Development of Method Validation for Related Substances In lenalidomide Capsules with HPLC Method

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ABSTRACT

A validated HPLC method was developed for the determination of Lenalidomide(LLE) in pharmaceutical formulation. Isocratic elution at a flow rate of 1.0ml/min was employed on Zorbax SB C8 3.5µm × 4.6 mm, 150mm, or similar is used for this chromatography analysis and the column temperature is maintained as ambient.The Mobile phase is a mixture of pH 6.1buffer and Methanol in the ratio of 95:5%v/v as mobile phase-A and the mixture of pH 6.1buffer and Methanol in the ratio of 5:95%v/v as mobile phase-B was used. Flow rate was identified at 1.0ml/min. a 5.0µl sample was injected. The run time is 52 minutes to Sample, blank, system suitability, furthermore 55 minutes to diluted standard. Retention time is noted for LLE is 18.1 minutes. The% R.S.D to LLE is measured. Mean percentage recovery to LLE is identified and it is found that within specification limit. The proposed HPLC processshould successfully applied to routine quality control analysis of formulations. The method developed in this article is more simple and is much better than the methods reported in the literature.

Keywords: Lenalidomide; HPLC method; validation and limit of quantization

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Introduction

The lenalidomide (LLE) molecular formulae is $C_{13}H_{13}N_3O_3$. This drug is available under trade name Revlimid along with others. It is a medication utilized to treat smoldering myeloma, as well as MDS, taken by mouth.[1] This drug has various mechanism of action.[2] It is on the WHO List of required medicines.[3]for the treatment of multiple myelomaLLE is utilized.[4] It is highly potent analog molecular of thalidomide, this inhibits angiogenesis of tumor , tumor-secreted cytokines, as well as tumor proliferation by apoptosis induction.[5-7] In 2017, FDA approved LLE as standalone maintenance therapy to

people.[8]Premanand Ranganathan et.al.,[9] got isocratic elution on a XTerra RP18, (4.6 × 50 mM, 5 µm) column. mobile phase is 0.1% Formic acid as 10 v/v: Methanol as 90%. LOQ is 9.999 ng/mL. Calibration curves obtained as 9.999 to 1010.011 ng/mL. PunnaVenkateshwarlu et.al., [10] used the column as Shimadzu LC -2010 HT by using C18 (250 X 4.6 X mm X 5µ). Mobile phase as Phosphate buffer 55v/v and Acetonitrile 45v/v. 1.00ml/min is the flow rate. 242nm is the wavelength. Rt is 2.5 min. 16199.817 is the theoretical plates. 1.128 is the tailing factor. 0.058 µg/ml is LOD and 0.174µg/ml is LOQ. Somana Siva Prasad et.al., [11] proposed X-bridge-C18 column (150 mm × 4.6 mm × 3.5 µ). Mobile phases as Potassium dihydrogen orthophosphate anhydrous buffer 90v/v and methanol 10v/v. 0.8 mL/minis the flow rate. 210 nm is wave length. Liu Q et.al.,[12] described C-18 column with H_2O and ACN, each with 0.1% formic acid. LLOQ for both drugs are 1nM and 0.3nM. 99% to 116% is the recovery. Nourah Z. Alzoman et.al.,[13] observed the separation of the LDM enantiomers on a LUX 5U cellulose-2 chiral column (250 × 4.6 mm i.d.). Mobile phase is methanol 100v, glacial acetic acid as 0.01v, triethylamine as 0.01v. 1.2 mL/min is the flow rate. 220 nm is the wavelength The calibration curve ranged as 2 to 1,000 ng/mL (r = 0.9999) for both LDM enantiomers. Different authors are performed the research by selecting different drugs with the help of different methods[14,15,16,17,18,19,20,21,22,23]. All the authors are concluded depending upon their results that their proposed methods are sample reanalysis and method is reproducible, very simple and precise, can be used for routine quantitative analysis, proven to be robust and accurate for quantitative analysis of residual solvent in neat materials, method suitable for its intended use, cleaning in cleaning validation for quality control purposes, specific and sensitive to routine analysis, selective and specific, stability-indicating, within the acceptable range, successfully be used for the analysis of Losartan Potassium and Hydrochlorothiazide in bulk or in combined dosage forms. By considering all these we are proposed this method.

EXPERIMENTAL

Gradient pump and UV-Visible detector with flexible wavelength 240nm.Zorbax C8 150 mm × 4.6 mm, 3.5µm, or similar is used for this chromatography analysis. The Mobile phase – A is pH 6.1 containing buffer and Methanol as 95:5%v/v and mobile phase-B is pH 6.1buffer and Methanol as 5:95%v/v were utilized in ultrasonic bath sonicator. Mobile phase containing gas is separated. Reference sample of LLE is procured from local market. Methanol,Acetonitrile and Orthophosphoric acid are AR grade.Mobile Phase-A is pH 6.1

containing buffer95%v/v and Methanol as 5%v/v. and mobile Phase-B is pH 6.1 contains buffer as 5%v/v, methanol 95%v/v.

METHOD DEVELOPMENT

Different related substances in drug product of LLE capsulesstrength which is taken as 0.2%.240nm is wavelength is noted for LLE. Authors are used Zorbax C8 150 mm × 4.6 mm, 3.5µm Column, or equivalent. Mobile phase-A is the mixture of pH 6.1buffer95%v/v and Methanol 5%v/v. Mobile phase-B is the mixture of pH 6.1buffer5%v/v, methanol as 95%v/v. 1.00mL/min. is the rate of flow. For this analysis authors are prepared stock-1, 2 as 500 ppm and standard solution (2ppm).

Validation of Proposed Method requirements

Specificity:

For this validation authors are prepared blank, standard solution, and sample solution as per method requirement. From the results, it is observed that no interference is noted due to blank,at Rt of known impurities peak and LLE peak. Moreover, peak purity of LLE and known impurities also meet the acceptance criteria, Hence, it is concluded as method is specific.

Forced Degradation studies:

From the forced degradation study, observed that in all stressed samples no obstructionis recognized due to blank at the RT of known impurities and LLE peak and hence concluded that the method is specific and stability indicative. For these studies all the chromatograms are noted. The tailing factor, theoretical plates, % RSD of six replicate injections for LLE peak in standard solution chromatograms are within the limit. For a stressed blank, any interference should be no more than 0.1% of sample concentration at the retention time of the impurities. All known foreign substances or degradable products are there shall be distantby LLE peak

System Suitability

Authors are designed blank as diluent, standard solution, Impurity – A, B, D, E standard stock solutions, Dimethylglyoxime impurity standard solution and spiked sample solution as per method description. Tailing factor to LLE peak in standard chromatograms is 0.99. The

theoretical plates for LLE peak in standard solution chromatograms is 52700. % RSD to LLE peak area response by six replicate injections of standard solution is 0.7. From this data table 1, finalized that the system is suitable to analytical approach validation.

Solution	**Peak purity**						
	LLE	**Impurity - A**	**Impurity - B**	**Impurity - C**	**Impurity - D**	**Impurity - E**	**DMGE impurity**
Std, Solution	997	NA	NA	NA	NA	NA	NA
LLE- 2.5mg	1000	999	997	1000	1000	NA	NA
LLE- 5mg	1000	996	999	1000	1000	NA	NA
LLE- 7.5mg	1000	999	1000	1000	1000	NA	NA
LLE-10mg	1000	992	998	1000	999	NA	NA
LLE-15mg	1000	995	1000	1000	1000	NA	NA
LLE-20mg	1000	999	998	1000	1000	NA	NA
LLE-25mg	1000	992	997	1000	1000	NA	NA
Spiked-25mg	1000	1000	1000	1000	1000	NA	998
Impurity – A	NA	NA	NA	1000	NA	NA	NA
Impurity – B	NA	1000	NA	NA	NA	NA	NA
Impurity – C	NA	NA	1000	NA	NA	NA	NA
Impurity – D	NA	NA	NA	NA	997	NA	NA
Impurity – E	NA	NA	994	NA	NA	NA	NA
Dimethylglyoxime	NA	NA	NA	NA	NA	991	999

Table 1: System suitability results

Precision:

The RT and area of total six measurements and %RSD is computed to LLE peak. %RSD to area LLE in six replicate injections of standard solution not more than 1.0. by the values

represented in table 2 it is finalized that both RT, area response to LLE peak is consistent which is unambiguous by RSD.

Injections	LLE	
	Retention time(min.)	Area Response
1	18.336	26809.889
2	18.321	27281.938
3	18.315	28091.405
4	18.286	26579.096
5	18.273	26536.080
6	18.283	27135.655
Mean	18.302	27.72.344
%RSD	0.14	2.1

Table 2: System Precision results

Method precision(MP)

Analysis is performed by taking sample of LLE injection 2.5mg, 5mg, 7.5mg, 10mg, 15mg, 20mg and 25mg six times of a single batch by following the analytical procedure. Computed %impurity in sample preparation and calculated the % of total impurities, moreover, performed the MP by spiking all known impurities in sample solution at specification level for LLE injection 2.5mg, 7.5mg, 10mg, 20mg and 25mg. From the data in table 3 it is observed that %RSD for impurities in six replicate preparation of as such samples and spiked sample 25mg strength solution is met acceptance criteria and hence concluded this method is precise.

ample olution	Content in %w/w LLE capsules 2.5mg				LLE capsules 5mg			LLE capsules 7.5mg			LLE capsules 10mg		
	Impurity – B	Impurity – C	Any unspecified impurity(at RRT 0.75)	Total impurities	Impurity – C	Any unspecified impurity(at RRT 0.75)	Total impurities	Impurity – C	Any unspecified impurity(at RRT 0.75)	Total impurities	Impurity – C	Any unspecified impurity(at RRT 0.75)	Total impurities
reparation - 1	BQL	0.116	0.086	0.471	0.105	0.085	0.419	0.105	0.090	0.418	0.110	0.087	0.419
reparation - 2	BQL	0.123	0.087	0.474	0.114	0.093	0.497	0.111	0.092	0.430	0.107	0.087	0.455
reparation - 3	BQL	0.117	0.085	0.421	0.122	0.093	0.497	0.109	0.084	0.404	0.105	0.090	0.465
reparation - 4	BQL	0.130	0.085	0.432	0.120	0.090	0.435	0.115	0.092	0.483	0.109	0.091	0.472
reparation - 5	BQL	0.126	0.087	0.445	0.118	0.085	0.426	0.106	0.086	0.362	0.108	0.091	0.421
reparation – 6	BQL	0.128	0.087	0.481	0.107	0.091	0.456	0.104	0.087	0.409	0.114	0.092	0.432
Mean	NA	0.123	0.086	0.454	0.114	0.090	0.455	0.108	0.089	0.418	0.109	0.089	0.444
%RSD	NA	4.7	1.1	5.5	6.1	4.1	7.7	3.9	3.8	9.4	2.8	2.5	5.2

Table 3: Spiked sample (LLE capsules 25mg) results

Intermediate Precision(IP)

Analyzed the sample of LLE injection 2.5mg, 5mg, 7.5mg, 10mg, 15mg, 20mg, and 25mg six times. Computed %impurity in sample preparation and calculated the % of total impurities. Moreover, performed the intermediate precision by spiking all known impurities in sample solution at specification level for LLE injection 2.5mg, 5mg, 7.5mg, 10mg, 15mg, 20mg, and 25mg. From the results obtained in table 4, intermediate precision results are meeting the compliance criteria, hence it is concluded that the above method is rugged.

Sample preparation	**Impurity – B content in %w/w**		**Impurity – C content in %w/w**		**Any unspecified Impurity (RRT 0.75)content in %w/w**		**Total impurities in %w/w**	
	MP	**IP**	**MP**	**IP**	**MP**	**IP**	**MP**	**IP**
1	BQL	ND	0.116	0.116	0.086	0.085	0.471	0.464
2	BQL	ND	0.123	0.117	0.087	0.085	0.474	0.478
3	BQL	ND	0.117	0.117	0.085	0.089	0.421	0.487
4	BQL	ND	0.130	0.119	0.085	0.096	0.432	0.504
5	BQL	ND	0.126	0.109	0.088	0.090	0.445	0.418
6	BQL	ND	0.128	0.111	0.087	0.091	0.481	0.426
Mean(12 determinations)	NA		0.119		0.088		0.458	
%RSD(12 determinations)	NA		5.4		3.8		6.3	

Table 4: Comparison between the method precision and intermediate precision for as such sample(LLE capsules 2.5mg)

Stability in analytical solution

Evaluated analytical solution at 25^0C and 2-8^0C by injecting both standard and sample solution. Analyzed standard, sample and spiked sample solution at initial time and latter regular time intervals up to 53 hrs. From the data obtained in table 5, it is noted that standard solution is stable for at least 51hrs. at 25^0C and 53hrs. at 2-8^0C. Sample solution is stable for 7 hrs. at 25^0C and 35hrs. at 2-8^0C.

Time interval in Hours	**Any unspecified impurity at RRT 0.72**		**Any unspecified impurity at RRT 0.75**		**Any unspecified Impurity RRT 0.77**	
	Response in area	**% Difference**	**Response in area**	**% Difference**	**Response in area**	**% Difference**
Initial	6212.422	NA	11629.693	NA	7063.811	NA
5	6640.376	6.9	11691.769	0.5	6686.578	5.3
11	6979.348	12.3	11764.231	1.2	7724.141	9.3
27	6098.630	1.8	12090.884	4.0	7028.115	0.5
49	6974.953	12.3	12091.291	4.0	7826.082	10.8

Table 5: Stability in analytical solution

Filter compatibility study:

The area difference between unfiltered sample and sample filtered through 0.45µm PVDF filter, 0.45 µm Nylon filter was calculated. By the data it is finalized that 0.45µm PVDF, 0.45µm Nylon and 0.45 µm PTFE filters are compatible for filtration of standard solution and sample solution by removing first 3.00ml filtrate. However, for routine analysis it is recommended to use 0.45 Nylon filter by removing first 3.00ml filtrate. Results are represented in table 6.

Name of the solution	**%Difference of area response with respect to unfiltered sample solution**				
	Impurity – B	**Impurity - C**	**Name of the solution**	**Area Response**	**% Difference**
Sample solution(0.45µm PVDF filter	0.6	2.1	Standard solution un filtered	27443.376	NA
Sample solution(0.45µm Nylon filter	**3.1**	4.9	Standard solution (0.45µm PVDF filter(Merck)	27443.376	1.1
Sample solution(0.45µm PTFE filter	**4.0**	6.3	Standard solution (0.45µm Nylon filter	27181.685	1.0
			Standard solution (0.45µm PTFE filter	28089.613	2.4

Table 6: % Difference of area response results of LLE capsules 25mg(Spiked sample)

Linearity:

Performed the linearity with impurity – B,C in the range of LOQ to 300% of specification. Performed linearity with LLE standard in the spectrum of LOQ to 300% of specification. Precision performed at higher levels and RRF values of each impurity entrenched. By linearity data of LLE, impurity – B, C, it is identified that this method is linear among LOQ to 300% of specification level for known impurities and LOQ to 300% of diluted standard concentration for LLE. Both correlation coefficient and regression coefficient observed as 0.995. %Y-intercept noted as ±5.0% of area response at 100% level. It is finalized that this process is linear by LOQ to 300% of specification level to well-known impurities with respect to sample strength and LOQ to 300% of diluted standard concentration for LLE. The linearity and impurity chromatograms are represented in figure 1to 7 and results are tabulated in table 7.

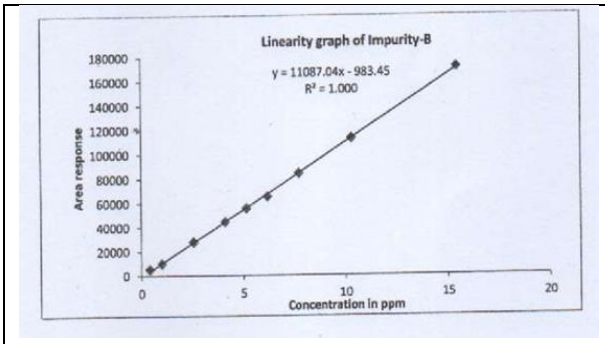

Figure 1: Linearity of Impurity - B

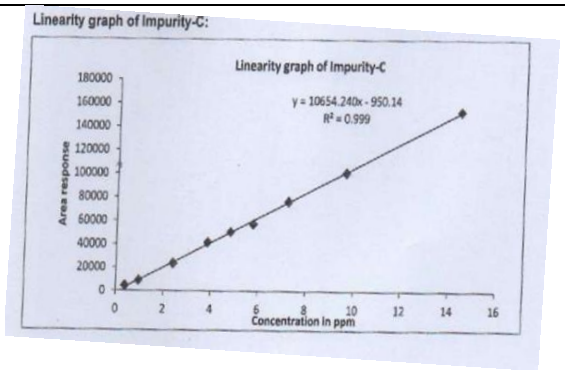

Figure2: Linearity of Impurity - C

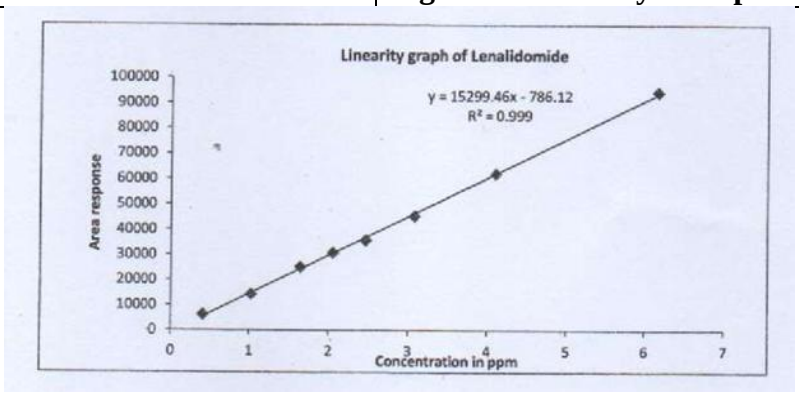

Figure 3: Linearity graph for LLE

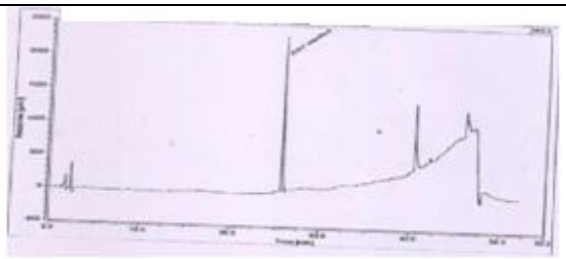

Figure 4: Impurity A Standard Solution

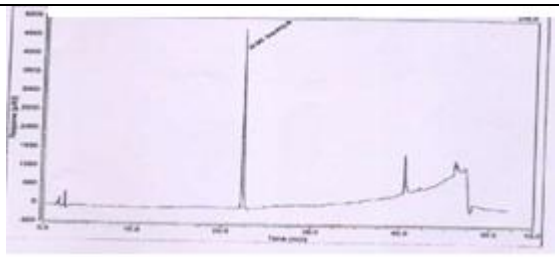

Figure 5: Impurity B Standard Solution

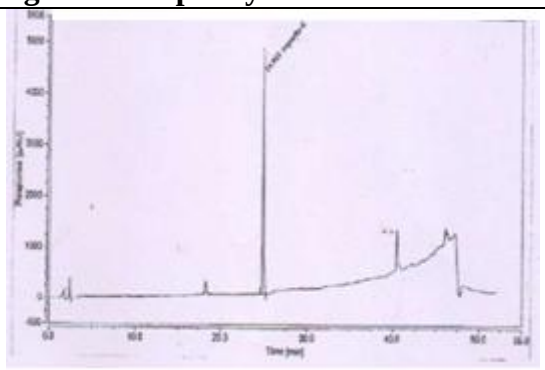

Figure 6: Impurity C Standard Solution

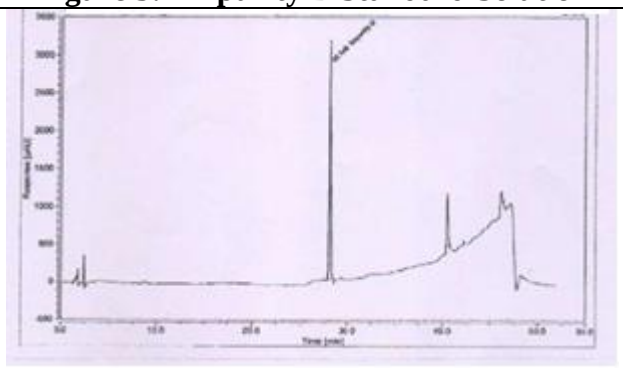

Figure 7: Impurity D Standard Solution

	Area Response		
Injection No.	**LLE**	**Impurity – B**	**Impurity - C**
1	90280.257	164044.828	147374.582
2	92133.245	165150.342	149834.040
3	91748.093	164991.801	150491.195
4	92281.083	164875.373	1499186.458
5	91418.674	167475.991	149898.089
6	90458.896	165427.301	148762.994
Mean	91386.708	165327.606	1492570893
%RSD	0.9	0.7	0.7

Table 7: Results of precision at higher level

Accuracy:

Prepared recovery samples by spiking known quantities of impurity – B, C of specification to sample. Prepared the recovery samples in triplicate for each level. Performed recovery for LLE(unknown recovery) from LOQ to 300% of diluted standard strength. By the results represented in table 8, it is finalized that % recovery is in the limit and concluded that this method is accurate.

	% Recovery						
Set		**Impurity - A**		**Impurity - B**		**Impurity - C**	
	Levels	**Mean**	**%RSD**	**Mean**	**%RSD**	**Mean**	**%RSD**
1	LOQ	112.4	2.3	102.5	10.5	103.0	10.6
2	50%	107.1	1.7	98.8	0.1	107.3	4.1

3	100%	102.4	0.6	97.2	0.9	106.2	1.3
4	300%	101.0	0.6	98.2	1.6	105.4	0.5

Table 8: Accuracy for unknown impurity, impurity - B and C

Range:

From the precision, linearity and accuracy data, it is assured that this process is more precise, linear and highly accurate in the range of LOQ to 300% of specification limit to well- known impurities and LOQ to 300% of diluted standard strengthto LLE

LOD and LOQ:

The LOD and LOQ of impurity – B, C and LLE are derived by using the S/N ratio values got from the LOD/LOQ prediction solution using software. LOQ concentration was determined for impurity – B and impurity – C and LLE with respect to specification limit. The results are tabulated in table 9,10 and related chromatogram in figure 8..

S.No	Name	LOD in ppm	LOD in %w/w	LOQ in ppm	LOQ in %w/w
1	LLE	0.136	0.014	0.412	0.041
2	Impurity - B	0.135	0.014	0.410	0.041
3	Impurity - C	0.122	0.012	0.371	0.037

Table 9: LOD and LOQ concentration results

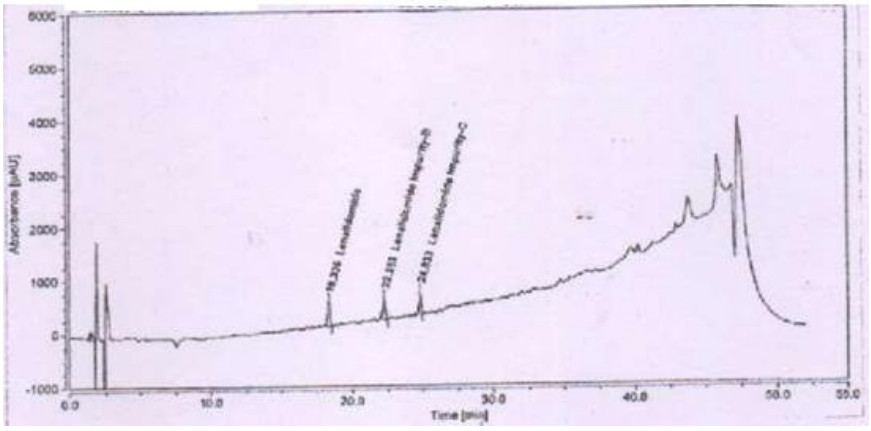

Figure 8:Chromatogram LOQ Solution

S.NO	Area response of LLE	S/N ratio of LLE	Area response of impurity - B	S/N ratio of impurity - B	Area response of impurity - C	S/N ratio of impurity - C

1	5424.184	24.2	4445.010	21.0	3710.693	21.8
2	5254.370	23.0	4228.659	21.0	4357.460	22.5
3	5541.141	22.7	4496.380	21.8	4391.319	21.7
4	5378.468	20.2	5028.455	21.4	3888.776	21.5
5	5552.753	19.3	4196.444	18.2	4080.725	17.5
6	5145.552	23.1	4070.852	20.6	3547.529	21.6
Average	5382.745		4410.967		3996.083	
%RSD	3.0		7.8		8.6	

Table 10: LOQ precision and signal to noise ratio results

Robustness:

Changed in mobile phase rate of flow by ±10%(0.9ml and 1.1ml). change in column oven temperature $\pm5^0$C(30^0C and 20^0C). change in mobile phase ±5%(Mobile phase – A: pH 6.1 buffer and methanol in the ratio 95:4.8 and 95:5.2 v/v, Mobile phase – B: pH 6.1 buffer and methanol in the ratio as 5:90 and 5:100v/v. change in buffer pH(±0.2) . From the above data, it is observed that system suitability criteria are meeting the acceptance criteria in all the robustness conditions. Hence, concluded that thisprocess is robust.

Results and Discussion

The system suitable parameter result for the tailing factor LLE peak in standard solution is 0.82. The theoretical plates for LLE peak in standard solution is 36552. The % RSD for LLE peak in standard solution is 1.0. in specificity parameter observed that no interference is because of blank, at Rt of known impurities peak and LLE peak. The forced degradation study observed that in all stressed no interference because of blank at the Rt of known impurities and LLE peak. The system precision is concluded that the retention timeand area response for LLE peak is consist of evidenced by the relative standard solution. The method precision is observed %RSD for impurities in six replicate preparation of as such samples and spiked sample 25mg strength solution is meeting the acceptance criteria. The intermediateprecision results are meeting the acceptance criteria. The solution stability solution parameter observed that standard solution is stable in 51hrs at 25^0c and 53 hrs at 2-8^0c. Sample solution is stable for 7hrs. at 25^0c. and 35hrs. at 2-8^0c. the filter compatibility study of LLE capsules using concluded that 0.45µm PYDF. 0.45 µm nylon and 0.45 m PITFE filters are compatible for the filtration of standard solution and sample solution by discarding at first 3ml of filtrate. The linearity data of LLE, impurity – B and impurity – C is found that the method is linear between LOQ to 300% of specification level for known

impurity and LOQ at 300% diluted concentration for LLE. The robustness is observed that system suitability criteria is meeting the acceptance criteria in all the robustness conditions.

Conclusion

It is concluded that the system is suitable for analytical method validation., Moreover, peak purity of LLE and known impurities peak also meeting the acceptance criteria. It is concluded hat the method is specific. Hence concluded that the method is specific and stability indicative, conduced that the system precision parameter meets the requirement of method validation. The method is precise, rugged. In this work authors are concluded that the use 0.45µm nylonfilterby discarding first 3ml of filtrate. This method is linear form LOQ to 300% of specification level for known impurities with respect to sample strength and LOQ to 300% of diluted standard concentration for LLE. By considering this that the method is robust.

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Conflicts of interest

There are no conflicts of interest among the authors who were done this present work.

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