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Original Article

DEVELOPMENT OF VALIDATED STABILITY INDICATING METHOD BY RP-HPLC FOR SIMULTANEOUS ESTIMATION OF MEROPENEM AND VABORBACTAM IN BULK AND PHARMACEUTICAL FORMULATION

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ABSTRACT

Objective: To develop a simple, rapid, precise and reproducible liquid chromatographic method for the estimation meropenem (MEP) and vaborbactam (VAB) in bulk and pharmaceutical formulation and study of the stability of the drugs in different stressed conditions.

Methods: The chromatographic separation was achieved on a Kromasil C18 column (250 × 4.6 mm) using a mobile phase composition of acetonitrile and 10 mmol phosphate buffer (pH 3.50) in a ratio 30:70 v/v, pumped at a flow rate of 1.0 ml/min with UV detection set at 260 nm.

Results: Symmetrical and sharp peaks of MEP and VAB were obtained at retention times of 2.29 and 3.10 min, respectively. The chromatographic method was validated for linearity, limits of detection and quantitation, precision, accuracy, system suitability and robustness. Calibration curves were obtained in the concentration ranges of 25–150 µg/ml for MEP and VAB. Stability tests done through the exposure of the analytes solution for different stress conditions and the obtained results indicate no interference of degradants with HPLC method.

Conclusion: The proposed method has been found to be selective, precise, linear, accurate, and sensitive. The method can be successfully applied to the assay determination of bulk drugs and combined dosage forms for routine analysis.

Keywords: Meropenem, Vaborbactam, Method development, RP-HPLC

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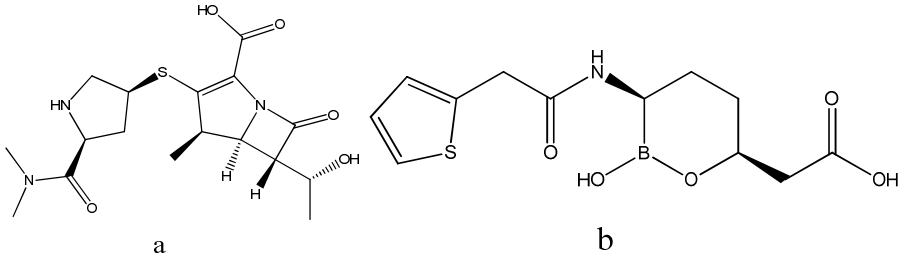
INTRODUCTION

The threat of antimicrobial resistance is rising globally at alarming rates, leading to a major hazard to patients. The Centers for Disease Control and Prevention (CDC) has categorized carbapenem-resistant *Enterobacteriaceae* (CRE) as the highest warning level of 'urgent' and the WHO has deemed CRE as one of the three vital pathogens in need of new antimicrobial options [1-3]. The mortality rates in patients with invasive infections which are caused by CRE have historically been reported to be as high as 70%. Therefore, the discovery and development of new regimen options are critical public health priorities, underlining the need for newer therapies for treating gram-negative pathogens. Meropenem–Vaborbactam (Vabomere™), a carbapenem and first-in-class boronic acid-based β-lactamase inhibitor combination product recently approved by the FDA for the treatment of complicated urinary tract infections, including pyelonephritis [4].

Meropenem (MEP), (4R,5S,6S)-3-(((3S,5S)-5-(dimethylcarbamoyl) pyrrolidin-3-yl)thio)-6-((R)-1-hydroxyethyl)-4-methyl-7-oxo-1-azabicyclo [3.2.0] hept-2-ene-2-carboxylic acid is a carbapenem class of antibiotics which have in common a carbon atom substituted for sulfur at place 1 and an unsaturated bond between C2 and C3 of the familiar penicillin nucleus (fig. 1a) [5]. The basic dimethyl-carbamoylpyrrolidinethio side chain at C2 on MEP enhances activity against gram-negative organisms. Carbapenems exert their bactericidal action through penicillin-binding proteins (PBPs) with subsequent inhibition of cell wall synthesis. TheMEP may give a stronger antibiotic backbone compared to cephalosporins when combined with carbapenemase inhibitors.

Vaborbactam (VAB), (3R, 6S)-2-hydroxy-3-[[2-(2-thienyl) acetyl] amino]-1, 2-oxaborinane-6acetic acid is a novel non-β-lactam, cyclic boronic acid inhibitor of β-lactamases, based on a cyclic boronic acid pharmacophore (fig. 1b)[5]. It has mainly potent action against *Klebsiella pneumoniae* carbapenemase (KPC). The cyclic boronate ester was designed to configure the structure in a preferred conformation to selectively enhance substrate activity against beta-lactamases. The potency of beta-lactamase inhibitor activity has increased by the addition of a 2-thienyl acetyl group in the structure of VAB [6]. The VAB largely has an action in molecular class A and C β-lactamase inhibitors, which also incorporate imipenem-resistant and *Serratia marcescens* enzyme [7]. The structures of MEP and VAB are shown in fig. 1.



CDC. Antibiotic Resistance Threats in the United States, 2013. http://www. cdc. gov/drug resistance/threat-report-2013/pdf/ar-threats-2013-508. p

Fig. 1: Chemical structures of (a) MEP and (b) VAB

The Code of Federal Regulations (CFR) 311.165c explicitly states that "the accuracy, sensitivity, specificity, and reproducibility of test methods employed by the firm shall be established and documented". The parameters performed for the development of analytical method validation can be used to establish the quality, consistency and reliability of analytical results. The combination MEP and VAB introduced as an injection, for intravenous use. The method used in the formulation of drug delivery system (DDS) undergoes into different processes such as solvent evaporation or solvent removal techniques may result in degradation of the drug content. The method validation of MEP and VAB has to be performed that it could attain at most precise and accurate therapeutic efficacy by the techniques and procedure of formulation is potential in resulting desired therapeutic effect.

Numerous analytical methods have been reported for the analysis of MEP in single or with the combination of other drugs in pharmaceutical formulations and several biological fluids, including human plasma using different sample preparation procedures: precipitation, liquid-or solid-phase extraction, generally followed by liquid chromatography coupled to UV detection or mass detection [8-14]. Spectrophotometric methods were also reported for the determination of MEP [15-16]. However, no stability indicating the method is available for simultaneous estimation of MEP and VAB in a combined pharmaceutical dosage form. Thus, the purpose of the present study was to develop and validate the Rp-HPLC method for the estimation of pure MEP and VAB, also the drug content of MEP and VAB in a pharmaceutical formulation as per ICH guidelines.

MATERIALS AND METHODS

Chemicals and reagents

Pure active pharmaceutical ingredients, MEP and VAB, were kindly supplied by Aurobindo Pharma Ltd. (Telangana, India). MEP and VAB Injection (VABOMERE) Manufactured by Facta Farmaceutici. Acetonitrile and methanol of HPLC grade were procured from Merck Specialties Pvt. Ltd. (Mumbai, India). AR grade Potassium dihydrogen phosphate and ortho-phosphoric acid were purchased from Spectrochem Pvt. Ltd. HPLC grade water was obtained by using a Millipore Milli-Q water purification system (Millipore, Milford, USA).

Instrumentation

The chromatographic analysis was carried out on the Waters HPLC Alliance 2695 separating module using photodiode array detector (waters 2998) with autosampler and column oven. The instrument was controlled by Empower 2 software installed with equipment for data collection and acquisition. UV-VIS spectrophotometer PG Instruments T60 with UV win 6 Software was used for measuring the absorbance of MEP and VAB solutions. A Kromasil C18 250 x 4.6 mm, 5µ analytical column was used.

Chromatographic conditions

The mobile phase consisted of buffer and acetonitrile (70:30 v/v). The buffer used in the mobile phase contained 10 mmol potassium dihydrogen phosphate, pH adjusted to 3.50±0.02 with ortho-phosphoric acid, filtered through 0.45 µm nylon filter and degassed in ultrasonic bath prior to use. Measurements were made with an injection of volume 10 µL and UV detection at 260 nm. All analyses were performed at ambient temperature.

Standard and sample solutions preparation

Standard stock solution

The standard solution was prepared by dissolving the drugs in the acetonitrile and diluting to the required concentration. The diluent water: acetonitrile (50:50 v/v) was used as a solvent system. Accurately weighed 1000 mg of dry powder (for injection) and transferred to 500 ml volumetric flask, to this 5 ml of acetonitrile was added and degassed in the ultrasonic bath. Volume was made up to 500 ml with diluents and filtered through a 0.45 µm nylon filter to get a solution of 2000µg/ml of each drug.

Preparation of sample solutions

5 ml of filtered sample stock solution was transferred to a 100 ml volumetric flask and filled the volume up to the mark with diluent to get a solution of concentration 100µg/ml of each drug.

Method validation

The optimized chromatographic conditions were validated in accordance with the ICH guidelines of Validation of Analytical Procedure: Q2 (R1) by evaluating the linearity, range, specificity, precision, accuracy, limit of detection (LOD), limit of quantification (LOQ), robustness and system suitability parameters. To assess the linearity and range of the developed method, six different mix standard concentrations (25, 50, 75, 100, 125 and 150 µg/ml) of MEP and VAB were prepared. The accuracy and precision were calculated by performing the assay of samples (spiked placebos) prepared at three concentration levels of 50%, 100% and 150% of the standard concentration, with three replicates for each concentration. The % recovery and %RSD were calculated for each of the replicate samples. The limit of detection (LOD) and limit of quantification (LOQ) of the proposed method were determined by the standard deviation of the response (σ) and slope approach as defined in ICH guidelines. The LOD was calculated using the formula 3.3*σ/slope, and the LOQ was calculated using the formula 10*σ/slope. The robustness of the method was established by the study of the effect of small modification of method parameters such as flow rate, the percentage of a buffer in the mobile phase and column oven temperature. The ruggedness of the method was tested by studying the effect of elapsed assay times and by an analyst on the method performance [17-18].

Forced degradation study

To evaluate the stability-indicating properties and specificity of the method, Forced degradation studies were carried out on MEP and VAB. Forced degradation was performed by exposing the drug substance and drug product to different stress conditions. The conditions illustrated in table 1 were followed in the stress study protocol. Stressed samples were analysed periodically and the presence of related peaks, retention time and peak purity for the active ingredients was checked [19-21].

Table 1: Forced degradation conditions

Stress type	Conditions
Acid hydrolysis	1 mg/ml in 1 N HCl at 80 °C for 2 h
Base hydrolysis	1 mg/ml in 2 N NaOH at 80 °C for 2 h
Oxidative degradation	1 mg/ml in 20% H2O2 at 80 °C for 2 h
Thermal degradation	1 mg/ml in 80 °C for 48 h
Photo degradation	Overall illumination of 200Wh/m2 at 25 °C for 7 d

RESULTS AND DISCUSSION

In present work stability indicating analytical method for the determination of MEP and VAB in bulk drug and injection formulation was developed and validated as per ICH guidelines for analytical method validation, Q2 (R1).

Method development

The main purpose of this work was to develop new stability indicating RP-HPLC method for estimation of MEP and VAB within a short run time. The mobile phases and stationary phase play an important role in theoretical plates, peak shape, symmetry and

resolution. To obtain symmetrical peaks with better resolution and peak purity, a variety of chromatographic conditions were investigated and optimized for the determination of MEP and VAB; such as mobile phases with different composition, pH and stationary phases with different packing material etc.

The UV spectrum showed that MEP and VAB have a maximum absorption at 260 nm. The MEP and VAB have a high ratio of carbon to heteroatom and has a conjugated bond. Therefore, MEP and VAB can be separated on a C18 column mainly based on their overall hydrophobicity. Trials were made by using three kinds of columns (Kromosil C18, XTerra C18 and Zorbax SB-C18, column) with different mobile phase compositions and ratios. In all of the proceeding columns, broad characteristic peaks were obtained through using different ratios (10:90, 30:70, 50:50, 70:30, 90:10) of methanol/acetonitrile and water. No improvement in peak shapes was obtained even when the temperature of the column was increased to 40 °C. The theoretical plates with the mixture of acetonitrile or methanol with water as a mobile phase were below 1000 which indicated poor column chromatography separation power. The peak shape and peak symmetry were all poor with the above two kinds of mixtures, which might be accredited to the low polarity of the mobile phase. So phosphate buffer with different concentration (10, 20 and 50 mmol) was used to enhance polarity of the mobile phase, which resulted in a narrowed peak shape. However, the peak shape was still not satisfactory. So acetonitrile was used in place of methanol.

So, the mobile phase mixture solution of phosphate buffer (10 mmol) and acetonitrile (70:30, v/v) was demonstrated to be the suitable for the improvement of peak shape and peak symmetry. With the exception of the composition of mixture solution, buffer pH was also found to be significant in the analyte separation and method optimization. The impact of buffer pH on retention time was related with the ionization form of the solute. A series of mixture solutions with different pH values (3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0) were used to investigate the retention time and resolution of MEP and VAB in which the rest of chromatographic parameters were kept unchanged.

Finally, the mobile phase containing phosphate buffer (10 mmol potassium dihydrogen phosphate) and acetonitrile in 70:30v/v ratio (pH 3.50±0.02, adjusted with orthophosphoric acid) was preferred and found to be optimal with more theoretical plates, narrow peak, high peak symmetry and short retention time. Based on the optimal mobile phase, a highly symmetrical and sharp characteristic peak of MEP and VAB was further obtained on a Kromasil C18 column with 1.0 ml/min flow rate. A typical HPLC chromatogram obtained during simultaneous estimation of MEP and VAB is given in fig. 2.

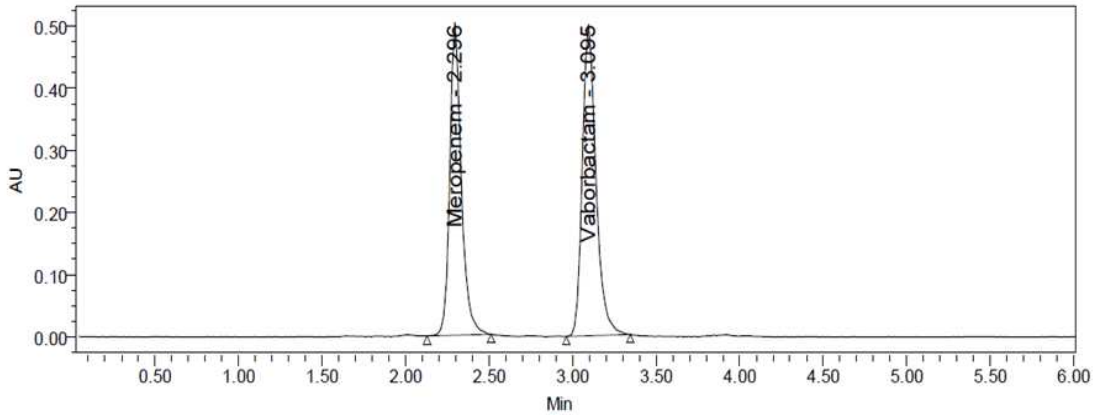


Fig. 2: HPLC chromatogram obtained during simultaneous separation of MEP and VAB. Chromatographic conditions: Kromasil C18 column (150 mm × 4.6 mm, particle size 5 µ); mobile phase phosphate buffer 10 mmol potassium dihydrogen phosphate and acetonitrile 70:30 v/v, (pH adjusted to 3.50±0.02 with orthophosphoric acid); at flow rate of 1.0 ml/min; and UV detection at 260 nm

Method validation

An optimized method must be validated before actual use. As per ICH guidelines for analytical method validation, Q2 (R1) the system suitability testing was performed. The validation studies were performed as given in the following sections.

Specificity

The specificity studies proved the no interference since no other peak appeared at the retention time (2.30 and 3.10 min) MEP and VAB. Moreover, interaction studies indicated that the analytes did not interact with each other and were well within the acceptable level 2.0% of RSD.

Range and linearity

Six different concentrations (25, 50, 75, 100, 125 and 150 µg/ml) of the mixture of MEP and VAB were prepared for linearity studies. The calibration curves obtained by plotting peak area against concentration showed a linear relationship. Calibration curves with corresponding residual plots of MEP and VAB were shown in fig. 3. The linear regression equations for MEP and VAB were found to be y = 4826. x+2593, and y = 4887. x+6194, respectively. The regression coefficient (R^2) values for MEP and VAB were noted 0.999 and 0.999, respectively. The results confirmed that there was an excellent correlation existed between concentration and peak area of the drug within the selected concentration range. The results established the linearity and the reproducibility of the assay method. Regression characteristics of the proposed HPLC method are given in table 2.

Precision

The intra-day precision of the developed method was determined by preparing the samples of the same batch with three concentrations and three replicate each. The inter-day precision was also determined by assaying the dosage form in triplicate every day for three consecutive days. The low value of RSD (%) represented that the method is precise within the acceptance limit of 2.0%. The intra and inter-day precision data are given in table 3. The results showed good precision of the developed method.

Accuracy

The recovery experiments were performed by adding known amounts of the drugs in the placebo at three levels: 50%, 100% and 150% of the label claim of the marketed formulation. Three samples were prepared for each recovery level. The solutions were then analyzed and the percent recoveries were calculated from the calibration curve. The mean recovery values were found to be 99.30% and 99.83%. The results showed that there was no interference of excipients. The results of accuracy are shown in table 4.

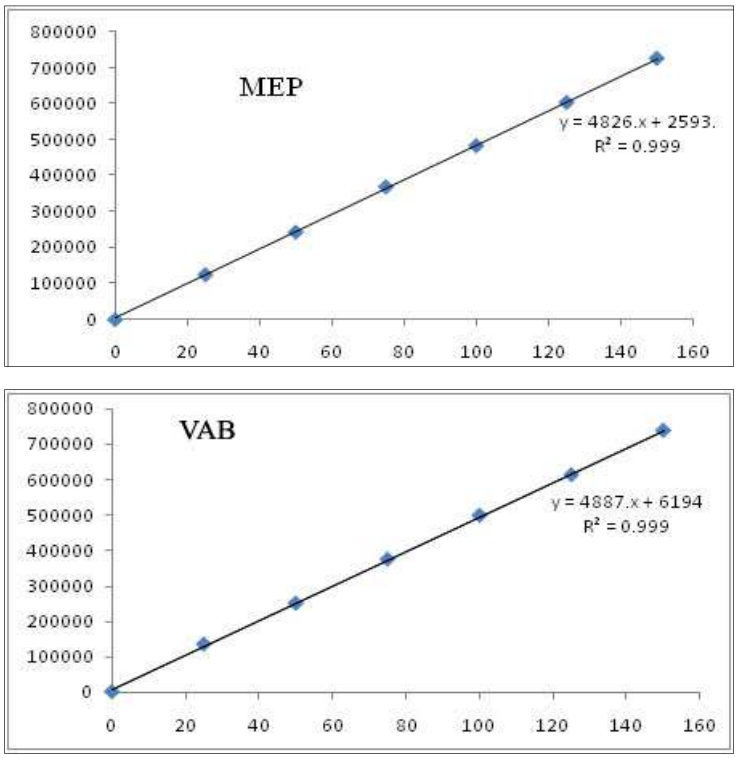


Fig. 3: Linearity plots for MEP (a) and VAB (b)

Table 2: Linearity parameters for the MEP and VAB

Linearity parameter	MEP	VAB
Range (%v/v)	25-150	25-150
Slope	4826±100	4888±114
Intercept	2594±211	6194±300
Regression Coefficient (r^2)	0.999±0.001	0.999±0.001

Table 3: Precision data MEP and VAB

Parameter	MEP	VAB
Intra-day (%RSD)	0.59	0.65
Inter-day (%RSD)	0.60	0.74

Table 4: Percent recovery data of MEP and VAB

Drug	% simulated dosage nominal	% Mean (n=3)	±SD	RSD (%)
MEP	50	98.50	0.967	0.98
VAB	50	99.58	0.662	0.66
MEP	100	99.85	0.676	0.68
VAB	100	99.93	0.614	0.61
MEP	150	99.57	0.684	0.69
VAB	150	99.98	0.532	0.53

Table 5: The values of LOD and LOQ

Drug	LOD (µg/ml)	LOQ (µg/ml)
MEP	0.34	1.03
VAB	0.44	1.32

Limit of detection (LOD) and limit of quantitation (LOQ)

The sensitivity of the method determined by LOD and LOQ. The LOD and LOQ for MEP and VAB were determined based on a signal-to-noise ratio of 3:1 and 10:1, respectively, by injecting a series of dilute solutions with known concentrations. The LOD for MEP and VAB were 0.34 and 0.44 µg/ml, respectively, whereas LOQ were 1.03 and 1.32 µg/ml, respectively. The values showed that the method is sensitive. The values of LOD and LOQ are summarized in table 5.

Robustness

The robustness of an analytical method is the capability to remain unaffected by minor changes in parameters. The experimental conditions were intentionally altered and the chromatographic

resolution of MEP and VAB was assessed. To study the effect of the organic solvent (acetonitrile) on the resolution, the concentration was changed 2 units on either side from 30 to 32 and 28, while other chromatographic conditions were kept constant. To study the effect of flow rate on the resolution, the flow rate was changed±0.1 units from 1.0 to 1.1 ml/min and 0.9 ml/min, while other conditions were kept constant. The resolution between MEP and VAB was not less than 1.5 in the study.

Forced degradation study

All the stress conditions applied were enough to degrade MEP and VAB in the pharmaceutical formulation. The results of stress studies of MEP and VAB are shown in table 6 and table 7, respectively. MEP and VAB were degraded and remained 90.87% and ~91.24% respectively when 1 N HCl was used at 80 °C for 2 h. The MEP and VAB were degraded and remained ~92.77% and ~92.84% respectively when 2 N NaOH was used at 80 °C for 2 h. The MEP and VAB were degraded and remained ~94.12% and ~95.82% respectively under 20% H2O2 at 80 °C for 2 h. The MEP and VAB were degraded and remained ~96.17% and ~96.80% respectively under 80 °C for 4 h. The MEP and VAB were degraded and remained ~98.81% and ~98.13% respectively under overall illumination of 200 Wh/m² at 25 °C in photostability chamber for 7 d. From these stress studies it was thus concluded that MEP and VAB were not stable in strong acidic, strong basic and oxidative conditions, but stable in thermal and photolytic conditions and developed method can be considered highly specific for the intended use. The chromatograms of stress studies of MEP and VAB are given in fig. 4 (b), (c), (d), (e) and (f).

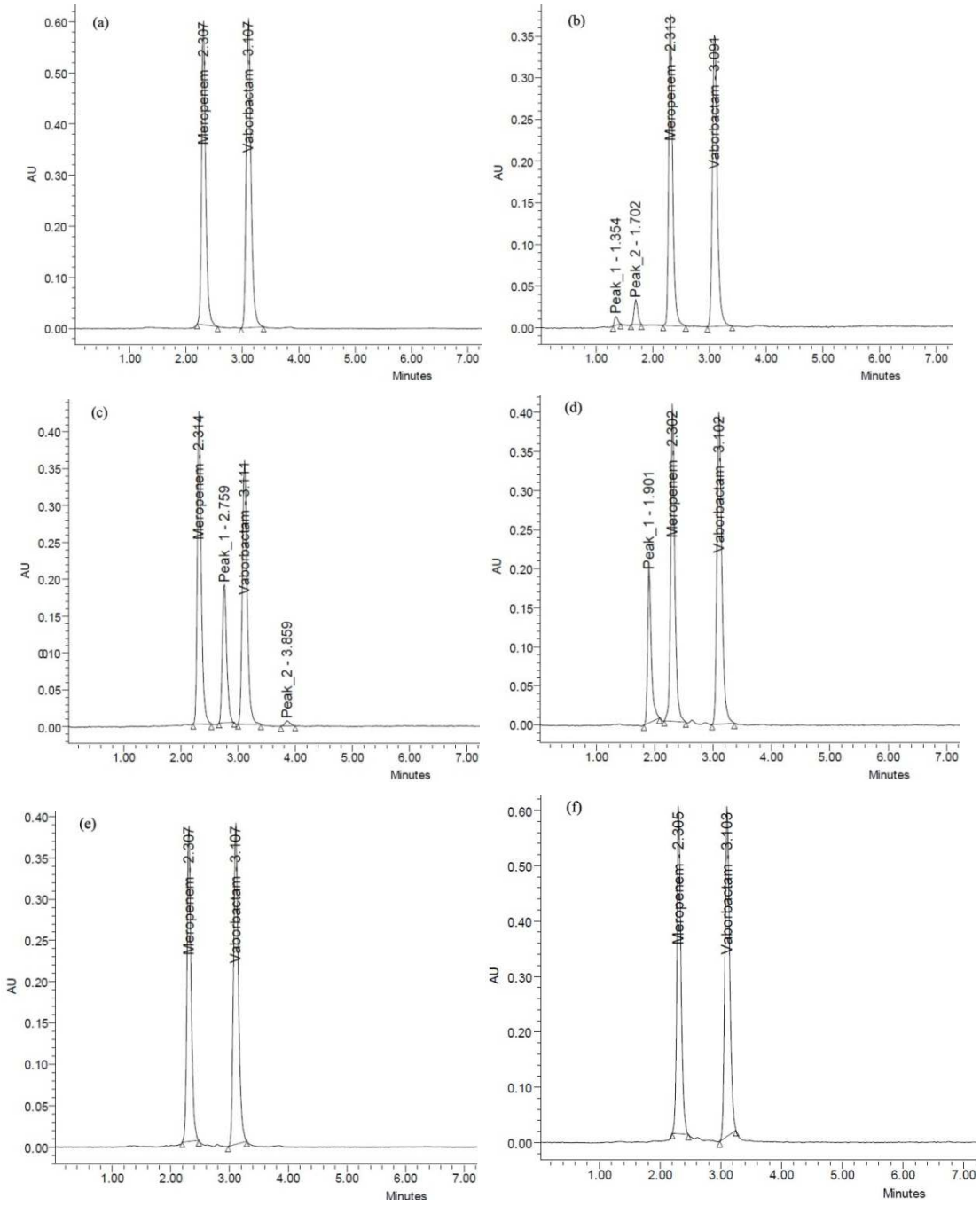


Fig. 4: (a) A typical HPLC chromatogram of a sample solution containing MEP and VAB. HPLC chromatogram of MEP and VAB obtained from degradation studies, (b) Acid hydrolysis (1 N HCl at 80 °C for 2 h); (c) Base hydrolysis (2 N NaOH at 80 °C for 2 h); (d) Oxidative degradation (20% H_2O_2 at 80 °C for 2 h); (e) Thermal degradation (80 °C for 48 h); (f) Photodegradation (overall illumination of 200Wh/m²at 25 °C for 7 d)

Table 6: Degradation study of MEP

Condition	RT	Purity angle	Purity threshold	% Drug degraded
Acid Hydrolysis	2.31	98.50	0.967	0.98
Base Hydrolysis	2.31	99.58	0.662	0.66
Oxidative (Peroxide)	2.30	99.85	0.676	0.68
Thermal	2.30	99.93	0.614	0.61
Photo (UV Light)	2.30	99.57	0.684	0.69

Table 7: Degradation study of VAB

Condition	RT	Purity angle	Purity threshold	% Drug degraded
Acid Hydrolysis	3.09	0.312	0.465	8.76
Base Hydrolysis	3.11	0.597	0.649	7.16
Oxidative (Peroxide)	3.10	0.226	0.418	4.18
Thermal	3.10	0.128	0.315	3.20
Photo (UV Light)	3.10	0.142	0.327	1.87

System suitability parameters

System suitability parameters were measured to make sure the system performance. For system suitability parameters, six replicates of mixed standard solution were injected.

All critical parameters met the acceptance criteria on all days [19]. Parameters such as resolution, capacity factor, tailing factor, theoretical plate, retention volume, and asymmetry factor of the peaks were calculated. The results are shown in table 8.

Table 8: System suitability data for MEP and VAB

Parameters	MEP	VAB
Retention time (min.)	2.29±0.03	3.09±0.02
Injection precision RSD (%)	0.11	0.15
Resolution	-	5.70
Tailing factor	1.22	1.25
Theoretical plates	5540	6982

CONCLUSION

A new, simple reversed-phase HPLC method for simultaneous estimation of MEP and VAB was developed and validated as per ICH guidelines. Validation exercise proved that the HPLC method is linear in the proposed working range as well as accurate, precise and specific. The good recovery percentage of dosage form suggests that the excipients have no interference in the determination. The RSD (%) was also less than 2 show a high degree of precision of the method. The proposed analytical method was also found to be robust with respect to the flow rate and composition of the mobile phase. In addition, easy isocratic elution and simple extraction procedure offered a rapid and cost-effective analysis of the drugs. The developed method is reliable as well as capable of demonstrating and detecting any expected change in the drug product assay during stability studies. Peak purity for MEP and VAB peaks was checked indicating that they are pure from any other excipients or impurities or derivative materials. Thus, the method of analysis is reliable and qualified to exhibit and identify any expected change in the drug product assay during stability studies. The proposed method can be used for routine analysis of MEP and VAB in the combined dosage form and the quality control in bulk manufacturing as well.

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AUTHORS CONTRIBUTIONS

All the author have contributed equally

CONFLICT OF INTERESTS

The authors declare no conflict of interest

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