC name of Lefamulin was mentioned in Figure-1[5-6].



IUPAC: [(1S,2R,3S,4S,6R,7R,8R,14R)-4-ethenyl-3-hydroxy-2,4,7,14-tetramethyl-9-oxo-6-tricyclo[5.4.3.01,8]tetradecanyl] 2-[(1R,2R,4R)-4-amino-2-hydroxycyclohexyl]sulfanylacetate

Molecular weight: 507.73 g/mol

Figure-1: Chemical structure of Lefamulin

As Lefamulin was recently approved by the US FDA in 2019, research data like various analytical methods, formulation methods and clinical data was very less. Analytical methods can be played significant role in the pharmaceutical industry to know the quality, quantity, and possible degradants of the specified analytes[5-7]. Analytical methods have a prominent role in pharmacokinetic studies of analytes in vitro and in-vivo studies ensure the bioavailability and drug release properties [5].

Bioanalytical methods have a significant role in clinical research to assess the pharmacokinetic parameters in in vivo studies [5-7]. From the literature survey, it was known that no analytical methods were officially reported for evaluation of Lefamulin in pharmacopeias. Only one LC-MS method was reported for estimation of Lefamuli and its metabolite hydroxy Lefamulin in human plasma where the elution time of lefamulin was observed to be 8min [6]. It was came to know that a significant LC-MS method with less elution time with good sensitivity and precision should be required for estimation of Lefamulin in plasma sample. To fulfill the gap, a competent bio analytical LC-MS method was developed for analysis of Lefamulin in rat plasma.

MATERIALS AND METHODS

The Lefamulin and an internal standard (D6-Lefamulin) were given by ICON laboratories, Hyderabad as gift sample, All HPLC grade solvents were procured from the Merck Indiad Limited, India. Waters 2695, SCIEX QTRAP 5500Series coupled with Elecro spray ionization and Quadrapole mass detector was used to develop and validate the method.

Method development by LC-MS

Preparation of Standard Stock Solution (40ng/ml)

Weigh 5mg of Lefamulin working standard and transferred into a 10ml volumetric flask diluted to volume with diluent. Further dilute 0.1mL to 10mL with diluent. Take 0.08 ml of the above solution into a 10 ml volumetric flask and made up to the mark with diluents.

Preparation of Standard Solution (10ng/ml)

Transfer 500µl of standard stock solution into 2ml centrifuged tube. To this add 200µl of plasma, 500µl of internal standard, 300µl of acetonitrile and 500µl of diluent. Centrifuge it to 20 min. Filter the

supernatant liquid and transfer into HPLC vial.

Preparation of Internal Standard Stock Solution (40ng/ml)

Weigh 5mg of D6-Lefamulin working standard and transferred into a 10ml volumetric flask diluted to volume with diluent. Further dilute 0.1ml to 10ml with diluent. Take 0.08 ml of above solution into 10 ml volumetric flask and made up to the mark with diluents.

Preparation of Sample Solution (10ng/ml of Lefamulin)

Transfer 500µL of sample stock solution into 2mL centrifused tube. To this add 200µL of plasma, 500µl of internal standard, 300µL of acetonitrile and 500µl of diluent. Centrifuge it to 20 min. Filter the supernatant liquid and transfer into HPLC vial.

Buffer Preparation:

1 ml of formic acid is dissolved in 1L of HPLC grade water and filter through 0.45µ membrane filter paper.

Extraction procedure

Label the Centrifuged and treated plasma samples accordingly to their time intervals. To about 200µl of plasma add 500µl of diluent and mix well. Further add 300µl of Acetonitrile to precipitate all the proteins and mix in vortex cyclo mixture. Centrifuge at 4000 RPM for 15 – 20 min. collect the supernatant solution in HPLC vial and inject into the chromatograph

Optimized LC-MS method conditions

The separation of Lefamulin was achieved by Zorbax Symmetry C18, (150mm x 4.6mm, 3.5µ) column and mobile phase of 0.1%v/v formic acid buffer : acetonitrile (70:30 v/v), which was pumped at l ml/min by Reciprocating quaternary pump. The ambient temperature was maintained in both auto sampler and column. MS parameters used in the method include electrospray ionization with 5500V, nitrogen as collision gas with 15V energy, drying gas temperature of 120-250°C, Declustering potential of 40V and Dwell time of 1 second.

Method validation

System suitability test

This was accomplished by injecting a subsequent injection of standard solution of Lefamulin consists of internal standard (10ng/ml) for 6 times. The %CV (coefficient of variation) was assessed for peak areas (CPS) of both Lefamulin and internal standard, %CV for peak area ratio of Lefamulin and internal standard were assessed and ensured their acceptance as per ICH.

Linearity

The linearity of stated method was ascertained by assessing the R^2 value for a series of concentrations ranged from 1 to 20ng/ml of Lefamulin by building a linear plot connected series of concentration and their peak area ratio, which are got by the help of peak area of internal standards.

Recovery

The recovery of Lefamulin is the detector response (CPS) attained from an amount of the Lefamulin added to and extracted from the biological matrix, compared to the detector response (CPS) attained for the pure authentic standard of Lefamulin. In the current LC-MS method, it was carried out by spiking or adding a certain amount of drug into plasma matrix at three QC levels namely low, medium and high quality control (LQC, MQC and HQC). Recovery of Lefamulin was calculated by the comparing extracted sample response in 6 replicates with that of neat standard solution (un extracted) responses. The % CV of the amount of Lefamulin spiked was assessed. The procedure was done for both extracted and un extracted spiked samples (9, 10).

Precision and Accuracy

The system precision (Intra-day) was estimated by analyzing six replicates containing Lefamulin at MQC (10ng/ml) levels. The inter-day precision was determined by analyzing the four levels QC samples in 6 replicates. Reproducibility was

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estimated by analyzing six replicates containing Lefamulin at three different levels [(HQC-15ng/ml), (MQC-10ng/ml) and (LQC-5ng/ml). The %CV and % mean accuracy were assessed to each level to ensure the confirmation of various precisions along with accuracy.

Specificity

10µL of standard blank (plasma), internal standard solution and placebo solution were injected separately. The chromatograms recorded were investigated to identify any interference at the RT of either Lefamulin or ISD by the any peaks of placebo and blank.

Sensitivity

Sensitivity was calculated using (LLOQ) QC "Lower Limit of Quantification Quality Control," which is the lowest concentration of analyte in a sample that can be consistently measured with sufficient accuracy and precision. Six QC samples of LLOQ and one set of linearity curve standards were prepared and spiked with respective aqueous dilutions in blank matrix that has acceptable interfering area. The % mean accuracy and %CV were calculated for the obtained peak responses.

Ruggedness

Ruggedness of the method was confirmed by evaluating the precision accuracy at three different concentration levels of Lefamulin standard solution (5, 10, 15ng/ml). Each concentration level was injected in six replicates. The mean accuracy in percentage was assessed to ensure the acceptance of the reported results.

Matrix effect

Ionization of the analytes due to plasma components was assessed by comparing the peak response of post-extracted MQC samples (10ng/ml of Lefamulin) (n = 6) with the peak response of drug substance from fresh samples at same concentrations [11-12]. The matrix effect intended method was assessed by using chromatographically screened rat plasma.

Stability studies

Stability methods should check how stable the analytes are during sample collection and handling, long-term and short-term storage, freeze-thaw cycles, and the analysis process. Conditions used in stability tests should be similar to what will happen when samples are actually handled and analyzed [9-10]. The procedure should also test how stable the analyte is in the stock solution. For all stability tests, you should use a set of samples made from a freshly made stock solution of the analyte in the right biological matrix that is free of the analyte and other things that could mess with it.

Bench top stability

In the current method it was done by storing the LQC, MQC and HQC sample in refrigerator for 12 hr. After that retrieved the sample and kept at room temperature on working bench for 6hr. The resultant each QC level sample was introduced in to LC-MS system in six replicates. The % mean accuracy and %CV was determined from the obtained responses.

Auto sampler injection stability

To avoid the bias in results prior to validation it is highly significant to confirm the reinjection or reproducibility of auto sampler stability. In the current method it was confirmed by determining the %mean accuracy of LQC, MQC and HQC sample, which were stored for 24 hr in auto sampler.

Freeze thaw stability

Freeze-thaw stability testing will be performed to determine the analyte's stability in biological fluids following repeated freezing and thawing cycles. In the current method it was done by storing the LQC, MQC and HQC sample in refrigerator and frozen for 24 hr at --20 ±5 ^{o}C. After that retrieved the sample and kept at ambient for thaw condition6hr. The resultant each QC level sample was introduced in to LC-MS system in six replicates. The % mean

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accuracy and %CV was determined from the obtained responses.

Short term stability

In the current LC-MS method it was done by storing the LQC, MQC and HQC sample in vials for 3 days at -20 ±5 °C. Six replicate injections of each level QC samples were introduced into LC-MS system on respective days. The % mean accuracy and %CV was determined from the obtained responses.

Long term stability

Long term stability of Lefamulin in biological samples were performed by storing the LQC, MQC and HQC sample in vials for freezing in refrigerator at -20 ±5 °C for 1, 7, 14,21 and 28 days. Six replicate injections of each level QC samples were introduced into LC-MS system on respective days. The % mean accuracy and %CV was determined from the obtained responses.

RESULTS

Optimized method conditions

The successful separation of Lefamulin from plasma samples were achieved with retention time (RT) of 2.65 min by the use of above mentioned LC conditions. D_6-Lefamulin was considers as internal standard, which was behaved as same as Lefamulin in separation. Both are separated with same RT (Figure-2). The separated Lefamulin spectrometrically characterized and confirmed by the mass spectrometric condition a mentioned above methodology. The mass spectrum of Lefamulin was shown in Figure-3.

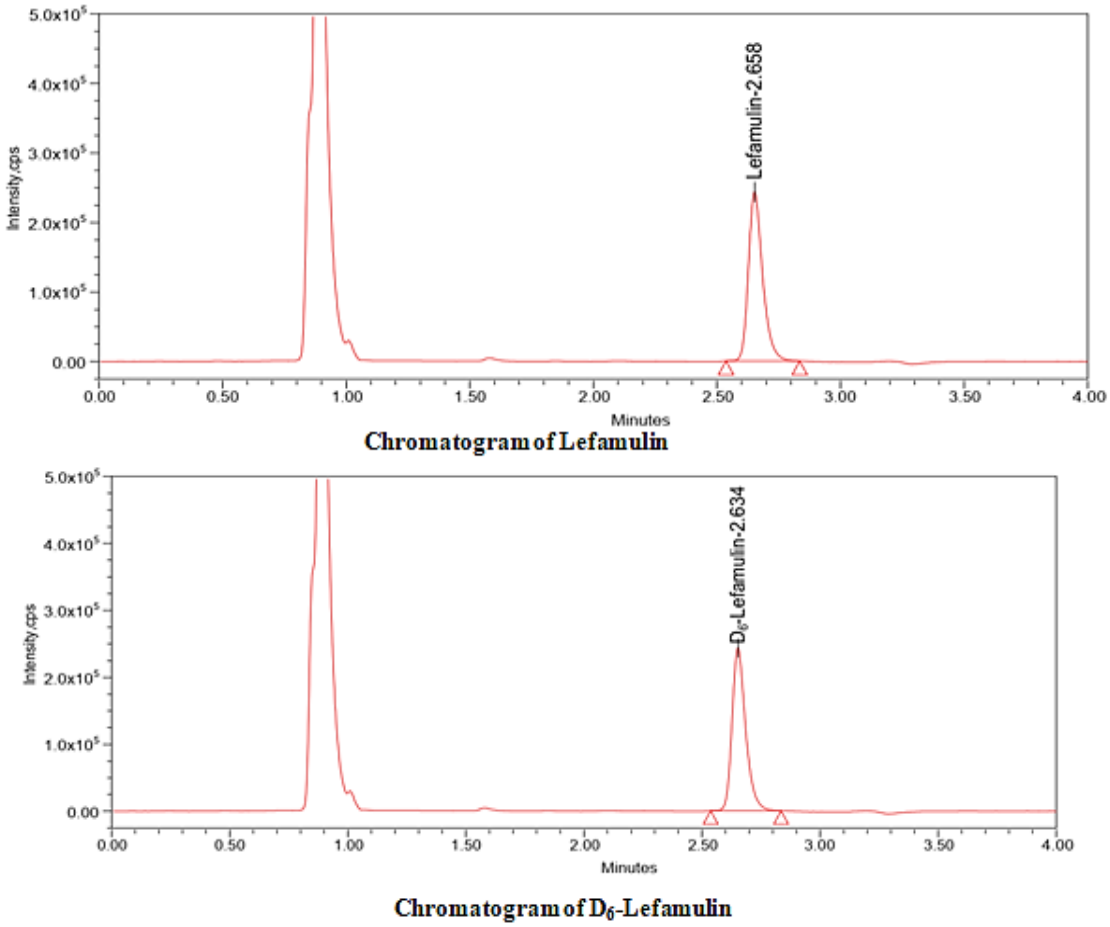
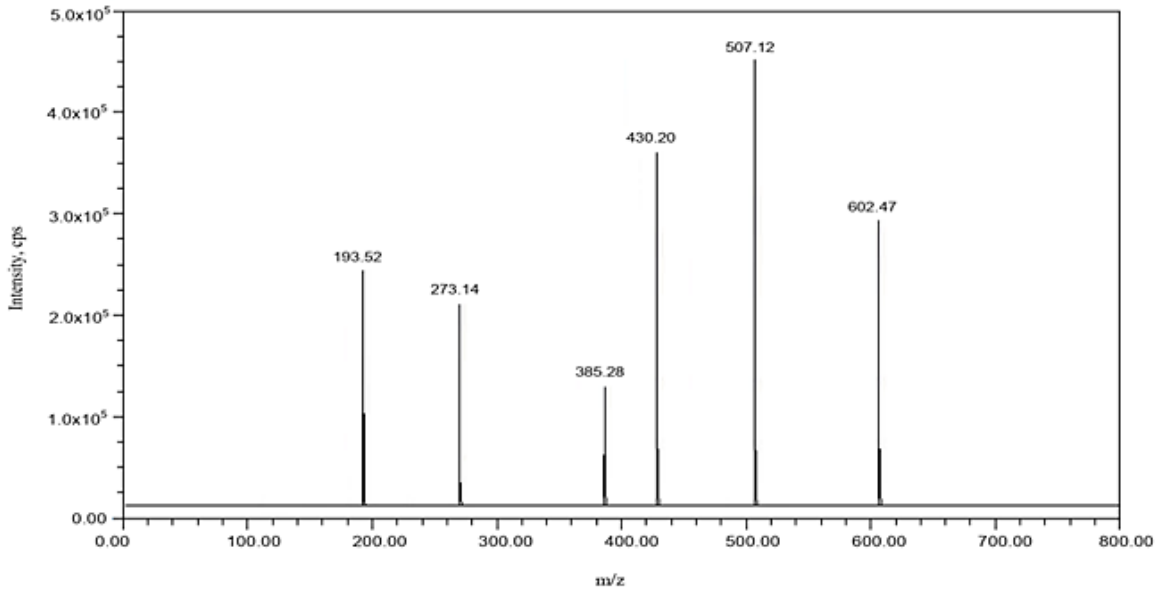


Figure-2: Optimized chromatogram of Lefamulin and D_6-Lefamulin



Multiple Reaction Monitoring-MRM of the Lefamulin using Positive Polarity

Analogue	Precursor Ion (m/z)	Daughter Ion with the Highest Intensity (m/z)
Lefamulin	507.12	430.20

Figure-3: Mass spectrum of Lefamulin

Method validation

Q2 specifications of the ICH were considered to validate the developed method.

System suitability

The % CV of the peak areas of the six injections of the standard solution of Lefamulin was calculated to be 0.4 and % CV of pear area ratio of Lefamulin and its internal standard was found to be 1.05 (Table-1). Those results within the allowable limits, ensures the system suitability of the method.

Table 1: System suitability Results of Lefamulin

Samlple Name MQC (10ng/ml)	**Analyte Area (cps)**	**Analyte RT (min)**	**ISTD Area (30ng/ml)**	**ISTD RT (min)**	**Area Ratio**
Mean (N=6)	$2.315x10^5$	2.655	$2.351x10^5$	2.643	0.985
SD	0.0092	0.00301	0.02443	0.01323	0.01031
%CV	0.4	0.11	1.04	0.50	1.05
Acceptance limit (%CV)	**≤ 15**	**≤ 2**	**≤ 15**	**≤ 2**	**≤ 5**

Linearity

The R^2 value addressed for given series of concentration was 0.999, which depicts the linear response of the proposed method as per the ICH guide lines (Table 2 and Figure-4).

Concentration (ng/ml)	**Lefamulin Response (CPS)**	**IS Response (CPS)**	**Ratio**
0	0	0	0
0	0	0	0

1.00	0.2466 x10^5	2.589 x10^5	0.095
2.50	0.5264 x10^5	2.546 x10^5	0.207
5.00	1.1258 x10^5	2.595 x10^5	0.434
7.50	1.6682 x10^5	2.564 x10^5	0.651
10.00	2.3752 x10^5	2.584 x10^5	0.919
12.50	2.8886 x10^5	2.523 x10^5	1.145
15.00	3.3842 x10^5	2.567 x10^5	1.318
20.00	4.6721 x10^5	2.559 x10^5	1.826

Table 2: Linearity results of Lefamulin

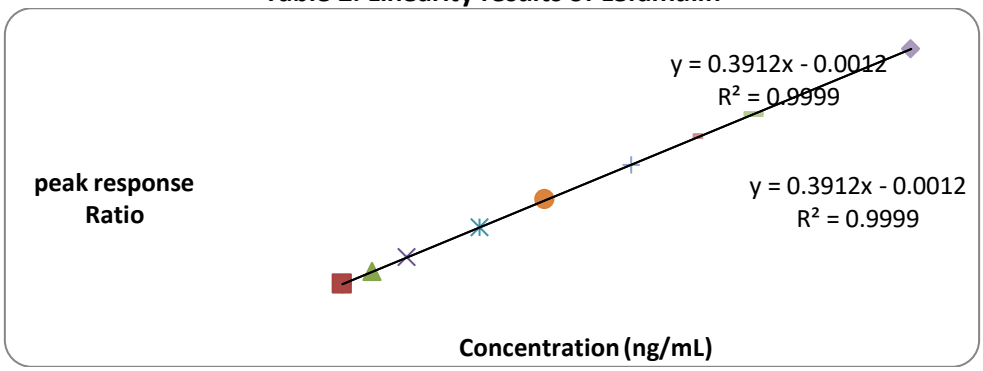


Figure-4: Linear graph of Lefamulin

Recovery

The % CV of recovery of Lefamulin at each QC level was ≤ 15.00 % and the overall mean recovery % CV for all QC levels were ≤ 20.00 % (Table 3). The obtained %CV of recovery of all QC samples has been ensured the accuracy of the current LC-MS method.

Table 3: Recovery results of Lefamulin at various levels

QC level	Parameter	Extracted Response	Un Extracted Response	Matrix Factor
HQC (15ng/ml)	Mean (n=6)	3.755x10^5	3.834x10^5	0.9816
	SD	0.03485	0.03455	0.00254
	%CV	0.93	0.90	0.26
	%Mean Recovery	99.85%	101.95%	-
MQC (10ng/ml)	Mean (n=6)	2.361x10^5	2.406x10^5	0.9812
	SD	0.02998	0.02522	0.00369
	%CV	1.27	1.05	0.38
	%Mean Recovery	101.99%	103.93%	-
LQC (5ng/ml)	Mean	1.169x105	1.208x105	0.968
	SD	0.01526	0.01660	0.00557
	%CV	1.31	1.37	0.58
	%Mean Recovery	100.99%	104.36%	

Precision and Accuracy

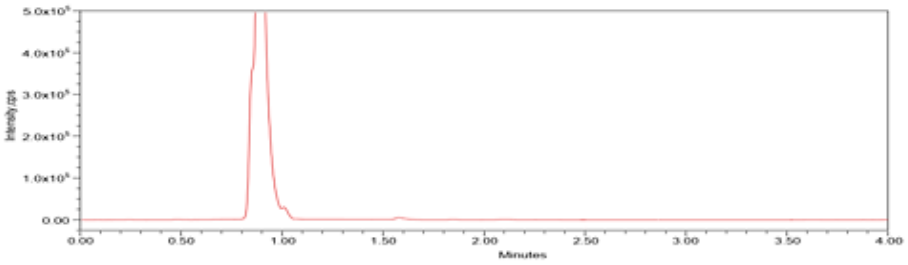
The % CV of peak responses of Lefamulin in MQC samples found to be 0.4 and 1.04% respectively (Table-1), The % CV of Lefamulin HQC, MQC, LQC, and LQC were observed to be 0.97%, 0.68%, 2.30%, and 7.23% respectively were obtained within the acceptable limit (Table 4), which strongly unveil the precision of the LC-MS method as per ICH limits. The mean accuracy of all QC levels were assessed to be 100±5%

Table 4: Precision and accuracy results of Lefamulin

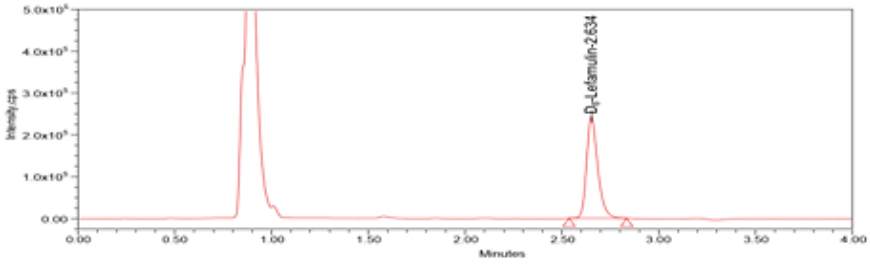
Precision		**HQC (15ng/ml)**	**MQC (10ng/ml)**	**LQC (5ng/ml)**	**LLQC (1ng/ml)**
		Nominal Concentration (ng/ml)			
		15.42	**10.15**	**5.43**	**1.24**
		Analyte peak area			
Inter day Precision and Accuracy	Mean	$3.338x10^5$	$2.322x10^5$	$1.157x10^5$	$0.24x10^5$
	SD	0.03228	0. 01572	0.02657	0.01736
	% CV	0.97	0.68	2.30	7.23
	% Mean Accuracy	96.13%	100.30%	99.96%	103.67%
Reproducibility and Accuracy	Mean	$3.338x10^5$	$2.356x10^5$	$1.137x10^5$	-
	SD	0.02257	0.02412	0.02454	-
	% CV	0.68	1.02	2.16	-
	% Mean Accuracy	96.13%	101.77%	98.23%	
	% CV limit	**%≤ 15%**	**≤ 15%**	**≤ 15%**	**≤ 20%**

Specificity

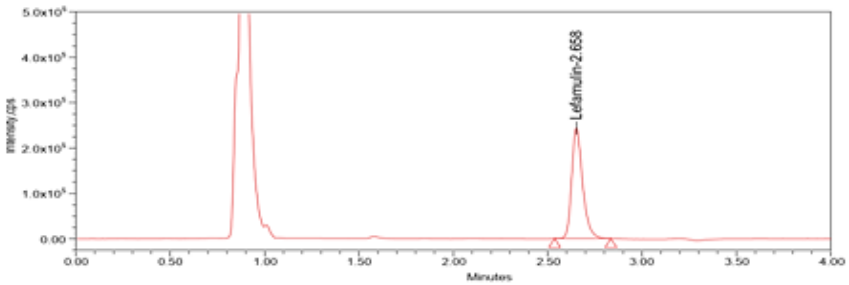
No interfering peaks were observed in six different random blank rat plasma samples at the retention times of either Lefamulin or ISD. **(Figure-5)**,which disclose the specificity of the LC-MS method towards the analysis of Lefamulin in biological samples.



Chromatogram representing blank



Chromatogram representing internal standard solution



Chromatogram representing Lefamulin standard solution

Figure-5 : Chromatograms reprsenting specificity of the method

Sensitivity

The %CV and % mean recovery of analyte from LLQC samples were 1.01% and 106.2% respectively indicating the methods sensitivity with good precision and accuracy

Ruggedness

The % mean accuracy for LQC, MQC and HQC samples should be within 100±15%. The obtained results of 96.24%,101.30% and 100.13% for HQC, MQC and LQC corrospondingly confirms the ruggedness of the LC-MS procedure.

Table 5: Ruggedness on reinjection reproducibility Results of Lefamulin

Injection no	HQC (15ng/ml)	MQC (10ng/ml)	LQC (5ng/ml)
	Nominal Concentration (ng/ml)		
	15.528	10.416	5.357
	Peak areas		
Mean	$3.342x10^5$	$2.345x10^5$	$1.159x10^5$
SD	0.02574	0.03071	0.01980
% CV	0.77	1.31	1.71
% Mean Accuracy	96.24%	101.30%	100.13%
Acceptance criteria :	±15%	±15%	±15%

Matrixeffect

Two out of three samples from a lot must be in the range of 85 to 115%, at the very least. At least 80% (5 of 6) of the matrix lot should meet the requirements for approval. The range of 85% to 115% percent should apply to the mean accuracy of back computed concentrations for LQC and HQC samples made from various biological matrix batches.The %mean accuracy at HQC and LQC was calculated to be 96.56% and 102.59% respectively (Table 6).

Table 6: Matrix effect Results of Lefamulin

S.No.	Plasma Lot No.	HQC	LQC
		Nominal Concentration(ng/ml)	
		15.42	5.43
		Analyte peak area	
Mean (n=18)		$3.353x10^5$	$1.127x10^5$
SD		0.02821	0.01716
%CV		0.84	1.52
% Mean Accuracy		96.56%	102.59%
No. of QC Failed		0	0

Stability studies

The mean accuracy and %CV of repeated injections were should not deviated the acceptance limit of 100±15% and ≤15% respectively (Table 7). The obtained results indicating that the concentration of the analyte is not changed by any of the steps taken to prepare, process, and analyze a sample or by the way it is stored.

Table 7: Stability studies of Lefamulin in various studies

Stability type	Parameter	HQC (15 ng/ml)	LQC (5 ng/ml)	MQC (10ng/ml)
		Nominal Concentration(ng/ml)		
		15.216	5.652	10.582

		Analyte peak area		
Bench Top	Mean (n=6)	$3.329x10^5$	$1.141x10^5$	$2.366x10^5$
	SD	0.00619	0.01458	0.02158
	%CV	0.19	1.28	0.91
	% Mean Accuracy	95.87%	98.57%	102.2%
Auto Sampler	Mean (n=24)	$3.348x10^5$	$1.161x10^5$	$2.342x10^5$
	SD	0.02207	0.00959	0.00944
	%CV	0.66	0.83	0.40
	% Mean Accuracy	96.41%	100.30%	101.17%
Freeze Thaw	Mean (n=6)	$3.357x10^5$	$1.156x10^5$	$2.356x10^5$
	SD	0.02696	0.01968	0.01804
	%CV	0.80	1.70	0.77
	% Mean Accuracy	96.67%	99.87%	101.77%
Short term	Mean	$3.172x10^5$	$1.082x10^5$	$2.155x10^5$
	SD	0.01167	0.00695	0.01282
	%CV	0.37	0.64	0.59
	% Mean Accuracy	91.35%	93.48%	93.09%
Long term (Day-1)	Mean	$3.367x10^5$	$1.168x10^5$	$2.346x10^5$
	SD	0.02082	0.00792	0.00794
	%CV	0.62	0.68	0.34
	% Mean Accuracy	96.96%	100.91%	101.34%
Long term (Day-7)	Mean	$3.142x10^5$	$1.080x10^5$	$2.125x10^5$
	SD	0.02699	0.01757	0.01334
	%CV	0.86	1.63	0.63
	% Mean Accuracy	90.48%	93.30%	91.79%
Long term (Day-14)	Mean	$3.028x10^5$	$0.958x10^5$	$2.029x10^5$
	SD	0.01717	0.01919	0.01619
	%CV	0.57	2.00	0.80
	% Mean Accuracy	87.2%	82.76%	87.65%
Long term (Day-21)	Mean	$2.988x10^5$	$0.908x10^5$	$1.940x10^5$
	SD	0.00697	0.00176	0.01481
	%CV	0.23	0.19	0.76
	% Mean Accuracy	86.05%	78.44%	83.80%
Long term (Day-28)	Mean	$2.868x10^5$	$0.869x10^5$	$1.879x10^5$
	SD	0.01873	0.00979	0.01250
	%CV	0.65	1.13	0.67
	% Mean Accuracy	87.59%	85.08%	85.17%

DISCUSSION

Bio analytical methods have significant role in preclinical and clinical studies of the drug substance and drug product to assess the pharmacokinetic properties of the analyte in biological fluids. An efficient LC-MS method provides the separation, identification and quantification of drugs by conforming the molecular weight. In previously only one single LC-MS method with longer retention time was reported [6]. The existed method has few drawbacks like longer RT and expensive solvent system leads to more expensive and more time consuming [6]. To overcome the drawbacks of the reported method, a new LC-MS method was created with shorter RT of

2.65min and a mobile system of 0.1%v/v formic acid: Acetonitrile (70:30 v/v). The developed method was validated in terms of ICH guidelines. The obtained results of validation parameters were within the acceptance limit. Based on the results it was confirmed that the matrix effect was very minimum within allowable limits. The developed method was superior to previously reported method in terms of retention time, simple mobile phase, sensitivity and specificity. Hence the proposed method has adoptability in analysis of biological samples of Lefamulin in quality control department of the pharmaceutical production sector.

CONCLUSION

A specific and sensitive LC-MS method was developed for estimation of Lefamulin in plasma samples. The proposed method has less retention time for Lefamulin and its ISD. The developed gas good accuracy, precision and sensitivity as of ICH limits. The obtained stability studies results indicating that the concentration of the analyte is not changed by any of the steps taken to prepare, process, and analyze a sample or by the way it is stored. Hence, the current method gas good adoptability to assess the pharmacokinetic parameters in in-vivo studies.

ACKNOWLEDGEMENTS

The authors are thankful to Department of pharmaceutical Analysis, GITAM University and Dr. Vasudha bakshi, Principal, Anurag University for her continuous support.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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