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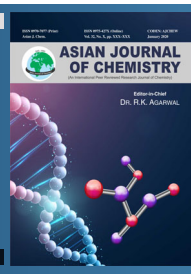

Determination of Possible Potential Genotoxic Impurities in Lenalidomide Drug Substance by Simple RP-HPLC Method

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This study is concerned with development and validation of HPLC method for the simultaneous detection and quantification of methyl 2-(chloromethyl)-3-nitrobenzoate (MCN), methyl 2-(bromomethyl)-5-nitrobenzoate (MMM), methyl 2-(bromomethyl)-6-nitrobenzoate (MON), methyl 2-(bromomethyl)-4-nitrobenzoate (MPN) and 2-methyl-3-nitrobenzoic acid methyl ester (MNM), which are the genotoxic impurities of lenalidomide. Chromatographic separation was accomplished using a Waters HPLC system equipped with Ascentis Express F5 (150 × 4.6 mm, 2.7 µm) using mobile phase composed of solvent A (0.1% perchloric acid): solvent B (methanol 80% and acetonitrile 20%); 55:45, vol/vol. The selected impurities were detected using UV detector set at 210 nm. The standard curves showed linearity in the range of concentrations 4.59-91.2 ppm (for MCN), 6.58-90.0 ppm (for MMM), 3.96-89.1 ppm (for MON), 6.47-89.7 ppm (for MPN) and 4.28-90.1 ppm (for MNM). The statistical results of method precision, system precision, specificity, accuracy, ruggedness was found to be within limits of acceptance. All the impurities were stable in lenalidomide test samples up to 24 h.

Keywords: Lenalidomide, Nitro benzoates, Genotoxic impurities, HPLC.

INTRODUCTION

Lenalidomide, a dicarboximide, chemically known as (*R*,*S*)-3-(4-amino-1-oxo-1,3-dihydro-2*H*-isoindol-2-yl) piperidine-2,6-dione. It has antineoplastic activity and prescribed for treating cancers of various types [1-3]. In patient populations with some selected blood or bone marrow disorders such as myelodysplastic syndromes, lenalidomide can also be used to manage anaemia [4-6].

An impurity is known in the pharmaceutical industry as any inorganic/organic material or any leftover solvents or additives arising during synthesis or undesirable chemicals that persist with active pharma ingredient [7]. The occurrence of these contaminants, even in trace levels, may affect the safety and effectiveness of the pharmaceutical final product (API or formulation) [8]. Impurities in pharmaceutical final product are governed by different regulatory organizations like ICH and USFDA [9-11]. Detection, determination and regulation of impurities in pharmaceutical final product (API or formulation) are crucial during drug development. To improve the efficacy of drug-based treatment, impurities must be detected and evaluated using specific analytical techniques.

Few HPLC based techniques were documented to quantify lenalidomide related impurities in bulk and formulations [12-16]. Prasad *et al.* [12] reported the separation of lenalidomide and its impurities A, B and C were carried out on an X-bridge C18 column employing potassium phosphate buffer and methanol (90:10, v/v) as mobile phase with flow rate of 0.8 mL/min and photodiode array detection at 210 nm. The detector response for impurities I, II and III was linear over the range of concentrations of 0.2 to 3.4 mg/L. The procedure was implemented to the oral dose formulations to determine lenalidomide impurities I, II and II.

Swetha *et al.* [13] described the separation of lenalidomide and its impurities A, B and C, which was carried out on an X-terra RP 18 column by employing acetonitrile and methanol (40:60, v/v) as mobile phase with flow rate of 1.0 mL/min and ultraviolet detection at 210 nm. The procedure was imple-

mented to the dosage forms and bulk to determine lenalidomide impurities A, B and C.

Using a Sunfire C-18 column as stationary phase and solvent system A (phosphoric acid buffer) and solvent system B (methanol-55% and acetonitrile-45%) in the ratio of 85:15 vol/vol as mobile phase, lenalidomide impurity B in lenalidomide capsules was determined by Reddy *et al.* [14] using HPLC. The procedure was implemented over a range of concentrations of 9.52 to 1.7456 µg/mL. Payab *et al.* [15] reported that lenalidomide and its acid, base and oxidative stress related impurities was chromatographed on endcapped C8 column using mobile phase consisted of solvent system A (potassium phosphate buffer) and solvent system B (methanol-10% and acetonitrile-50%) pumped in gradient mode at flow rate of 1.0 mL/min. The procedure was implemented to the dosage forms of lenalidomide.

The separation of lenalidomide enantiomers were carried out on a chiral LUX 5U cellulose 2 column employing glacial acetic acid: methanol: triethyl amine (0.01:100:0.01, v/v/v) as mobile phase [16]. The procedure was implemented to the dosage forms and bulk forms to determine lenalidomide enantiomers.

The genotoxic impurities of lenalidomide *viz*. methyl 2-(chloromethyl)-3-nitrobenzoate (MCN), methyl 2-(bromomethyl)-5-nitrobenzoate (MMM), methyl 2-(bromomethyl)-6-nitrobenzoate (MON), methyl 2-(bromomethyl)-4-nitrobenzoate (MPN) and 2 -methyl-3-nitrobenzoic acid methyl ester (MNM) are produced during the lenalidomide manufacturing process. The structures of all the impurities were verified in Nexus software by using two complementary models (Derek-Knowledge data base approach and Sarah-Statistical data approach) to find any mutagenicity alerts. It was found that MCN, MMM, MON, MPN and MNM impurities were predicted as Class-3 genotoxic impurities as per ICH M7 classification [17]. Therefore, the specification concentration limit for the selected impurities were determined. A comprehensive review of literature revealed that no analytical technique has been used to date for simultaneous determination of MCN, MMM, MON, MPN and MNM in lenalidomide drug substances. To the best of our knowledge, this study was concerned with the development followed by validation of a novel HPLC method to simultaneously detect and determine the genotoxic impurities of lenalidomide (MCN, MMM, MON, MPN and MNM) in lenalidomide drug substances.

EXPERIMENTAL

The reference samples of lenalidomide, methyl 2-(chloromethyl)-3-nitrobenzoate (MCN, 96.3% purity), methyl 2-(bromomethyl)-5-nitrobenzoate (MMM, 97.5% purity), methyl 2-(bromomethyl)-6-nitrobenzoate (MON, 97.8% purity), methyl 2-(bromomethyl)-4-nitrobenzoate (MPN, 94.9% purity) and 2-methyl-3-nitrobenzoic acid methyl ester (MNM, 99.96% purity) was obtained as gift samples from Hetero R & D (Hyderabad, India). Perchloric acid (Sigma-Aldrich Chemicals Pvt. Ltd., India), methanol (SD Fine-Chem Ltd., India) and acetonitrile (Rankem Chemical, India) were employed in this study. Milli-Q water was utilized throughout the study.

Detection and quantification of MCN, MMM, MON, MPN and MNM was done on Waters HPLC system (model e2695) with Photodiode array detector (model 2998) and Waters HPLC system (model e2695) with UV detector (model 2489). Ascentis Express F5 (150 × 4.6 mm, 2.7 µm) was the main column employed for separation and analysis MCN, MMM, MON, MPN and MNM in LLE drug substances and Security Guard Cartridges Phenyl (4 × 3.0 mm) was employed as guard column.

Conditions of HPLC system: The analysis was done under constant column and autosampler temperatures of 40 and 10 ºC, respectively. The separation of MCN, MMM, MON, MPN and MNM was performed in isocratic mode with mobile phase composed of solvent A (0.1% perchloric acid): solvent B (methanol 80% and acetonitrile 20%); 55:45, vol/vol. The combination of acetonitrile and water (80:20, v/v) was utilized as a diluent for solution preparations. After preparation, the mobile phase and diluent were filtered with 0.45 µm filter membrane paper and degassed. The ideal flow rate was 0.8 mL/min and impurities were detected at 210 nm. Samples of 10 µL were injected for analysis into the HPLC system.

Solutions of impurities and test sample: Impurities stock solution of 600 ppm in diluent was prepared by accurately weighed (each 60 mg) MCN, MMM, MON, MPN and MNM and dissolve in 100 mL diluent. Working standard with 60 ppm concentration of each impurity was produced by diluting the stock solution using diluent. Solutions of impurities in the range of concentrations 4.59-91.2 ppm (MCN), 6.58-90.0 ppm (MMM), 3.96-89.1 ppm (MON), 6.47-89.7 ppm (MPN) and 4.28-90.1 ppm (MNM) were prepared by aptly diluting stock impurities solution (600 ppm) with diluent for studying linearity. Lenalidomide test solution of 8 mg/mL in diluent was developed by dissolving accurate weighed (80 mg) lenalidomide in 100 mL diluent.

Quantification of impurities in test sample: The system was allowed to equilibrate for 40 min. The system was injected separately with diluent blank (10 µL, n = 1), working impurity solution (60 ppm concentration 10 µL, n = 5) and lenalidomide test sample (10 µL, n = 1). The above solutions were chromatographed using the proposed HPLC conditions and peak areas of MON, MNM, MCN, MPN and MMM were determined in standard and lenalidomide test samples. The MON, MNM, MCN, MPN and MMM content in ppm in LLE test sample was calculated by using the formula as shown below:

$$\text{Impurity (ppm)} = \frac{AT}{AS} \times \frac{CS}{CT} \times P \times 10000$$

where, AT = impurity area response in lenalidomide test sample, AS = impurity area response in standard impurity solution, CS = impurity concentration in standard impurity solution (mg/mL), CT = lenalidomide concentration of test sample (mg/mL) and P = purity impurity standard (%).

RESULTS AND DISCUSSION

Method development: The chromatographic parameters were standardized and included the selection of elution mode, solvent mixture as mobile phase and kind of column. Several

mobile phases systems [0.1% perchloric acid with methanol; 0.1% perchloric acid with methanol/acetonitrile mixture; 0.1% phosphoric acid with methanol/acetonitrile mixture], columns [ACE PFP C18 (150 × 4.6 mm, 3.0 µm); ACE PFP C18 (250 × 4.6 mm, 3.0 µm), YMC Tri art Ex RS C 18 (150 × 4.6 mm, 3.0 µm) and Ascentis Express F5 (l50 × 4.6 mm, 2. 7 µm)] and mode of elution (gradient and isocratic) were studied during the preliminary optimization studies. The most apt mobile phase for separation and analysis of MON, MNM, MCN, MPN and MMM was identified to be the solvent mixture of solvent A (0.1% perchloric acid) and solvent B (methanol 80% and acetonitrile 20%) in 55:45 v/v ratio in isocratic elution mode. The Ascentis Express F5 (l50 × 4.6 mm, 2. 7 µm) column provided the utmost acceptable MON, MNM, MCN, MPN and MMM separation. The flow rate (0.8 mL/min) and column temperature (40 ºC) during the optimization were not varied. The full run time for single analysis with the conditions optimized was 40 min. The retention time for MON, MNM, MCN, MPN and MMM were 15.748 min, 18.054 min, 21.004 min, 24.426 min and 25.543 min, respectively (Fig. 1).

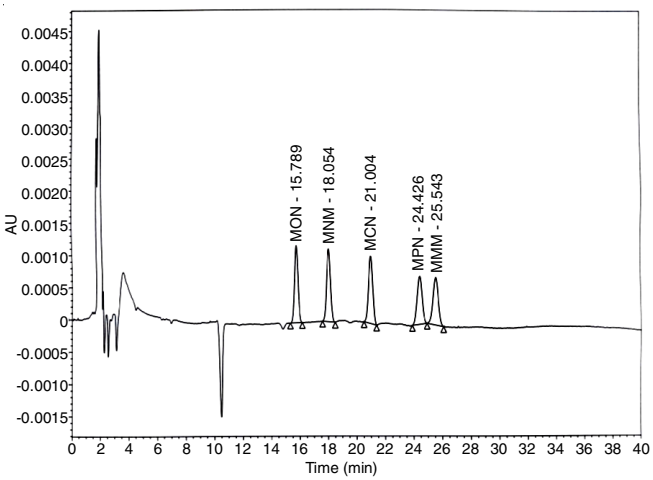

Fig. 1. Chromatogram of MON, MNM, MCN, MPN and MMM obtained with conditions optimized

Validation: Validation of the novel HPLC method to simultaneously detect and determine MCN, MMM, MON, MPN and MNM contents in lenalidomide drug substances was done by following guidelines of ICH [18].

System suitability: To ascertain system suitability, relative standard deviation for peak area counts of MCN, MMM, MON, MPN and MNM were checked by repetitively (n = 6) injecting the working impurity solution (concentration level-60 ppm). The findings of system suitability experiments are summarized in Table-1. The relative standard deviation for peak area counts of MCN, MMM, MON, MPN and MNM were less than 10%, indicated the suitability of HPLC system for determining the selected impurities by the proposed procedure.

Specificity: To ascertain specificity, the variation between the retention times of MCN, MMM, MON, MPN and MNM obtained by analyzing the working impurity solution (concentration level-60 ppm) and lenalidomide sample spiked with selected impurities at 60 ppm concentration was determined. The findings of specificity experiments are summarized in Table-2. The variation in the elution order and the retention times of MCN, MMM, MON, MPN and MNM obtained from working impurity solution and lenalidomide impurity spiked sample was insignificant (Fig. 2a and b).

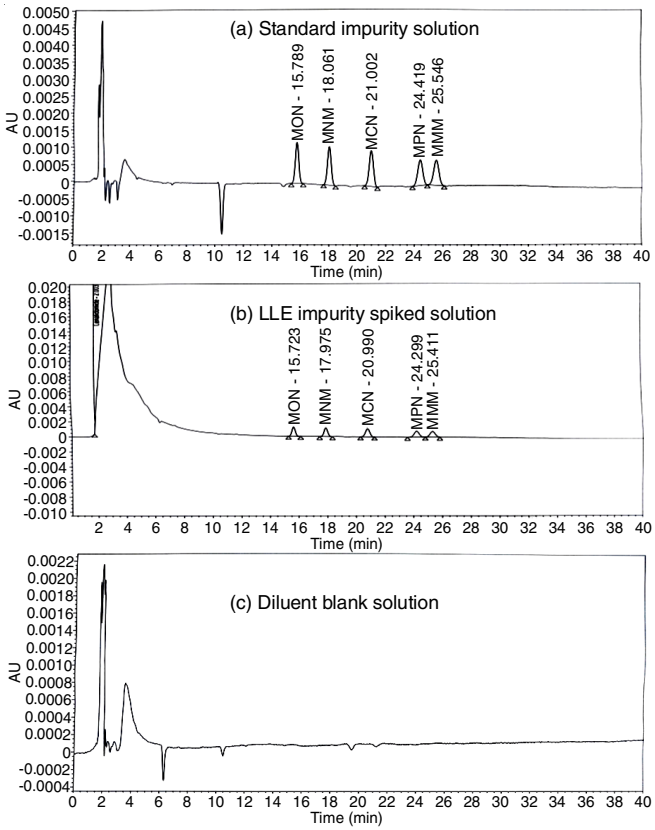

Fig. 2. Chromatograms of specificity experiments

The diluent blank was infused and analyzed using proposed method. The chromatogram collected (Fig. 2c) did not display any peak at the MCN, MMM, MON, MPN and MNM retention times.

The purity angle and purity threshold values of MCN, MMM, MON, MPN and MNM in working impurity solution (concentration level-60 ppm) and lenalidomide sample impurity spiked solution (concentration level-60 ppm) were determined

TABLE-1
SYSTEM SUITABILITY RESULTS FOR MCN, MMM, MON, MPN AND MNM

Statistical evaluation values	Peak area counts				
	MON	MNM	MCN	MPN	MMM
Mean value*	20996	21521	20779	17348	17609
% RSD	2.80	2.15	5.97	4.41	2.74

*Mean value for six peak area counts determined

TABLE-2
SPECIFICITY RESULTS FOR MCN, MMM, MON, MPN AND MNM

Impurity	Retention time (min)			Peak purity test	
	Working solution	LLE impurity spiked solution	Variation	Angle value	Threshold value
MON	15.799	15.859	0.06	4.008	4.715
MNM	18.050	18.080	0.03	4.319	4.817
MCN	21.013	21.047	0.03	4.311	4.918
MPN	24.450	24.464	0.01	6.389	7.188
MMM	25.568	25.595	0.03	5.292	5.964

using photodiode array detector. The findings of this experiments are summarized in Table-2. The results show the successful implementation. In all cases the purity threshold values were greater than the angle of purity. This showed that the MCN, MMM, MON, MPN and MNM peaks were pure, without interruption from all other substances. The results of all the above experiments indicated the specificity of method for determining the selected impurities in lenalidomide.

Detection limit: Detection limit was ascertained based on signal to noise proportion. The detection limit was described as the lowest quantity for which the impurity response was about three times more than baseline noise (Fig. 3). The findings of these experiments are summarized in Table-3. The results indicated the method's sufficient sensitivity for the detection of MCN, MMM, MON, MPN and MNM in lenalidomide drug substance.

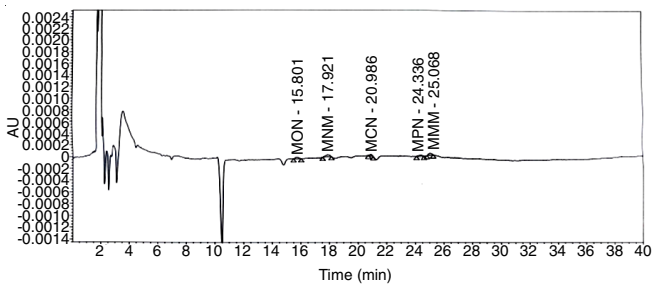

Fig. 3. Chromatogram of selected impurities at detection limit concentration

Quantification limit: Quantification limit was also ascertained based on signal to noise proportion. The quantification limit was described as the lowest quantity for which the impurity response was about ten times more than baseline noise (Fig. 4). The quantification limit values of the impurities were confirmed through determining the mean percent recovery (n = 3) of MCN, MMM, MON, MPN and MNM contents in lenalidomide test solution spiked with selected impurities at quantification limit concentration level. The quantification limit values of the impurities were also confirmed through determining relative standard deviation for peak area counts (n = 6) of MCN, MMM, MON, MPN and MNM at quantification limit concentration level. The findings of these experiments are summarized in Table-3. The percent recovery of MCN, MMM, MON, MPN and MNM were within the range of 80-120%, relative standard deviation for peak area counts of MCN, MMM, MON, MPN and MNM were less than 10% and indicated the method's sufficient sensitivity for analysing MCN, MMM, MON, MPN and MNM in lenalidomide drug substances.

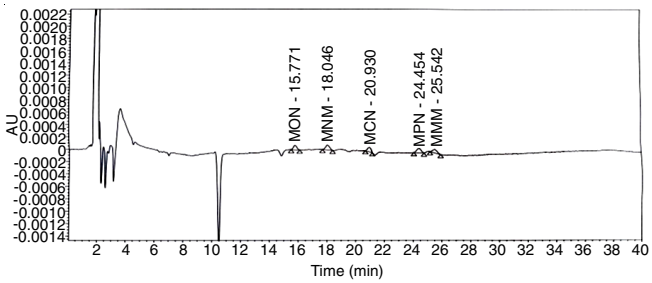

Fig. 4. Chromatogram of selected impurities at quantification limit concentration

Linearity: Linearity studies were performed for impurities in the range of quantification limit level to 150% level. Solutions of impurities in the range of concentrations 4.59-91.2 ppm (MCN), 6.58-90.0 ppm (MMM), 3.96-89.1 ppm (MON), 6.47-89.7 ppm (MPN) and 4.28-90.1 ppm (MNM) were prepared and subjected to analysis using proposed method. The calibration curve included six calibration points. The calibration plot for impurities were constructed as peak area count of impurity (*y*-axis) *versus* impurity concentration in ppm (*x*-axis). Every calibration standard was evaluated three times and the slope (m), intercept (Y) and correlation coefficient (r^2) were measured (Table-4). The determined values of RSD (limit ≤ 5%), correlation coefficient (limit ≥ 0.995) and percentage Y intercept (limit ≤ 5%) of selected impurities were within the limit of acceptance. The test results revealed the linearity of the method.

TABLE-3
SENSITIVITY RESULTS FOR MCN, MMM, MON, MPN AND MNM

Impurities	Detection limit		Quantification limit			
	DL value (ppm)	s/n proportion	QL value (ppm)	s/n proportion	RSD (%)*	Recovery (%)**
MON	1.19	3.8	3.96	13.5	4.89	98.7
MNM	1.30	5.1	4.28	14.4	3.52	106.0
MCN	1.40	4.0	4.59	13.5	6.45	106.1
MPN	1.95	3.1	6.47	13.3	5.67	98.4
MMM	2.00	4.8	6.58	10.3	6.48	102.6

*Relative standard deviation for six values determined; **Mean values of three recovery values determined

TABLE-4
LINEARITY RESULTS FOR MCN, MMM, MON, MPN AND MNM

Parameters	MON	MMM	MCN	MPN	MMM
Linearity (ppm)	3.96-89.1	4.28-90.1	4.59-91.2	6.47-89.7	6.58-90.0
RSD* (%)	0.61-3.65	0.17-3.18	0.04-6.66	0.59-6.38	0.70-2.48
Trend line equation	y = 352 *x* + 189	y = 360 *x* + 283	y = 368 *x* + 193	y = 318 *x* + 97	y = 333 *x* + -17
Correlation coefficient (r^2)	0.9995	0.9996	0.9993	0.9980	0.9975
Intercept (Y)	189	283	193	97	-17
Y intercept percentage (%)	0.91	1.28	0.86	0.52	-0.09
Slope (m)	352	360	368	318	333

*Range of relative standard deviation for three peak area counts; y = Peak area counts; *x* = Concentration of impurity in ppm

Precision: System and method precision were assessed by repeated analysis (n = 6) of standard impurity solution (60 ppm) and lenalidomide sample spiked with selected impurities (60 ppm), respectively. The relative standard deviation for impurity peak area counts (n = 6) was calculated for system precision (Table-5). The relative standard deviation for impurity content determined (n = 6) was calculated for method precision (Table-5). The RSD values and method precision studies were in limits of acceptance (≤ 5% for system precision and ≤ 10% for method precision), indicated the accuracy of the method and system for determining the selected impurities in lenalidomide drug.

Accuracy: Accuracy of the method was ascertained by checking the percent recovery of selected impurities in lenalidomide test solution spiked with selected impurities at 50, 100 and 150% level. For each level of concentration three replicates have been injected and analyzed. The impurity concentrations were measured once again and presented as recovery percentage (Table-6). The percent recoveries of MCN, MMM, MON, MPN and MNM obtained were within the limits of acceptance (85 to 115%) and indicated the accuracy of method for determining the MCN, MMM, MON, MPN and MNM in lenalidomide without any interference from LLE.

Ruggedness: Ruggedness was ascertained by repeated analysis (n = 6) of lenalidomide sample spiked with selected impurities (60 ppm), on a different day with different column, different instrument and different analyst. The relative standard deviation for impurity content determined (n = 6) was calculated (Table-7). The RSD values of the results obtained from ruggedness studies were in limits of acceptance (≤ 15%), indicated the ruggedness of method for determining MCN, MMM, MON, MPN and MNM in lenalidomide drug.

Stability of impurities spiked LLE test sample: In order to check the stability of impurity spiked (60 ppm) lenalidomide test sample solution during experimentation, the lenalidomide solution was kept at 10 ºC and subjected to analysis at 0, 12 and 24 h. The content of MCN, MMM, MON, MPN and MNM spiked was determined. The variation (%) of content of MCN, MMM, MON, MPN and MNM obtained from initial (fresh) sample and each time interval was calculated (Table-8). The percent variation of impurity content was within limits of acceptance (± 30%) up to 24 h and indicated that the impurity spiked lenalidomide test sample was stable up to studied time period.

Conclusion

The proposed HPLC method is proved to be an appropriate analytical method for detection and measurement of impurities (methyl 2-(chloromethyl)-3-nitrobenzoate, methyl 2-(bromomethyl)-5-nitrobenzoate, methyl 2-(bromomethyl)-6-nitrobenzoate, methyl 2-(bromomethyl)-4-nitrobenzoate and 2-methyl-3-nitrobenzoic acid methyl ester) in lenalidomide

TABLE-5
PRECISION RESULTS FOR MCN, MMM, MON, MPN AND MNM

Precision study	Statistical evaluation	MON	MNM	MCN	MPN	MMM
	Peak area counts of impurity					
System precision	Mean value*	21184	21268	21732	19094	19614
	% RSD	0.38	0.70	0.49	0.79	0.79
	Content of impurity determined (ppm)					
Method precision	Mean value**	58.0	58.0	61.0	60.0	57.0
	% RSD	0.81	0.77	0.68	1.81	1.09

*Mean value for six peak area counts determined; * Mean values of six content values determined

TABLE-6
ACCURACY RESULTS FOR MCN, MMM, MON, MPN AND MNM

Spiked level	Parameters	MON	MNM	MCN	MPN	MMM
50	Spiked amount (ppm)	29.7	30.0	29.8	30.1	30
	Recovery (%)	100.06	97.4	104.1	92.7	96.4
100	Spiked amount (ppm)	59.5	60.3	59.6	60.2	59.9
	Recovery (%)	97.0	97.4	101.1	100.9	95.6
150	Spiked amount (ppm)	89.2	90.4	89.4	90.2	89.9
	Recovery (%)	100.2	98.0	1.3.4	93.6	92.8

TABLE-7
RUGGEDNESS RESULTS FOR MCN, MMM, MON, MPN AND MNM

Ruggedness study	Statistical evaluation	Content of impurity determined (ppm)				
		MON	MNM	MCN	MPN	MMM
Condition 1	Mean value*	58.0	58.0	61.0	60.0	57.0
	% RSD	0.81	0.77	0.68	1.81	1.09
Condition 2	Mean value*	58.0	62.0	60.0	57.0	54.0
	% RSD	1.04	0.92	1.53	3.43	1.19
Overall results	Mean value**	58.0	60.0	60.0	58.0	55.0
	% RSD	0.89	3.29	1.22	4.01	2.33

Condition 1: Day 1, column 1, instrument 1 and analyst 1; Condition 2: Day 2, column 2, instrument 2 and analyst 2
*Mean value for six content values determined; **Mean value for conditions 1 and 2

TABLE-8
STABILITY OF MCN, MMM, MON, MPN AND MNM SPIKED IN LLE TEST SAMPLE

Impurity	Content of impurity determined (ppm)		Variation (%)	Content of impurity determined (ppm)		Variation (%)
	At 0 h	After 12 h		At 0 h	After 24 h	
MON	58.0	60.7	2.7	58.0	63.9	5.9
MNM	58.9	56.1	2.8	58.9	56.2	2.7
MCN	60.7	59.2	1.5	60.7	59.7	1.0
MPN	61.5	53.2	8.3	61.5	47.9	13.6
MMM	57.2	48.9	8.3	57.2	44.0	13.2

drug substance. The developed HPLC method was sensitive, selective, precise, accurate and robust and fulfilled the criteria of the International Conference on Harmonization standards for validation.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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