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A novel validated eco-friendly RP-UHPLC method for assay and related substances in Meropenem

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A simple, rapid, sensitive, specific, eco-friendly and stability-indicating linear gradient liquid chromatographic method (RP-UHPLC) for simultaneous estimation of assay and its related compounds in Meropenem API samples is developed and validated. Chromatographic separation was achieved on Zorbax Eclipse plus C18, (100 x 4.6) mm, 3.5 µm RRLC short column and 10 mM potassium dihydrogen orthophosphate is used as buffer, buffer solution used as eluent A and buffer and acetonitrile combination 30: 70 *v/v* ratio used as eluent B and Agilent RRLC (UHPLC) system is used for analysis. The mobile phase flow rate was 1.0 ml/min, and the eluted compounds have been monitored at 220 nm for related substance method and 290 nm for assay method. Excellent resolution is obtained between Meropenem and its related compounds which were eluted within 10 min. The correlation co-efficient(r) is > 0.995 for both the methods from linearity data and percentage of recovery is 98.0 to 102.0 and 80.0 to 120.0 % for assay method and for related substance method, respectively. Sensitivity of the method is found to be less than 0.316 µg/ml. Peak homogeneity data for Meropenem in the chromatograms from the stressed samples are obtained by using photodiode array detector demonstrated the specificity of the method for analysis of Meropenem in presence of the degradation compounds. The performance of the method is validated according to the present ICH guidelines for specificity, limit of detection, limit of quantification, linearity, accuracy, precision, and robustness.

Keywords: Meropenem, Related substances, Assay, Method development, Validation, UHPLC, RRLC

Meropenem, is (4R,5S,6S)-3-[[(3S,5S)-5-(dimethyl carbamoyl)-3-pyrrolidinyl]thio]-6-[(1R)-1-hydroxyethyl] -4-methyl-7-oxo-1-azabicyclo [3.2.0] hept-2-ene-carboxylic acid, trihydrate (Fig. 1). It is a broad-spectrum carbapenem antibiotic with wide range of activity. It is used mainly for the treatment of serious bacterial infections, including lower respiratory tract, intra-abdominal, obstetric/gynecological, urinary tract, skin structure, meningitis, cystic fibrosis and in febrile neutropenia[1,2]. Meropenem is metabolized inside the human body through hydrolysis to form the opened-lactam ring product which is pharmacologically inactive. In healthy volunteers, 70 % of the administered dose is excreted unchanged in urine and 20 % as the open-ring metabolite and it is a new parenteral carbapenem antibiotic with a very broad spectrum of antibacterial activity against the majority of gram-positive and gram-negative pathogens[3-6]. Meropenem is frequently used in intensive care units for treating severe infections caused by organisms resistant to other antibiotics, such Pseudomonas aeruginosa. As a β-lactam, its activity is dependent upon the time during which plasma concentrations stay above the MIC of the offending organism, triggering its use by extended infusion administration[7-9]. It is more active in vitro than imipenem against *Enterobacteriaceae* and *Pseudomonas aeruginosa*, but less active against gram positive *cocci*. *Meropenem* is more stable to ring opening by human renal dehydropeptidase -I (DHP-I) than imipenem and consequently does not require concomitant administration of a DHP-1 inhibitor. This antibiotic has shown clinical efficacy in the treatment of a wide range of serious infections such as

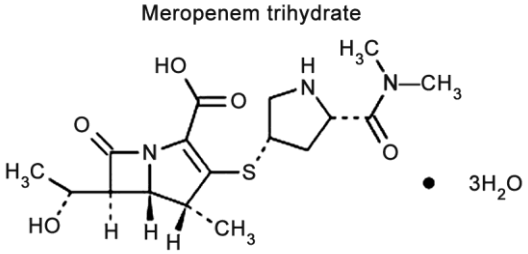


Fig. 1 — Chemical structure of Meropenem.

intra-abdominal infections, urinary tract infections and lower respiratory tract infections including patients with cystic fibrosis. These sterile carbapenems are in very high demand and the innovator of this Meropenem is M/S Astra Zeneca, brand name MERONEM®.

Meropenem was patented in 1983 and it was approved for medical use in the United States in 1996. It is on the World Health Organization's List of Essential Medicines, the most effective and safe medicines needed in a health system. The wholesale cost in the developing world is between 3.44 and 20.58 USD per one gram vial as of 2015. In the United Kingdom this amount costs the NHS about £16 in 2015[10-15]. The Meropenem was synthesized by various route of synthesis and selected compound synthesized by using MAP and Thiol intermediates compound by preparing protected Meropenem and followed by deprotection. The route of synthesis is given below in Fig. 2. An extensive literature survey revealed that there are certain methods reported for determination of Meropenem and its main metabolite (ICI-213689) in biological fluids, including high-performance liquid chromatography (HPLC), capillary zone electrophoresis, microbiological assay and few quantitation of Meropenem in pharmaceutical dosage form by combination of HPLC and UV-visible spectrophotometer[16-26]. An official compendia method is available for related substances and assay determination in United States Pharmacopoeia (USP). This method was based on the mobile phase containing triethyl amine as organic modifier which required more stabilization time at lower wavelengths and shortens the column life, more over impurity C is not eluting in stated method[27].

In our knowledge and after an exhaustive literature survey, there is no evidence of availability of methods on stability indicating gradient UHPLC method for estimation of assay and its related compounds of Meropenem in bulk and its formulation. Consequently, the implementation of novel analytical methodology to determine the assay and its related compounds in API and its formulation is a challenge of the pharmaceutical analysis. The objectives of the research work is to develop a suitable gradient stability-indicating short run time UHPLC method for analysis of Meropenem in API samples and to validate the method for specificity, LOD, LOQ, linearity, precision, accuracy, and robustness to show the stability-indicating power of the method and to ensure compliance with ICH guidelines.

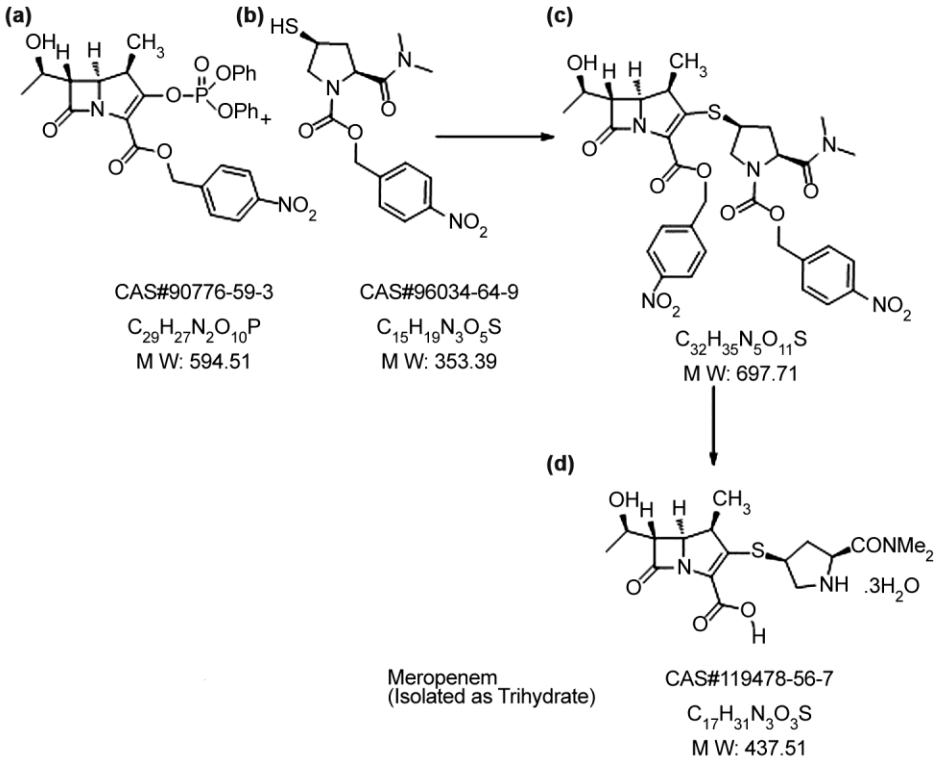


Fig. 2 — Route of synthesis of Meropenem.

Material and Methods

Materials

Meropenem reference standard was purchased from USP catalogue number 1392454, lot number was IOJ244 (USP Rockville, MD). Meropenem test sample and known impurity compounds (for specificity) were synthesized (impurity structures are shown in Fig. 3) at ecoLogic Technologies limited, Hyderabad, India. Acetonitrile and Potassium dihydrogen orthophosphate (HPLC–grade) were purchased from Merck Fine Chemicals (Mumbai, India). Sodium hydroxide (NaOH), hydrochloric acid (HCl) and hydrogen peroxide (H_2O_2) GR grade chemicals were also obtained from Merck Fine Chemicals (Mumbai, India). Milli-Q water is obtained from Millipore direct 8 l/h system.

Apparatus

The instrument Agilent 1260 Infinity series with a diode array detector (quaternary pump: G1311B, column thermostat: G1316B, Auto sampler with cooler: G1329B and G1330B and detector: G4212B) was used for the development of ultra-high performance liquid chromatographic method (UHPLC). The chromatographic data was recorded by using Chemstation (Agilent Technologies, Clara, US), peak purity of Meropenem was tested by using Waters Empower 3 software (Waters, US). A column, Zorbax Eclipse plus C18, (100 x 4.6) mm 3.5 µm RRLC, manufactured by Agilent (Agilent Technologies, Clara, US) was procured from LCGC India.

Optimized chromatographic conditions

The chromatographic separation was achieved on Zorbax Eclipse plus C18, (100 x 4.6) mm 3.5 µm RRLC column. In house prepared 10 mM potassium dihydrogen orthophosphate was used as buffer, eluent A is buffer solution and eluent B is a combination of buffer and acetonitrile in 30:70 v/v ratio with a linear gradient programme:time min / % B is 0/30, 5/90, 9.5/90,10/30 and 15/30 at flow rate of 1.0 ml/min. The analysis was carried out at UV detection of 220 nm for related substances determination and 290 nm for assay determination and column temperature was maintained at 25 °C with 10.0 µl injection volume. Samples were diluted in eluent-A and B in 1:1 ratio.

Preparation of solutions

Preparation of standard solution: Meropenem Standard solution is prepared about 1000 µg/ml for both assay and related compounds methods.

Preparation of impurities stock solutions: Meropenem related compound stock solutions were prepared using an estimation of about 100 µg/ml for related compounds.

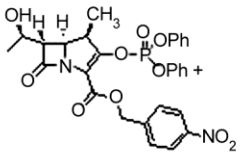
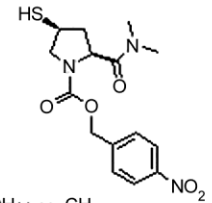
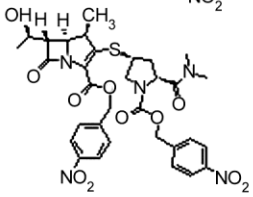
Impunity	Structure	Chemical Name	Molecular formula and Molecular weight
Impunity-A (MAP)		4-nitrobenzyl (4R,5R,6S)-3-((diphenoxyphosporyl)oxy) -6-((R)-1-hydroxyethy1)-4-methl1-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylate.	$C_{29}H_{27}N_2O_{10}P$ M W: 594.51
Impunity-B (Thiol)		4-nitrobenzyl (2S,4S)-2-(dimethylcarbamoyl)-4-mercaptopyrrolidine-1-carboxylate.	$C_{15}H_{19}N_3O_5S$ 353.39
Impunity-C (Protected Meropenem)		4-nitrobenzyl (4R,5S,6S)-3-(((3S,5S)-5-(dimethylcarbamoyl)-1-(((4-nitrobenzyl)oxy)carbonyl) pyrrolidin-3-yl)thio)-6-((R)-1-hydroxyethyl)-4-methyl-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylate.	$C_{32}H_{35}N_5O_{11}S$ 697.72

Fig. 3 — Chemical structure of related compounds of Meropenem.

Preparation of sample solutions: Sample solutions were prepared about 1000 µg/ml solution for both assay and related compounds methods.

Preparation of spike sample solutions: Spike sample solutions were prepared by spiking impurities about 1 µg/ml.

Specificity/Selectivity

Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities and a study was conducted to demonstrate the effective separation of degradants and stress studies were divided into two parts one is in liquid state and another one was solid state as described manner.

- Liquid state stress study conditions were optimized as given below

a) Acid hydrolysis using 0.1 N HCl at room temperature
b) Base hydrolysis using 0.1 N NaOH at room temperature
c) Oxidative hydrolysis using 3% H_2O_2 at room temperature

- Solid state stress study conditions were optimized as given below

a) Thermal stress at temperature 60 °C for 5 days
b) Photolytic degradation under
Ultraviolet light (200 watt hours/square meter),
Visible light (1.2 million Lux h)

Precision

For related compound method six solutions containing Meropenem (100 µg/ml) were spiked with related compounds solutions 0.5 µg/ml (0.10% of Meropenem concentration). Chromatography was performed and value of %RSD was calculated considering peak area for Meropenem and each related compounds. Similarly, intermediate precision of the method was also evaluated by another analyst, on a different day in the same laboratory.

For assay method six individual sample solutions were prepared Meropenem (1000 µg/ml) and calculated assay of the compound against standard solution and also checked % RSD for assay values for six determinations. Similarly, intermediate precision of the method was also evaluated by another analyst, on a different day in the same laboratory.

Limit of detection and Limit of quantitation

The limit of detection (LOD) and limit of quantitation (LOQ) for Meropenem and related compounds were determined. Precision study was also carried out at the LOQ level by injecting six (n=6) individual preparations and calculated the %RSD considering peak area for Meropenem and each related compounds.

Linearity

For the related compound determination method linearity was checked for related compound and Meropenem at lower concentration levels of 0.03% to 0.2% (i.e., 0.3 µg/ml to 2 µg/ml). The responses were measured as peak areas and plotted against concentration. The similar experiment was performed for assay method linearity by preparing the standard concentrations 80% to 120% at assay concentration level (800 µg/ml to 1200 µg/ml). The calibration curve was drawn by plotting the each impurity peak area versus its corresponding concentration. The correlation co-efficient, slope and Y-intercept for each impurity were determined.

Accuracy

The accuracy of the assay method was evaluated in triplicate (n=3) at the concentration levels of Meropenem 800, 1000 and 1200 µg/ml (80%, 100% and 120%) and the % recovery was calculated at each level. Similarly accuracy of the related substances method evaluated in triplicate (n=3) at the concentration levels of each related compound about 0.03%, 0.1% and 0.2% level and the %recovery was calculated for each related compound.

Stability of the solution

A sample solution of assay method and related substance method were checked at different time intervals up to 48 h by keeping solution at 5 °C and checked cumulative %RSD for the peak area of Meropenem and its related compounds.

Results and Discussion

Method development

The main aim of the method is to develop a rapid and single chromatographic method for estimation of assay and its related compounds in Meropenem API samples. Meropenem UV spectrum was shown in Fig. 4. As Meropenem and related impurities are containing betalactum groups, based on pKa value and solubility of the compound, various compositions of mobile phase were screened. Meropenem and its degraded products peak shapes were not improved below pH 4, various pH and different brand C18

columns were tried for the development and optimization, the best peak shapes and good retention were observed about pH 4.5. The chromatographic conditions were optimized in view of a stability indicating assay method, which can separate the drug from its degradation peaks with good resolution. Mobile phase consisting of phosphate buffer and acetonitrile, at a flow rate of 1.0 ml/min, was found to be satisfactory to obtain well-resolved peaks with better reproducibility and repeatability for Meropenem.

Specificity/Selectivity

All degradant peaks were resolved from Meropenem peak in the chromatograms of all stressed samples. The chromatograms of the stressed samples were evaluated for peak purity of Meropenem using Waters Empower software. For all forced degradation samples, the peak purity of Meropenem was passed as purity angle is less than the purity threshold with no purity flag. Thus, this method is considered to be stability indicating. According to stress study data, significant degradation was observed at acid and base hydrolysis and mild degradation was observed in oxidative hydrolysis. Similarly, mild degradation was observed in thermal and photolytic conditions and peak purity for Meropenem peak was passing in all the stressed samples and also there was no interference from degradation products from the analyte peak which are formed throughout the study. The assay of the Meropenem was quantitatively determined in all stress sample solutions against reference standard and the mass balance (%assay + % sum of all impurities + %sum of all degradation products) was tabulated in Table 1 and System suitability results are given in Table 2. A Typical chromatogram of system suitability & selectivty and stress study chromatograms (acid & base stress) are shown in Fig. 5 & 6.

Table 1 — Forced degradation results

Stress conditions	Duration	Assay (% w/w)	Total impurities (%)	Mass balance (% w/w)
Acid hydrolysis using 0.1N HCl (at 25°C)	2 h	91.9	7.8	99.7
Base hydrolysis using 0.1 N NaOH (at 25°C)	24 h	65.8	32.5	98.3
Oxidative degradation using 3 % H_2O_2 (at RT)	24 h	97.2	3.5	100.8
Photolytic degradation (controlled)	4 days	99.4	0.44	99.8
Photolytic degradation (un-controlled)	4 days	98.5	0.88	99.4
Thermal degradation at 60°C	5 days	99.3	0.34	99.6

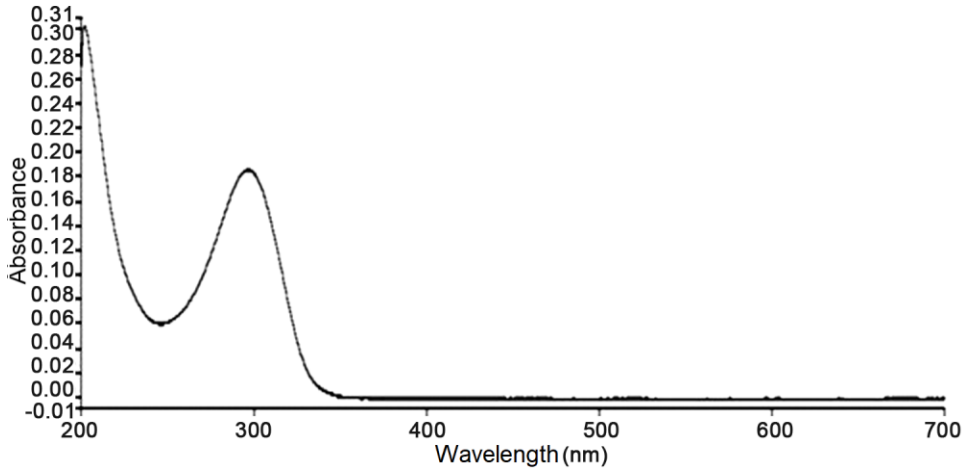


Fig. 4 — UV spectrum of Meropenem.

Table 2 — System suitability results

Name	Retention time (min)	Resolution (R_s) by Tangent method (USP)	USP Theoretical Plates	USP Tailing factor (T)
Meropenem	4.86	-	110000	1.07
Impurity-B	6.75	-	48000	1.19
Impurity-C	7.54	1.9	98000	1.02
Impurity-A	8.49	2.2	83567	1.02

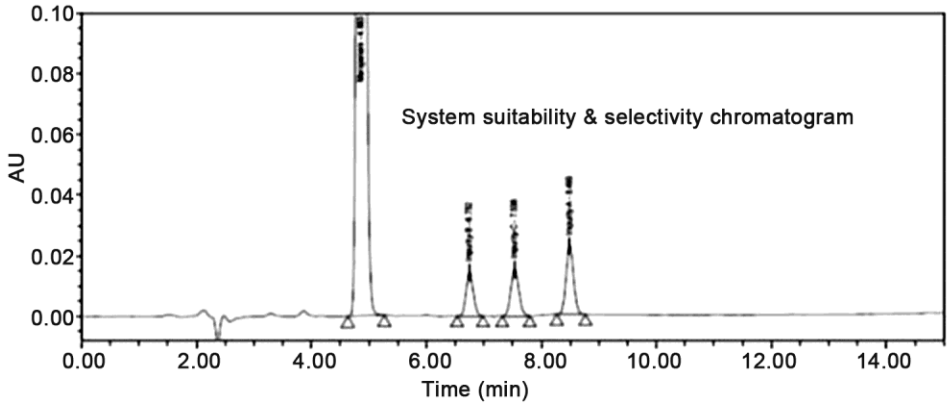


Fig. 5 — System suitability & selectivity chromatogram of Meropenem.

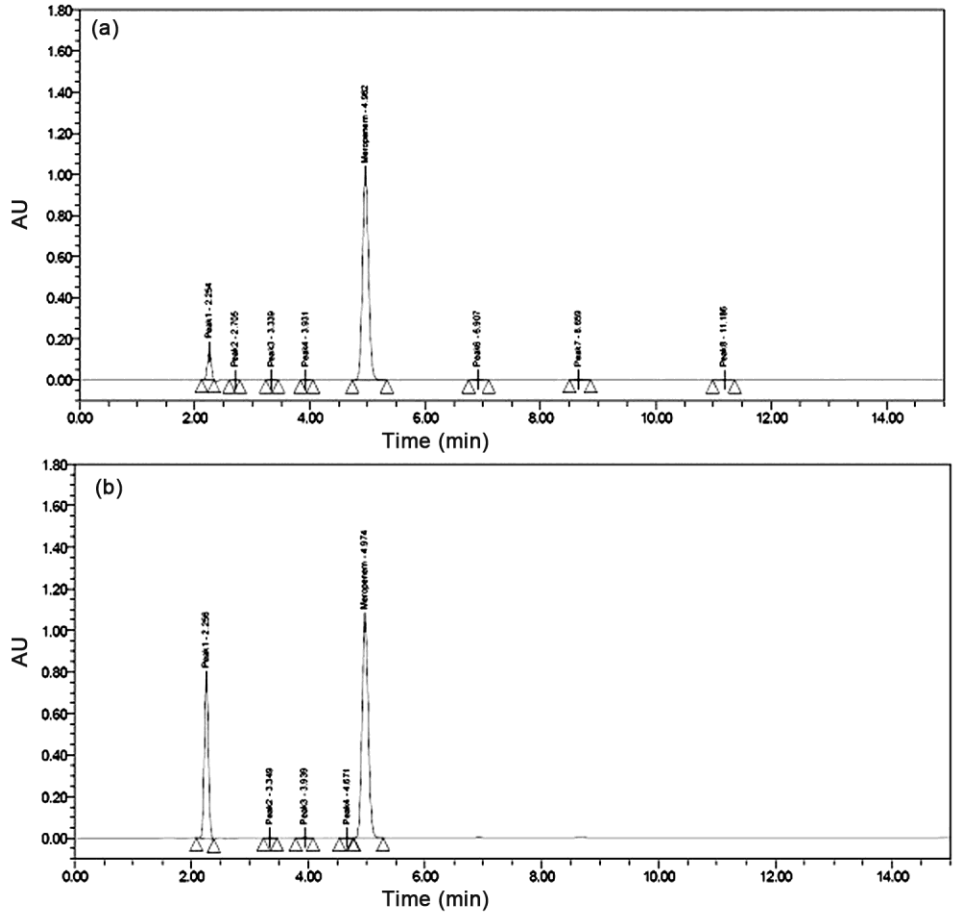


Fig. 6 — Stress study chromatograms.

Precision

The precision of the method for assay determination, the %RSD is below 0.47 and it was well within the acceptance range. The precision of the method for related compound determination, the %RSD is less than 4.8%. The %RSD of Meropenem assay results obtained in the intermediate precision is below 1.0 and related substance method is found be less than 15%. The method precision results are given in Table 3 and 4.

Limit of detection and Limit of quantitation

The LOD and LOQ for Meropenem and related compounds are found to be 0.09 and 0.32 µg/ml

respectively. The results are given in Table 5. Method sensitivity chromatograms (LOD and LOQ) were shown in Fig. 7.

Linearity

Calibration curve obtained by least square regression analysis between peak areas versus

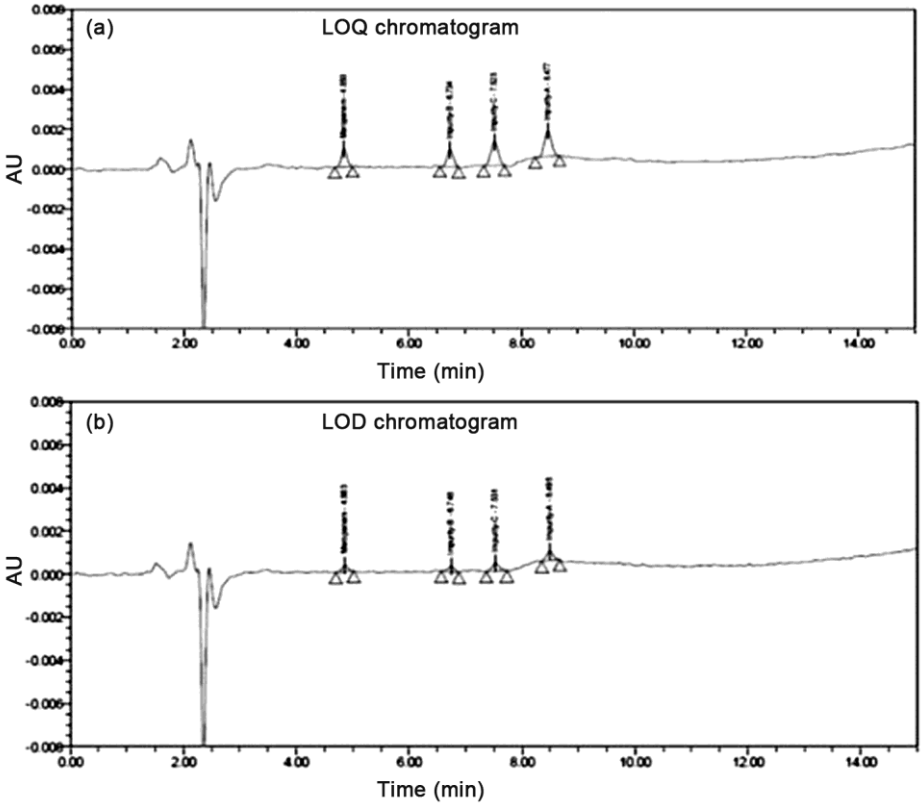


Fig. 7 — Method sensitivity chromatograms (a) LOQ and (b) LOD.

Table 3 — Method precision results for related substances method

Preparation	Impurity-A	Impurity-B	Impurity-C	Total Impurities
Preparation-1	0.112	0.105	0.093	0.712
Preparation-2	0.109	0.101	0.096	0.701
Preparation-3	0.111	0.104	0.089	0.719
Preparation-4	0.113	0.111	0.099	0.722
Preparation-5	0.108	0.103	0.092	0.718
Preparation-6	0.115	0.106	0.101	0.714
Average	0.111	0.105	0.095	0.714
Std dev	0.003	0.003	0.005	0.007
%RSD	2.32	3.24	4.75	1.04

Table 4 — Method precision results for assay method

Name	Meropenem
Preparation-1	99.1
Preparation-2	99.9
Preparation-3	99.7
Preparation-4	98.9
Preparation-5	99.1
Preparation-6	98.7
Average	99.2
Std dev	0.468
%RSD	0.47

Table 5 — Method sensitivity results

Parameter	Meropenem	Impurity-A	Impurity-B	Impurity-C
LOD (µg/ml)	0.0948	0.0894	0.0882	0.0714
LOD S/N	4.5	6.0	4.0	8.2
LOQ (µg/ml)	0.316	0.298	0.294	0.238
LOQ S/N	13.8	16.9	14.1	24.2
LOQ Precision %RSD (n=6)	2.1	3.8	2.3	1.2

concentration showed linear relationship with regression coefficient of 0.999 for Meropenem and ≥ 0.997 for related compounds respectively over the calibration ranges tested. The results are demonstrated that an excellent correlation between the peak area and concentration. The linearity results are given in Table 6 and 7 and the obtained calibration curve is given in Fig. 8 and 9.

Accuracy

The accuracy of the assay method is determined in percentage recovery of Meropenem from bulk drug samples ranged from 99.9% to 100.0%. The percentage recovery of the five impurities from bulk drug samples ranged from 95.9% to 104.2% and the results are shown in Table 8 and 9.

Solution stability

The %RSD of Meropenem and its related compounds peak areas were found be less than 2% and 10% respectively. The stability of Meropenem

Table 7 — Linearity results of assay method

Conc. (µg/ml)	Meropenem
834	35997367
938	40512214
1042	44968603
1146	49667822
1250	54011789
Correlation (r)	0.9999
Regression (r^2)	0.9999
slope (m)	43363
Y-intercept (c)	-152893
% Y-intercept	-0.3

Table 6 — Linearity results of related substances method

Conc. (µg/ml)	Meropenem	Conc. (µg/ml)	Imp-A	Conc. (µg/ml)	Imp-B	Conc. (µg/ml)	Imp-C
0.316	12909	0.298	18572	0.294	16430	0.238	20492
0.537	21238	0.507	30554	0.500	27029	0.405	33712
1.074	41643	1.013	59909	1.000	52999	0.809	66102
1.613	66213	1.521	95255	1.500	86268	1.215	106102
2.137	88284	2.015	123413	1.988	109588	1.609	130526
Correlation (r)	0.9994	Correlation (r)	0.9994	Correlation (r)	0.9987	Correlation (r)	0.9978
Regression (r^2)	0.9988	Regression (r^2)	0.9989	Regression (r^2)	0.9975	Regression (r^2)	0.9957
slope (m)	41600	slope (m)	61783	slope (m)	56048	slope (m)	82320
Y-intercept (c)	-1174.7	Y-intercept (c)	-614.7	Y-intercept (c)	-746.5	Y-intercept (c)	988.6
% Y-intercept	-2.8	% Y-intercept	-1.0	% Y-intercept	-1.4	% Y-intercept	1.5

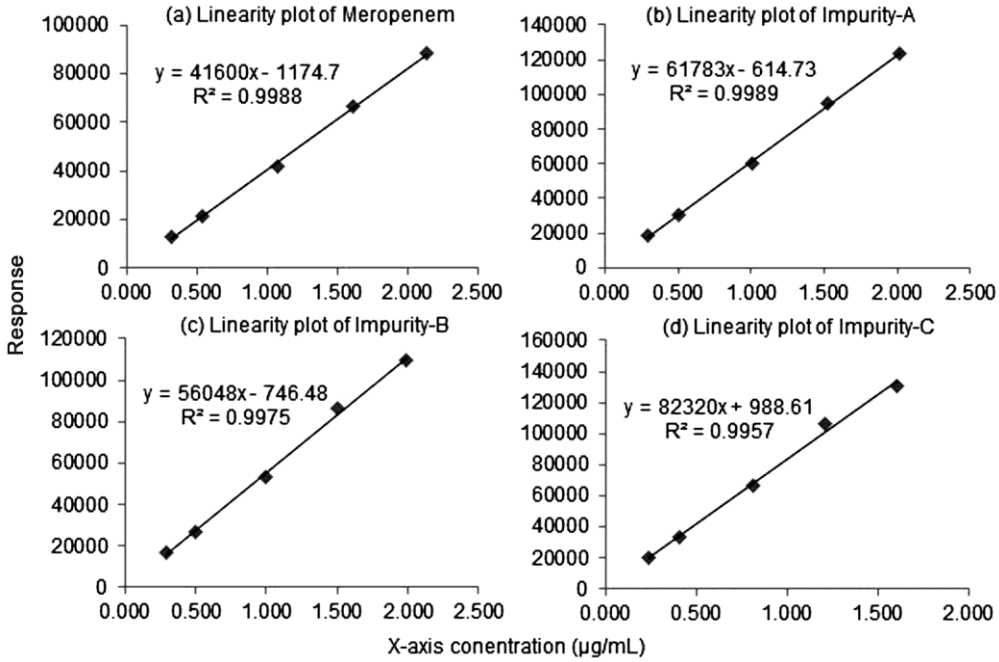


Fig.8 — Linearity plots of related substances.

Table 8 — Results of accuracy for assay method

Compound Name	Level (%)	Amount added (in µg/ml)	Amount recovered (in µg/ml)	% Recovery
Meropenem[a]	80	833.6	833.7	100.0
	100	1042.0	1040.5	99.9
	120	1250.4	1249.1	99.9

[a]*Assay of Meropenem concentration (1000 µg/ml).*

Table 9 — Results of accuracy for RS method

Compound Name	Amount added (in µg/ml)	Amount recovered (in µg/ml)	% Recovery
Impurity-A	0.298	0.311	104.2
	1.013	0.980	96.7
	2.015	2.007	99.6
Impurity-B	0.294	0.306	104.2
	1.000	0.959	95.9
	1.988	1.969	99.0
Impurity-C	0.238	0.237	99.5
	0.809	0.791	97.7
	1.609	1.574	97.8

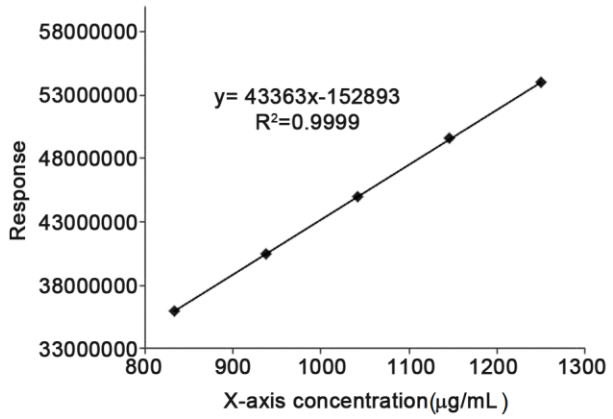


Fig. 9 — Linearity plot of Meropenem at assay concentration level.

sample solution by assay method and also related substances method are stable up to 48 h at 5 °C.

Robustness

The robustness of the method was determined as a measure of the analytical method capability, which is to be unaffected by small variation in method parameters[15]. The variations such as flow rate by ± 0.2 ml/min, variation in column temperature by ± 5°C and slight variation in wavelength ± 2 nm. At these changed conditions the system suitability was evaluated at each condition. In all the conditions, the resolution between critical pair was greater than 1.7 and tailing factor of Meropenem peak is found be less than or equal to 1.5 **(**Results are given in Table 10).

Table 10 — Results of robustness evaluation data

Chromatographic changes	Resolution[a]	Tailing factor[b]	Theoretical plates[b]
Flow rate (ml/min)			
0.8	2.3	1.42	89000
1.2	1.6	1.03	123657
Temperature (°C)			
20	2.2	1.36	98234
30	1.8	1.13	110498
Wavelength(nm)			
218	1.9	1.09	106256
222	1.9	1.09	108381

[a] *Resolution between impurity-B and C peaks*
[b]*Meropenem peak*

Conclusions

The developed gradient stability-indicating reversed phase high performance liquid chromatographic method (RP-UHPLC) method has shown excellent selectivity between impurities along with Meropenem and objective of the development is achieved on short column i.e., RRLC Zorbax eclipse plus C18 100 x 4.6 mm, 3.5 µm to reduce the run time of the method to have a quick turnaround time for the routine samples analysis, which was more economic and environment friendly to minimize the HPLC effluent waste when compare to other reported conventional HPLC methods. The developed method is 15 min of the run time and the Meropenem and its related compounds were eluted within 10 min. The present method was found to be stability-indicative for assay determination and validation data indicates, method was simple, linear gradient method and it was found to be specific, precise, linear and accurate. The main advantage of developed method is suits eco-friendly method than published methods in terms of analysis time, cost, effluent waste and outcome of the analysis. Hence, it can be used successfully for the routine analysis of Meropenem API samples and for analysis of stability samples obtained during accelerated stability study.

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