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Original Article

A UPLC-MS/MS METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF POMALIDOMIDE FROM HUMAN PLASMA

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ABSTRACT

Objective: The present work aimed to develop a simple, rapid, specific and precise liquid chromatography-tandem mass spectrophotometric (LC–MS/MS) validated method for quantification of pomalidomide and internal standard (ISTD) Fluconazole in human plasma.

Methods: 50 µl of 0.1% formic acid was added to plasma samples prior to liquid-liquid extraction (LLE) using 2.5 ml of ethyl acetate. Chromatographic separation was achieved on Xterra, RP_{18}, 5 µ (50 x 4.6 mm) column using a mixture of 0.1% (v/v) formic acid in water to methanol at a ratio of 12:88, v/v as the mobile phase. The flow rate was 0.50 ml/min. The LC eluent was split, and approximately 0.1 ml/min was introduced into Tandem mass spectrometer using turbo Ion Spray interface at 325 °C. Quantitation was performed by transitions of *m/z 260.1* precursor ion to the *m/z 148.8* for pomalidomide and *m/z* 307.1/238.0 for fluconazole.

Results: The concentrations of nine working standards showed linearity between 9.998 to 1009.650 ng/ml ($r^2 \geq 0.9968$). Chromatographic separation was achieved within 2 min. The average extraction recoveries of three quality control concentrations were 53.86% for pomalidomide and were within the acceptance limits. The coefficient of variation was ≤15% for intra-and inter-batch assays. The %CV of ruggedness ranges 1.32 to 4.03. The % stability of short term and long term stock solution stability studies was found to be 99.01% and 98.49% respectively.

Conclusion: The results obtained for specificity, linearity, accuracy, precision, ruggedness and stability studies were within limits. Thus the validated economical method was applied for pharmacokinetic studies of pomalidomide.

Keywords: Pomalidomide, LC-MS/MS, Human plasma, Liquid-liquid extraction

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INTRODUCTION

Pomalidomide chemically 4-amino-2-(2, 6-dioxopiperidin-3-yl) isoindoline-1, 3-dione, the newest immune-modulatory drugs (IMiD), was designed to be more potent and less toxic than thalidomide and lenalidomide [1-2]. It is used for the treatment of relapsed and refractory multiple myeloma. Dr. Rober D'Amato's labs led to the first report [3-4] stating that 3-amino-thalidomide was able to directly inhibit both the tumor cell and vascular compartments of myeloma cancers. An HPLC-UV method was reported for inversion of pomalidomide in phosphate-buffered saline and human plasma in µg/ml range [5]. The LC-MS methods were also published for the Pharmacokinetic study of pomalidomide but no validation details were presented [6-7]. A validated UPLC-MS method was reported using negative ionisation mode for determination of pomalidomide from rat plasma [8], Described here is a simple, sensitive, and selective LC-MS/MS method for pomalidomide in the human plasma concentration range of 9.998 to 1009.650 ng/ml. As there is no literature on stability data of pomalidomide in human plasma, this study performed assay validations, according to the FDA guidelines [9]. While this method with validation details were economical and applied for pharmacokinetic studies of pomalidomide.

MATERIALS AND METHODS [8]

Apparatus and software

The HPLC pump (Agilent 1200 series Binary SL) with an autosampler (Agilent 1200 series Hip-ALSSL) was coupled with Agilent 6460 Triple Quad Tandem mass spectrometer. The column oven was Agilent 1200 series TCC SL. The chromatographic integration was performed by Agilent mass hunter software.

Chemicals and reagents

Pomalidomide and Fluconazole (IS) were procured from NATCO Pharma Ltd., Hyderabad, Formic acid, Methanol and ethyl acetate was procured from Merck Specialities Pvt. Ltd, Mumbai, India. Water used was collected from water purification systems (Milli Q, Milli Pore, USA) installed in the laboratory. Pooled drug-free expired frozen human plasma (K2-EDTA as anticoagulant) was obtained froma Blood Bank, Hyderabad, was used during validation and study sample analysis. The plasma was stored into-70±5 °C.

Standards and working solutions

Calibration standard solutions

Stock solutions of pomalidomide and Fluconazole internal standard (IS) were prepared in methanol. Further dilutions were carried out in 50% methanol. Calibration standards of nine concentration levels were prepared freshly by spiking drug-free plasma with pomalidomide stock solution to give the concentrations of 9.998, 25.241, 50.281, 150.438, 301.885, 503.815, 705.745, 906.666 and 1009.650 ng/ml.

Quality control standards

Lowest quality control standards, Median quality control standards and highest quality control standards were prepared by spiking drug-free plasma with pomalidomide to give a solution containing 26.248, 323.056 and 807.640 ng/ml respectively. They were stored at-20 °c till the time analysed.

Chromatographic conditions

Chromatographic separation was performed on Xterra, RP18, 5 µ (50 x 4.6 mm), analytical column and the mobile phase was a mixture of 0.1% (v/v) formic acid in water to methanol at a ratio of 12:88, v/v. Injection volume was 10µL. The flow rate was 0.50 ml/min. Total analysis time of single injection was 2.0 min. Column oven temperature and autosampler temperature was set to 30 °C and 10 °C, respectively.

Mass spectrometric conditions

The LC eluent was split, and approximately 0.100 ml/min was introduced via electrospray ionisation using a Turbo Ion Spray interface set at 325 °C to generate positive ions [M+H]+. The Mass spectrometric parameters were optimised as shown in table no 1.

Table 1: Mass spectrometric conditions

Capillary voltage		3500V
Nozzle voltage		1500V
Delta EMV(+)		500 Positive
Gas flow		5 L/min
Gas temperature		350 °C
Nebulizer pressure		25 psi
Sheath gas temperature		300 °C
Sheath gas flow		11L/min
Acquisition		
Parameters	**Pomalidomide**	**ISTD**
Transition	260.1/148.8 (m/z)	307.1/238.0 (m/z)
Polarity	Positive	Positive
MS1 resolution	Unit	Unit
MS2 resolution	Unit	Unit
Dwell time (millisec)	200	200
Fragmentor (V)	100	100
Collision energy (V)	8	10

Sample preparation method

To 250 µl of plasma, 50 µl of ISTD (1µg/ml) and 50 µl of 0.1% formic acid was added and vortexed. The drug was extracted with 2.5 ml of ethyl acetate, followed by centrifugation at 2000 rpm/min on a cooling centrifuge for 15 min at 4 °C. The supernatant of 2 ml was withdrawn and evaporated at 50 °C 15 psi of nitrogen until dryness at LV evaporator. The residue was reconstituted with 500 µl of mobile phase and respective samples were injected into the column.

Validation

Specificity

A solution containing 9.999 ng/ml was injected onto the column under optimised chromatographic conditions to show the separation of pomalidomide from impurities and plasma. The specificity of the method was checked for the interference from plasma.

Linearity

Spiked concentrations were plotted against peak area ratios of pomalidomide to the internal standard, and the best fit line was calculated. Wide range calibration was determined by solutions containing9.998 to 1009.650ng/ml.

Recovery studies

The % mean recoveries were determined by measuring the responses of the extracted plasma Quality control samples at HQC, MQC and LQC against un-extracted Quality control samples at HQC, MQC and LQC.

Precision and accuracy

The between-run (Inter-day) accuracy and precision evaluation were assessed by the repeated analysis of human K_3 EDTA plasma samples containing different concentrations of pomalidomide on separate occasions. A single run consisted of a calibration curve plus six replicates of the lower limit of quantitation, low, medium and high-quality control samples.

Within-run (Intraday) accuracy and precision evaluations were performed by analysing replicate concentrations of pomalidomide in human K_3 EDTA plasma. The run consisted of a calibration curve plus a total of 24 spiked samples, six replicates of each of the LLOQ, lower, medium and higher quality control samples.

Matrix effect

Blank plasma samples of 6 different human K3 EDTA plasma sources were processed and spiked with aqueous low-quality control and high-quality control (post extraction addition) and analysed in a single run along with diluted pure standard at each concentration level.

Ruggedness

The ruggedness of the method was assessed by analysing a precision and accuracy batch using a different column, by the different analyst in another instrument.

Stability studies

Short-term stock solution stability of pomalidomide

Solutions of pomalidomide were prepared in methanol (Stability Samples) and were kept at room temperature for 6 h 30 min. A freshly prepared solution of pomalidomide (Comparison Samples) and stability samples were diluted at approximately the same analyte concentration and analysed in a single run; analyte responses were used to determine % stability over time.

Short-term stock solution stability of internal standard

Solutions of internal standard (Fluconazole) were prepared in methanol (Stability Samples) and were kept at room temperature for 6 h 30 min. A freshly prepared solution of internal standard (Comparison Samples) and stability samples were diluted at approximately the same analyte concentration and analyzed in a single run; Analyte responses were used to determine % stability over time.

Freeze-thaw stability

Samples were prepared at low and high-quality control levels, aliquoted and frozen at-70 °C. Some of the aliquots of quality control samples were subjected to five freeze-thaw cycles (stability samples). A calibration curve and quality control samples were freshly prepared (Comparison Samples) and processed with 6 replicates of stability samples and analysed in a single run.

Long-term stock solution stability of pomalidomide

Solutions of Pomalidomide were prepared in methanol (Stability Samples) and were kept at refrigerator (2-8 °C) for 10 D 02 H. A freshly prepared solution of pomalidomide (Comparison Samples) and stability samples were diluted at approximately the same analyte concentration and analysed in a single run.

Long-term stock solution stability of internal standard

Solutions of Internal standard were prepared in methanol (Stability Samples) and were kept at refrigerator (2-8 °C) for 10 D 02 H. A freshly prepared solution of internal standard (Comparison Samples) and stability samples were diluted at approximately the same analyte concentration and analysed in a single run

RESULTS AND DISCUSSION

The chromatography observed during the course of validation was acceptable and representative chromatograms of, LLOQ, LQC, MQC, HQC, internal standard (ISTD) and standard blank samples are shown in (fig. 1).

The method developed was validated for specificity, accuracy and precision, linearity, ruggedness and stability as per USFDA guidance [10-12]. The results of validating parameters are given below.

Specificity

Nine different lots of plasma were analysed to ensure that no endogenous interferences were present at the retention time of pomalidomide and fluconazole. Nine LLOQ (9.999 ng/ml) level samples along with plasma blank from the respective plasma lots were prepared and analysed. (table 2) shows results of specificity. In all plasma blanks, the response at the retention time of pomalidomide was less than 20% of LLOQ response, and at the retention time of IS, the response was less than 5% of mean IS response in LLOQ. The typical chromatogram of plasma blank and chromatogram of LLOQ was shown in (fig. 1).

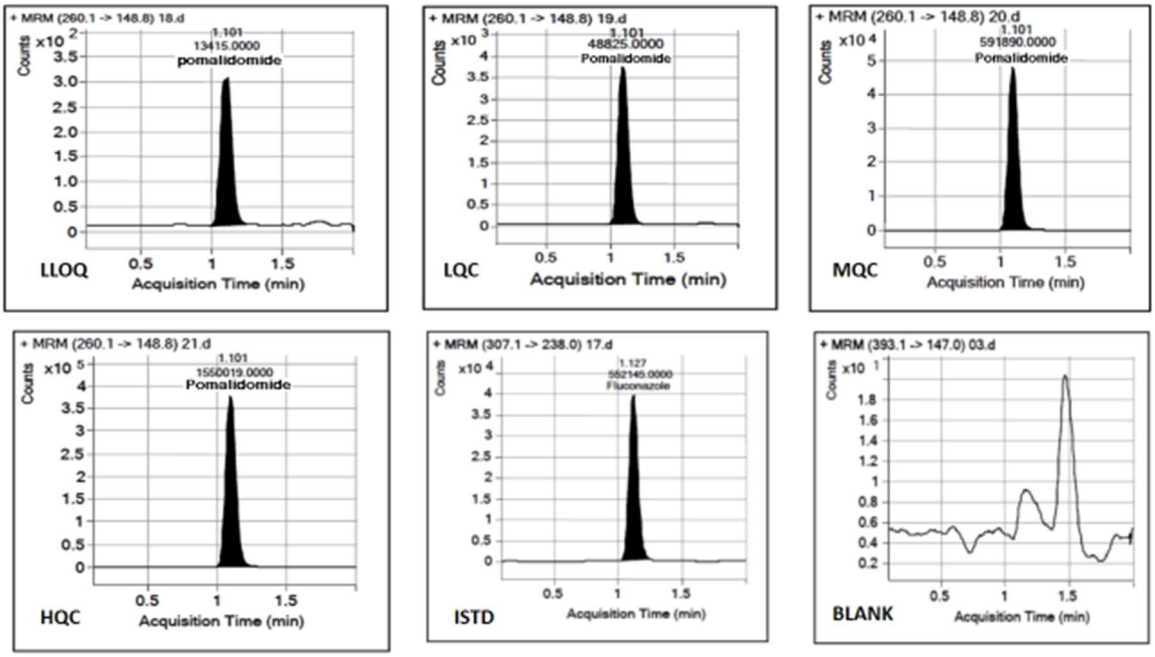

Fig. 1: Chromatograms of LLOQ, quality control samples, ISTD and blank matrix

Table 2: Results of specificity for pomalidomide and fluconazole (ISTD)

	Analyte			IS		
	Area of interfering peak at RT of analyte	Area observed for extracted LLOQ	% Interference at RT of analyte	Area of interfering Peak at RT of ISTD	Area observed for extracted ISTD	% Interference at RT of ISTD
01	0	15286	0	0	575244	0
02	0	13288	0	0	582214	0
03	0	11110	0	0	578922	0
04	0	11440	0	0	564562	0
05	0	11402	0	0	558925	0
06	0	11059	0	0	589001	0
07	0	11215	0	0	591285	0
08	0	11580	0	0	578862	0
09	0	12089	0	0	588638	0
Mean		12052.111	MEAN		180201.89	

Linearity

The calibration curve (peak area ratio Vs Concentration) was linear over working a range of 9.998 to 1009.650 ng/ml with nine point calibration used for quantification by linear regression, shown in (fig. 2). The regression equation for the analysis was Y=0.0039x-0.0089 with coefficient of correction (r^2) = 0.99686.

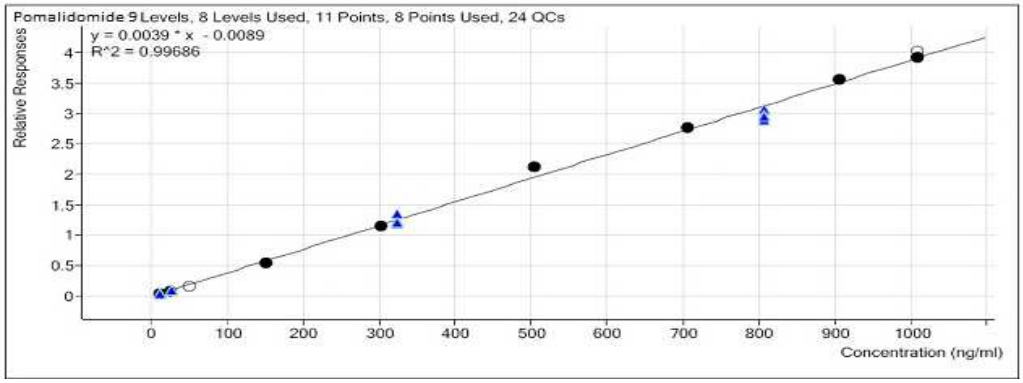

Fig. 3: Spiked concentrations (9.998 to 1009.650 ng/ml) were plotted against calculated concentration Vs concentration with ten point calibration used for quantification by linear regression

Recovery

The % mean recovery for pomalidomide in LQC, MQC and HQC was 50.68%, 53.43% and 57.49% respectively (table 3).

Table 3:% Mean recovery of pomalidomide for LQC, MQC and HQC

	LQC 26.248ng/ml		MQC 323.056 ng/ml		HQC 807.640 ng/ml	
	Aqueous analyte response	Extracted analyte response	Aqueous analyte response	Extracted analyte response	Aqueous analyte response	Extracted analyte response
01	240986	49902	2785844	599122	6694089	1549621
02	245029	49079	2784946	595935	6765199	1550584
03	244894	48906	2792062	593232	6782112	1564921
04	243537	48828	2785487	591894	6835789	1571431
05	240892	49190	2779585	593165	6838061	1568923
06	238416	48789	2785210	598329	6870744	1573663
Mean	242292.3	49115.6	2785522.3	595279.5	6797665.6	1563190.5
SD (+)	2626.8	414.3	3963.6	2987.2	63952.7	10549.7
CV (%)	1.08	0.84	0.14	0.50	0.94	0.67
Conc. Factor	250		250		250	
Mean Recovery	50.68		53.43		57.49	
Global Recovery	53.86					

Intraday (within run) and inter-day (between run) precision and accuracy

The within-run coefficients of variation ranged between 1.47% and 4.57% for pomalidomide. The within-run percentages of nominal concentrations ranged between 97.95% and 107.58% for pomalidomide. Results are presented in table 4.

The between-run coefficients of variation ranged between 2.88% and 4.22% for pomalidomide. The between-run percentages of nominal concentrations ranged between 99.41% and 106.97% for pomalidomide. Results are presented in table 5.

Matrix effect

The percentage matrix effect of analyte was found to be-0.25 and 2.74 for pomalidomide for low and high-quality control samples. Results are presented in table 6.

Ruggedness

The coefficients of variation ranged between 1.32% and 4.03% for pomalidomide. The percentages of nominal concentrations ranged between 101.06% and 110.08% for pomalidomide. Results are presented in table 7.

Table 4: Intraday precision and accuracy of quality control standard

S. N0.	LLOQ 9.999 ng/ml		LQC 26.248ng/ml		MQC 323.056 ng/ml		HQC 807.640 ng/ml	
	Conc. found (ng/ml)	% nominal Conc	Conc. found (ng/ml)	% nomi Conc nal	Conc. found (ng/ml)	% nominal Conc	Conc. found (ng/ml)	% nominal Conc
1	10.705	107.06	28.081	106.98	315.669	97.71	762.368	94.39
2	10.569	105.70	27.895	106.27	355.681	110.10	782.178	96.85
3	10.411	104.12	27.857	106.13	318.659	98.64	775.543	96.03
4	11.487	114.88	27.942	106.45	324.372	100.41	810.934	100.41
5	10.61	106.11	27.841	106.07	317.699	98.34	805.069	99.68
6	10.758	107.60	28.915	110.16	327.842	101.48	810.606	100.37
N	6	6	6	6	6	6	6	6
Mean	10.757	107.58	28.089	107.01	326.654	101.11	791.116	97.95
SD(±)	0.38		0.41		14.93		20.57	
CV(%)	3.51		1.47		4.57		2.60	

Table 5: **Inter day precision and accuracy of quality control standard**

Batch ID	LLOQ 9.999 ng/ml		LQC 26.248ng/ml		MQC 323.056 ng/ml		HQC 807.640ng/ml	
	Conc. found (ng/ml)	% nominal conc	Conc. found (ng/ml)	% nominal conc	Conc. found (ng/ml)	% nominal conc	Conc. found (ng/ml)	% nominal conc
	10.705	107.06	28.081	106.98	315.669	97.71	762.368	94.39
	10.569	105.70	27.895	106.27	355.681	110.10	782.178	96.85
PandA-01	10.411	104.12	27.857	106.13	318.659	98.64	775.543	96.03
	11.487	114.88	27.942	106.45	324.372	100.41	810.934	100.41
	10.610	106.11	27.841	106.07	317.699	98.34	805.069	99.68
	10.758	107.60	28.915	110.16	327.842	101.48	810.606	100.37
	10.322	103.23	27.867	106.17	311.270	96.35	762.021	94.35
	10.385	103.86	26.741	101.88	355.184	109.95	783.091	96.96
PandA-02	10.609	106.10	26.569	101.22	315.344	97.61	762.157	94.37
	10.896	108.97	27.498	104.76	312.676	96.79	798.395	98.86
	10.268	102.69	27.581	105.08	313.590	97.07	786.741	97.41
	10.307	103.08	27.235	103.76	317.332	98.23	793.101	98.20
	10.729	107.30	28.810	109.76	330.628	102.34	775.171	95.98
	10.867	108.69	28.880	110.03	318.223	98.50	838.267	103.79
PandA-03	10.309	103.10	29.561	112.62	326.177	100.97	830.304	102.81
	10.321	103.22	29.126	110.97	328.816	101.78	874.482	108.28
	10.805	108.06	29.018	110.55	327.572	101.40	854.598	105.81
	10.443	104.44	27.968	106.55	327.423	101.35	846.592	104.82
N:	18	18	18	18	18	18	18	18
Mean:	10.600	106.01	28.077	106.97	324.675	100.50	802.868	99.41
SD(±):	0.31		0.83		12.76		33.89	
CV (%):	2.88		2.95		3.93		4.22	

Table 6: Results of matrix effect obtained by preparing LQC and HQC with six different lots of plasma

S. No.	AQS LQC response	PEX LQC response	AQS HQC response	PEX HQC response
1	195688	203204	5680638	5635662
2	200736	197892	5694436	5694225
3	200626	197423	5637040	6355785
4	198973	198785	5647571	5716531
5	200778	199084	5680208	5753628
6	201077	198505	5655245	5772353
Mean	199646.333	199148.833	5665856.333	5821364
SD	2078.29	2076.23	22443.71	266171.21
%CV	1.04	1.04	0.4	4.57
%ME	-0.25		2.74	

Table 7: **Results of ruggedness**

S. No.	LLOQ 9.999 ng/ml		LQC 26.248ng/ml		MQC 323.056 ng/ml		HQC 807.640 ng/ml	
	Conc. found (ng/ml)	% nominal conc	Conc. found (ng/ml)	% nominal conc	Conc. found (ng/ml)	% nominal conc	Conc. found (ng/ml)	% nominal conc
1	10.729	107.30	28.810	109.76	330.62	102.34	775.171	95.98
2	10.867	108.69	28.880	110.03	318.22	98.50	838.267	103.79
3	10.309	103.10	29.561	112.62	326.17	100.97	830.304	102.81
4	10.321	103.22	29.126	110.97	328.81	101.78	874.482	108.28
5	10.805	108.06	29.018	110.55	327.57	101.40	854.598	105.81
6	10.443	104.44	27.968	106.55	327.42	101.35	846.592	104.82
N	6	6	6	6	6	6	6	6
Mean	10.579	105.80	28.894	110.08	326.47	101.06	836.569	103.58
SD(±)	0.25		0.53		4.31		33.69	
CV (%)	2.37		1.82		1.32		4.03	

Stability studies

Short-term stock solution stability of pomalidomide

Pomalidomide is found to be stable in methanol for 6 h 30 min at room temperature with a % stability of 99.01%. Results are presented in table 8.

Short-term stock solution stability of internal standard

The internal standard is found to be stable in methanol for 6 h 30 min at room temperature with a % stability of 99.15%. Results are presented in table 9.

Freeze-thaw stability

Pomalidomide is found to be stable in human K_3 EDTA plasma after five freeze-thaw cycles at-70 °C with coefficients of variation of 3.27% (LQC) and 3.86% (HQC) for pomalidomide, and the percentages of nominal concentrations for pomalidomide were found to be 103.17% (LQC) and 101.23% (HQC). Results are presented in table 10.

Long-term stock solution stability of pomalidomide

Pomalidomide is found to be stable in methanol 10 D 02 H at refrigerator (2-8 °C) with a % stability of 98.49% for pomalidomide. Results are presented in table 11.

Long-term stock solution stability of internal standard

The internal standard is found to be stable in methanol 10 D 02 H at refrigerator (2-8 °C) with a % stability of 97.74%. Results are presented in table 12.

Table 8: Short-term stock solution stability of analyte

S. No.	Analyte	
	SS	CS
1	2564444	2606664
2	2597482	2616699
3	2606795	2630379
4	2611068	2627041
5	2598633	2630048
6	2608998	2629280
Mean	2597903.333	2623351.833
SD	17295.66	9651.08
%CV	0.67	0.37
% stability	99.01	

Table 9: Short-term stock solution stability of internal standard

S. No.	ISTD	
	Stability solution	Comparison solution
1	2504198	2521525
2	2510082	2524682
3	2498215	2537401
4	2487925	2519696
5	2512367	2504040
6	2491004	2526432
Mean	2500631.833	2522296.000
SD	9997.37	10877.47
%CV	0.40	0.43
% stability	99.15	

Table 10: Freeze-thaw stability at-70 °C

S. No.	Freshly spiked		Freeze-thaw	
	LQC	HQC	LQC	HQC
	Nominal Con (ng/ml)		Nominal Con (ng/ml)	
	26.249	**807.648**	**26.248**	**807.64**
1	28.019	791.749	29.380	801.458
2	27.816	767.862	30.051	839.017
3	27.327	789.495	28.055	824.962
4	27.830	821.854	27.867	754.842
5	27.819	842.104	28.198	791.864
6	27.272	770.159	27.791	830.043
Mean	27.680	797.204	28.557	807.031
SD	0.31	29.34	0.93	31.17
%CV	1.10	3.68	3.27	3.86
% stability			103.17	101.23

Table 11: Long-term stock solution stability of analyte

S. No.	Analyte	
	SS	CS
1	2280682	2314048
2	2287758	2311173
3	2291699	2307848
4	2288329	2312386
5	2300006	2324564
6	2287308	2353235
Mean	2280682	2314048
SD	6355.03	16985.98
%CV	0.28	0.73
% stability	98.49	

Table 12: Long-term stock solution stability of internal standard

S. No.	ISTD	
	Stability solution	Comparison solution
1	2458845	2520014
2	2448795	2531462
3	2431526	2512650
4	2484415	2526423
5	2478462	2513284
6	2460025	2500186
Mean	2460344.7	2517336.5
SD	19359.133	11151.5822
%CV	0.79	0.44
% stability	97.74	

CONCLUSION

Chromatographic separation was achieved on Xterra, RP_{18}, 5 µ (50 x 4.6 mm) column using a mixture of 0.1% (v/v) formic acid in water to methanol at a ratio of 12:88, v/v as the mobile phase. The drug was extracted with 2.5 ml of ethyl acetate. The specificity of the method was checked for the interference from plasma. The calibration curve (peak area ratio Vs Concentration) was linear over working a range of 9.998 to 1009.650 ng/ml with nine point calibration used for quantification by linear regression. The % mean recovery for pomalidomide in LQC, MQC and HQC was 50.68%, 53.43% and 57.49% respectively. The within-run coefficients of variation ranged between 1.47% and 4.57% for pomalidomide. The between-run coefficients of variation ranged between 2.88% and 4.22% for pomalidomide. The percentage matrix effect of analyte was found to be-0.25 and 2.74 for pomalidomide for low and high-quality control samples. The stability test was performed to assess the long term and short term stability of sofosbuvir sample solutions, internal standard solutions. The developed method was validated for the quantitative determination of sofosbuvir from plasma was simple, rapid, specific, sensitive, accurate and precise. Hence, the method is quite suitable to detect the drug from plasma samples of human volunteers.

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CONFLICT OF INTERESTS

Declared none

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