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A NEW STABILITY INDICATING RP-HPLC METHOD FOR THE DETERMINATION OF RELATED SUBSTANCES IN DORIPENEM MONOHYDRATE: BROAD-SPECTRUM CARBAPENEM ANTIBIOTIC DRUG

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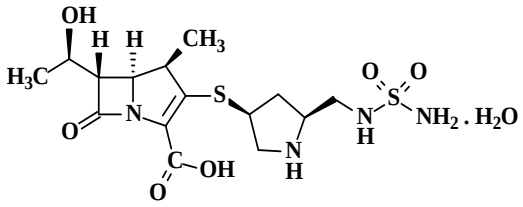
ABSTRACT
A new stability indicating RP-HPLC method has been developed, optimized and validated for the determination of related substances in Doripenem monohydrate (DORIBAX) drug substance. Successful chromatographic separation of Doripenem monohydrate from its related substances, namely Doripenemic acid was achieved on octadecyl silane chemically bonded to silica particles stationary phase i.e. Inertstil ODS-3V, (250 x 4.6) mm, 5µm column, maintained at temperature 40°C by using phosphate 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. UV detection: 230nm and 210nm for Doripenemic acid, flow rate: 1.0 ml/min and injection volume: 20µl. The proposed method is validated as per ICH guidelines in terms specificity, linearity, limit of detection (LOD) and limit of quantification (LOQ), precision, accuracy and robustness. Limit of detection of Doripenemic acid was 0.016(%w/w) indicating the high sensitivity of the developed method. The proposed method is useful in bulk drug analysis.

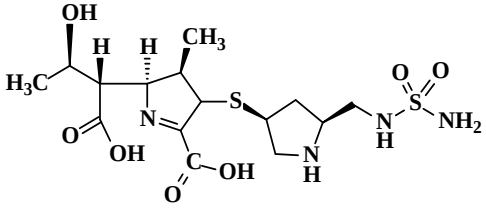
1. INTRODUCTION
Doripenem monohydrate, chemically known as (4*R*, 5*S*, 6*S*)-3-[[(3*S*, 5*S*)-5[[(Amino sulfonyl) amino] methyl]-3-pyrrolidinyl]thio]-6-[(1*R*)-1-hydroxyethyl]-4-methyl-7-oxo-1-aza bicycle [3.2.0] hept-2-ene-2-carboxylicacid monohydrate, molecular formula is $C_{15}H_{24}N_4O_6S_2.H_2O$ and molecular weight is 438.52. Doripenem comes under the carbapenem class drugs[1] with broad spectrum antibiotic activity. It is a ß-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 current a lower risk of comprising seizures than other carbapenems. It is sold under the brand name Doribax,[5] it is the fourth member of the carbapenem class. The recommended dosage of this drug is 500 mg and to be administered for every 8 hours by intravenous infusion.

The present work is aimed to develop and validate a RP-HPLC method for the determination of related substances in Doripenem monohydrate. The chemical structure of Doripenem monohydrate and its related impurity Doripenemic acid was shown in Figure 1.



Doripenem monohydrate



Doripenemic acid

Figure 1: Chemical structures of doripenem Monohydrate and Its related substance (Doripenemic acid).

Few analytical methods have been reported in literature for the determination of Doripenem monohydrate drug products and its related substances. Jose kurien described a stability indicating HPLC method for the determination of doripenem drug substance in pharmaceutical dosage.[6] Katarzyna reported the method for the determination of doripenem and its related substances using capillary electrophoresis.[7] In literature there is no method available for the determination of related substances in Doripenem monohydrate by HPLC. Hence the present work is a novel approach for the determination of related substances in Doripenem monohydrate by RP-HPLC.

Many pharmaceutical industries develop a drug impurity profile, which is defined as "a description of the identified and unidentified impurities present in a new drug substance". Impurities can be formed during drug synthesis, manufacturing and / or storage. Impurity of the drug is defined as 'any component of the new drug substance that is not the chemical entity defined as the new drug substance'. The drug impurities should be identified, qualified and/or quantified whether or not certain threshold (concentration) limits are exceeded. Impurity profile analyses are required to demonstrate the ability to detect a wide range of impurities which may occur in pharmaceuticals.[8-9] The chromatographic impurity profile should allow detecting and separating all (un) identified impurities in each new active compound. High performance liquid chromatography (HPLC) is routinely used for determination of both assay and impurities in both bulk active and formulated drug products.

The present research work represents the determination of related substances in Doripenem monohydrate by RP-HPLC. This method is very much useful in the bulk drugs analysis.

2. MATERIALS AND METHODS

2.1 Materials

Investigated samples of Doripenem monohydrate and Doripenemic acid were received as a gift from APL Research Centre-II Laboratories (A division of Aurobindo Pharma Ltd., Hyderabad). Diammonoium hydrogen orthophosphate, Ortho phosphoric 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).

2.2 Instrumentation and Chromatographic conditions

The HPLC systems used was Waters Alliance e2695 separation module equipped with 2489UV detector, Waters Alliance e2695 separation module with 2996 PDA detector with Empower data handling system *i.e.* Empower 3 software, [Waters Corporation, MILFORD, MA 01757, 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 5µm diameter [Inertsil ODS-3V, (250 × 4.6) mm, 5µm, Make: Inertsil], column oven temperature maintained at 40°C. A degassed mixture of pH 6.0 Buffer (2.64 g of diammonium hydrogen orthophosphate in 1000ml of water and adjusted pH 6.0 (± 0.05) with orthophosphoric acid, filtered through 0.45 µ or finer porosity membrane filter) and acetonitrile (98: 2 v/v) used as mobile phase-A, acetonitrile as mobile phase-B. A degassed mixture of mobile phase-A and mobile phase-B (98:2v/v) used as diluent. Flow rate: 1.0 ml/min, injection volume: 20 µl, UV detection: 230 nm, 210 nm for Doripenemic acid, chromatographic data acquisition time: 50 min. The pump adjusted in gradient mode and programmed as: Time (min)/ A (v/v): B (v/v); $T_{0.01}$/98:2, T_{15}/90:10, T_{30}/70:30, T_{40}/40:60, T_{50}/40:60, T_{52}/98:2, T_{60}/98:2.

2.3 Preparation of system suitability, Standard and Sample solutions

2.3.1 System suitability solution: 0.4 mg/mL concentration of Doripenem monohydrate dissolved in diluent.

2.3.2 Standard solution: 0.0012 mg/mL concentration level of Doripenem monohydrate used as standard. *i.e.* 40 mg of Doripenem monohydrate dissolved in 100 ml of diluent. Further diluted 5 ml to 100 ml and 3 ml to 50 ml using diluent.

2.3.3 Sample solution: 1.2 mg/mL concentration of solution using Doripenem monohydrate sample in diluent.

2.3.4 System suitability evaluation: The column efficiency was determined as Doripenem peak is not less than 10000 USP Plate count, USP tailing for the same peak is not more than 2.0 at 230nm. Relative standard deviation (RSD) for peak areas obtained from six injections of the standard solution was not more than 5.0% at 230nm and at 210nm. Chromatograms shown in **Figure 2**.

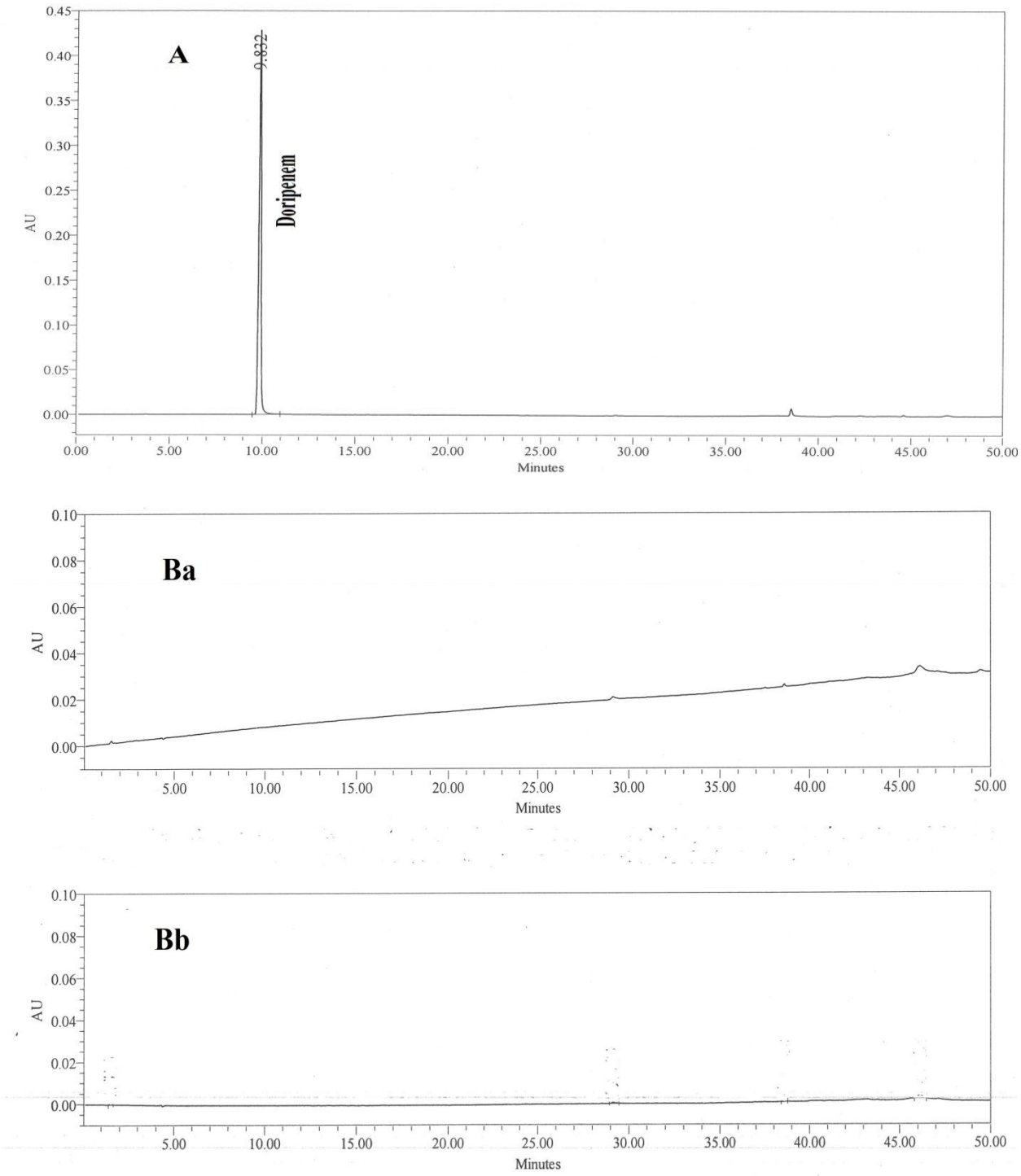


Figure 2: A, Chromatogram of SST and B, Chromatogram of diluent.

3. RESULTS AND DISCUSSION

3.1 Method development

The main aim of this method was to separate Doripenem monohydrate with its impurities. Various chromatographic parameters were tested and optimized in order to achieve the optimum separation between the Doripenem and its related substances.

3.1.1 Selection of column

Reverse phase compatible column was selected to separate the above said related impurities on HPLC. Trials were carried out on Symmetry shield RP18, 5µm (250mm x 4.6mm), X Terra MS C18, 5µm (250mm x 4.6mm); X Terra RP18, 5µm (250mm x 4.6mm); YMC Pack ODS-AQ, 5µm (250mm x 4.6mm); Inertsil ODS-3V, 5µm (250mm x 4.6mm). Though, the tested columns resulted optimistic separation, Inertsil ODS-3V, 5µm (250mm x 4.6mm) was found to be more suitable, reason for this criteria was believed due to its end capping 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, 5µm (250mm x 4.6mm) reverse phase column.

3.1.2 Optimization of buffer solution

The calculated pKa of Doripenem monohydrate is approximately at 2.8 and 7.9. Based on the chemical structure and pKa values of Doripenem monohydrate, optimization of buffer solution and effect of pH on retention time, separation parameters were detailed over a pH range between 2.0 and 8.0. Several buffer solutions were trialed for the separation of all said components. Aqueous mono potassium phosphate (KH_2PO_4) of 0.02 M solution afforded moderate separation and the peak shapes were not impressive. Another trial was performed using aqueous ortho phosphoric acid solution but the retention and separation was not achieved. Subsequently, another experimental trial was performed using 0.02M Ammonium dihydrogen orthophosphate ((NH_4) H2PO4) with as such pH, but the retention and separation were not achieved. Finally, the best separation was achieved using 0.02M Diammonium hydrogen orthophosphate ($(NH_4)_2HPO_4$) adjusted to pH 6.0 using orthophosparic acid.

3.1.3 Optimization of the 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 (0.02M Diammonium hydrogen orthophosphate ($(NH_4)_2HPO_4$) adjusted pH 6.0 with orthophosporic acid) and acetonitrile in the ratio of 98:2 v/v used as mobile phase-A and acetonitrile used as mobile phase-B. In these specified conditions, best separation was observed with shorter time.

3.1.4 Selection of UV detection

The response was studied on PDA detector under different nanometers, Doripenem has response at 295nm, 230nm and Doripenemic acid has response at 210nm. Hence, quantified the Doripenemic acid at 210nm and any other impurities quantified at 230nm including Doripenem.

3.1.5 Optimization of column oven temperature

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

3.2 Method validation

Optimized method validation has been done based on ICH guidelines[10] to ensure the method capability for determination of related substances in Doripenem monohydrate. The streamlined method validation parameters were specificity or selectivity, linearity, LOD & LOQ, recovery, precision (repeatability and intermediate precision) and robustness. Validation experiments were discussed in 3.2.1 to 3.2.8 sections.

3.2.1 System suitability

Injected the system suitability and standard solutions into the HPLC system and monitored the system suitability parameters such as USP plate count and USP tailing at 230nm, achieved 31756 and 1.0 respectively. Doripenem standard %RSD considered at 210nm and 230nm achieved 1.3 and 1.3 respectively.

3.2.2 Specificity

Specificity is the ability of assess unequivocally of analytic in the presence of components which may be expected to be present. For determination of specificity, injection of blank, individual related substances were prepared and injected to confirm the retention time. The solutions of Doripenem drug substance (control sample) and Doripenem monohydrate spiked with all known related substances (spiked sample) were prepared and injected into HPLC. Peak purity was established by using empower 3 software. The specificity results were tabulated in **Table 1a** and representative chromatograms shown in **Figure 3**.

Table 1a: Specificity results.

Identification	**Standard RT**	**Sample RT**
Doripenem	10.098	10.172
Spiked sample at 210nm	**Purity angle**	**Purity threshold**
Doripenemic acid	1.606	2.895
Doripenem	0.038	0.262
Spiked sample at 230nm	**Purity angle**	**Purity threshold**
Doripenem	0.029	0.237
Individual impurities for RT		
Doripenemic acid		3.907

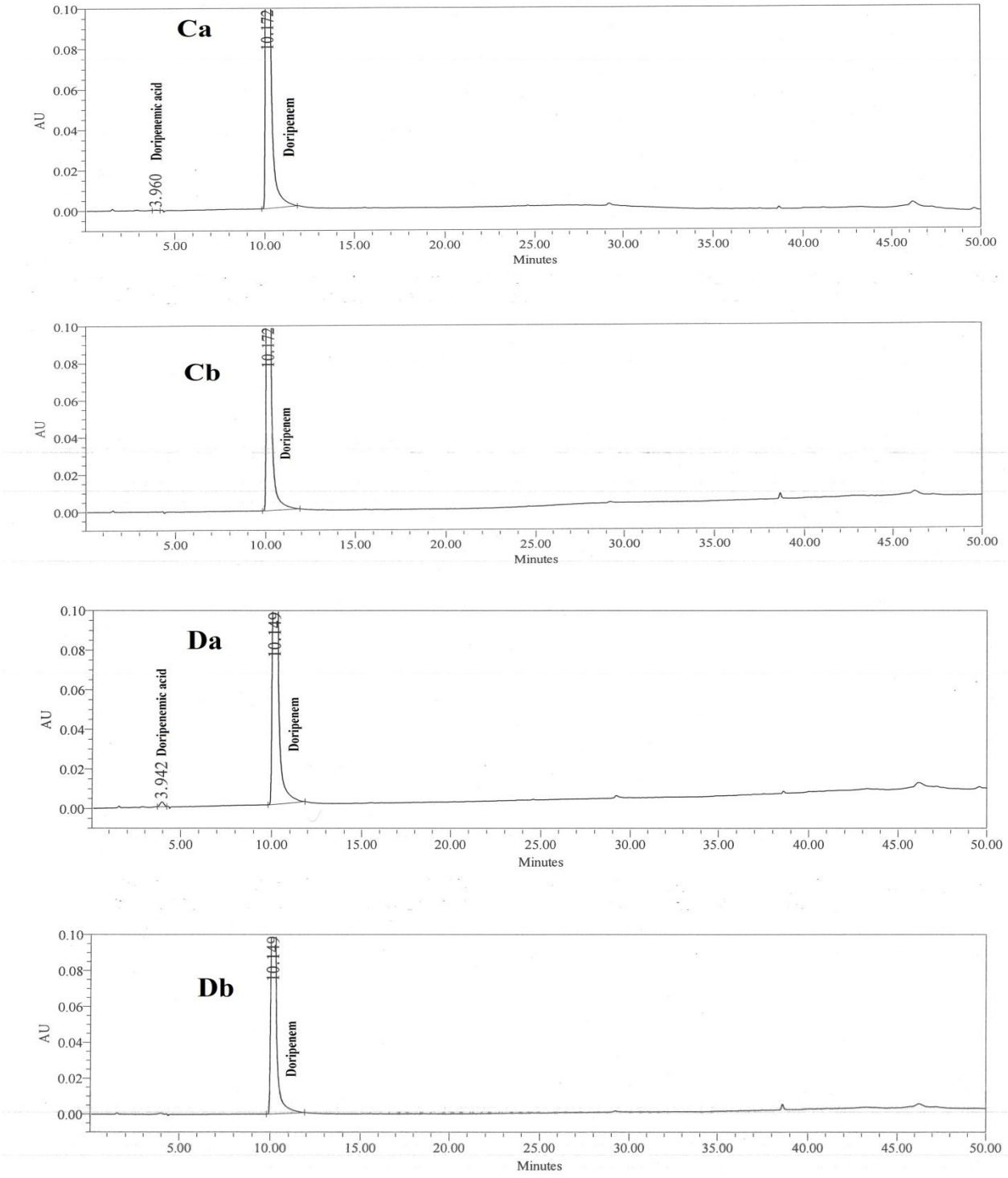


Figure 3: Ca & Cb, Chromatogram of Doripenem monohydrate drug substance at 210 & 230nm, Da & Db, Chromatogram of spiked sample at 210nm & 230nm.

3.2.3 Forced degradation

Forced degradation experiments were performed to establish the stability indicating nature of this method. Doripenem monohydrate sample was subjected to different stress conditions i.e alkaline/ acidic hydrolysis [0.1M NaOH/85°C/30min & 1M HCl/RT/Initial], peroxide degradation under oxidative stress [10%w/v hydrogen peroxide solution, RT/Initial], thermal degradation [105°C/120Hours], humidity degradation [90%RH/25°C/120Hours] and photolytic degradation [white fluorescent light, 1.2million Lux hours and UV light, 200watt hours per meter square] w.r.t ICH option 2 of Q1B.[11] In all stress conditions peak homogeneity of Doripenem was established by using PDA detector with empower 3 software ensured the stability indicating nature (specificity) of this method. Forced degradation results shown in T**able 1b**.

Table 1b: Specificity-Forced degradation results.

Degradation condition	At 210nm			At 230nm		
	Degr. (%)	Peak purity of Doripenem		Degr. (%)	Peak purity of Doripenem	
		Purity angle	Purity threshold		Purity angle	Purity threshold
- Undegraded Sample	-	0.006	0.253	-	0.048	0.240
Acid degradation (1M HCl/Initial)	8.9	0.009	0.264	9.5	0.077	0.215
Base degradation (0.1MNaOH/85°C/30min)	7.1	0.011	0.252	7.7	0.004	0.222
Peroxide degradation (10% H2O2/ RT/Initial)	5.5	0.013	0.253	6.1	0.004	0.215
Thermal degradation (105°C/120 hours)	6.6	0.012	0.252	7.5	0.049	0.219
Photolytic degradation Fluorescentlight & UV light[a]	1.3	0.010	0.256	2.1	0.055	0.219
Humidity degradation (90% RH/25°C/120 hours)	2.2	0.010	0.251	3.0	0.049	0.221

3.2.4 Linearity

Detector linearity of this method was determined by preparing the Doripenemic acid solutions ranging from LOQ to 150%, this data was applicable to statistical calculations by using linear- regression model, graphs plotted between peak area verses concentration of related substances (Linearity graphs shown in **Figure 4**). The Doripenemic acid correlation coefficient (R^2) was achieved to be 0.9997.

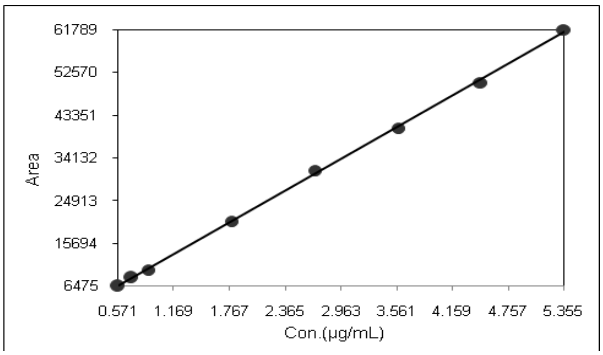


Figure 4: Linearity graph of doripenemic acid.

3.2.5 LOD and LOQ

LOD, LOQ values of Doripenemic acid was determined as 0.571µg/mL and 5.362µg/mL, respectively by using the linearity curve slope value. Precision was established by analysing six replicates of Doripenemic acid and %RSD for LOD and LOQ areas were found to be 3.1 and 2.3, respectively. These values indicate the tolerable sensitivity of the method for the determination of Doripenemic acid in Doripenem drug substance. LOD, LOQ data tabulated in **Table 2**.

Table 2: LOD, LOQ results.

S. No.	Doripenemic acid	
	LOD	LOQ
1	2069	6475
2	2014	6598
3	1957	6824
4	1942	6779
5	2092	6844
6	2062	6858
Mean area	2023	6730
SD	62	157
%RSD	3.1	2.3
Concentration (µg/mL)	0.188	0.571
Concentration (%w/w)	0.016	0.048

3.2.6 Accuracy

Accuracy of this method was performed by using standard addition procedure; it was determined by spiking the Doripenemic acid to Doripenem monohydrate sample at four concentrations i.e. LOQ, 50%, 100% and 150%level of specification and analyzed in triplicate. The Doripenemic acid recovery results laid between 94.0% and 100.0%. Overall four concentration levels average recovery was achieved 100.0 percentage, these results were shown the accuracy of this method (Results shown in **Table 3**)

Table 3: Accuracy results.

Recovery results of Doripenemic acid				
%Level/ sample ID Test conc.(2mg/mL)	**Conc. spiked in sample (%w/w)**	**Amount found (%w/w)**	**%Recovery**	**Statistical Analysis**
LOQ level sample-1	0.0490	0.0491	100.2	Mean 100.0
LOQ level sample-2	0.0487	0.0489	100.4	SD 0.53
LOQ level sample-3	0.0487	0.0484	99.4	%RSD 0.5
50%level sample-1	0.146	0.140	95.9	Mean 95.7
50%level sample-2	0.146	0.141	96.6	SD 1.01
50%level sample-3	0.148	0.140	94.6	%RSD 1.1
100%level sample-1	0.293	0.282	96.2	Mean 97.0
100%level sample-2	0.292	0.287	98.3	SD 1.12
100%level sample-3	0.293	0.283	96.6	%RSD 1.2
150%level sample-1	0.436	0.437	100.2	Mean 99.2
150%level sample-2	0.438	0.434	99.1	SD 0.91
150%level sample-3	0.439	0.432	98.4	%RSD 0.9
Over all mean				97.3
SD				1.78
%RSD				1.8

3.2.7 Precision

System precision solution was prepared by using Doripenem standard at 0.1% level to the test concentration, injected six replicates in to HPLC and calculated the system preision %RSD by using peak areas, it was found to be 1.3. Repeatability and intermediate precision peak areas %RSD were calculated by using the same batch of Doripenem monohydrate sample spiked with Doripenemic acid at 0.3% level, it was found to be 2.1 for repeatability and 1.2 for intermediate precision respectively (Results shown in **Table 4**).

Table 4: Precision results.

Name	**Repeatability (Set-I) (% w/w)**					
	Sample 1	**Sample 2**	**Sample 3**	**Sample 4**	**Sample 5**	**Sample 6**
Doripenemic acid	0.412	0.421	0.419	0.430	0.435	0.416
Intermediate precision (Set -II) (% w/w)						
Doripenem acid	0.409	0.405	0.407	0.395	0.399	0.404
Overall statistical analysis (Set-I and Set-II)						
Name	Overall mean	Overall SD	Overall % RSD		Overall 95% CI(±)	
Doripenem acid	0.413	0.012	2.9		0.008	

3.2.8 Robustness

Robustness of this method was checked by using variations in flow, column oven temperature, wavelength, pH of the buffer and acetonitrile variation in mobile phase-A. There is no change in the method even after changing by these parameters. Hence, under these conditions this RP-HPLC related substances method is robust (Results shown in **Table 5**).

Table 5: Robustness results.

Parameter	**Variation**	**USP plate count**	**USP tailing**	**RRT of**	
				Dorepenemic acid	**Doripenem**
Standard conditions	-	25363	1.3	0.39	1.00
Flow	-10%	26955	1.3	0.39	1.00
	+10%	21946	1.3	0.38	1.00
Column oven temperature	-5°C	26316	1.3	0.38	1.00
	+5°C	22146	1.4	0.39	1.00
Organic variation in	-2% absolute	39527	1.3	0.38	1.00

mobile phase-A	+2% absolute	15585	1.3	0.43	1.00
pH of Buffer	-0.2 units	27155	1.0	0.40	1.00
	+0.2 units	26660	1.1	0.38	1.00
Wavelength	-5nm	25449	1.0	0.39	1.00
	+5nm	25441	1.0	0.39	1.00

3.2.9 Solution stability

Solution stability was generated by using Doripenem monohydrate sample spiked with Doripenemic acid at specification level. Solution was injected into equilibrated HPLC system by keeping the sample cooler temperature at 5°C±3°C, generated at different time intervals and it was found to be stable up to 1hour at this condition.

4. Concluding remarks

The present research work describing the determination of related substances in Doripenem monohydrate drug substance. A simple and sensitive reversed phase gradient RP-HPLC method has been developed optimized and validated the method in terms of ICH guidelines. The validation experimental results demonstrated that the developed related substances method is stability indicating (*i.e.* forced degradation), accurate, sensitive, precise, linear, rugged and robust for the determination of related substances in Doripenem monohydrate and very much useful in the analysis of bulk drugs.

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