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# Development of an HPLC method for the determination of doripenem in human and mouse serum

Christina Sutherland [a], David P. Nicolau [a,b,*]

[a] *Center for Anti-Infective Research and Development, Hartford Hospital, Hartford, CT, United States*
[b] *Division of Infectious Diseases, Hartford Hospital, Hartford, CT, United States*

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**Abstract**

A HPLC method utilizing solid phase extraction was developed to analyze doripenem (formerly S-4661) in human and mouse serum. A reversed-phase column was used with a UV detector set at 295 nm. The mobile phase consisted of methanol and phosphate buffer at a flow rate of 1.5 ml/min. Meropenem was used as the internal standard. The standard curve was linear over a range of 0.5–40 μg/ml. The assay is simple, reproducible, and accurate and has been used successfully to analyze doripenem concentrations from a murine pharmacokinetic study.


*Keywords:* Doripenem; HPLC; Murine; Human

## 1. Introduction

Doripenem (formerly S-4661, Fig. 1A) is a new parenteral 1β-methyl carbapenem being developed for the treatment of hospitalized patients with moderate to severe bacterial infections. Doripenem has been shown to have broad-spectrum activity and to be more potent than other carbapenems versus wild type *P. aeruginosa* [1] Against ESBL-producing *E. coli*, doripenem had low MIC values when compared to ertapenem [1]. In another study, doripenem exhibited the lowest 50% inhibitory concentration ($MIC_{50}$) and lowest 90% inhibitory concentration ($MIC_{90}$) values against 600 multidrug-resistant clinical strains of *P. aeruginosa* and *B. cepacia* complex isolated from patients with cystic fibrosis when compared with seven other antipseudomonal antibiotics [2]. Similar to imipenem, doripenem displayed greater activity against Gram-positive cocci compared to meropenem [3].

To date, no analytical methods for the quantification of doripenem using HPLC have been reported in Medline journals. The purpose of this present study was to develop a simple, reproducible, and selective HPLC method. This assay was successfully used to analyze doripenem concentrations obtained from ICR (CD-1) mice during pharmacokinetic studies.

## 2. Experimental

### 2.1. Chemicals

Doripenem standard powder with purity of 990 μg/mg was provided by Peninsula Pharmaceuticals Inc. (Mountain View, CA). Meropenem (internal standard, Fig. 1B) was supplied by Astra Zeneca (Wilmington, DE). Sodium phosphate was purchased from Sigma (St. Louis, MO). HPLC grade methanol (Mallinckrodt Baker Inc., Phillipsburg NJ) and HPLC grade dichloromethane (Mallinckrodt) were used without further purification. Deionized water was obtained from a Milli-Q analytical deionization system (Bedford, MA).

### 2.2. Instrumentation and chromatographic conditions

A HPLC system consisting of a Waters 510 pump (Waters Associates, Milford, MA) and 717 plus autosampler (Waters) was equipped with a 5 μm phenyl hypersil column (4.6 mm × 100 mm, Thermo Electron Corp., Bellefonte, PA) coupled to a μBondapak $C_{18}$ 10 μm Guard-pak precolumn (Waters). The autosampler was cooled to 10 °C. The column was

* Corresponding author at: Center for Anti-Infective Research and Development, Hartford Hospital, 80 Seymour Street, Hartford, CT 06102, United States. Tel.: +1 860 545 3941; fax: +1 860 545 3992.
*E-mail address:* dnicola@harthosp.org (D.P. Nicolau).

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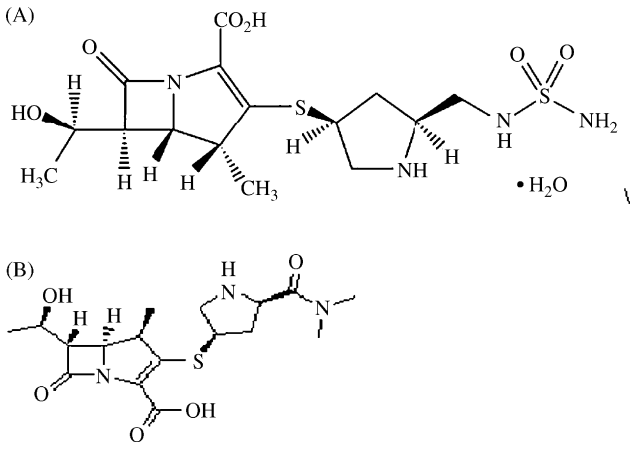


Fig. 1. Chemical structure of doripenem (formerly S-4661) (A) and meropenem (B).

maintained at room temperature. A programmable UV detector set at 295 nm (Model 526; ESA Inc., Chelmsford, MA) was used to detect the analytes. EZChrom Elite chromatography data system (Scientific Software Inc., Pleasanton, CA) was used to quantify the peak heights.

The mobile phase consisted of a mixture of 0.026 M phosphate buffer with 4.35% methanol. The flow rate was 1.5 ml/min. The column pressure was 1500 psi. The running time for one sample was 16 min. All chromatographic procedures were performed at room temperature.

### 2.3. Standard solutions and controls

Doripenem stock solution of 2000 μg/ml was made in a volumetric flask according to Clinical and Laboratory Standards Institute guidelines using 0.85% physiological saline to dissolve and dilute the stock standard [4]. The internal standard, meropenem 80 μg/ml was prepared in water.

Drug free human and ICR mouse serum was purchased from Bioreclamation Inc. (Hicksville, NY). Additionally, freshly obtained drug free ICR mouse blood was collected at our site and centrifuged at 2400 × *g* for 10 min. The pooled blank serum was stored at −20 °C prior to use. Doripenem was spiked into human serum to make 8 standard solutions (0.5, 2, 4, 5,10,15, 20, and 40 μg/ml) and three quality controls (1, 8, and 30 μg/ml). The range of the standard curve was based on the anticipated concentration-versus-time profile for doripenem as compared to similar agents within the carbapenem class. Additionally three quality controls (1, 8, and 30 μg/ml) were prepared for a cross-matrix validation in mouse serum. Aliquots of the standards and internal standard were store at −80 °C until analysis.

### 2.4. Sample extraction

A Waters Oasis 1cc HLB Extraction Cartridge with extension needle tip was used along with the extraction manifold for sample preparation. A vacuum pump was used to draw the fluid through the cartridge. One milliliter of methanol conditioned the cartridge. One milliliter of water was used to equilibrate the cartridge. While the manufacturer recommends 5% methanol to equilibrate the cartridge, preliminary studies revealed that 1 ml of water further improved recovery (data not shown). A 200 μl sample of standard, quality control, or unknown sample along with a 50 μl aliquot of internal standard was loaded into the cartridge. The cartridge was washed with 1 ml of water. The waste tube underneath was removed and replaced with a clean blue polyethylene sample tube. The analytes were then eluted from the cartridge with 1 ml methanol. The eluate was dried under a stream of nitrogen for 30 min at 40 °C. The residuals were reconstituted with 200 μl of mobile phase, vortexed for 30 s, and placed into an autosampler vial for injection.

### 2.5. Assay validation

Calibration curves in human serum were generated by plotting the peak height ratio of doripenem to that of the internal standard. Weighted (1/concentration) least square regression analyses were applied to generate the linear regression equation. This equation was used to calculate the concentrations of the quality controls and unknown samples. Linearity of the standard curve was assessed with the correlation coefficient.

A full validation ($n = 6$ runs) was performed in human serum. A calibration curve consisted of a blank sample (matrix sample processed without internal standard), a zero sample (matrix sample processed with internal standard), and eight standards. Quality controls with low, middle, and high concentrations were used to evaluate the precision and accuracy. The precision was determined by the relative standard deviation (RSD) and the accuracy was determined by the relative error from the theoretical concentrations. The lower limit of quantification (LLQ) for the assay was evaluated on five samples with 0.5 μg/ml of doripenem. Recovery experiments were performed in triplicate by comparing the analytical results for extracted serum samples at the quality control concentrations with unextracted controls prepared in saline that represent 100% recovery. Values of percent recoveries of doripenem in the quality controls samples were calculated by comparing the peak height ratio of doripenem and the internal standard in serum to that of nonextracted aqueous solutions. The recovery of the internal standard was estimated by comparing the peak height of meropenem in human serum to that in aqueous solution.

The room temperature, autosampler, and freeze and thaw stability of doripenem were determined in triplicate on each quality control sample. The freeze and thaw stability was performed by completely thawing the quality controls at room temperature and refreezing at −80 °C for 24 h. The freeze–thaw cycle was repeated two more times, then analyzed on the third cycle. Stability of internal standard at 80 μg/ml was assessed at room temperature.

A partial or cross-matrix validation was performed to ensure that the mouse serum was comparable to the original biological matrix of human serum. Quality controls in mouse serum with low, middle, and high concentrations were used to evaluate the precision and accuracy against the human standard curve.

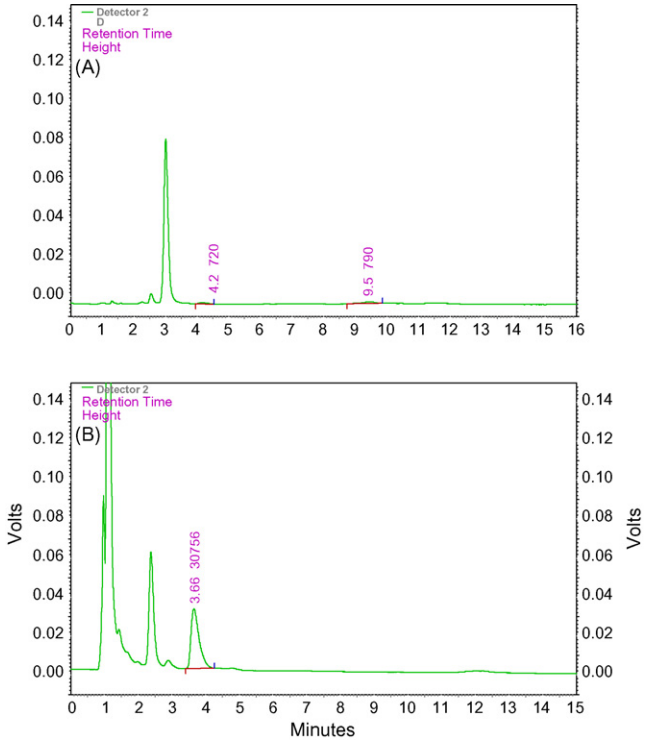


Fig. 2. Chromatogram of blank human serum (A) and mouse serum (B).

## 3. Results

### 3.1. Chromatography

Several mobile phases based on methanol, phosphate buffer with different pH, ion-pairing agents were tried along with different extraction methodologies. It was noticed that lowering the methanol concentration increased the retention time of both doripenem and meropenem, however the methanol concentration had a greater effect on meropenem. Adjusting the pH or adding an ion-paring agent of tetrapentyl ammonium hydroxide or tetrapentyl ammonium bromide did not effect the retention time. Endogenous peaks interfered with the doripenem using liquid–liquid extractions.

Six sources of human and mouse serum were tested for interference using the solid phase method described. Fig. 2A represents a typical chromatogram with blank human serum. Fig. 2B represents a typical chromatogram with blank mouse serum. The chromatograms show no interfering peaks with doripenem or the internal standard. The retention time of doripenem and the internal standard were 5.4 and 12.7 min, respectively. Three out of the 410 mouse samples assayed for the PK study had an interfering peak seen at the doripenem retention time.

### 3.2. Linearity

Plotting the peak height ratio of doripenem and the internal standard versus the theoretical concentrations generated the six standard curves. The correlation coefficient ($r$) for each calibration curve was $\geq$0.998. The slope was 0.0575 ± 0.0037 (mean ± SD) and the intercept was 0.0013 ± 0.0057 (mean ± SD). While we did note some tailing of the internal standard, the use of peak heights for quantitation, minimized the impact of this event as peak height remained consistent through out the assay.

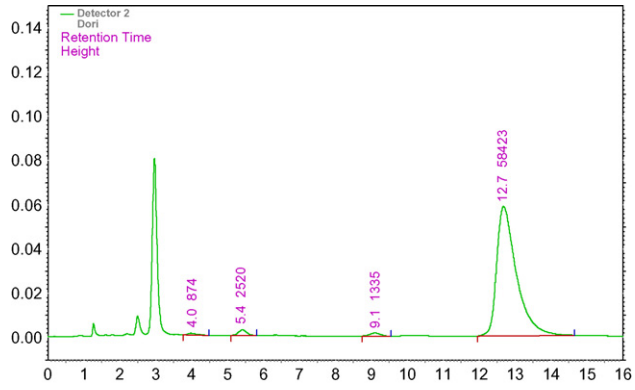


Fig. 3. Chromatogram of LLQ in human serum. The retention time of doripenem and the internal standard were 5.4 and 12.7 min, respectively.

### 3.3. Lower limit of quantification

The LLQ of 0.5 μg/ml doripenem was chosen as the concentration for the lowest standard sample Fig. 3. The precision and accuracy of LLQ ($n$ = 5) was 5.50 and 4.94%, respectively. There was no response seen from the blank human serum at the doripenem retention time.

### 3.4. Precision and accuracy

The summary data for the inter- and intra-day precision and accuracy in both the human and murine matrices are shown in Tables 1 and 2. In human serum the inter-run ($n$ = 6 runs of 1 sample each) precision of the 1, 8, and 30 μg/ml samples was 7.83, 4.86, and 5.71% respectively. The intra-run ($n$ = 10 samples) precision of the 1, 8, and 30 μg/ml samples was 4.80, 4.96, and 5.44%, respectively. The inter-run accuracy of the 1, 8, and 30 μg/ml samples was 0.77, 2.57, and 2.46%, respectively.

Table 1
Precision and accuracy of doripenem in human serum

| | Theoretical concentration (μg/ml) | | |
|---|---|---|---|
| | Low (1) | Medium (8) | High (30) |
| Inter-run ($n$ = 6) | | | |
| Mean | 1.01 | 8.21 | 29.26 |
| SD | 0.079 | 0.398 | 1.672 |
| RSD (%) | 7.83 | 4.86 | 5.71 |
| Relative error (%) | 0.77 | 2.57 | 2.46 |
| Intra-run ($n$ = 10) | | | |
| Mean | 1.02 | 8.10 | 30.03 |
| SD | 0.049 | 0.402 | 1.634 |
| RSD (%) | 4.80 | 4.96 | 4.96 |
| Relative error (%) | 2.19 | 1.26 | 0.10 |

Table 2
Precision and accuracy of doripenem in mouse serum

| | Theoretical concentration (μg/ml) | | |
|---|---|---|---|
| | Low (1) | Medium (8) | High (30) |
| Inter-run ($n=6$) | | | |
| Mean | 1.06 | 7.99 | 30.68 |
| SD | 0.051 | 0.422 | 1.476 |
| RSD (%) | 3.30 | 3.41 | 5.13 |
| Relative error (%) | 4.16 | 0.47 | 2.12 |
| Intra-run ($n=8$) | | | |
| Mean | 1.04 | 8.04 | 30.64 |
| SD | 0.034 | 0.274 | 1.572 |
| RSD (%) | 4.86 | 5.28 | 4.18 |
| Relative error (%) | 5.86 | 0.12 | 2.27 |

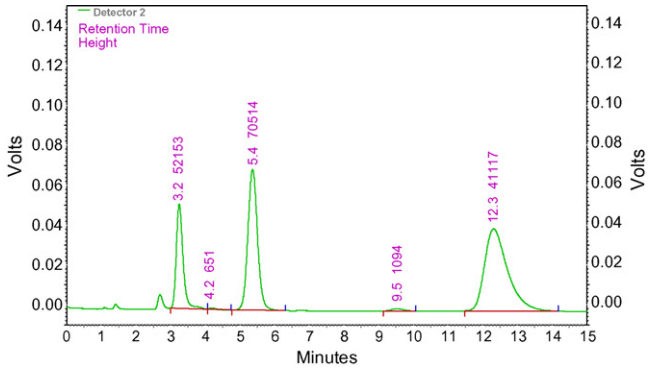


Fig. 4. Chromatogram of doripenem high quality control (30 μg/ml) sample in mouse serum. The retention time of doripenem and the internal standard were 5.4 and 12.3 min, respectively.

The intra-run accuracy of the 1, 8, and 30 μg/ml samples was 2.19, 1.26, and 0.10%, respectively.

The mouse serum inter-run ($n=6$ runs of 1 sample each) precision of the 1, 8, and 30 μg/ml samples was 3.30, 3.41, and 5.13%, respectively. Fig. 4 depicts the chromatogram of the high quality control sample in this matrix. The intra-run ($n=8$ samples) precision of the 1, 8, and 30 μg/ml samples was 4.86, 5.28, and 4.18%, respectively. The inter-run accuracy of the 1, 8, and 30 μg/ml samples was 4.16, 0.47, and 2.12%, respectively. The intra-run accuracy of the 1, 8, and 30 μg/ml samples was 5.86, 0.12, and 2.27%, respectively.

### 3.5. Recovery

The recovery of doripenem in the 1, 8, and 30 μg/ml human matrix samples was 61.5% ± 0.5, 52.9% ± 0.7, and 47.3% ± 0.4%, respectively. The recovery of the internal standard was 54.2 ± 3.6%.

### 3.6. Stability

Doripenem and meropenem stock solutions were stable at room temperature at 23 °C for at least 5 h with ≤10% degradation. Doripenem quality controls were stable at room temperature at 23 °C for 5 h with ≤10% degradation. Doripenem was stable for three freeze–thaw cycles with <10% degradation. The quality control samples after extraction in the 10 °C autosampler were stabile for 24 h with ≤7% degradation.

## 4. Conclusions

A new validated HPLC method has been developed to assess doripenem concentrations in both human and mouse serum. The inter-run and intra-run precision and accuracy for both the human and mouse quality samples were within the 10% acceptance criteria used in our laboratory. The correlation coefficient also exceeded our group's acceptable limit of 0.995. The LLQ (0.5 μg/ml) was well below our acceptable limit (20%) for both precision and accuracy. These data support a methodology that results in a precise, selective and accurate assay. This assay has been successfully utilized to determine doripenem concentrations in mouse serum collected within 1 week of sample assay with minimal interference of the host matrix. Additional study is required to assess the long-term sample stability under various duration and temperature conditions.

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