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Development of an LC-MS/MS Approach to Detect and Quantify Three Impurities of Darunavir

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KEYWORDS

Process impurities, Darunavir, LC-MS/MS, X-Bridge C18 column, MRM mode,genotoxic impurities.

ABSTRACT:

The In the Darunavir (DAR) synthesis tert-Butyl (2S,3S)-4-chloro-3-hydroxy-1-phenylbutan-2-ylcarbamate (IMP-1), (2S,3S)-1,2-Epoxy-3-(Boc-amino)-4-phenylbutane (IMP-2) and (S)-tert-Butyl 4-chloro-3-oxo-1-phenylbutan-2-ylcarbamate (IMP-3) are generated as intermediates. The presence of Imp-1, Imp-2 and Imp-3 in DAR could potentially affect its effectiveness. The purpose of this investigation was to establish a LC-MS/MS methodology to identify and evaluate Imp-1, Imp-2 and Imp-3 in DAR samples. The method for Imp-1, Imp-2 and Imp-3 analysis was developed on X-Bridge C18 (150 x 4.6 mm, 3.5µm) column with gradient elution using mobile phase consisted of 0.1% formic acid (mobile phase A) and acetonitrile (mobile phase B). Mass spectrometer with electrospray ionization operated in the MRM mode was used in the analysis of Imp-1 (m/z300.2 > 244.1), Imp-2 (m/z264.300 > 120.100) and Imp-3 (m/z298.100 > 241.900). The LC-MS/MS methodology proposed showed a good linearity (0.38 to 1.9ppm), good system precision (RSD = 2.7%, 1.6% and 3.2%), good method precision (RSD = 5.2%, 2.8% and 2.3%), acceptable accuracy (91.8-105.2%, 87.7%-95.3% and 92.8-98.4%), low detection limit (0.127 ppm, 0.125 ppm and 0.12 ppm) and low quantitation limit (0.38 ppm, 0.375 and 0.38 ppm) for Imp-1, Imp-2 and Imp-3 respectively. The LC-MS/MS methodology proposed can be utilized to assess the quality of DAR sample for the presence of Imp-1, Imp-2 and Imp-3.

1. Introduction

Darunavir (DRV), sold under the brand name Prezista among others, is an antiretroviral medication used to treat and prevent HIV/AIDS.[1] It is generally recommended for use with other antiretrovirals.[1][3] It is often used with low doses of ritonavir or cobicistat to increase darunavir levels.[1] It may be used for prevention after a needlestick injury or other potential exposure.[1] It is taken by mouth once to twice a day.[1] Common side effects include diarrhea, nausea, abdominal pain, headache, rash and vomiting.[1][3] Severe side effects include allergic reactions, liver problems, and skin rashes such as toxic epidermal necrolysis.[1] While poorly studied in pregnancy it appears to be safe for the baby.[2] It is of the protease inhibitor (PI) class and works by blocking HIV protease.[1]

tert-Butyl (2S,3S)-4-chloro-3-hydroxy-1-phenylbutan-2-ylcarbamate (IMP-1), (2S,3S)-1,2-Epoxy-3-(Boc-amino)-4-phenylbutane (IMP-2) and (S)-tert-Butyl 4-chloro-3-oxo-1-phenylbutan-2-ylcarbamate (IMP-3) are intermediates generated during DRV chemical synthesis process [6]. The chemical structures of DRV, IMP-1, IMP-2 and IMP-3 are given in Figure 1. Using the Derek nexus software programme, IMP-1, IMP-2 and IMP-3 were determined to belong to genotoxic compounds of class 3. The detection and quantitation of IMP-1, IMP-2 and IMP-3 throughout production of DRV is extremely difficult. Impurities, IMP-1, IMP-2 and IMP-3, have a significant impact on the purity and effectiveness of DRV. It is also difficult to completely remove IMP-1, IMP-2 and IMP-3 from the DRV product. Significant decrease of IMP-1, IMP-2 and IMP-3 impurities to the lowest level possible in DRV is therefore important. A novel and valid approach for the detection and quantitation of trace impurities in DRV must therefore be established.

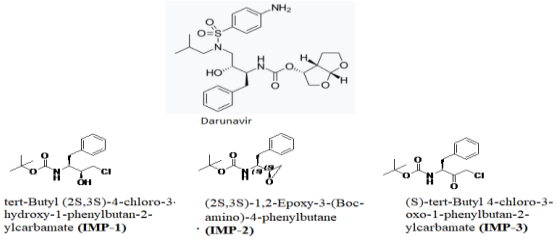

Figure 1. Chemical structures

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The biggest limitation of HPLC and UPLC for trace-level impurities is the poor sensitivity of the UV detector, that could not satisfy the detection and quantitation needs. To quantify impurities, advanced techniques such as LC-MS, GC-MS, UHPLC-MS/MS are being used because of high sensitivity and selectivity [4-11]. A number of LC-MS [4-7], GC-MS [8-10] and UHPLC-MS/MS [11] methods for the detection and quantitation of impurities in bulk medications and formulations have recently been reported. However, no LC-MS method is reported to detect and quantitate trace levels of IMP-1, IMP-2 and IMP-3 simultaneously in the DRV sample. This work was aimed to develop and validate an LC-MS method to detect and quantitate trace levels of IMP-1, IMP-2 and IMP-3 simultaneously. After development and validation, the method was applied to detect and quantify IMP-1, IMP-2 and IMP-3 in DRV batch samples.

2. Experimental

2.1. Materials

The standard impurities IMP-1, IMP-2 and IMP-3 were obtained from API Pharma Tech Pvt Ltd, Hyderabad, India and DRV sample of purity 99.9% was also from the same company. The percent purities of IMP-1, IMP-2 and IMP-3 were 98.0%, 99.1% and 98.5%, respectively. Milli Q (Bedford, USA) water has been used throughout investigation. Formic acid of AR grade, and acetonitrile of HPLC grade were obtained from Merck (Darmstadt, Germany).

2.2. Instrumentation

AB Sciex LC-MS/MS system model API 4500 (MA, USA), X-Bridge C18 (150 x 4.6 mm, 3.5µm) column, AB Sciex Analyst software (MA, USA) and Mettler Toledo analytical balance (Switzerland) were employed for analysis of IMP-1, IMP-2 and IMP-3.

2.3. Conditions

X-Bridge C18 (150 x 4.6 mm, 3.5µm) column and auto-sampler port were operated at temperatures of 45°C and 25°C, respectively for separation. Mobile phases A and B were 0.1% formic acid and acetonitrile, respectively. The procedure for gradient elution was as follows: 40% volume of mobile phase A and 60% volume of mobile phase B in isocratic elution from 0 - 12 min; The flow rate, total analysis time and injection size was 0.5 mL and 10 µL, respectively. Water and acetonitrile (20:80, *v/v*) was used for diluents and needle wash.

Mass spectrometer fitting with positive type of electro spray ionization operated in the MRM mode was used in IMP-1, IMP-2 and IMP-3 analysis. The pressure of collision gas, curtain gas, neubilizing gas and drying gas were set at 6 psi, 25 psi, 50 psi, and 45 psi, respectively. The ion spray voltage and ion source temperature were set at 5000 V and 500°C. The ion transitions for concentration determination were *m/z*300.2 > 244.1 for IMP-1 *m/z*264.3 > 120.1 for IMP-2, and *m/z* 298.1 > 241.9 for IMP-3.

2.4. IMP-1, IMP-2 and IMP-3 solutions

Stock IMP-1, IMP-2 and IMP-3 solution (100 ppm) was prepared in water and acetonitrile (20:80, v/v) solvent blend. Working IMP-1, IMP-2 and IMP-3 solution (1.25 ppm) was prepared from stock IMP-1, IMP-2 and IMP-3 solution (100 ppm) through apt dilution with water and acetonitrile (20:80, v/v) solvent blend. Calibration IMP-1, IMP-2 and IMP-3 solutions at 0.38 to 1.90 ppm concentration range were prepared for IMP-1, IMP-2 and IMP-3.

2.5. DRV sample solution

A solution of DRV was prepared by direct weighing (200 mg) of DRV substance with subsequent dissolution in water and acetonitrile (20:80, *v/v*) solvent blend by sonication at 26 ºC for 2 min in 10 mL flask. Volume was made after sonication to mark with the same solvent system. The concentration of the DRV sample solution was 20000 ppm.

2.6. Procedure to analyze IMP-1, IMP-2 and IMP-3 in DRV samples

Equilibrated the LC-MS system for at least 2 hr. Aliquots (10 µL) of blank diluent (n=1), working IMP-1, IMP-2 and IMP-3 solution (n=6) and DRV sample solution (n=2) into the system and analysed with proposed conditions of LC-MS/MS. The IMP-1, IMP-2 and IMP-3 contents of the DRV sample were measured using the formula beneath:

$$\text{Impurity content (ppm)} = \frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times P \times 10000$$

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Where, AT - average area of IMP-1/IMP-2/IMP-3 in DRV sample solution; AS - average area of IMP-1/IMP-2/IMP-3 in working IMP-1, IMP-2 and IMP-3 solution; WS - weight of IMP-1/IMP-2/IMP-3 (mg) in working IMP-1, IMP-2 and IMP-3 solution; DS - dilution factor of IMP-1, IMP-2 and IMP-3 in working IMP-1, IMP-2 and IMP-3 solution; WT - weight of DRV sample (mg); DT - dilution factor of DRV in DRV sample solution; and P - potency of IMP-1/IMP-2/IMP-3.

3. Results and Discussion

3.1. Method establishment for IMP-1, IMP-2 and IMP-3 analysis

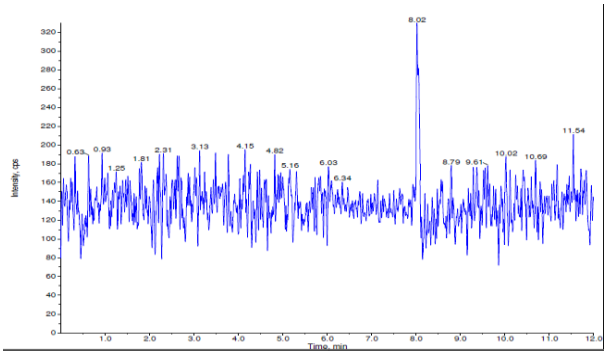
Initially X- Select CSH column C18 (100 mm length, 3.0 mm ID & 2.5 µm particle magnitude) and X-Bridge column C18 (150 mm length, 4.6 mm ID & 3.5 µm particle magnitude) using mobile phase blend of 0.1% ammonia solution with methanol/acetonitrile were tried. When used X- Select CSH column C18 and mobile phase blend of 0.1% ammonia solution with acetonitrile, resolution among IMP-1, IMP-2 and IMP-3 was <1.5. But with Select X-Bridge column C18 and mobile phase blend of 0.1% formic acid solution with Acetonitrile, acceptable resolution (>2.0) was obtained. Consequently, the same was chosen to solve recovery problems owing to the overlap of peaks moving to the mass source along with targeted analytes, resulting in lower level recovery problems. Further improved the gradient mode, flow stream to get better resolution amongst Imp-1, IMP-2, IMP-3 and DRV. Finally, formic acid and acetonitrile procedure for gradient elution was improved as follows:40 % volume of 0.1% fromic acid solution and 60% volume of acetonitrile from 0 - 12 min; As the concentration of the sample is used further to achieve a sensitivity of 1.25 ppm, the diverter value programme has applied as precautionary measure to prevent contamination of the sample at the mass source, so if the high sample is moved through the mass source due to the deposition of the sample at the source repeatability and recovery problems would occur.

3.2. Validation

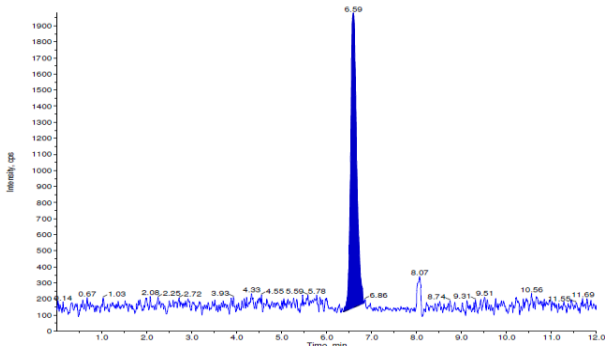
The proposed LC-MS/MS approach was verified according to ICH strategies to determine the reliability, consistency and evenness of the analytical outcomes [12].

3.2.1. Specificity

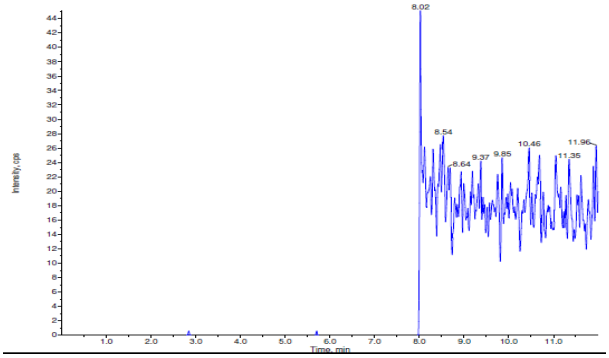
Specificity was appraised by injecting (10 µl) separately the individual working solutions of IMP-1 (1.25 ppm), IMP-2 (1.25 ppm) and IMP-3 (1.25 ppm), and diluent blank in to X-Bridge C18 (150 x 4.6 mm, 3.5µm) and analysed with proposed conditions of LC-MS/MS. Chromatograms are given in Figure 2. No interference was found with diluent component at the IMP-1 (6.59 min), IMP-2 (9.91 min) and IMP-3 (5.94 min), retention times. This proved specificity of LC-MS/MS approach to analyse IMP-1, IMP-2 and IMP-3.



Chromatogram a



Chromatogram b



Chromatogram c

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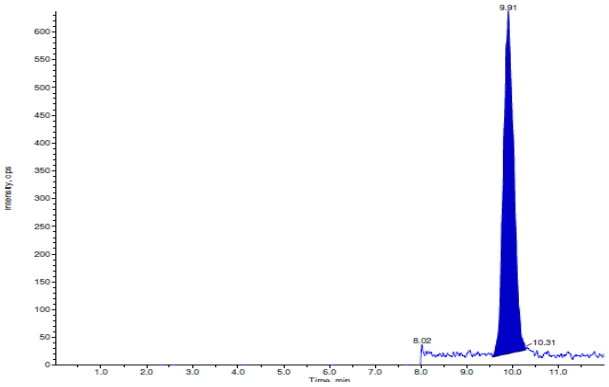
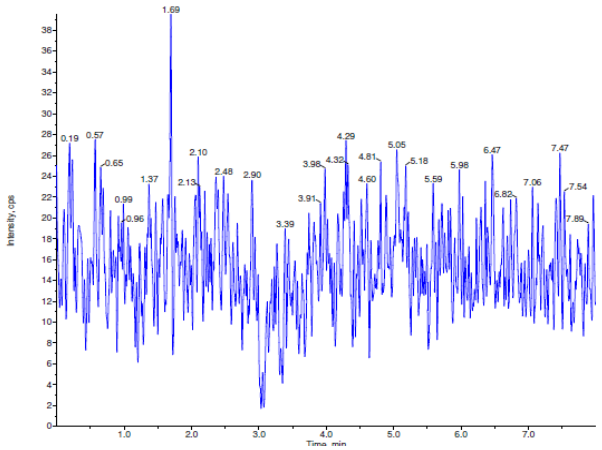
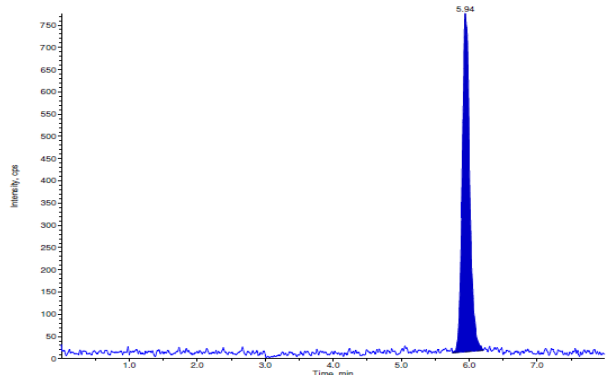


Chromatogram d



Chromatogram e



Chromatogram f

Figure 2 Chromatogram a – diluent blank for IMP-1; Chromatogram b – working IMP-1 solution; Chromatogram c - diluent blank for IMP-2; Chromatogram d - working IMP-2 solution Chromatogram e - diluent blank for IMP-3; Chromatogram f - working IMP-3 solution

3.2.2. Limit of Quantification (LOQ) and Limit of Detection (LOD)

Diluted the IMP-1, IMP-2 and IMP-3 solution quantitatively and stepwise with diluent. The diluted solutions were separately injected into X-Bridge C18 column and analysed with proposed conditions of LC-MS/MS. LOQ and LOD were described as IMP-1, IMP-2 and IMP-3 concentrations (ppm) which could be detected and give signal to noise proportion values of ≥10 and ≥3, respectively. The LOQ and LOD values (Table 1) evidenced the sensitivity of LC-MS/MS approach to analyse IMP-1, IMP-2 and IMP-3 at trace levels.

Table 1 LC-MS/MS methodology sensitivity results

Content	LOQ		LOD	
	S/N Proportion	ppm	S/N Proportion	ppm
IMP-1	11.3	0.38	5.6	0.13
IMP-2	11.4	0.38	4.5	0.13
IMP-3	11.8	0.38	4.7	0.12

S/N - signal to noise

3.2.3. Linearity

Five calibration IMP-1, IMP-2 and IMP-3 solutions with IMP-1 at 0.38 to 1.9 ppm concentration range, IMP-2 at 0.38 to 1.9 ppm concentration range and IMP-3 at 0.38 to 1.9 ppm concentration range were analysed thrice. The calibration curves of IMP-1, IMP-2 and IMP-3 were developed by mapping the mean area obtained against concentrations. The regression equation and linearity correlation coefficient were calculated for IMP-1, IMP-2 and IMP-3 curves (Table 2). The linearity correlation coefficient values (>0.99) evidenced the linearity of LC-MS/MS approach.

Table 2 LC-MS/MS methodology linearity and line equation results

Parameter	IMP-1	IMP-2	IMP-3
Linearity	0.38 to 1.9 ppm	0.38 to 1.9 ppm	0.38 to 1.9 ppm
Line Equation	Y=12717x+183.05	Y=7425.1+632.35	Y=4078.7+65.4
Y-Intercept	183.1	632.4	65.4
Slope	12716.9	7425.1	4078.7

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% Y-Intercept	1.1	6.8	1.2
Linearity Correlation Coefficient	0.998	0.99	0.998

Y – peak area; *x* – concentration of IMP-1/IMP-2/IMP-3 in ppm

3.2.4. System precision

Separately injected (10 µL) working IMP-1, IMP-2 and IMP-3 solution (1.25 ppm) into X-Bridge C18 column, analysed with proposed conditions of LC-MS/MS in six replicates and noted the peak areas of IMP-1, IMP-2 and IMP-3. The percent RSD between the peak areas of IMP-1, IMP-2 and IMP-3 from six replicates were 2.7%. 1.6% and 3.2% respectively (Table 3). This proved system precision to analyse IMP-1, IMP-2 and IMP-3.

3.2.5. Method precision

Separately injected (10 µL) DRV sample spiked with pure IMP-1 (1.25 ppm), IMP-2 (1.25 ppm) and IMP-3 (1.25 ppm) into X-Bridge C18 column and analysed with proposed conditions of LC-MS/MS in six replicates. The concentration of IMP-1, IMP-2 and IMP-3 were determined. The percent RSD between the concentrations of IMP-1, IMP-2 and IMP-3 from six replicates were 5.2%. 2.8% and 2.3% respectively (Table 3). This proved LC-MS/MS method precision to analyse IMP-1, IMP-2 and IMP-3 in DRV sample.

Table 3 LC-MS/MS methodology precision results

Samples	**IMP-1**		**IMP-2**		**IMP-3**	
	System precision	**Method precision**	**System precision**	**Method precision**	**System precision**	**Method precision**
	Peak area	**Amount obtained (ppm)**	**Peak area**	**Amount obtained (ppm)**	**Peak area**	**Amount obtained (ppm)**
1	16921	1.294	9776	1.153	5675	1.171
2	15880	1.235	9586	1.139	5708	1.202
3	16718	1.148	9388	1.165	5553	1.231
4	15917	1.187	9499	1.191	5658	1.224
5	16280	1.278	9752	1.096	5622	1.192
6	16675	1.315	9485	1.142	6071	1.163
Average	16398.5	1.243	9581	1.148	5714.5	**1.195**
SD	439.61	0.065	155.23	0.032	182.5	**0.027**
SD	**2.7**	**5.2**	**1.6**	**2.8**	**3.2**	**2.3**

3.2.6. Ruggedness

Ruggedness was appraised by conducting a precision analysis in six prepares of the DRV samples spiked with pure pure IMP-1 (1.25 ppm), IMP-2 (1.25 ppm) and IMP-3 (1.25 ppm) by different analysts (n=2) and on different days (n=2). All of the results disclosed percent RSD not above than 5.0% (4.02% for IMP-1, 2.51% for IMP-2 and 2.96% for IMP-3 2.9%, Table 4). This proved LC-MS/MS method ruggedness to analyse IMP-1, IMP-2 and IMP-3 in DRV sample.

Table 4 LC-MS/MS methodology ruggedness results

Day and analyst	**IMP-1 amount obtained (ppm)**	**IMP-2 amount obtained (ppm)**	**IMP-3 amount obtained (ppm)**
Day 1 and analyst 1	1.294	1.153	1.171
	1.235	1.139	1.202
	1.148	1.165	1.231
	1.187	1.191	1.224
	1.278	1.096	1.192
	1.315	1.142	1.163
	1.251	1.162	1.161
Day 2 and analyst 2	1.242	1.201	1.232
	1.211	1.195	1.245
	1.179	1.181	1.271
	1.201	1.152	1.189
	1.214	1.171	1.174
Overall Average	1.23	1.16	1.20
Overall SD	0.05	0.03	0.04
Overall % RSD	4.02	2.51	2.96

3.2.7. Recovery

Accuracy was tested by undertaking a recovery analysis in compliance thru ICH guidance. Known concentration of IMP-1, IMP-2 and IMP-3 standard solutions corresponding to LOQ level, 100% and150% of specification limit quantity (1.25 ppm) was add up to the DRV sample solution (20000 ppm). The accuracy was measured for these concentrations by three times sample solution injection, and the findings were given in Table 5. As seen (Table 5), the recoveries of IMP-1, IMP-2 and IMP-3 were found as 94.1%–106.0% and 96.2% - 99.2%, respectively which proved LC-MS/MS method accuracy in analysing IMP-1, IMP-2 and IMP-3 in DRV sample.

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Table 5 LC-MS/MS methodology accuracy results

Accuracy level	Amount Add up (ppm)	Amount obtained (ppm)	Recovery (%)	Average of Recovery (%)
IMP-1 recovery				
	0.38	0.360	94.7	
LOQ	0.38	0.349	91.8	96.0
	0.38	0.386	101.5	
	1.25	1.294	103.5	
100%	1.25	1.235	98.8	98.0
	1.25	1.148	91.8	
	1.90	1.941	102.2	
150%	1.90	1.853	97.5	96.8
	1.90	1.722	90.6	
IMP-2 recovery				
	0.375	0.311	82.9	
LOQ	0.375	0.364	97.0	90.7
	0.375	0.346	92.2	
	1.25	1.153	92.2	
100%	1.25	1.139	91.1	92.2
	1.25	1.165	93.2	
	1.90	1.730	91.0	
150%	1.90	1.709	89.9	91.0
	1.90	1.748	92.0	
IMP-3 recovery				
	0.38	0.39	102.6	
LOQ	0.38	0.39	102.6	100.9
	0.38	0.37	97.4	
	1.25	1.171	93.6	
100%	1.25	1.202	96.0	96.0
	1.25	1.231	98.4	
	1.90	1.757	92.4	
150%	1.90	1.803	94.9	94.8
	1.90	1.847	97.2	

3.2.8. Robustness

The robustness had been tested by modifying column oven temperature. Robustness was appraised by analysis in three prepares of the DRV samples spiked with pure MP-1 (1.25 ppm), IMP-2 (1.25 ppm) and IMP-3 (1.25 ppm) with modified and optimized column oven temperature. The percent relative difference for the mean content of IMP-1, IMP-2 and IMP-3 observed from the results obtained with modified and optimized column oven temperature were 0.0% - 2.8% and 1.7% - 1.8%, respectively (Table 6). This proved LC-MS/MS method robustness for the variation studied in analysing IMP-1, IMP-2 and IMP-3 in RDV sample.

Table 6 LC-MS/MS methodology robustness results

	IMP-1		IMP-2		IMP-3	
Temperature	Mean amount obtained* (ppm)	Relative difference (%)	Mean amount obtained* (ppm)	Relative difference (%)		
43 ºC	1.255	2.37	1.162	0.87	1.195	0.50
45 ºC	1.226		1.152		1.201	
47 ºC	1.271	3.67	1.151	0.09	1.211	0.83
45 ºC	1.226		1.152		1.201	

* mean of three values

3.2.9. Solution stability

The stability of IMP-1, IMP-2 and IMP-3 solution was judged by analysing the working IMP-1, IMP-2 and IMP-3 solution at 1.25 ppm concentration. The stability of the IMP-1, IMP-2 and IMP-3 in stored (room temperature) stock solution during 24 hr and 48 hr was determined by comparing it with the fresh stock IMP-1, IMP-2 and IMP-3 solution. The percent relative difference for the content of IMP-1, IMP-2 and IMP-3 between results obtained in initial and at predetermined intervals were given Table 7. The values proved stability of IMP-1, IMP-2 and IMP-3 in solution upto 48 hr.

Table 7 Stability of IMP-1, IMP-2 and IMP-3 in solution

	IMP-1		IMP-2		IMP-3	
Time	Mean amount obtained * (ppm)	Relative difference (%)	Mean amount obtained * (ppm)	Relative difference (%)	Mean amount obtained * (ppm)	Relative difference (%)
0 hr	1.252	2.5	1.211	2.4	1.262	5.3
24 hr	1.221		1.182		1.195	
0 hr	1.252	3.5	1.211	3.0	1.262	6.4
48 hr	1.296		1.175		1.181	

* mean of three values

3.3. Application of LC-MS/MS methodology developed

The LC-MS/MS methodology developed and validated was applied to detect and quantify IMP-1, IMP-2 and IMP-3 in six batches of DRV samples. The results are provided in Table 8. The IMP-1, IMP-2 and IMP-3 content was below detection limit (0.13 ppm) in all batches of DRV.

Table 8 Batch analysis of IMP-1, IMP-2 and IMP-3

Batch number of	IMP-1 content	IMP-2 content	IMP-3 content

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DRV sample	quantified (ppm)	quantified (ppm)	quantified (ppm)
A19000009	BDL	BDL	BDL
A19000010	BDL	BDL	BDL
A19000011	BDL	BDL	BDL
A19000012	BDL	BDL	BDL
A19000013	BDL	BDL	BDL
A19000014	BDL	BDL	BDL

BQL – below quantification limit; BDL - Below detection limit

4. Conclusion

The developed LC-MS/MS methodology provides accurate, sensitive, specific and precise analysis for the concurrent quantitation of two impurities, IMP-1, IMP-2 and IMP-3, in DRV samples. This method had the benefits of reduced LD and LQ values for IMP-1, IMP-2 and IMP-3. This LC-MS/MS methodology has been shown to be effective for quality evaluation of the DRV sample for IMP-1, IMP-2 and IMP-3 impurities.

Author contribution

Arthanareeswari. M, SRM Institute of Science and Technology, Kattankulathur, Department of Chemistry, Tamilnadu, 603203, India. Ravi Uppala, Biocon Limited, Analytical Research, Bangalore 560 100, India. Designed the experiment; Arthanareeswari. M, SRM Institute of Science and Technology, Kattankulathur, Department of Chemistry, Tamilnadu, 603203, India. Ravi Uppala, Biocon Limited, Analytical Research, Bangalore 560 100, India. Carried them Out; Arthanareeswari. M, SRM Institute of Science and Technology, Kattankulathur, Department of Chemistry, Tamilnadu, 603203, India. iocon Limited, Analytical Research, Bangalore 560 100, India. Venkatasubbaiah. B, Dr Reddys laboratories limited, Analytical Research, Hyderabad 500 072, India. Prepared the manuscript with contribution from all co-authors; Arthanareeswari. M, SRM Institute of Science and Technology, Kattankulathur, Department of Chemistry, Tamilnadu, 603203, India. Venkatasubbaiah. B, Dr Reddys laboratories limited, Analytical Research, Hyderabad 500 072, India. Reading of manuscript.

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