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Development and validation of a liquid chromatography-tandem mass spectrometric method for the determination of lenalidomide in human plasma and its application on bioequivalence studies

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Abstract

A simple, sensitive, and specific liquid chromatography-tandem mass spectrophotometry (LC-MS/MS) method was developed and validated for the quantification of lenalidomide in human plasma. The separation was carried out on a symmetry, C₁₈, 5- μ m (50 \times 4.6 mm) column as stationary phase and with an isocratic mobile phase of 0.1% formic acid in water-methanol in the ratio of (15:85, v/v) at a flow rate of 0.5 mL/min. Protonated ions formed by electrospray ionization in the positive mode were used to detect analyte and fluconazole (internal standard). The mass detection was made by monitoring the fragmentation of m/z 260.1/148.8 for lenalidomide and m/z 307.1/238.0 for internal standard on a triple quadrupole mass spectrometer. The developed method was validated over the concentration range of 10–1000 ng/mL for lenalidomide in human plasma with a correlation coefficient (r^2) was 0.9930. The accuracy and precision values obtained from six different sets of quality control samples analyzed on separate occasions ranged from 99.41 to 106.97% and 2.88 to 4.22%, respectively. Mean extraction recoveries were 98.06% and 88.78% for the analyte and IS, respectively. The developed method was successfully applied for analyzing lenalidomide in human plasma samples.

Keywords: Lenalidomide, LC-MS/MS, Human plasma, Electrospray ionization, Pharmacokinetics

Introduction

Lenalidomide is a [derivative](#) of [thalidomide](#); it was initially intended as a treatment for [multiple myeloma](#) for which thalidomide is an accepted therapeutic treatment. Lenalidomide has also shown efficacy in the class of hematological disorders known as [myelodysplastic syndromes](#) (MDS). Lenalidomide has significantly improved overall survival in myeloma (which generally carries a poor prognosis), although toxicity remains an issue for users (Gupta et al. 2001; Hideshima et al. 2006; McCarthy et al. 2012; Palumbo

et al. 2008; Shah Tran 2007; Tariman 2007). Lenalidomide is rapidly absorbed following oral administration. Following single and multiple doses in patients with [multiple myeloma](#) or [MDS](#), the maximum plasma concentrations occurred between 0.5 and 6 h post-dose. The single- and multiple-dose pharmacokinetic disposition of lenalidomide is linear with AUC and C_{\max} values increasing proportionally with dose. Lenalidomide undergoes limited [metabolism](#). Unchanged lenalidomide is the predominant circulating component in humans. It is eliminated primarily in renal, and the mean half-life of lenalidomide is 3 h in healthy subjects and 3 to 5 h in patients (MYOPATHY n.d.; Richardson et al. 2002). Few analytical methods have been reported for the estimation of lenalidomide in bulk form and capsule dosage forms (Darwish et al. 2012; Reddy et al. 2012;

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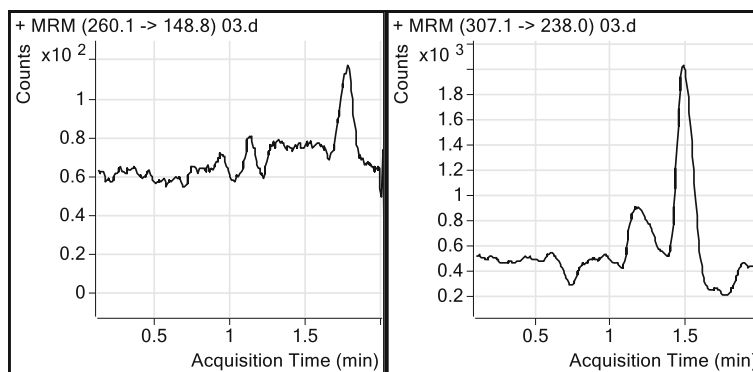


Fig. 1 Representative chromatogram of human blank plasma sample of lenalidomide and IS

Saravanan et al. 2007; Sastry et al. 2009); besides few analytical methods, determination of lenalidomide in human plasma and other biological matrices have been reported (Hasnain et al. 2013; Khalil et al. 2013; Ranganathan et al. 2018; Saha 2019; Veeraraghavan et al. 2014; Veeraraghavan et al. 2015). Some of these reported methods have their individual advantages of short runtime and improved method sensitivity and sample treatment. However, it is essential to have one method which is economical and has significant advantages over a group of methods. Hence, the aim of the present study is to develop an economical, reliable, sensitive, and validated method for the estimation of lenalidomide in human plasma by tandem mass spectrometry using liquid-liquid extraction method.

Experimental

Chemicals and reagents

Lenalidomide working standards (99.9%) were obtained from Vivian Life Sciences Pvt. Ltd. (Mumbai, India), and fluconazole working standards (98.85%) were obtained from Tablets India Ltd. (Chennai, India). Methanol (LC-MS CHROMA-SOLV[®]) and formic acid (LC-MS ultra) were obtained from Sigma Aldrich (St. Louis, MO, USA). Ethyl acetate (HPLC grade) was obtained from Rankem Chemicals (India). All other reagents were of analytical grade unless stated otherwise, and all solutions were prepared using a Milli-Q water purification system from Millipore (Bangalore, India). Blank human plasma (K_2EDTA as an anticoagulant) was obtained from Sri Venkateshwara Blood Bank, Chennai, India.

Instrument and conditions

The LC-MS/MS analyses were performed using an Agilent 6460 triple quadrupole LC-MS system (Agilent Technologies, Palo Alto, CA) with a column of Symmetry C_{18} (50×4.6 mm) $5 \mu m$ (Waters, USA). The mobile phase was 0.1% formic acid in water-methanol (15:85, v/v) at a flow rate of 0.5 mL/min. The column temperature was maintained at 30 °C. A quadrupole mass spectrometer equipped with an electrospray ionization source was set with a capillary voltage 3500 V, Nozzle voltage 1500 V, drying gas (N_2) flow of 5 L/min, nebulizer pressure of 25 psi, drying gas temperature of 350 °C, sheath gas temperature 300 °C, sheath gas flow 11 L/min, collision energy 8 and positive ion mode. Quantitation was performed in multiple reaction monitoring modes (MRM) with the transition of parent ions to the product ions m/z 260.1/148.8 for lenalidomide and m/z 307.1/238.0 for fluconazole; fragment or voltage was 100 V; resolution was maintained at unit resolution, and dwell time was set at 100 ms. System control and data analysis were performed by MassHunter workstation (version B.05.01).

Preparation of calibration standards and quality control samples

Stock solutions of lenalidomide and fluconazole (IS) were prepared in methanol at a concentration of 1000 $\mu g/mL$, respectively (Tohny et al. 2004). A series of lenalidomide working standard solutions in the concentration range of

Table 1 Precision and accuracy results for lenalidomide in human plasma

Spiked conc. (ng/mL)	Intra-day			Inter-day		
	Calculated conc. (mean \pm SD)	Precision (% CV)	Accuracy (% RE)	Calculated conc. (mean \pm SD)	Precision (% CV)	Accuracy (% RE)
10.00	10.76 \pm 0.38	3.51	- 7.58	10.60 \pm 0.31	2.88	- 6.01
26.25	28.09 \pm 0.41	1.47	- 7.0	28.08 \pm 0.83	2.95	- 6.96
323.06	326.65 \pm 14.93	4.57	- 1.11	324.68 \pm 12.76	3.93	- 0.50
807.64	791.12 \pm 20.57	2.60	2.05	802.87 \pm 33.89	4.22	0.59

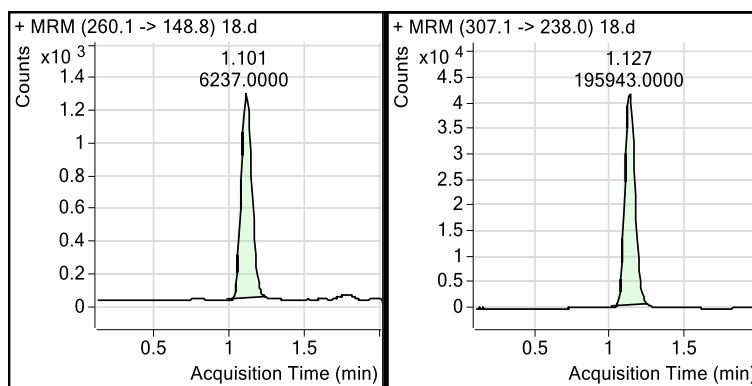


Fig. 2 Representative chromatogram of blank plasma sample spiked with lenalidomide at the LLOQ of 10 ng/mL and I.S (1000 ng/mL)

500–50000 ng/mL were obtained by further diluting the stock solution with 50% methanol in water solution. The fluconazole working solution of a final concentration of 1000 ng/mL was obtained by the same method. All solutions were stored at 4 °C until analysis. The plasma calibration standards of lenalidomide with final concentrations of 10, 25, 50, 150, 300, 500, 700, 900, and 1000 ng/mL were prepared by spiking a 980 μ L aliquot of blank plasma with a 20 μ L aliquot of lenalidomide working standard solutions. Quality control samples (LLOQ, lower limit of quantification; LQC, low-quality control; MQC, middle-quality control; HQC, high-quality control) of 10, 25, 325, and 800 ng/mL were prepared in the same way as calibration standards. The bulk samples were aliquoted (300 mL) into polypropylene tubes and stored at – 80°C for further analysis.

Extraction of plasma samples

An aliquot of 200 μ L plasma sample and 50 μ L of IS solution (1000 ng/mL fluconazole) were added into 5 mL polypropylene tube. Add 50 μ L of 0.1% formic acid and vortex and add 2.5 mL of ethyl acetate, the sample was placed on a platform shaker for 10 min at 2000 rpm, followed by centrifugation at 4000 rpm for 10 min. The organic layer was separated from the aqueous and evaporated to dryness at 50 °C under a nitrogen stream. The residue was reconstituted in 400 μ L of the mobile phase, and 10 μ L was injected for LC-MS/MS for analysis.

Optimization of MS condition

A standard solution of lenalidomide and fluconazole (IS) was directly infused along with the mobile phase into the mass spectrometer in the ESI source. The mass spectrometer was tuned in positive ionization mode for the optimum response of lenalidomide and fluconazole. In the positive precursor ion full-scan spectra, the most abundant ions were protonated ions $[M+H]^+$, m/z 260.1 and 307.1 for lenalidomide and fluconazole, respectively. Parameters such as gas flow, gas temperature, nebulizer

pressure, fragmentation, and capillary voltage were optimized for fine-tuning to obtain the best response. The product ion scan spectra showed high abundance fragment ions at m/z 148.8 for lenalidomide and m/z 238 for fluconazole. The fragmentation voltage and collision energy were optimized for the maximum response of the fragmentation of m/z 148.8 for lenalidomide and m/z 238 for fluconazole. The ion transitions of m/z 260.1 \rightarrow 148.8 and m/z 307.1 \rightarrow 238.0 were chosen for lenalidomide and fluconazole, respectively.

Optimization of LC condition

The chromatographic conditions were modified to obtain high sensitivity and sample throughput. The separation and ionization of lenalidomide and fluconazole were affected by the composition of the mobile phase. The chromatographic conditions were optimized with different compositions of the mobile phase system using formic acid, ammonium acetate, methanol/acetonitrile being used for the determination. The highest ionization efficiency of lenalidomide and fluconazole was achieved when the ration of 0.1% formic acid in water and methanol (15:85 v/v). The chromatographic column, Agilent Zorbax Eclipse XDB-C18 (4.6 \times 50 mm) 5 μ m, and Symmetry C₁₈ (50 \times 4.6 mm) 5 μ m were investigated in the chromatographic separation. The Symmetry C₁₈ (50 \times 4.6 mm) 5 μ m column was proved to be better than the others, which was tested to achieve shorter run time (2.0 min), better resolution, and symmetric peak shapes for lenalidomide and fluconazole.

Table 2 Absolute and IS normalized matrix effect of lenalidomide in human plasma

Spiked conc. (ng/mL)	Absolute matrix factor	IS normalized matrix factor
26.25	0.97	0.89
807.64	1.02	0.91

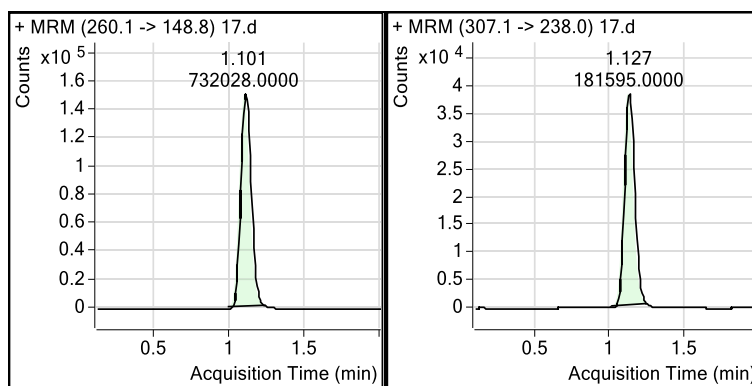


Fig. 3 Representative chromatogram of blank plasma sample spiked with lenalidomide at the HQC of 800 ng/mL and IS (1000 ng/mL)

Clinical study design

Twelve healthy Indian female volunteers participated in the study following Helsinki declaration (Protocol No: 1924 version 1.0 Feb 26 2016). After an overnight fasting, each volunteer received one capsule containing 25 mg lenalidomide, either test or reference formulation. Blood was sampled pre-dose and at 0.25, 0.5, 0.75, 1.0, 1.25, 1.50, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 7.0, 8.0, 10.0, 12.0, 16.0, and 24.0 h post-dose for the determination of plasma concentration of lenalidomide (Iqbal et al. 2013; Liu et al. 2008).

Pharmacokinetic analysis

Model-independent pharmacokinetic parameters were calculated for lenalidomide. The maximum plasma concentrations (C_{max}) and the time to those (t_{max}) were noted directly. The elimination rate constant (k_{el}) was calculated by linear regression of the terminal points of the semi-log plot of plasma concentration against time. Elimination half-life ($t_{1/2}$) was calculated from the formula $t_{1/2} = 0.693/k_{el}$. Area

under the plasma concentration-time curve (AUC_{0-24}) was calculated by the linear trapezoidal rule.

Results and discussion

Validation of the method

Full method validation was performed according to guidelines set by the US Food and Drug Administration (USFDA) (USFDA 2001; Gopinath et al. 2011; Hasnain et al. 2013). Specificity was determined by analyzing six blank human plasma samples without adding an internal standard to determine the interference of the analyte. Matrix effects for lenalidomide were evaluated by comparing the peak areas of analyte in extracted blank plasma samples from six different drug-free volunteers spiked the known concentrations with the corresponding peak areas obtained by direct injection of standard solutions, IS normalized matrix effects were also investigated. Linearity was analyzed by weighted linear regression ($1/X^2$) of analyte-internal standard peak area ratios. The calibration curves ($n = 8$) were prepared by spiking blank human plasma with a standard solution of

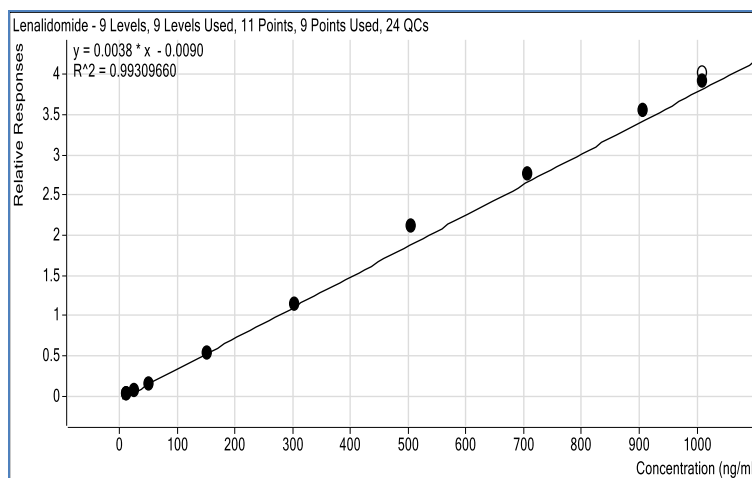


Fig. 4 Representative calibration curve for lenalidomide in matrix

Table 3 Extraction recoveries of lenalidomide and IS in human plasma

Spiked conc. (ng/mL)	Extraction recoveries (%)	% CV
26.25	97.32	2.15
323.06	98.58	0.59
807.64	98.28	2.12
Mean	98.06	1.62
Internal standard	88.78	4.00

lenalidomide. The LLOQ was the smallest analytical concentration at which the precision expressed by the coefficient of variation (CV) was lower than 20% and the accuracy evaluated by the deviation of the mean from the true value (Dev) was lower than 20%.

Intra-day, inter-day precision and accuracy were determined by analyzing QC samples ($n = 6$) on three different days. The extraction recoveries of lenalidomide were determined by comparing the results obtained from the spiked plasmas to standard solutions. Stability tests including five freeze-thaw cycles, dry extract stability in room temperature, autosampler stability at 4 °C, bench-top stability, and long-term stability in plasma at – 20 °C and – 80 °C were evaluated by QC samples at two different concentrations.

Method validation

Specificity and selectivity

Specificity and selectivity were evaluated by comparing chromatograms of six blank plasma lots with the spiked

plasma to make sure there were no significant interfering peaks at retention time at LLOQ of the analytes. The representative chromatograms are shown in Fig. 1, which indicated that no endogenous interferences were observed at the retention time for lenalidomide and fluconazole. Typical retention times were 1.10 and 1.12 min for lenalidomide and fluconazole, respectively, with a total runtime of 2.0 min (Figs. 2 and 3).

Linearity of calibration curves and lower limits of quantification

The linearity was determined in the range of 10.0 to 1000.0 ng/mL by plotting the peak area ratio (y) of lenalidomide to IS versus the nominal concentration (x) of lenalidomide in plasma. The average regression equation for the calibration curve ($n = 9$) was $y = 0.0038x - 0.0090$, $r^2 = 0.9930$, which is represented in Fig. 4. The slopes of the regression equations were consistent for the calibration curves prepared on five separate days. The LLOQ was set at the level of the lowest calibrators for the analyte (10 ng/mL) and was evaluated for the other QC samples individually spiked to at least 6 different lots of plasma, with precision and accuracy reported in Table 1 with RE % within $\pm 20\%$ and % CV lower than 20%.

Precision and accuracy

Validation runs were conducted on three consecutive days. In each run, QC samples were evaluated in sets of six replicates to evaluate the intra-day and inter-day precision and accuracy. The percentage deviation of the

Table 4 Stability of lenalidomide in plasma samples

Nominal concentration (ng/mL)	Obtained concentration (ng/mL)	% CV	% RE
Autosampler stability			
26.25	26.63 \pm 0.89	3.34	– 1.46
807.64	815.27 \pm 29.37	3.6	– 0.94
Freeze-thaw stability			
26.25	27.56 \pm 0.70	2.54	– 4.99
807.64	805.36 \pm 29.96	3.72	0.28
Bench top stability			
26.25	27.02 \pm 1.17	4.33	– 2.94
807.64	808.84 \pm 6.81	0.84	– 0.15
Dry extract stability in room temperature			
26.25	26.26 \pm 1.26	4.81	– 0.04
807.64	792.79 \pm 30.55	3.85	1.84
Long term stability in plasma at 65 days in – 80 °C			
26.25	25.47 \pm 0.36	1.42	2.95
807.64	786.65 \pm 6.01	0.76	2.60
Long term stability in plasma at 65 days in – 20 °C			
26.25	25.01 \pm 0.49	1.96	4.71
807.64	785.27 \pm 0.82	0.82	2.75

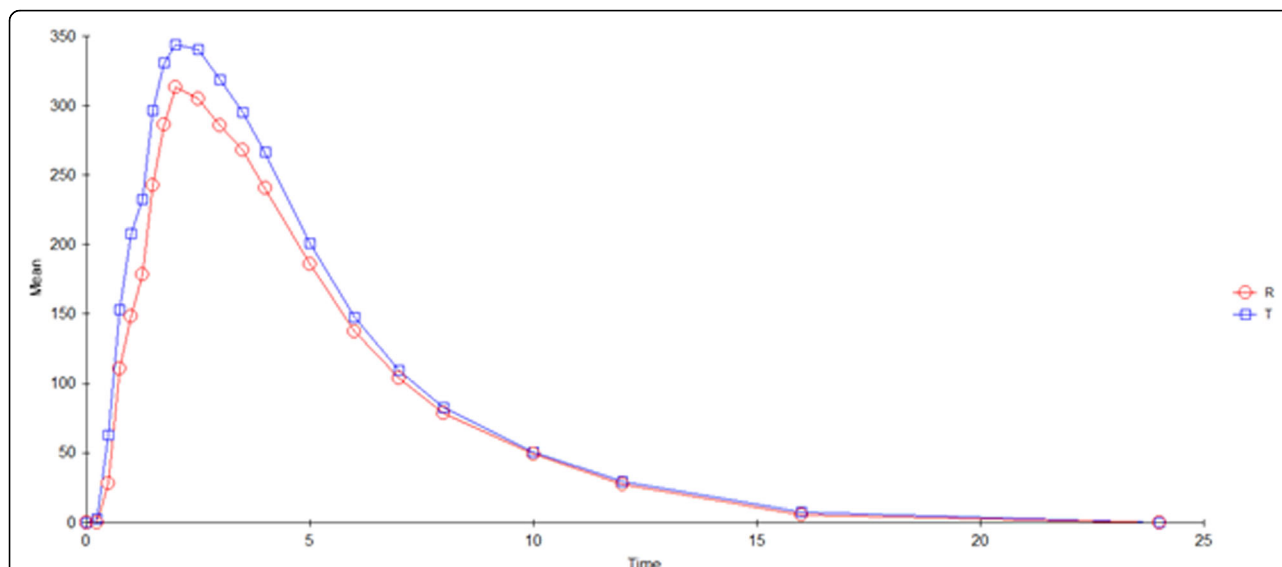


Fig. 5 Mean plasma concentration (ng/mL) profile of lenalidomide following 25 mg oral dose of test (T) and reference (R) preparation to 12 healthy human volunteers

mean from true values, expressed as a relative error (RE), and the % CV served as the measure of accuracy and precision, respectively. Summary of precision and accuracy data are presented in Table 1. The values were all within the acceptable range.

Matrix effect

The absolute matrix effects for lenalidomide were assessed at low and high concentrations. It was assessed by comparing the mean peak areas of the analyte spiked at two concentrations into the extracts originated from six different blank human plasma samples the mean peak areas for neat solutions of the analytes in 50% methanol in water. Summary of matrix effect data are presented in Table 2 and shown in Figs. 2 and 3. It is demonstrated that the matrix effect was not independent of the concentration.

Extraction recovery

Ethyl acetate was chosen as the extraction solvent for its higher extraction efficiency to the two target compounds.

The extraction recoveries of lenalidomide and fluconazole can be promoted by acidifying the plasma samples with formic acid, so 50 μ L of 0.1% formic acid was added to 200 μ L of plasma sample before extraction. Recovery was calculated by comparison of the peak areas of lenalidomide extracted from plasma samples with those of injected standards. The mean recovery of lenalidomide and IS from human plasma with ethyl acetate was $98.06 \pm 1.62\%$ and $88.78 \pm 4.0\%$. The results are shown in Table 3.

Stability

QC samples (LQC and HQC) of six replicates were subjected to the conditions below. Autosampler stability was assessed by analyzing processed QC samples kept in the autosampler at 4 $^{\circ}$ C for 47 h 33 min to evaluate post-preparative stability, with -1.46 and -0.94% and precision of 3.34 and 3.60%. Freeze-thaw stability was investigated after five freeze-thaw cycles (-80 $^{\circ}$ C), with RE of -4.99 and 0.28% and precision (% CV) of 2.54 and 3.72%. Bench stability was assessed by analyzing QC plasma samples kept at room temperature for 15 h 45 min which exceeded the routine preparation time of samples, with RE of -2.94 and -0.15% and precision of 4.33

Table 5 Mean pharmacokinetic parameters of lenalidomide in 12 human volunteers

Parameters	Reference (mean \pm SD)	Test (mean \pm SD)
C_{\max} (h)	338.73 \pm 87.44	390.50 \pm 144.47
AUC_{0-t} (ng.h/mL)	1669.63 \pm 620.29	1883.66 \pm 757.12
$AUC_{0-\infty}$ (ng.h/mL)	1792.35 \pm 681.77	1982.24 \pm 791.43
t_{\max} (h)	2.03 \pm 0.70	2.08 \pm 0.61
k_{el}	0.26 \pm 0.05	0.26 \pm 0.05
$t_{1/2}$ (h)	2.80 \pm 0.64	2.79 \pm 0.58

and 0.84%. Dry extract stability was assessed by analyzing extracted QC plasma samples (without reconstitution) kept at room temperature for 69 h 25 min, with RE of -2.94 and -0.15% and precision of 4.33 and 0.84%.

Long-term stability was determined by assaying QC samples after storage at -80 °C and -20 °C for 65 days, with RE of 2.95 and 2.60% and precision of 1.42 and 0.76% for LQC and HQC samples at -80 °C, with RE of 4.71 and 2.75% and precision of 1.96 and 0.82% for LQC and HQC samples at -20 °C. The stock solutions' stability was investigated at mid-quality control sample levels (323.06 ng/mL) after storage at 4 °C for 20 days. The results were obtained by a comparison with freshly prepared solutions, lenalidomide, and IS stock solutions was stable at refrigerated condition. Lenalidomide proved to be stable under all testing conditions. No significant degradation of lenalidomide was observed under any of these conditions. Results are summarized in Table 4.

Application

The method described above was successfully applied to the bioequivalence study to determine the plasma concentration of lenalidomide up to 24 h after an oral administration of the reference and the test formulations of lenalidomide to 12 healthy Indian female volunteers. This study was approved by the Madras ethical committee, Chennai, India. All volunteers gave their signed informed consent to participate in the study according to the principles of the Declaration of Helsinki. The mean plasma concentration-time curve of lenalidomide is shown in Fig. 5. The mean pharmacokinetic parameters of lenalidomide in 12 volunteers were calculated and summarized in Table 5. Both the mean values and standard deviations of the main pharmacokinetic parameters such as C_{max} , t_{max} , AUC_{0-24} , and $AUC_{0-\infty}$ were found to be close, between test and reference preparations, indicating that plasma profiles generated by test preparations were comparable with those produced by reference preparations. In addition, the calculated 90% CIs for mean C_{max} , AUC_{last} , and $AUC_{0-\infty}$ of two drugs lay within the FDA's accepted range of 80–125%. Therefore, it could be concluded that the two lenalidomide preparations (test and reference) analyzed were bioequivalent in terms of rate and extent of absorption.

Conclusion

The above-developed method achieved good sensitivity and specificity for the determination of lenalidomide in human plasma. No significant interferences and matrix effect caused by endogenous compounds were observed. This simple and sensitive assay is suitable for bioequivalence and bioavailability studies of lenalidomide in human subjects. The characteristics of this analytical method are suitable for the routine analysis in a clinical

laboratory for pharmacokinetic and bioequivalence studies in humans in a single analytical run.

Abbreviations

AUC: Area under a curve; C_{max} : Maximum concentration; CV: Coefficient of variance; HQC: High-quality control; IS: Internal standard; k_{el} : Elimination rate constant; LC-MS/MS: Liquid chromatography-tandem mass spectrophotometry; LLOQ: Lower limit of quantification; LOQ: Limit of quantification; LQC: Low-quality control; MDS: Myelodysplastic syndromes; MQC: Middle-quality control; MRM: Multiple reaction monitoring; QC: Quality control; RE: Relative error; $t_{1/2}$: Half-life; t_{max} : Maximum time; USFDA: US Food and Drug Administration

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Authors' contributions

All the authors have contributed equally to this research work. All authors read and approved the final manuscript.

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Availability of data and materials

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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