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Detection and quantification of meropenem in broiler bird's plasma using high performance liquid chromatography with diode array detector

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Abstract
In this investigation, meropenem (MEM) in broiler bird's plasma was determined using a novel, sensitive and selective high-performance liquid chromatographic technique. In the devised procedure, methanol: glacial acetic acid solution mixture was employed as the mobile phase on a C18 column (Enable, 5µ, 250 X 4.60 mm) with a Phenomenex C18 (4.0 × 3.0 mm, 5 µm) guard column at 40 °C. Chromatographic separation was done at a flow rate of 1 mL/min and a diode array detector was used for detection at 300 nm. The analytes were extracted from plasma using the protein precipitation method with orthophosphoric acid in methanol with doripenem used as the internal standard. The calibration curve was linear over the concentration range of 0.1-1 µg/mL with a correlation coefficient of 0.9989. The values for the limits of detection and quantification were discovered to be 0.31 µg/mL and 0.94 µg/mL, respectively. The validated method can be effectively used to study the pharmacokinetic behavior of meropenem in broiler birds.

Keywords: Carbapenem, broiler bird, plasma, accuracy, extraction efficiency

1. Introduction

Carbapenems a novel class of β-lactam antibiotics are the best medication for the treatment of resistant microbes. These medications have advantages over other β-lactams due to their bactericidal and post-antibiotic actions. Additionally, they have higher resistance to many of the β-lactamases including certain extended-spectrum enzymes (Bidgood and Papich, 2002; Li *et al.*, 2007; Steffens *et al.*, 2021) [1, 2, 3].

Among carbapenems, meropenem is the second carbapenem marketed in the United States after imipenem and approved in July 1996 by the United States Food and Drug Administration (USFDA). It is primarily used to treat severe infections including sepsis and meningitis caused by resistant microbes and can prevent the production of the cell wall in the majority of gram-positive and gram-negative bacteria by binding to penicillin-binding proteins which cause cell lysis and ultimately cell death by preventing the cross-linking of peptidoglycan chains. Due to their limited oral bioavailability, it is available only in parenteral formulations i.e., intravenous (i.v.) infusion or bolus, intramuscular and subcutaneous.

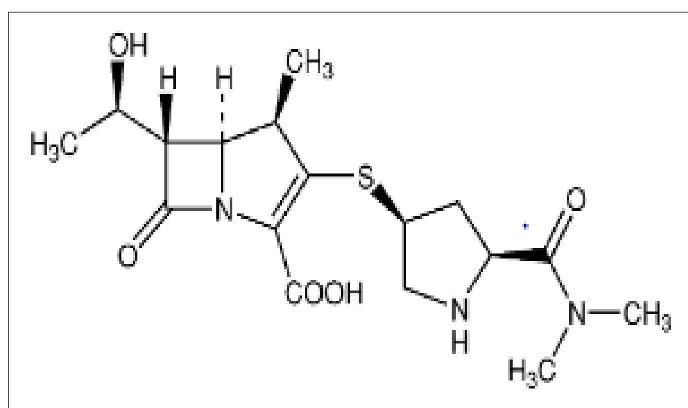

Fig 1: Chemical structure of meropenem (Courtesy: Chang *et al.*, 2002 [4])

In comparison to conventional analytical techniques like microbiological, radioimmunoassay and enzyme linked immunosorbent assays (ELISA), the high-performance liquid chromatography (HPLC) approaches have many benefits, including quick analysis times, high specificity and sensitivity. On the other side, it also necessitates high-end equipment and specialized employees. Further**,** researchers have used the chromatographic methods for the quantification of meropenem in dissimilar matrices *viz*., plasma, sputum, urine, bile, cerebrospinal fluid, peritoneal fluid, aqueous humor, bronchoalveolar fluid, epithelial lining fluid, interstitial fluid, renal replacement therapy effluents, blister fluids etc. by utilizing wide diversity of mobile phases. The present study was aimed to develop a sensitive and selective method for determining meropenem in plasma of broiler birds so that it can be effectively applied to study the pharmacokinetic behavior and residual retention of the drug in poultry birds.

2. Materials and Methods

2.1 Chemicals and reagents

The pure standards meropenem and doripenem were purchased from M/s Sigma Aldrich India Ltd., Bangalore and used without further purification. The purity of the standards was ≥ 99%. The HPLC grade methanol, ortho-phosphoric acid and glacial acetic acid were procured from M/s Sigma Aldrich India Ltd., Bangalore. Meropenem Injection IP Meromac® 1g was procured from M/s Macleod's Ltd Mumbai. HPLC-grade water used for chromatography and other experiments was purified on a Milli-Q® system from M/s Millipore (Bedford, MA, USA).

2.2 High-Performance Liquid Chromatography

A high-performance liquid chromatography (HPLC; Shimadzu Corporation, Tokyo, Japan) machine equipped with LC- 30 AD quaternary gradient pump, DGU- 20 A_{5R} Vacuum degasser, SIL- 30 AC autosampler, CTO-20 AC column oven and SPD-M20A PDA detector was used. Lab Solutions version 2.0 software was used for data analysis. The chromatography column was an Inertsil C_{18} (150 × 3.9 mm, 5 µm) column with a Phenomenex C_{18} (4.0 × 3.0 mm, 5 µm) guard column, maintained at 40 °C. The mobile phase was a mixture of 0.01 percent glacial acetic acid and methanol, in the ratio of 85:15, v/v, operated at a flow rate of 1 mL/min. The injection volume was set at 20 µL, with a detector wavelength of 300 nm.

2.3 Preparation of standard stock solutions and working solutions

2.3.1 Meropenem and Doripenem

The master stock solution of meropenem was prepared in Milli-Q® water by dissolving 10 mg of meropenem in one milliliter of Milli-Q® water to obtain 10000 µg/mL. From this stock solution, further, a diluted stock solution of 1000µg/mL, 100 µg/mL and 10 µg/mL was prepared.

Similarly, the master stock solution of doripenem was prepared in Milli-Q® water by dissolving 1 mg of doripenem in one mL of Milli-Q® water to obtain 1000 ppm. From this stock solution, further, a diluted stock solution of 100 ppm and10 ppm was prepared.

Composite working standard solutions of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1ppm were prepared by diluting the stock solutions of both with suitable quantities of Milli-Q® water and transferred to auto-sampler vials (M/s Shimadzu, Japan; 1.5 mL Short Thread Vial, 32 x 11.6 mm, amber glass with ultraclean closure: 9 mm PP Short thread cap, blue, center hole; Silicone white/PTFE red, 55° shore A, 1.0mm) for immediate analysis by HPLC. The standard stock solutions were stored at -80 ºC freezer (M/s Thermo Fisher Scientific, Asheville, USA) in cryovials until analysis by HPLC.

2.3.2 Selection of suitable mobile phase for HPLC

The analytical methodology described by Dincel *et al*. (2020) [5] was used for the standardization of meropenem and doripenem, with certain modifications. Reversed-phase HPLC was performed using the mobile phase comprising of methanol (solvent A) and 0.01% glacial acetic acid in water (solvent B) in a ratio of 15:85 at a flow rate of 1 mL per minute. The solvent B was prepared by adding 100 µL of glacial acetic acid (1.75mM) in one litter of Milli-Q® water. Both the Lichrosolv® methanol and solvent B were filtered separately through a 0.45 µm membrane filter (Durapore® PVDF 0.45 µm) using a solvent filtration assembly (M/s Riviera Glass Pvt. Ltd., Mumbai).

2.4 Method validation

2.4.1 Selectivity

The selectivity of the method was evaluated by injecting three different blank plasma samples. The data obtained from the blank plasma samples were examined for interference at the retention time of the analyte by comparing them with those data obtained from spiked plasma samples. In addition, meropenem standard peak purities were investigated by PDA. The peak purity index values of the meropenem and doripenem (internal standard) samples were found as 0.999.

2.4.2 Response Linearity

Ascending concentrations of meropenem and doripenem *viz.,* 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1 ppm in Milli-Q® water were analysed using HPLC. The peak with the area were calculated and plotted in an excel sheet. The same procedure was repeated three times to verify the reproducibility of the results and the average of these samples was taken to reduce the error. Finally, linear regression was calculated by plotting the concentration versus peak area curve in MS-Excel® and derived the regression equation and R^2 values.

The regression coefficient (R^2) of meropenem was calculated from the response linearity graph of the meropenem standard. The concentration of meropenem in plasma was quantified using the regression equation.

$$y = mx + C$$

Where,
y = peak area
C = Y-intercept
m = Slope of the calibration curve
x = Concentration (ppm)

The concentration of meropenem in plasma and tissues was quantified using the following linear regression equation:

Peak area = Slope x (Concentration of meropenem in ppm) + y-intercept

2.4.3 Precision and accuracy
Intra-day and inter-day precision and accuracy were determined in plasma samples by determining quality control samples at three concentration levels (1, 2.5 and 5 ppm). For intra-day assay precision and accuracy, three replicates of samples at each concentration were processed and assayed all at once within a day. The inter-day assay precision and accuracy were determined by processing and analyzing the samples on two different days. Three replicates at each concentration were assayed per day.

2.5 Recovery studies of meropenem
Absolute recoveries of meropenem at different concentration levels (0.5, 1, and 2.5 ppm) (n=3) were measured by comparing the peak area of the drug obtained from the plasma with the peak area obtained by the direct administration of the pure standard drug. The mean recovery of the drug at three concentration levels (0.5, 1, and 2.5 ppm) was calculated by comparing the concentration obtained from the drug-supplemented plasma to the added concentration.

2.5.1 Recovery from Plasma
The extraction efficiency of meropenem was determined using *in-vitro* studies. Blood was collected from a slaughterhouse in heparinized blood collection vials. Blood was immediately transported to the laboratory in an ice-bucket (M/s Tarsons, Kolkata) and was centrifuged at 6000 rpm for 10 min at 4ºC in a refrigerated micro centrifuge (M/s Eppendorf AG, Germany). The separated plasma was transferred to new cryovials and stored at -80°C. Later, 0.50 mL plasma was diluted to 1 mL with 0.90 percent physiological normal saline solution. To the test samples of 0.50 mL diluted plasma samples, 50 µL of 1 ppm IS (doripenem) and 50 µL meropenem at different concentrations (0.5, 1, and 2.5 ppm; three replicates for each concentration) were added. Then 400 µL ortho-phosphoric acid solutions in methanol (75 µL/10mL) were added and the final solution was mixed by a vortex mixer for 2 min. The samples were centrifuged at 6000 rpm at 4 °C for 10 min. After centrifugation, the supernatant was filtered through a 0.22 µm PVDF Durapore® membrane filter (M/s Merck Life Science Pvt. Ltd., Mumbai) into 1.5 mL autosampler vials (M/s Shimadzu Asia Pacific Pte. Ltd., China) and 20 µL was injected into the HPLC system for analysis. The mean recovery percentage was calculated from the spiked concentrations. Method validation was done by calculating the precision, accuracy and sensitivity of the method.

Plasma unspiked with meropenem was also prepared using the above procedure to ensure the absence of meropenem in the poultry bird plasma.

Finally, AUCs of spiked and standard meropenem samples analyzed by HPLC were compared to determine the recovery percentage of meropenem. The mean and standard deviation of each sample was calculated by using the formula,

$$\text{RSD} = \frac{\text{The standard deviation of replicates of each concentration}}{\text{Mean of replicates of each concentration}} \text{ X } 100$$

2.6 Statistical analysis
The mean and standard error of the data obtained were determined as per the formulae described by Snedecor and Cochran (1994) [6].

3. Results
3.1 Detection of meropenem and doripenem
Meropenem pure standard and Meromac® (commercial meropenem) were detected at the wavelength of 300 nm with a retention time of 6.8 and 6.9 min, respectively. Doripenem pure standard was detected at the wavelength of 300 nm with a retention time of 4.3 min. The chromatograms meropenem pure standard and doripenem standard (1 ppm) shown in Fig. 2.

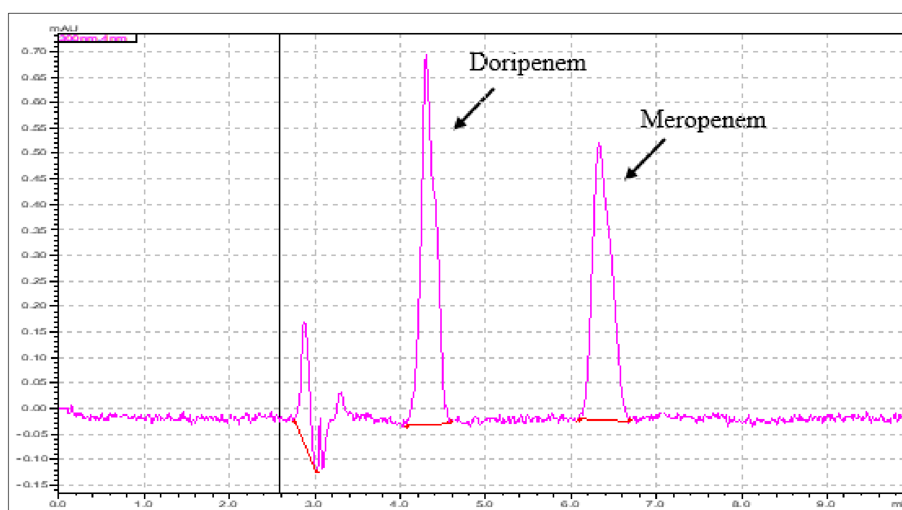

Fig 2: Chromatogram of analytical standard meropenem (1 ppm) and doripenem (1 ppm).

3.2 Response linearity
The calibration curve for meropenem was linear over the concentrations ranging from 0.1 to 1 µg/mL with the regression coefficient (r^2) of 0.9989. The reproducibility of the results was verified at least thrice with each concentration of meropenem. A graphical representation of the calibration curve for meropenem is depicted in fig. 3.

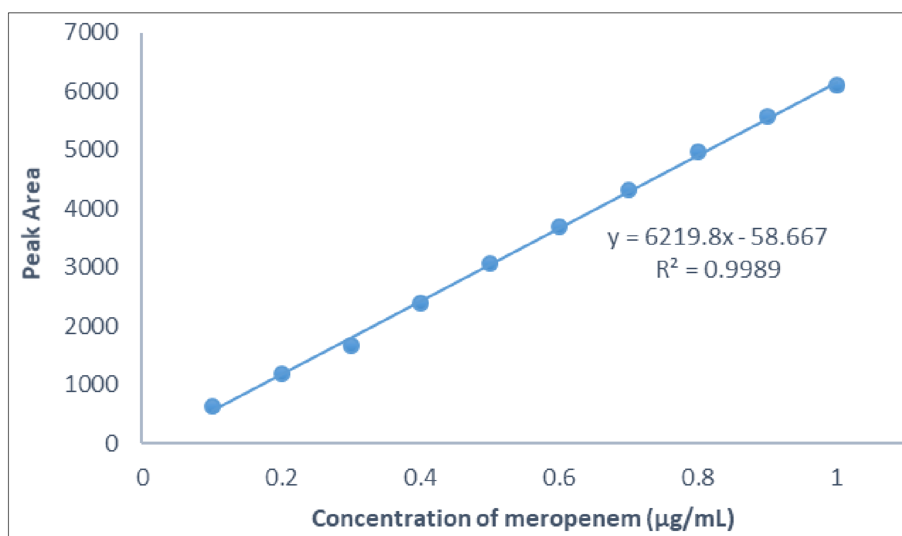

Fig 3: Plot of response linearity of concentrations of meropenem ranging from 0.1-1 µg/mL

Similarly, the calibration curve for doripenem for concentrations ranging from 1 to 5 µg/mL with the regression coefficient (r^2) = 0.9996 is depicted in fig 4. The reproducibility of the results was verified at least thrice with each concentration.

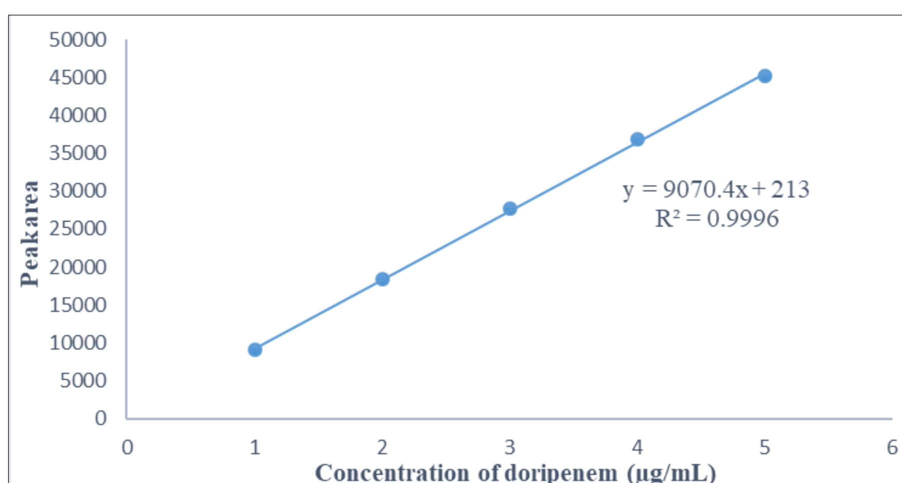

Fig 4: Plot of response linearity of concentrations of doripenem ranging from 1-5 µg/mL

3.3 Sensitivity

The limit of detection (LOD) and limit of quantification (LOQ) of the method was 0.31 µg/ mL and 0.94 µg/ mL, respectively for meropenem for the concentration ranging from 0.1 to 1 ppm, 0.62 µg/ mL and 1.08 µg/ mL for IS doripenem for the concentration ranging from 0.5 to 1 ppm, with acceptable accuracy and precision.

Table 1: Linear correlation parameters and LOQs of meropenem and doripenem

Compound	Slope	SE of Intercept	Concentration Range (µg/ mL)	r^2	LOD (µg/ mL)	LOQ (µg/ mL)
Meropenem	621.98	58.66	0.1-1	0.9989	0.31	0.94
Doripenem	10825	1176.59	0.5-1	0.9875	0.62	1.08

3.4 Recovery of meropenem from plasma

The maximum recovery of meropenem after fortifying plasma with different concentrations like 0.5, 1 and 2.5 ppm of meropenem was obtained using ortho-phosphoric acid (OPA) in methanol for protein precipitation. Hence, OPA in methanol is used in the experiment.

Table 2: Recovery percent of meropenem in plasma samples

Concentration (ppm)	Apparent recovery of meropenem (%)		SD OF RA	RSD of RA
0.5	R1	90.86	6.50	6.72
	R2	103.70		
	R3	95.46		
	Mean	96.67		
1	R1	110.48	2.72	2.47
	R2	113.07		
	R3	106.45		
	Mean	110.0		
2.5	R1	97.35	6.98	6.73
	R2	111.19		
	R3	102.60		
	Mean	103.71		

3.4 Precision and accuracy of the method

The samples spiked with meropenem at three different concentrations (0.5, 1 and 2.5 µg/ mL) were analyzed to get precision and accuracy with three replicates. The values of precision and accuracy are summarized in table 3 which shows better accuracy and precision of the method with relative error (RE) and relative standard deviation (RSD) both within the limit of 20 percent.

Table 3: Accuracy of extraction recovery of meropenem in plasma (n=3)

Spiked concentration of meropenem	Detected concentration of meropenem from spiked samples			Absolute error			Mean error	Relative error percent
	R1	R2	R3	R1	R2	R3		
0.5	0.48	0.34	0.50	0.02	0.16	0	0.09	18
1	0.77	0.93	0.79	0.23	0.07	0.21	0.17	17
2.5	2.35	2.32	2.06	0.15	0.18	0.44	0.25	10

4. Discussion

Analysis of meropenem and doripenem (Internal standard) by high-performance liquid chromatography was done according to the parameters described by Dincel *et al.* (2020) [5] with certain modifications. The results indicated good linearity within the described range of meropenem (analytical and commercial) and doripenem with a regression coefficient of 0.9989 and 0.9996 respectively for concentrations ranged from 0.1-1 ppm (meropenem) and 1-5 ppm (doripenem). The peak observed for the standard meropenem was in accordance with the one reported by Utapal (2011) [7] and Dincel *et al.* (2020) [5], for both pure and commercial standards. Roth *et al.* (2017) [8] quantified meropenem with a retention time of 7.8 min on a C_{18} column using a mobile phase mixture of 100 mM Tris/hydrochloric acid buffer (pH 8.5) containing 15 percent methanol at a flow rate of 1.0 mL/min. Recently, Peng *et al*. (2021) [9] analyzed meropenem on a C_8 column of 2 x 100 mm with a mobile phase of acetonitrile and 0.2 percent formic acid (30:70) at the flow rate of 0.3 mL/min and reported a retention time of 1.19 min. In another study, Legrand *et al.* (2008) [10] separated three carbapenems on a C_{18} column using a mobile phase of methanol and phosphate buffer (pH 6.8) in a gradient elution mode at a flow rate of 0.5 mL/min and reported a retention time of 22.5min in spiked human plasma samples. Meropenem was also eluted on a C_{18} (250 x 4.6 mm) with a retention time of 6.9 min when the mixture of methanol: water (15:85, v/v) at a flow rate of 1 mL/min (Kazanova *et al.,* 2020) [11]. However, we could not obtain a better-resolved peak of meropenem using the same mobile phase and run conditions. Variation in the run time observed in the above studies could be due to the column size and the different mobile phases used for its separation. The above studies further strengthened the fact that the presence of acid in aqueous phase improved the sensitivity of meropenem (Peng *et al.,* 2021) [9]. For the method validation and pharmacokinetic analysis, the retention time and the area of meropenem and doripenem were taken into consideration. The analytical method was validated by determining the accuracy and precision. Precision and accuracy of the method were also found satisfactory wherein the relative error (RE) and relative standard deviation (RSD) calculated were within the acceptable range (< 20%). The apparent recovery was calculated to evaluate the performance characteristics of the method as mentioned by Dincel *et al.* (2020) [5]. The Relative Standard Deviation (RSD) for meropenem ranged from 2.47 to 6.73 percent which met the criteria set forth by the Codex Alimentarius Commission for residue analysis-recovery of 70 to 110 percent and RSD of 20 percent. The limit of quantification (LOQ) and limit of detection (LOD) for meropenem were 0.94 µg/mL and 0.31µg/mL. Al-Jumaili and Ibrahim (2021) [12] analyzed meropenem using microbiological assay and reported the LOD and LOQ values of 0.19 µg/mL and 0.66 µg/mL respectively for meropenem in plasma.

Rancic (2022) [13] reviewed the chromatographic methods used by different researchers utilizing wide diversity of mobile phases for the quantification of meropenem in dissimilar matrices *viz.,* plasma, sputum, urine, bile, cerebrospinal fluid, peritoneal fluid, aqueous humor, bronchoalveolar fluid, epithelial lining fluid, interstitial fluid, renal replacement therapy effluents, blister fluids etc. The mixture of methanol and glacial acetic acid was used by the earlier researchers as either elution fluid or mobile phase for the detection of meropenem in serum, plasma, and urine (Amlashi *et al*., 2019; Dincel *et al.*, 2020) [14, 5]. In the present study, both meropenem and doripenem were best separated with retention times of 6.9 and 4.3 min respectively in the plasma with the mobile phase comprising of methanol: glacial acetic acid (15: 85, v/v) at the flow rate of 1 mL/min with UV absorbance at 300 nm with oven temperature maintained at 40 °C.

5. Conclusion

The described and validated method of high-performance liquid chromatography for the determination of meropenem from chicken plasma had good sensitivity, precision and accuracy were found to be satisfactory. The relative error (RE) and relative standard deviation (RSD) calculated were in an appropriate range within the acceptable limits. Hence, HPLC method can be effectively used to study the pharmacokinetic behavior of meropenem in broiler birds.

6. Acknowledgment

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7. Conflicts of interest

The authors declare that there are not any conflicts of interests

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