RESEARCH ARTICLE

Response Surface Methodology Assisted RP-HPLC Method for the Determination of Meropenem in Human Plasma: Application to a Pharmacokinetic Study

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ABSTRACT

A simple, accurate and precise development of an reversed-phase high-performance liquid chromatography (RP-HPLC) method for the quantification of meropenem in human plasma samples for therapeutic drug monitoring (TDM) was developed and validated after optimization of various chromatographic parameters. The best recovery of meropenem was achieved through sample preparation based on a simple extraction method that included deproteinization and extraction with acetonitrile. The developed method reported acceptable values for selectivity, linearity, accuracy and precision, carry-over, dilution integrity and stability. The developed method was efficiently shown to be applicable to a real-time monitoring and pharmacokinetic study of meropenem in critically ill patients.

Keywords: Meropenem, Human plasma, Bioanalytical method, RP-HPLC, Pharmacokinetics.

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INTRODUCTION

Meropenem ((4*R*,5*S*,6*S*)-3-[(3*S*,5*S*)-5-(dimethylcarbamoyl) pyrrolidin-3-yl]sulfanyl-6-[(1*R*)-1-hydroxyethyl]-4-methyl-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid) is acarbapenem-class semisyntheticβ-lactam antibiotic with a broad spectrum of action and low toxicity. This antibiotic gives protection against a variety of bacteria and is commonly used to treat severe and nosocomial infections in hospitalized patients.[1,2] Meropenem monotherapy is an appealing choice for the empirical treatment of moderate to severe bacterial infections due to its resistance to renal dehydropeptidase-I (DHP-I) metabolism, better activity against Enterobacteriaceae and *Pseudomonas aeruginosa*, and better tolerance by the gastrointestinal tract in terms of nausea and vomiting and the central nervous system.[3] The 1-β-methyl substitution is responsible for protecting the compound from hydrolysis by human kidney DHP-I[4] and the 2' side chain enhances its efficacy against *P. aeruginosa*.[5] Apart from these, the major metric related to therapeutic success is the percentage of time that antibiotic levels at the infection site surpass the pathogen's minimum inhibitory concentration (MIC) (%fT > MIC). Thus, the longer blood concentrations remain above the MIC, the greater the likelihood of clinical therapeutic effectiveness.[1,6] Considering the growing threat of antimicrobial resistance and the scarcity of novel antimicrobials for clinical use, dose optimization for existing medication regimens has become increasingly crucial in achieving optimum therapeutic efficacy.[1,7] As a consequence, therapeutic drug monitoring (TDM) has proven to be a significant tool for enabling therapy individualization, as well as for optimizing exposure, which is linked to increased clinical success.[1,8] TDM use in clinical practice with the goal of supporting treatment by minimizing failures and adverse events is already a reality in numerous countries for certain medication classes, such as immunosuppressants and antimicrobials.[1,9] Bioanalytical approaches are critical in TDM and pharmacokinetic (PK) research because they provide quantitative data on drug concentrations in biological samples.[10] Bioanalysis is frequently used for drug concentration quantification in biological samples, customized dose evaluations, pharmacokinetic profiling, drug interaction assessment, therapeutic efficacy and safety assessment, and drug development and clinical

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trials. Chromatographic techniques such as high-performance liquid chromatography (HPLC), gas chromatography (GC), and supercritical fluid chromatography (SFC) have grown in use in both routine and research laboratories for clinical analysis.[11] HPLC is the most extensively used chromatographic method, while GC is limited to a few particular applications and SFC is only slightly employed due to its relatively recent return to the analytical arena.[11] Analytical methods designed for the efficient, cost-effective, simple quantification of meropenem in biological matrices such as plasma are generally thought to be desirable in clinical analysis.

The present research comprises to development and validate a simple, accurate, and precise RP-HPLC method validated for the quantification of meropenem in human plasma samples using the DoE approach. The technique was applied in a pharmacokinetic investigation of critically ill subjects.

MATERIALS AND METHODS

Reference Standards, Solvents, Buffer Salts and Acids

Meropenem trihydrate (analytical standard) and ertapenem sodium (internal standard) were a kind gift from Subtle Pharmaceuticals Pvt. Ltd., Bengaluru, India. Acetonitrile (ACN) and methanol (MeOH) of HPLC grade were obtained from Merck India Pvt. Ltd., Mumbai, India. SD Fine Chem Ltd. was provided the ammonium formate of AR grade and formic acid water for HPLC was obtained from Milli-Q ultrapure water systems (Merck India Pvt. Ltd., Mumbai, India).

Standard Solutions

Meropenem trihydrate and internal standard (IS) stock solutions were prepared independently at 2400 and 1200 µg mL^{-1} concentrations, respectively, with water and methanol (80:20) as diluent. A working standard solution containing meropenem (60 µg mL^{-1}) and internal standard (30 µgmL^{-1}) was prepared for method development by diluting aliquots of appropriate volumes drawn from working stock solutions with mobile phase as a diluent.

Reversed-Phase High-Performance Liquid Chromatography

The RP-HPLC separation and quantification of meropenem and internal standard was performed using Agilent 1220 Infinity Gradient LC System (Agilent Technologies UK Ltd., Stockport, UK) equipped with photodiode array (PDA) detector, dual-channel gradient pump with integrated degassing unit, column compartment and autosampler injector. Chromatographic separation of analytes was performed on an Agilent Zorbax SB-C18 column (4.6 × 150 mm, 5 µm; Agilent Technologies Inc., Santa Carla, CA) maintained at 35°C (± 0.5°C). The mobile phase components were 10 mM aqueous ammonium formate adjusted to pH 3 with formic acid (A) and methanol (B) delivered isocratically in the ratio of 83:17 at a flow rate of 1-mL min^{-1} (± 0.08 $mLmin^{-1}$). The chromatographic separation and detection at 270 nm lasted for 6 minutes. The injection volume of sample was 10 µL. OpenLAB CDS ChemStation C.01.07 SR3 and OpenLAB CDS 3D UV add-on software (Agilent Technologies Inc., Santa Carla, CA) was used to control the instrument, modify the separation and acquisition parameters, generate and integrate the chromatograms.

Optimization of Chromatographic Parameters

Central composite design

A response surface study type with CCD design was considered to evaluate the influence of the independent variables i.e., methanol content (A) and the flow rate (B) of the mobile phase, on the peak height (R1) and resolution (R2) using a quadratic model. Factors A and B are regarded as vital factors based on preliminary experimentation in which they were shown to influence responses. A sample set of 13 experimental runs were suggested by the Design Expert software version 12.0 (Stat-Ease Inc., Minneapolis, MN) with each factor was analyzed for five levels (-α, -1, 0, +1, +α) of methanol content (%; R1) and flow rates (mL min^{-1}; R2). The data corresponding to the responses R1 and R2 were obtained after detection at 270 nm and the obtained data was fitted in a polynomial equation (Equation 1) that quantifies the relationships among the measured responses (Y_{R1} and Y_{R2}) and experimental variables (X_A and X_B), where A and B are the factors considered, β_0 is the intercept, β_n (β_A, β_B, β_{AA}, β_{BB} and β_{AB}) are the regressors and ε is an error associated with the model.

$$Y_R = \beta_0 + \beta_A X_A + \beta_B X_B + \beta_{AA} X_A^2 + \beta_{BB} X_B^2 + \beta_{AB} X_A X_B + \varepsilon \quad \text{[Equation 1]}$$

Statistical analysis of data

The F test and lack of fit test were used to determine the statistical significance (p-value) of the quadratic models in terms of the two-way ANOVA of each response. The statistically significant (p-value) model terms were assessed for their influence on the responses. To demonstrate the quadratic model fitness, coefficient of determination (R^2) values such as the model R^2 value, as well as the predicted and adjusted R^2 values, were used. The prediction of the response is further confirmed by HPLC-UV experimentation (n = 5) with the optimal values provided by the desirability function and evaluating the observed values of the responses to be within the 95% confidence range of the prediction.

Diagnostic plots, response surface plots and desirability function

Diagnostic plots such as normal probability, residuals versus predicted values, residual versus factors, Cook's distance, Box-Cox, leverage, difference in fits (DFFITS) and difference in betas (DFBETAS) were used for evaluating the reliability and efficacy of the CCD model, as well as identifying any potential flaws or outliers that may alter the interpretation of the observed responses. Response surface plots were analyzed to study the association between the responses and the independent factors, identify any interaction effects and optimization of the CCD model based on the desired responses. Furthermore, Derringer's desirability function was utilized to determine the most optimal values for each factor that are predicted to produce a desired response. The attributes of optimal values for each factor are graphically represented by desirability surface plots.

Sample Collection and Storage

Protocols for experimentation on human subjects were approved by the Institutional Ethics Committee (IEC), Jubilee Mission Medical College and Research Institute (JMMC&RI), Thrissur, Kerala. Written informed consent was obtained from the study subjects who were recruited as per the set of inclusion and exclusion criteria. 36 adult critically –ill patients were enrolled in the present study and from each subject 5 mL of venous blood was collected at the window of 1 to 8 hours post infusion at pre-dose, 0.5 hours and at 8, 12 and 24 hours post-dose from the subjects undergoing of 500 mg or 1 g of meropenem over 30 minutes or 3 hours infusion. The collected blood samples were aliquoted into polypropylene tubes pre-coated on the inner wall with spray-dried K2EDTA, then mixed by inversion and centrifuged for 10 minutes at 7000 rpm to separate the plasma. The separated plasma was collected into polypropylene eppendorf tubes, labeled, and stored at -80ºC until further processing.

Sample Preparation

The meropenem and IS was extracted by using protein precipitation approach. A sample of 500 µL of plasma, 50 µL of meropenem trihydrate standard, and 50 µL of IS was constituted and vortexed for 2 minutes. The precipitating agent of 1400 µL of acetonitrile was further added to the mixture and vortexed for 2 minutes. The precipitated sample was centrifuged for 10 min at 4ºC at 15000 x *g*. The supernatant was transferred to a new centrifuge tube and evaporated to dryness under a moderate stream of nitrogen. The dried residue was reconstituted with methanol to makeup to a volume of 2 mL and vortexes for 2 minutes and then centrifuged at 15000 x *g* for 10 minutes at 4ºC. The supernatant was collected, filtered through a 0.45 µm PVDF membrane filter, and injected into the HPLC-UV instrument for analysis.

Calibration standards and quality control samples

Freshly spiked calibration standards and quality control samples (QCs) were prepared from separate stock solutions. Calibration standards were prepared from stock solutions with concentrations of 20, 40, 200, 600, 1200, 1800, and 2400 µg mL^{-1} for meropenem and 1200 µg mL^{-1} for IS. Plasma (1.9 mL) was spiked with 50 µL of each stock solution of meropenem and IS to prepare seven calibration standards with meropenem concentrations of 0.5 (LLoQ), 1.0, 5.0, 15, 30, 45, and 60 (ULoQ) µg mL^{-1} and IS concentration of 30 µg mL^{-1}. A zero sample, which does not contain standard but does contain IS at a concentration of 30 µg mL^{-1} spiked in plasma, was also prepared. Each calibration standard, including the zero samples, was analyzed in triplicate (within run) to determine the regression equation (without taking the response from the zero sample into account) and plot calibration curves.

A series of four QCs were prepared from stock solutions with concentrations of 20, 30, 800 and 2000 µg mL^{-1} for meropenem and 1200 µg mL^{-1} for IS. Plasma (1.9 mL) was spiked with 50 µL of each stock solution of meropenem and IS to prepare QCs with concentrations of 0.50 (LLoQ), 0.75 (LQC), 20.0 (MQC) and 50.0 (HQC) µg mL^{-1} within the calibration range of meropenem and IS concentration of 30 µg mL^{-1}.

A series of three dilution QCs with concentrations of 30 (QC1), 15 (QC2) and 6.0(QC3) µg mL^{-1} for meropenem and 30 µg mL^{-1} for IS were prepared by diluting different volumes of single stock solution of meropenem (1200 µg mL^{-1}) and IS (1200 µg mL^{-1}) with blank plasma.

Method validation

The developed method was used for quantifying meropenem in human plasma was validated in compliance with the International Conference on Harmonization Guidance (ICH. Harmonised Guideline Bioanalytical Method Validation and Study Sample Analysis M10 (2022). QCs prepared in plasma were analyzed to assess validation parameters such as selectivity, matrix effect, calibration curve and range, accuracy and precision, carry-over, dilution integrity, stability, and reinjection reproducibility to comply with the comprehensive validation requirements.

HPLC-UV method selectivity was confirmed by the presence of meropenem and IS peaks in the chromatograms of LLoQ samples without any peaks in the chromatograms of blank samples (n = 6). The matrix effect was determined by evaluating the percentage difference between the observed and nominal concentration along with the %CV for LQC and HQC samples prepared in plasma (n = 6). The method's linearity was evaluated by plotting calibration curves with nominal concentrations of the calibration standards against the obtained response factors ((peak area of meropenem)/(peak area of IS)) through triplicate analyses using unweighted linear regression analysis. The attributes of linear regression equations such as slope and intercept values were considered for back-calculating the concentration of meropenem among the analyzed samples. The within-run (3 times a day) and between-run (once a week for four weeks) accuracy and precision for all QCs were determined by evaluating the percentage difference between the observed (n = 5) and nominal concentration along with the %CV (n = 5).

Analyte carry-over was demonstrated by the presence of residual analyte peak in the blank sample which was injected after the calibration standard at the ULoQ within-run. Dilution integrity was determined using replicate analyses of dilution QCs, with each dilution QC being analysed in replicates (n = 5) as a sample of three different dilution factors. As a measure of accuracy and precision, dilution integrity was reported as the percentage difference between measured concentrations and the nominal concentration, and as %CV.

Stability tests such as freeze-thaw, bench-top, long-term, and processed samples stability of meropenem in plasma were performed using LQC and HQC standards, with three aliquots of each QC analyzed at time zero and after the storage conditions were applied. The %RSD of the observed concentrations (n = 3) from the nominal concentration at time zero was determined for the stability parameters tested. Freeze-thaw stability test was performed by thawing the frozen QCs (stored at -20ºC for 12 hours) in three cycles. Bench-top

(short-term) stability test was performed on thawed QCs that were kept at 20ºC for 4 hours. A long-term stability test was performed on QCs that were stored in -80ºC for one week. The processed sample stability was evaluated by storing the QCs in an autosampler (maintained at 15ºC) for 24 hours and then analysed in triplicate at 0, 8, 16, and 24 hours. Meropenem stability in whole blood was evaluated by spiking meropenem into freshly collected blood and analyzing the meropenem content without storing the blood after processing.

Reinjection reproducibility was determined by reinjecting calibration standards and all QCs after three days of storage at -80ºC. To verify the viability of the treated samples, the precision and accuracy of the reinjected QCs were determined.

Pharmacokinetic study

The proposed technique was examined using a pharmacokinetic analysis of 36 critically ill patients who received 500 and 1000 mg of meropenem. The study's protocol was approved by the JMMC & RI in Thirissur's ethical committee. Before blood was sampled, each individual signed a written informed consent form. Following intravenous meropenem administration, three consecutive blood samples were taken from patients during the time window mentioned earlier. The concentration of meropenem was quantified, and the obtained concentration versus time data was plotted using a specialized programme known as PK-Solver. Several pharmacokinetic characteristics, including the maximum plasma concentration (C_{max}), the time it takes to reach the maximum plasma concentration (T_{max}), the area under the concentration-time curve, the half-life (T1/2), the clearance (CL), and the volume of distribution were calculated.

RESULTS

Optimization of Chromatographic Separation using CCD

Experimental design

Table 1 displays the responses obtained for the sample set of 13 experimental runs are reported. The obtained data set was examined using specialized software known as Design Expert software version 12.0 (Stat-Ease Inc., Minneapolis, MN). The response data were fitted into a quadratic model and analyzed with ANOVA to demonstrate the validity of the quadratic model through fit statistics and checking the statistical significance.

Statistical analysis of data

ANOVA and fit statistics of the quadratic models for the responses R1 and R2 is reported in Table 2. The quadratic model for R1 was determined to be significant with a model *p*-value of <0.0001 and lack of fit *p*-value of 0.6289. The quadratic model for R2 was determined to be significant with a model *p*-value of <0.0001 and a lack of fit *p*-value of 0.6375. Significant model values (*p*-value <0.0500) and insignificant lack of fit values (*p*-value >0.0500) show that the quadratic polynomial equation employed to analyse observed response values for prediction is appropriate. Higher quadratic model R^2 values for both responses, i.e., 0.9933 for R1 and 0.9937 for R2, and a difference between the adjusted R^2 and predicted R^2 of less than 0.2000 suggest a stronger correlation between the actual and predicted values.

Table1 : Experimental runs to study the quadratic interactions of factors on each response

	Factor 1	*Factor 2*	*Response 1*	*Response 2*
Run	*A:Methanol Content*	*B:Flow rate*	*Peak Height*	*Resolution*
	%	mL/min		
1	20	1.2	100	1.95
2	18	0.717157	131	4.26
3	15.1716	1	118	5.31
4	18	1	128	4.72
5	18	1	128	4.74
6	16	1.2	113	4.71
7	18	1	128	4.74
8	18	1	127	4.72
9	16	0.8	130	5.03
10	20.8284	1	101	1.95
11	18	1.28284	106	3.51
12	18	1	125	5.02
13	20	0.8	118	3.14

Table 2: ANOVA and fit statistics of the quadratic models for the responses R1 and R2

Parameter	*R1*	*R2*
Model	Quadratic	Quadratic
Significant ($p < 0.05$) model terms	A, B, A^2 and B^2	A, B, AB, A^2 and B^2
Lack of fit *p*-value	0.6289	0.6375
Model R^2	0.9933	0.9937
Adjusted R^2	0.9885	0.9891
Predicted R^2	0.9774	0.9789

The statistical significance of the model terms (*p*-value <0.0500) shown in Table 2 demonstrates their influence on the responses and are eligible for inclusion in the quadratic polynomial equation. The quadratic polynomial equations for the responses R1 (equation 2) and R2 (equation 3) are shown below.

$$Peak\ height\ (Y_{R1}) = 127.2 - 6.130*A - 8.790*B - 8.540*A^2 - 4.040*B^2 \text{ [Equation 2]}$$

$$Resolution\ (Y_{R2}) = 4.790 - 1.180*A - 0.321*B - 0.591*A^2 - 0.464*B^2 - 0.217*AB \text{ [Equation 3]}$$

Preliminary trials, diagnostic plots, response surface plots and desirability function

Meropenem and IS were separated chromatographically using a Zorbax SB-C18 column (4.6 × 150 mm, 5 µm), which was chosen based on the type of the analyte molecules. Various isocratic mobile phases composed of MeOH, ACN, or ACN: MeOH (50:50, v/v) was used as the organic phase, and a 0 to 50 mM NH_4HCO_2 buffer with a pH adjustment of 85% formic acid (pH 2.0–3.0) was used as the buffered aqueous component. MeOH resulted in superior peak shapes for the two analytes among the different organic components than ACN or the two

together. The two analytes' greater solubility in MeOH could be the cause. Meropenem peak and IS peak in plasma samples were optimally separated by MeOH composition of 15% and 20% in the mobile phase. Better peak resolution between the meropenem and IS peaks was observed with an aqueous phase adjusted to pH 3.0 with 85% formic acid and 10 mM NH_4HCO_2. This was accompanied by improved meropenem peak and IS peak symmetry. Peak resolution between the meropenem and IS peaks did not increase after switching from a 20 to a 50 mM NH_4HCO_2 buffer with a pH 3.0 adjustment, an aqueous phase constituted of no more than 85%. A range of flow rates between 0.7 and 1.5 mL min^{-1} were used to pump the mobile phase, but the flow rate between 0.8 and 1.0 mL min^{-1} resolved the two analytical peaks in less than 7 minutes without creating an excessive amount of backpressure on the column. At temperatures ranging from 22 to 35ºC in the column oven, the analytes were separated. Temperature increase had no effect on peak sizes, heights, areas, retention times, or resolution but reduced the back pressure. Therefore, 35ºC was chosen as the ideal temperature for the separation of meropenem and IS based on these factors. Key instrumental parameters influencing the chromatographic separation of meropenem and IS were found to be the mobile phase composition and flow rate based on the observation of these preliminary trials. In order to achieve more efficient peak intensities and resolution between meropenem and IS, these two parameters were optimized using CCD.

The diagnostic plots mentioned in section 2.4.3 demonstrated a good relationship between predicted and actual values for the responses R1 and R2, indicating the adequate fit of the quadratic model. The fact that the data points in these plots are relatively close to the straight line indicates that the actual values and the quadratic model's predicted values for each response concur strongly. The influence of independent factors on responses was depicted using two-dimensional contour plots (Figure 1) and three-dimensional response surface plots (Figure 2), which are graphical representations of the regression coefficients for each response. Figures 1a and 2a shows the interaction between methanol composition and mobile phase flow rate on peak height. Maximum peak height (>130 AU) was observed with methanol concentrations ranging from 16.1 to 18.5% and flow rates ranging from 0.80 to 0.95 mL min^{-1}. Figures 1b and 2b shows the interaction between methanol composition and mobile phase flow rate on peak resolution. Maximum resolution (>5 units) was observed with methanol concentrations ranging from 16.0 to 17.7 % and flow rates ranging from 0.80 to 1.15 mL min^{-1}.

The optimal levels of the factors, namely 17.0% methanol concentration and 1-mL min^{-1} flowrate of the mobile phase,

Table 3: Confirmation of the desirable levels of each factor i.e., 17.0% methanol concentration and 1-mL min^{-1} flow rate

Response	*Predicted Mean*	*Std Dev*	*n*	*SE Pred*	*95% PI low*	*Data Mean*	*95% PI high*
Peak height	128	1.19	3	0.86	126	127	130
Resolution	5.22	0.12	3	0.09	5.02	5.20	5.43

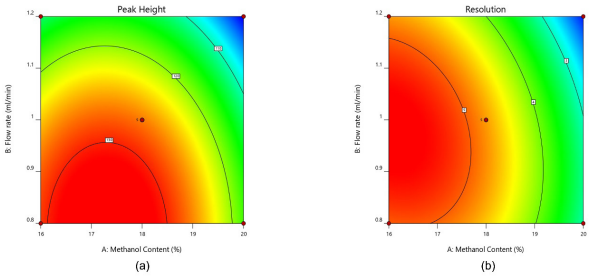

Figure 1: Contour plots (a) peak height and (b) resolution

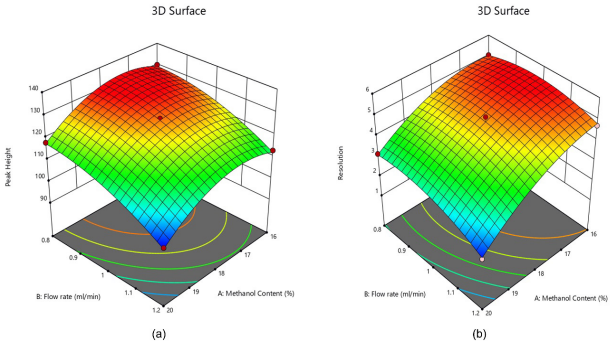

Figure 2: 3D response surface graphs (a) peak height and (b) resolution

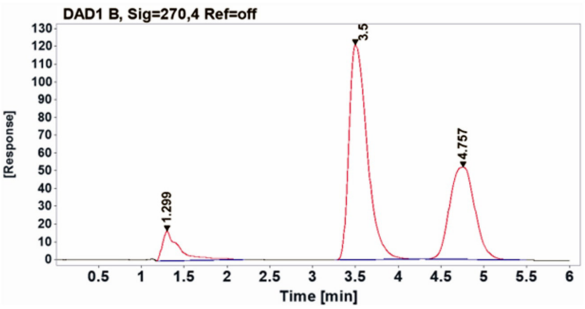

Figure 3: Representative chromatogram of analyte and IS spiked human plasma sample

demonstrated a higher desirability function (1.00), and triplicate analyses of these variables confirmed that the observed response values were well within the 95% CI of the prediction through a single sample t test (Table 3).

In conclusion, the optimal chromatographic separation of meropenem and IS from the range of chromatographic conditions investigated were found with the subsequent set of parameters: Mobile phase: 10 mM aqueous ammonium formate adjusted to pH 3 with 85% formic acid (A) and methanol (B) delivered isocratically in the ratio of 83:17 (v/v) at a flow rate of 1-mL min^{-1} (± 0.08 mL min^{-1}); 35ºC Column oven temperature; and an Injection volume of 10 µL. Stationary phase: Zorbax SB-C18 column. Figure 3 shows representative chromatograms of the meropenem (ULoQ) and IS (30 µg mL^{-1}) spiked plasma sample and the blank plasma sample under ideal chromatographic conditions. Meropenem and IS had mean retention times of 3.5 (± 0.1) and 4.7 (± 0.2) minutes under the given conditions.

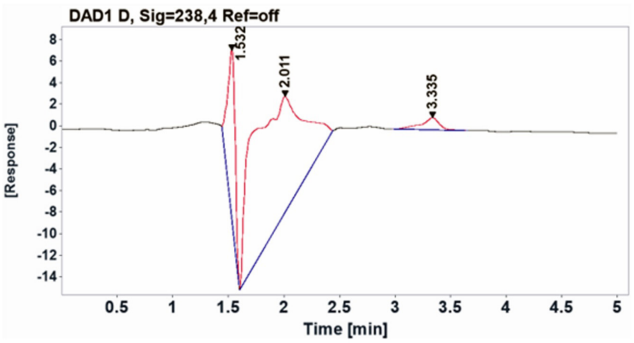

Figure 4: Representative chromatogram of drug free human plasma sample

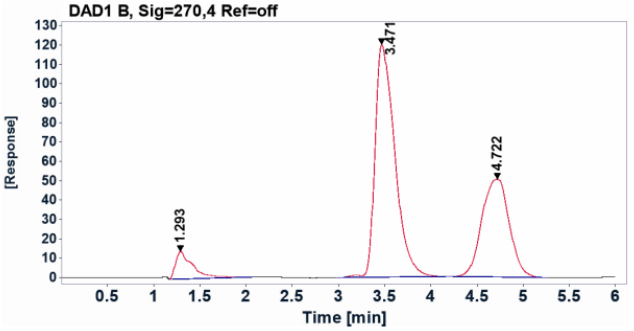

Figure 5: Patient's sample chromatogram

Table 4: Selectivity, linearity and sensitivity

Parameter	*Value*
Selectivity	
Meropenem (Rt; min)	3.5
IS (Rt; min)	4.7
Linearity	100, 500, 1000,3500,7000,10000
Regression equation	y = 0.0001x + 0.0465
r^2	0.9994
Sensitivity	
LLoQ (µg mL-1)	0.1

Method Validation

Selectivity

The target peaks (meropenem an IS) were well separated from one another, from other peaks of extraneous and endogenous compounds in analyte spiked plasma samples (Figure 3), and from the peak of the blank plasma sample (Figure 4), indicating that the method was selective for the determination of meropenem. Additionally, the patient's sample chromatogram is represented in Figure 5.

Linearity

Within the tested concentration range of 0.5 to 60 g mL^{-1} in spiked plasma samples, the suggested technique was linear. Table 4 presents the regression equation describing the calibration curve for spiked plasma samples and the correlation coefficient (r2) of meropenem.

Sensitivity

The LLoQ for meropenem was also included in Table 4, indicating that the suggested approach was sensitive for examining meropenem in plasma samples and that it could be used to calculate the concentration of meropenem in the plasma even after 24 hours after a single 500 or 1000 mg IV infusion.

Accuracy and precision

The recovery of the two analytes was greater than 95% at each of the three nominal concentration levels for plasma samples, according to the findings of the recovery tests using the chosen extraction method, which are presented in Table 5.

Table 5 also provides a summary of the results for repeatability (injection and analysis) and intermediate precision (reproducibility within and between days), demonstrating perfect agreement between repeated injections (both retention times and peak areas), repeated analyses, and intra- and inter-days studies.

Matrix effect

Matrix effect was not observed with no significant difference between the observed and nominal concentrations. Results of the matrix effect are represented in Table 6.

Carry over

Analyte carry-over was found to be less than 20% of the LLoQ and less than 5% of the IS suggesting that there was no significant carryover observed. The results of carryover studies are shown in Table 7.

Dilution integrity

Significant difference between the observed and nominal concentrations of the dilution QCs was not observed which indicates the accuracy and precision of dilution protocol. The results of dilution integrity are given in the Table 8.

Table 5: Accuracy and precision

QC level	*Intra-day precision*						*Inter-day precision*							
	Day 1		*Day 2*		*Day 3*		*Week 1*		*Week 2*		*Week 3*		*Week 4*	
	R	*SD*	*R*	*SD*	*R*	*SD*	*R*	*SD*	*R*	*SD*	*R*	*SD*	*R*	*SD*
LLOQ (100 ng/mL)	99.6	0.47	100	0.58	99.7	0.44	99.6	0.37	100	0.52	101	0.77	100	0.78
LQC (250 ng/mL)	99.8	0.69	99.0	0.15	99.9	0.80	100	0.63	99.8	0.67	100	0.93	100	1.20
MQC (3000 ng/mL)	98.3	0.05	98.4	0.16	98.8	0.50	98.7	0.57	98.75	0.57	99.1	0.57	98.8	0.63
HQC (8500 ng/mL)	98.6	1.69	99.9	0.21	102	1.61	99.3	0.92	100	0.44	100	0.76	99.3	0.71

*R = Mean recovery (%); SD = standard deviation

Table 6: Results of Matrix effect

Level	*Conc (ng/mL)*	*Amount of drug recovered in plasma sample (ng/mL),(n=6)*	*%RSD*
LLoQ	100	99.67 ± 0.455	0.456
HQC	8500	8390.541 ± 52.845	0.629

Table 7: Results of Carryover

Level	*% Carryover*
BLANK	0.02393617
IS	0.59

Table 8: Results of Dilution Integrity

Level	*Conc (ng/mL)*	*Mean %Recovery ± SD (ng/mL),(n=3)*	*%RSD*
LLoQ	100	99.140 ± 2.342	2.363
LQC	250	99.777 ± 0.780	0.782
MQC	3000	98.353 ± 0.925	0.941
HQC	8500	98.844 ± 1.451	1.468

Table 9: Freeze-thaw stability of plasma samples

Level	*Conc (ng/mL)*	*Actual amount of drug recovered in Plama (Mean%recovery ± SD) (ng/mL),(n=3)*	*%RSD*
1st cycle			
LLoQ	100	95.087 ± 3.462	3.641
LQC	250	99.328 ± 0.817	0.822
MQC	3000	97.812 ± 0.602	0.615
HQC	8500	98.974 ± 1.425	1.440
2nd Cycle			
LLoQ	100	95.888 ± 4.165	4.344
LQC	250	98.730 ± 1.195	1.210
MQC	3000	98.247 ± 0.983	1.0006
HQC	8500	96.870 ± 3.208	3.312
3rd Cycle			
LLoQ	100	93.243 ± 5.509	5.908
LQC	250	96.221 ± 3.153	3.277
MQC	3000	97.632 ± 0.681	0.697
HQC	8500	96.81 ± 3.622	3.741

Table 10: Short term stability for plasma samples

Level	*Conc (ng/mL)*	*Actual amount of drug recovered in Plama (Mean%recovery ± SD) (ng/mL),(n=3)*	*%RSD*
1st hour			
LLoQ	100	90.313 ± 2.550	2.823
LQC	250	95.765 ± 1.277	1.333
MQC	3000	97.174 ± 1.042	1.073
HQC	8500	97.992 ± 1.901	1.940
2nd hour			
LLoQ	100	96.143 ± 3.750	3.901
LQC	250	96.351 ± 1.908	1.980
MQC	3000	96.979 ± 0.984	1.014
HQC	8500	97.009 ± 2.093	2.158
3rd hour			
LLoQ	100	86.372 ± 3.374	3.906
LQC	250	94.646 ± 0.960	1.014
MQC	3000	96.390 ± 1.057	1.097
HQC	8500	98.638 ± 1.623	1.646

Table 11: Long term stability for plasma samples

Level	*Conc (ng/mL) (n = 3)*	*Actual amount of drug recovered in Plama (Mean%recovery ± SD) (ng/mL),(n=3)*	*%RSD*
1st Week			
LLoQ	100	96.680 ± 3.715	3.843
LQC	250	96.045 ± 2.857	2.974
MQC	3000	95.127 ± 0.813	0.855
HQC	8500	97.921 ± 1.409	1.439
2nd Week			
LLoQ	100	88.496 ± 7.669	8.666
LQC	250	94.271 ± 1.779	1.888
MQC	3000	96.061 ± 1.860	1.936
HQC	8500	91.275 ± 2.075	2.273
3rd Week			
LLoQ	100	79.854 ± 8.116	10.164
LQC	250	93.066 ± 1.3005	1.397
MQC	3000	89.211 ± 1.137	1.275
HQC	8500	91.931 ± 3.433	3.734

Stability

According to a study on freeze-thaw stability, spiked plasma samples hold their stability for three freeze-thaw cycles. Even at 20ºC., spiked plasma samples remained stable for at least 4 hours, according to a bench-top or short-term stability investigation. Based to a long-term stability investigation, spiking plasma samples were stable at -80 ºC for at least one week. In accordance to the processed sample stability research, spiking plasma samples were stable for at least 24 hours at 15ºC. Free-thaw stability studies of plasma samples including short term stability studies and Long-term stability studies are represented in Tables 9, 10 and 11 respectively.

Reinjection reproducibility

Reinjection reproducibility indicated that there was no variability of the processed samples even after three days of storage at -80ºC.

Pharmacokinetic study

The TDM was carried out using 108 Meropenem samples. In order to assess the lower limit of the therapeutic range (between ≤2 to ≥8 µg/mL), EUCAST and CLSI breakpoints were utilized. The average trough levels of 500 mg and 1 g of Meropenem at q8, q12, and q24 hours were observed to be less than or equal to 2 µg/mL (1.28+/- 0.1882µg/mL). This finding

Table 12: C_{max} and C_{min} of meropenem at different doses

*Dose (*mg*)*	*Frequency of Dose administration (hours)*	*MTC (µg/mL)*	*Dose (*mg*)*	*Frequency of Dose administration (hours)*	*Cmin*	*MIC (≤2 µg/mL-(≥8 µg/mL)*
500	12	24.716	500	12	1.315	S
	8	24.161		8	1.376	S
1000	24	50.15	1000	24	1.371	S
	12	49.05		12	1.253	S
	8	45.31		8	1.257	S

*MIC: Minimum Inhibitory Concentrations; MTC: Maximum Tolerable Concentration; S: susceptible category

Table 13: Pharmacokinetic profiles

Dosage (IV infusion)	*Pharmacokinetic parameters (mean ± SD)*								
500 mg (n = 7)	Lambda_z	t1/2	Tmax	Cmax	AUC 0-t	AUC 0-inf_obs	AUMC 0-inf_obs	Cl_obs	Vz_obs
Every 12 hours	0.235 ± 0.005	2.939 ± 0.066	0.5	24.716 ± 1.0001	88.302 ± 2.263	93.885 ± 2.112	296.012 ± 10.552	5.327 ± 0.119	22.596 ± 0.791
Every 8 hours	0.371 ± 0.015	1.869 ± 0.078	0.5	24.161 ± 1.133	68.605 ± 3.493	72.330 ± 4.008	165.637 ± 16.694	6.929 ± 0.404	18.659 ± 0.554
1000mg (n = 29)	Lambda_z	t1/2	Tmax	Cmax	AUC 0-t	AUC 0-inf_obs	AUMC 0-inf_obs	Cl_obs	Vz_obs
Every 24 hours	0.122 ± 0.008	5.682 ± 0.390	0.5	50.151 ± 4.153	160.111 ± 10.050	171.447 ± 13.578	942.826 ± 215.216	5.851 ± 0.463	47.835 ± 0.504
Every 12 hours	0.267 ± 0.015	2.6001 ± 0.150	0.5	49.053 ± 6.575	101.578 ± 26.910	106.271 ± 27.239	266.183 ± 62.249	9.964 ± 2.754	37.722 ± 11.834
Every 8 hours	0.435 ± 0.026	1.598 ± 0.095	0.5	45.312 ± 5.796	83.070 ± 6.870	85.993 ± 6.924	151.011 ± 18.998	11.702 ± 0.969	27.012 ± 2.975
Mean	0.370	2.106	0	42.03447	88.750	92.697	224.705	10.074	28.360
SD	0.098	1.003	0	10.27279	22.281	24.019	189.672	2.683	8.386
%CV	26.543	47.639	0	24.438	25.106	25.912	84.409	26.636	29.569

shows that (Table 12) the microorganisms are susceptible to the treatment. Additionally, it was found that there was significant inter-individual variability in the present study when the coefficient of variation (%CV) was calculated using the mean of the observed primary pharmacokinetic parameters, such as clearance (26.636%) and volume of distribution (29.569%). Hence, TDM need to be performed for the dosage optimization of meropenem to optimize the outcomes in critically ill patients. Table 13 depicts the mean, standard deviation and %CV of pharmacokinetic parameters.

DISCUSSION

Previous studies have demonstrated the use of HPLC technique for the quantitative determination of meropenem as mono-analyte[12–14] and simultaneous quantification with other antibiotics[15–19] in biological samples and most of the detection techniques employed were ultraviolet (UV) and mass spectrometric (MS) detectors. Meropenem has been measured in a number of biological matrices, with plasma and serum being the most common.[20] Various strategies for sample preparation, including dilution,[21] protein precipitation using methanol[22–24] and acetonitrile[14,18] and also a combination of both,[25,26] protein precipitation can be done by using acids like trichloroacetic acid,[27] solid phase extraction (SPE) using variety of sorbents including C18,[28] C8,[29] amino,[30] strata-X[12] and HLB,[31] liquid-liquid extraction (LLE) with organic solvents,[32] liquid-liquid microextraction (LLME),[33] on-line HPLC extraction,[34] and semi-automated sample preparation[35] have been applied previously. Protein precipitation is the most often used sample preparation procedure, with methanol extraction being the appropriate method of quantification of meropenem in human plasma samples.[29] The majority of meropenem chromatographic separation methods have relied on reversed-phase columns such as C18,[36–38] C8,[15,39] phenyl,[35] pentafluoro phenyl,[40] and HILIC[22,32] columns. Cost-effective, sensitive, reproducible, repeatable, and simple bioanalytical methods are typically regarded as efficient. Bioanalytical method development typically entails monitoring

a plethora of intrinsic factors such as extraction conditions (sample size, sample-to-solvent ratio, sonication frequency and time, centrifugation speed and time), chromatographic parameters (pH of the mobile phase components, ratio of aqueous and organic phases, column oven temperature), and likewise. The traditional concept of method development relied on a classic trial and error technique according to the opinions of a specialist with only one factor that could be modified at a time while the others remained constant, which unusually resulted in real optimum conditions.[41-46] Recently, there has been an increase in the use of statistical design of experiments with response surface methodology (RSM) and Design of Experiments (DoE) strategy to facilitates a deeper understanding of multiple variables for the enhancement of chromatographic parameters. When compared to Box-Behnken Design (BBD), Central Composite Design (CCD) is considered advantageous for its operation at extreme conditions (factorial points, axial points, and centre points) with minimal risks for practical concerns outweighing statistical problems in studying the relationship between multiple input variables (factors) and one or more response variables.[47, 48]

CONCLUSION

The present developed RP-HPLC method is currently being used for the quantification of meropenem in critically ill patients' plasma samples by using the DoE approach. Despite this, the response surface methodology was employed to investigate the impact of various chromatographic conditions and other experimental parameters on the chromatographic separation of meropenem, which led to the development of a method that is more effective and superior to previously reported methods for the determination of meropenem in terms of simplicity and economy. To examine the pharmacokinetic profile of meropenem in critically ill patients, the developed and validated RP-HPLC method was used in the TDM of meropenem at three dosages. The current RP-HPLC technology can be easily used to analyze meropenem in a variety of pharmaceutical dosage forms as well as for pharmacokinetic research of the drug. In the context of clinical research, it was confirmed that, the suggested method might be effectively used for the analysis of meropenem in pharmaceuticals and in other biological matrices.

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