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A Novel Reverse Phase HPLC Method for the Quantification of Potential Genotoxic Impurities in Doripenem Monohydrate: A Broad-Spectrum Carbapenem Antibiotic Drug

SUDHAKAR YERRA[1,2,*], P.N. KISHORE BABU[1], B. SREENIVASULU[1], HEMANT KUMAR SHARMA[1], K. MOHANA NAIDU[1] and B. VENKATESWARA RAO[2]

[1]APL Research Centre-II (A Division of Aurobindo Pharma Ltd), Survey No. 71 & 72, Indrakaran Village, Kandi Mandal, Sangareddy, Hyderabad-502329, India
[2]Department of Organic Chemistry, AU College of Science and Technology, Visakhapatnam-530003, India

*Corresponding author: E-mail: ysr10633@gmail.com

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A novel sensitive gradient reverse phase high performance liquid chromatographic (RP-HPLC) method has been developed and validated for the quantification of potential genotoxic impurities in doripenem monohydrate (DORIBAX) drug substance, namely mono-*p*-nitrobenzyl malonate, 1β-methyl bicyclic ketoester, desilylated β-keto ester, 1β-methyldiazoazetidinone, deprotected doripenem side chain, *N*-protected mercapto alcohol, protected doripenem and doripenem side chain. The analysis performed on Alliance Waters e2695 separation module on C18 (250 × 4.6) mm, 5 µm (make: Inertsil) column, oven temperature maintained at 40 ºC and UV detection at 270 nm. The separation was accomplished using buffer (pH 6.0 ± 0.05) and acetonitrile in the ratio of 98:2 v/v as mobile phase-A and acetonitrile as mobile phase-B, flow rate: 1.0 mL/min and injection volume: 50 µL. The proposed method was validated as per ICH guidelines in terms of limit of detection (LOD), limit of quantification (LOQ), linearity, precision, accuracy and specificity.

Keywords: RP-HPLC, Genotoxic impurities, Doripenem monohydrate.

INTRODUCTION

Doripenem monohydrate, chemically known as (4R,5S,6S)-3-[[(3S,5S)-5[[(amino sulfonyl)amino]methyl]-3-pyrrolidinyl]-thio]-6-[(1R)-1-hydroxyethyl]-4-methyl-7-oxo-1-azabicycle [3.2.0]hept-2-ene-2-carboxylicacid monohydrate (Fig. 1), molecular formula is $C_{15}H_{24}N_4O_6S_2 \cdot H_2O$ and molecular weight is 438.52. Doripenem comes under the carbapenem class drugs [1] with broad spectrum antibiotic activity. It is a β-lactum antibiotic drug, which is able to kill pseudomonas aeruginosa and used for various bacterial infections [2], such as complex abdominal infections, pneumonia and complicated infections of urinary tract including kidney infections with sepsis.

The greater stability of doripenem in aqueous solution compared to earlier members of the carbapenem class allows it to be administered as an infusion, which may be advantageous in the treatment of certain difficult infections [3,4]. It may lower risk of comprising seizures than other carbapenems. It is marketed under the brand name Doribax [5] and is the fourth member of the carbapenem class.

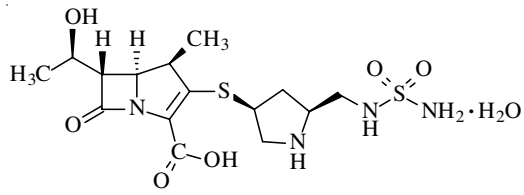

Fig. 1. Chemical structure of doripenem monohydrate drug substance

Synthesis of doripenem monohydrate drug substance, raw materials and intermediates may ascend the impurities. The chemical activity of these impurity components may frequently convert into biological reactivity and can often transformed as carcinogens or mutagens. It has been recognized that the fate of the several genotoxic agents were not entitled their retention with the final API due to elevated chemical reactivity, especially if the formation is separated from the final API by various synthetic steps. Some of these known impurities may be potential mutagens or carcinogens. But the formed impurities may not be completely possible to eliminate totally from synthetic route. In doripenem monohydrate, several impurities namely mono-

p-nitro-benzyl malonate, 1β-methyl bicyclic ketoester, desilylated β-ketoester, 1β-methyldiazoazetidinone, deprotected doripenem side chain, *N*-protected mercapto alcohol, protected doripenem and doripenem side chain are the possible genotoxic impurities. The current regulatory guidelines for genotoxic impurities indicate to develop the analytical methods to meets the required daily intake limit of 1.5 mg/day of any individual impurity [6,7]. The limit of each impurity considered as 80 μg g^{-1} with respect to doripenem monohydrate daily dose, *i.e.* 500 mg/day. The possible structure of genotoxic impurities formed are shown in Fig. 2.

There is no pharmacopeial monograph available for these impurities and no HPLC method available in literature for the quantification of doripenem monohydrate genotoxic impurities. However, few methods have been reported in literature for the determination of doripenem monohydrate drug products and its related substances. Kurien & Jayasekhar [8] reported a stability indicating HPLC method for the determination of doripenem drug substance in pharmaceutical dosage. Michalska *et al*. [9] determined doripenem and its related substances using capillary electrophoresis. However, there is constraint for the chromatographic methods in the determination of potential genotoxic impurities formed during the synthesis of doripenem monohydrate drug substance. HPLC techniques have advantageous for the determination of genotoxic impurities in pharmaceutical industry. In this perspective view, a simple and novel RP-HPLC method for the determination of potential genotoxic impurities in doripenem monohydrate drug substance is developed. In addition, the method is validated to meet the requirements of ICH validation guidelines [10].

EXPERIMENTAL

Investigated samples of doripenem monohydrate reference sample and analyzed impurities (for specificity experiment) were received as a gift from APL Research Centre-II Laboratories (A division of Aurobindo Pharma Ltd., Hyderabad, India). Diammonoium hydrogen orthophosphate, orthophosphoric acid and HPLC grade acetonitrile used were purchased from Merck Research Laboratories, India. Pure milli-Q water was used with the help of millipore purification system (Millipore®, Milford, MA, USA).

High performance liquid chromatography: The HPLC systems used was Waters Alliance e2695, separation module equipped with 2489UV detector, Waters Alliance 2695 separation module with 2996 PDA detector with Empower data handling system *i.e*. Empower 3 software, [Waters Corporation, Milford, USA]. The analysis performed on a stainless steel column (250 mm long, 4.6 mm internal diameter) filled with octadecylsilane groups chemically bonded to porous silica particles of 5 μm diameter [Inertsil ODS-3V, 250 mm × 4.6 mm, 5 μm, Make: Inertsil], column oven temperature maintained at 40 ºC.

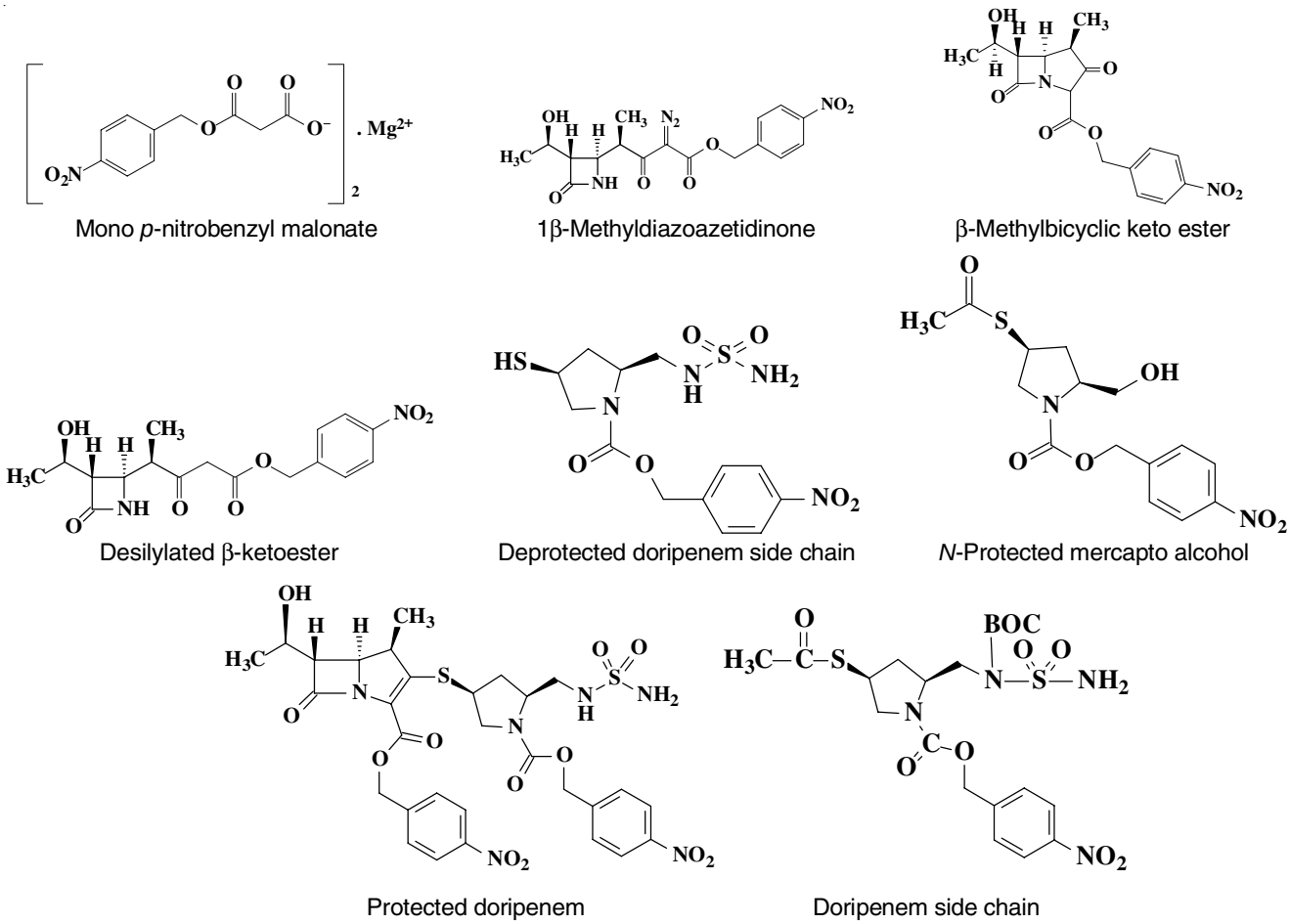

Fig. 2. Chemical structures of genotoxic impurities in doripenem monohydrate

Mobile phase: Mobile phase A: Degassed mixture of pH 6.0 buffer (2.64 g of diammonium hydrogen orthophosphate in 1000 mL of water and adjusted pH 6.0 with orthophosphoric acid, filtered through 0.45 µ or finer porosity membrane filter) and acetonitrile (98:2 v/v). Mobile phase-B:acetonitrile. Diluent used was a mixture of degassed mobile phase-A and mobile phase-B (98:2 v/v). Flow rate: 1.0 mL/min, injection volume: 50 µL, UV detection: 270 nm, chromatographic data acquisition time: 55 min. The pump adjusted in gradient mode and programmed as: Time (min)/A (v/v): B (v/v); $T_{0.01}$/80:20, T_{30}/45:55, T_{45}/30:70, T_{55}/30:70.

Preparation of standard solution: A concentration level of all impurity solutions (0.0004 mg/mL) were used as standard *i.e*. 10 mg each of mono-*p*-nitrobenzyl malonate magnesium salt, 1β-methylbicyclic ketoester, desilylated β-ketoester, 1β-methyldiazoazetidinone, deprotected doripenem side chain, *N*-protected mercapto alcohol, protected doripenem and doripenem side chain reference samples dissolved in 100 mL acetonitrile. Further dilute 5 mL to 100 mL and 4 mL to 50 mL using diluent.

Sample solution: Doripenem monohydrate sample solution of 5 mg/mL (50 mg of sample in 10 mL clean dry volumetric flask) added 7 mL of diluent and sonicated to dissolve. Finally, make upto the mark with diluent. Filtered the solution through 0.45 µ (or finer) porosity membrane filter.

System suitability evaluation: The column efficiency was determined as mono-*p*-nitrobenzylmalonate and was not less than 10000 USP plate count. USP Tailing for the same peak was not more than 1.5. Relative standard deviation (RSD) for peaks areas obtained from six injections of the standard solution was not more than 10.0%.

Procedure: Inject 50 µL of diluent, standard solution and sample solution into the chromatograph and recorded the chromatogram.

Method development: The main aim for this method was to separate and quantify the genotoxic impurities present in the doripenem monohydrate drug substance. Various chromatographic parameters were tested and optimized in order to achieve the optimum separation between the genotoxic impurities.

Selection of column: Reverse phase compatible column was selected to separate the above said genotoxic impurities on HPLC. Trials were carried out on × terra RP18, 5 µm (150 mm × 4.6 mm); × terra RP18, 5 µm (250 mm × 4.6 mm); YMC Pack pro C18, 5 µm (250 mm × 4.6 mm); Symmetry C18, 5 µm (250 mm × 4.6 mm); Inertsil ODS-3V, 5 µm (250 mm × 4.6 mm). Though, the tested columns resulted optimistic separation, Inertsil ODS-3V, 5 µm (250 mm × 4.6 mm) was found to be more suitable, reason for this criteria was believed due to its end caping technology. In addition, these columns were compatible with mass spectroscopy applications proving sharp peaks, high sensitivity, batch to batch reproducibility and symmetrical peak shapes with improved resolution. Finally, the desired separation was achieved with Inertsil ODS-3V, 5 µm (250 mm × 4.6 mm) reverse phase column.

Optimization of buffer solutions: Optimization of buffer solution and the effect of pH on retention time, separation parameters were studied over a pH range between 2 and 8. Several buffer solutions were tried for the separation of all said components. Aqueous monosodium phosphate (NaH_2PO_4) of 0.02 M solution afforded moderate separation and the peak shapes were not impressive. Another trial was performed using aqueous perchloric acid solution but the retention and separation was not achieved. Subsequently, another experimental trial was performed using 0.02 M ammonium dihydrogen orthophosphate ($(NH_4)H_2PO_4$), but again the retention and separation was not achieved. Finally, the best separation was achieved using 0.02 M diammonium hydrogen orthophosphate ($(NH_4)_2HPO_4$) adjusted to pH 6.0 using orthophosphoric acid.

Optimization of mobile phase: Optimization trials were carried out with gradient program by using different aqueous buffers and with acetonitrile. In order to achieve shorter run time with good separation and peak shape, we opted for gradient mode, buffer (0.02 M diammonium hydrogen orthophosphate having pH 6.0 adjusted with orthophosporic acid) and acetonitrile in the ratio of 98:2 v/v was used as mobile phase-A while acetonitrile used as mobile phase-B. In these specified conditions, best separation was observed with shorter time.

Selection of UV detection: The response was studied on PDA detector under different nanometers including 210, 230, 254, 270 and 295. At all these nanometers, best response was achieved at 270 nm.

Optimization of column oven temperature: The development trials to optimize the column oven temperature were carried out at column temperature between 20-50 ºC. Considering the better separation and good peak shape, the column temperature was fixed at 40 ºC.

Method validation: After optimizing the suitable conditions, method validation parameters were streamlined as per ICH guidelines, individually in terms of specificity or selectivity, LOD, LOQ, linearity, accuracy and precision.

RESULTS AND DISCUSSION

Specificity: For the determination of specificity, analytes of doripenem monohydrate (mono-*p*-nitrobenzyl malonate, 1β-methyl bicyclic ketoester, desilylated β-ketoester, 1β-methyldiazoazetidinone, deprotected doripenem side chain, *N*-protected mercapto alcohol, protected doripenem and doripenem side chain) were prepared and injected to confirm the individual retention times. Subsequently diluent, solutions of doripenem monohydrate drug substance (control sample), doripenem monohydrate drug substance spiked with mono-*p*-nitrobenzyl malonate, 1β-methyl bicyclic ketoester, desilylated β-ketoester, 1β-methyldiazoazetidinone, deprotected doripenem side chain, *N*-protected mercapto alcohol, protected doripenem and doripenem side chain were prepared and injected into HPLC to confirm any co-elution with analyte peaks from respective diluents. The chromatographic representation of potential genotoxic impurities in doripenem monohydrate is shown in Fig. 3.

All related substance peaks and the peak homogeneity was verified for each analyte injected in waters system with PDA detector using empower software and found to be pure (purity angle should be less than purity threshold). The specificity results are shown in Table-1.

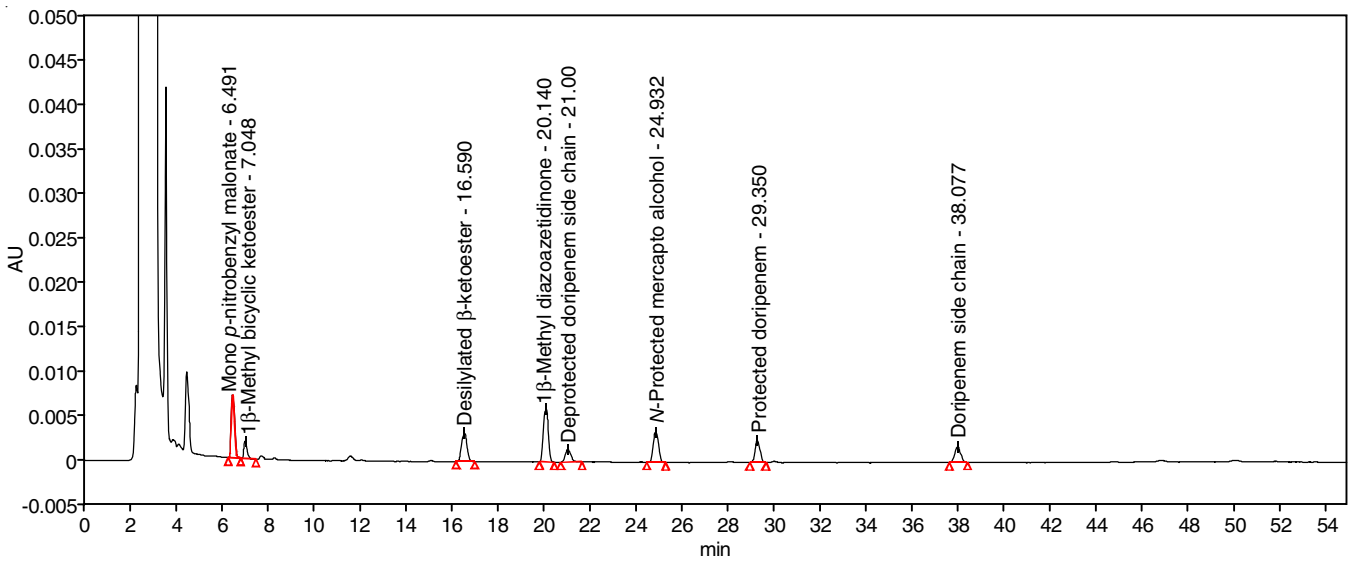

Fig. 3. Chromatographic representation of potential genotoxic impurities in doripenem monohydrate

TABLE-1
SPECIFICITY EXPERIMENTS RESULTS

Name of the genotoxic impurity	Retention time (min)	Spiked sample peak purity	
		Purity angle	Purity threshold
Mono *p*-nitrobenzyl malonate	6.389	0.048	0.620
1β-Methyl bicyclic ketoester	8.124	0.022	0.913
Desilylated β-ketoester	16.220	0.021	0.830
1β-Methyl diazoazetidinone	19.713	0.009	0.626
Deprotected doripenem side chain	20.646	0.026	1.398
N-Protected mercapto alcohol	24.453	0.025	0.953
Protected doripenem	28.827	0.035	1.002
Doripenem side chain	37.412	0.031	1.363
Other impurities for information:			
Doripenem acid	2.318		
Doripenem dimmer	3.588		
tert-Butyl doripenem	4.414		
Diphenyl phosphate	9.629		
Methyl vinyl phosphate	38.731		
Azetidinone malonate ester	46.442		

Limit of detection (LOD) and limit of quantification (LOQ): To quantify the limit of detection (LOD) and limit of quantification (LOQ) for mono-*p*-nitrobenzyl malonate, 1β-methyl bicyclic ketoester, desilylated β-ketoester, 1β-methyldiazoazetidinone, deprotected doripenem side chain, *N*-protected mercapto alcohol, protected doripenem and doripenem side chain impurities were predicted on the basis of response of analytes. The predicted concentrations of LOD and LOQ for these eight impurities were verified and the same were injected individually six times into HPLC system as per method conditions. Results are shown in Table-2.

Detector linearity: Linearity of the detector was determined by preparing a series of solutions using mono-*p*-nitrobenzyl malonate, 1β-methyl bicyclic ketoester, desilylated β-ketoester, 1β-methyldiazoazetidinone, deprotected doripenem side chain, *N*-protected mercapto alcohol, protected doripenem and doripenem side chain at concentration levels from LOQ to 150% level. The obtained data was subjected to statistical analysis by using a linear regression model. The statistical values like slope, intercept, STEYX and correlation coefficient data are shown in Table-3.

TABLE-2
RESULTS OBTAINED FOR LOD AND LOQ

Name of the impurity	Concentration (μg g^{-1})		RSD (%)	
	LOD	LOQ	LOD	LOQ
Mono *p*-nitrobenzyl malonate	3.1	9.3	1.7	0.8
1β-Methyl bicyclic ketoester	4.4	13	1.9	0.6
Desilylated β-ketoester	3.5	11	2.5	0.4
1β-Methyl diazoazetidinone	3.0	9.0	3.7	0.9
Deprotected doripenem side chain	9.3	28	2.4	0.7
N-Protected mercapto alcohol	4.4	13	3.2	0.6
Protected doripenem	6.8	21	5.6	6.0
Doripenem side chain	6.7	20	2.2	1.7

Accuracy: Accuracy of the method was performed by recovery experiments using standard addition technique. The recoveries was determined by spiking mono-*p*-nitrobenzyl malonate, 1β-methyl bicyclic ketoester, desilylated β-ketoester, 1β-methyldiazoazetidinone, deprotected doripenem side chain, *N*-protected mercapto alcohol, protected doripenem and doripenem side chain at four different concentration levels (*i.e.* LOQ, 40, 80 and 120 μg g^{-1}) into doripenem monohydrate drug substance. These samples were prepared as per respective test procedure and analyzed in triplicate. Percentage recoveries were calculated and the average percentage recoveries of four levels (twelve determinations) were 88.5, 94.8, 104.6, 108.3, 85.5, 112.7, 105.5 and 113.6, respectively. Fully validated accuracy results are shown in Table-4.

Precision: System precision was established by preparing the standard solutions of individual analytes as per the methodology and analyzed by injecting six replicates. Method precision (MP) and intermediate precision (IP) were demonstrated by preparing six sample solutions individually using a single batch of doripenem monohydrate drug substance spiked with mono-

TABLE-3
LINEARITY RESULTS

Name of the impurity	Conc. range (μg mL^{-1})	Slope	Intercept	STEYX	Correlation coefficient	RSQ (r^2)	Residual sum of squares
Mono-*p*-nitrobenzyl malonate	0.050-0.604	112092	394	473	0.9998	0.9996	1119513
1β-Methyl bicyclic ketoester	0.060-0.596	32382	377	172	0.9997	0.9994	147712
Desilylated β-ketoester	0.060-0.590	87997	352	248	0.9999	0.9998	3062568
1β-Methyl diazoazetidinone	0.050-0.602	145947	207	951	0.9995	0.9991	4517104
Deprotected doripenem side chain	0.140-0.571	48593	829	467	0.9986	0.9973	873189
N-Protected mercapto alcohol	0.070-0.594	92693	86	655	0.9994	0.9989	2145948
Protected doripenem	0.104-0.623	68639	1223	654	0.9990	0.9980	1710539
Doripenem side chain	0.104-0.623	55040	202	495	0.9991	0.9982	980983

TABLE-4
ACCURACY RESULTS

Accuracy parameter	LOQ level	40 μg g^{-1} level	80 μg g^{-1} level	120 μg g^{-1} level	LOQ level	40 μg g^{-1} level	80 μg g^{-1} level	120 μg g^{-1} level
	Mono *p*-nitrobenzyl malonate (Average of 3 replicates)				1β-Methyl bicyclic ketoester (Average of 3 replicates)			
Added (μg g^{-1})	9.6	40	81	122	13.5	39	79	119
Recovered (μg g^{-1})	8.5	41	83	126	12.8	37	76	117
Recovery (%)	88.5	102.5	105.7	103.2	94.8	94.9	96.2	98.3
RSD (%)	1.2	1.8	1.6	1.4	1.3	1.8	1.7	2.1
Average recovery (%)	99.9 (4 levels average)				96.1 (4 levels average)			
	Desilylated β-ketoester (Average of 3 replicates)				1β-Methyl diazoazetidinone (average of 3 replicates)			
Added (μg g^{-1})	10.1	40	81	120	10.0	41	79	121
Recovered (μg g^{-1})	10.6	41	83	122	10.9	44	84	127
Recovery (%)	104.6	102.5	102.5	101.7	108.3	107.3	108.9	105.0
RSD (%)	1.1	1.9	1.4	1.5	1.4	1.9	1.7	1.2
Average recovery (%)	102.8 (4 levels average)				107.4 (4 levels average)			
	Deprotected doripenem side chain (Average of 3 replicates)				*N*-Protected mercapto alcohol (Average of 3 replicates)			
Added (μg g^{-1})	29.6	40	79	119	13.4	41	81	121
Recovered (μg g^{-1})	25.3	36	72	114	15.1	44	85	126
Recovery (%)	85.5	90.0	91.1	95.7	112.7	107.3	104.9	104.1
RSD (%)	5.3	5.4	5.8	4.8	3.5	2.3	1.6	1.3
Average recovery (%)	90.6 (4 levels average)				107.3 (4 levels average)			
	Protected doripenem (Average of 3 replicates)				Accuracy of doripenem side chain (Average of 3 replicates)			
Added (μg g^{-1})	21.4	40	81	120	17.9	42	79	122
Recovered (μg g^{-1})	22.6	44	87	125	20.4	43	81	124
Recovery (%)	105.5	110.0	107.4	104.1	113.6	102.3	102.5	101.6
RSD (%)	1.7	1.4	1.3	1.2	2.0	2.4	2.2	1.9
Average recovery (%)	106.8 (4 levels average)				105.0 (4 levels average)			

p-nitrobenzyl malonate, 1β-methyl bicyclic ketoester, desilylated β-ketoester, 1β-methyldiazoazetidinone, deprotected doripenem side chain, *N*-protected mercapto alcohol, protected doripenem and doripenem side chain at a known concentration levels (about 80 μg g^{-1}) and injected each solution and determined the content of analytes. Achieved results of %RSD and 95% confidence interval for six determinations are summarized in Table-5.

Stability of solutions: Standard solution and sample solution spiked with these impurities at specification level were prepared and analyzed initially and different time intervals by keeping the solutions at 5 ± 3 ºC. Based on experimental data, the standard and sample solutions are stable up to 455 min at 5 ± 3 ºC.

Conclusion

A simple and novel optimized RP-HPLC method was developed and validated for the quantification of potential genotoxic impurities namely mono-*p*-nitrobenzyl malonate, 1β-methyl bicyclic ketoester, desilylated β-ketoester, 1β-methyldiazoazetidinone, deprotected doripenem side chain, *N*-protected mercapto alcohol and protected doripenem of doripenem monohydrate drug substance. The results of various validation parameters proved that the method is specific, selective, precise and accurate in doripenem monohydrate drug substance.

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CONFLICT OF INTEREST

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

TABLE-5
PRECISION RESULTS

Sample ID	Results (ppm)															
	Mono *p*-nitrobenzyl malonate		1β-Methyl bicyclic ketoester		Desilylated β-ketoester		1β-Methyl diazo azetidinone		Deprotected doripenem side chain		*N*-Protected mercapto alcohol		Protected doripenem		Doripenem side chain	
	MP	IP	MP	IP	MP	IP	MP	IP	MP	IP	MP	IP	MP	IP	MP	IP
Sample-1	85	84	75	71	82	78	83	82	79	84	84	80	86	94	78	86
Sample-2	86	83	75	71	82	77	84	79	75	78	85	78	86	91	80	85
Sample-3	86	83	75	71	82	78	84	79	72	72	85	79	87	87	80	85
Sample-4	86	83	75	70	82	77	84	79	70	69	85	78	87	83	80	84
Sample-5	86	83	77	71	82	77	84	79	68	69	85	78	87	86	82	82
Sample-6	89	82	78	70	85	76	87	78	69	71	88	77	89	85	83	84
Statistical analysis																
Mean	86	83	76	71	83	77	84	79	72	74	85	78	87	88	81	84
SD	1.4	0.6	1.3	0.5	1.2	0.8	1.4	1.4	4.2	6.0	1.4	1.0	1.1	4.1	1.8	1.4
RSD (%)	1.6	0.7	1.7	0.7	1.4	1.0	1.7	1.8	5.8	8.1	1.6	1.3	1.3	4.7	2.2	1.7
95% Confidence interval	1	1	1	1	1	1	1	1	4	6	1	1	1	4	2	1
Overall statistical analysis																
Overall mean	85		73		80		82		73		82		87		82	
Overall SD	2.0		2.9		2.9		2.9		5.0		3.8		2.9		2.5	
Over all RSD (%)	2.4		4.0		3.6		3.5		6.8		4.6		3.3		3.0	
Overall 95% confidence interval	1		2		2		2		3		2		2		2	

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