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Short communication

A simple and rapid determination of biapenem in plasma by high-performance liquid chromatography

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Abstract

A simple, rapid and precise HPLC method using ultrafiltration to remove plasma protein was developed to determine biapenem concentrations in human plasma. Plasma was separated by centrifugation at 4 °C from blood collected in heparinized vacuum tubes, and biapenem was stabilized by immediate mixing the plasma with 1 M 3-morpholinopropanesulfonic acid (MOPS) buffer (pH 7.0) (1:1). Biapenem was detected by ultraviolet absorbance at 300 nm with no interfering plasma peak. The calibration curve of biapenem in human plasma was linear from 0.04 to 50 μg/mL. The limit of detection was 0.01 μg/mL, which was more than 40-fold lower than that of conventional plasma protein precipitation using ammonium sulfate. The assay has been clinically applied to pharmacokinetic studies in patients.
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Keywords: Biapenem; Ultrafiltration

1. Introduction

Biapenem is a new parenteral carbapenem (Fig. 1) that has antibacterial activity against a wide range of Gram-positive and -negative bacteria [1]. The MIC of biapenem for 90% (MIC_{90}) of *Pseudomonas aeruginosa* strains is 3.13 μg/mL, and it is two-fold more active than imipenem. Biapenem inhibits *Haemophilus*, *Neisseria* and *Branhamella* species at MIC_{90}s of 3.13, 0.1 and 0.1 μg/mL, respectively. Biapenem is two- to four-fold less active than imipenem against methicillin-susceptible *Staphylococcus aureus* and *Staphylococcus epidermidis* at MIC_{90} values of 0.1 and 0.39 μg/mL. However, biapenem is two-fold more active than imipenem against *Bacteroides fragilis* at an MIC_{90} of 1.56 μg/mL [1]. Biapenem is stable against human renal dehydropeptidase-I (DHP-I) [1,2], so co-administration of a DHP-I enzyme inhibitor such as cilastatin is not required as it is for imipenem. The efficiency of carbapenem [3,4] and biapenem [5] is related to the length of time that the antibiotic concentration remains above the minimum inhibitory concentration (time above MIC). Pharmacokinetic studies can help to establish the optimal dosage regimen for clinical use.

The few methods that can measure plasma levels of biapenem include a microbiological assay [6] and an HPLC method with ammonium sulfate precipitation of plasma protein [6–8]. The HPLC method is specific, whereas microbiological assays cannot differentiate biapenem from other antibiotics that could be co-administered. Generally, the limit of detection using HPLC and ammonium sulfate precipitation is not low enough, because of dilution with the ammonium sulfate and interference by plasma blank peaks. Furthermore, reproducibility is often low because of co-precipitation with plasma proteins, so an internal standard is required. Previous reports describing biapenem determination using ammonium sulfate precipitation [6–8] did not include validation of the method, rendering details obscure. Other carbapenems such as imipenem and ertapenem have been measured in plasma using HPLC and ultrafiltration [9–11].

We describe here a sensitive HPLC method of measuring biapenem that uses ultrafiltration for plasma deproteinization.

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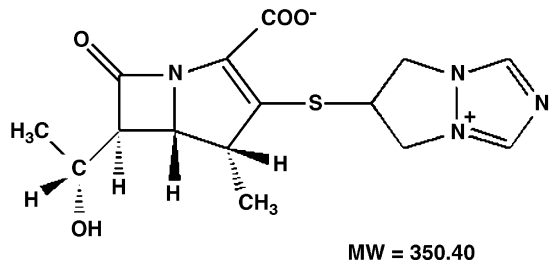

Fig. 1. Structure of biapenem.

2. Experimental

2.1. Reagents and materials

Standard biapenem was provided by Meiji Seika Kaisha Ltd. (Tokyo, Japan) and 3-morpholino-propanesulfonic acid (MOPS) buffer (pH 7.0) was purchased from Dojindo (Kumamoto, Japan). Acetic acid and sodium acetate were purchased from Wako (Osaka, Japan) and acetonitrile was purchased from Sigma–Aldrich Japan (Tokyo, Japan). All chemicals were of analytical grade. The Nanosep® 10K Centrifugal Filter Device was provided by the PALL Corporation (New York, USA).

2.2. Equipment

The HPLC system comprised 600E system controller, 700 Satellite WISP auto-sampler (Waters, Milford, MA, USA), UV spectrophotometric detector SPD-6A (Shimadzu, Kyoto, Japan), Chromatocorder 21 (System Instruments, Tokyo, Japan) and a column heater U-620 Type30 (Sugai Chemical Industry, Wakayama, Japan).

2.3. Chromatographic conditions

The samples were separated by chromatography on a μBondasphere C_{18} 5 μm (3.9 mm × 150 mm) column (Waters). The mobile phase was a mixture of 0.1 M sodium acetate buffer (pH 4.6) and acetonitrile (197:3, v/v), and the pump flow rate was 1.0 mL/min. The auto sampler was set to 4 °C, and the injection volume was 20 μL. The column temperature was 40 °C. The biapenem peak was detected by ultraviolet absorbance at 300 nm.

2.4. Plasma samples

Blood samples were collected into heparinized vacuum tubes (Nipro, Osaka, Japan), and separated by centrifugation at 1000 × *g* for 10 min at 4 °C. Plasma samples were mixed with the same volume of 1 M MOPS buffer (pH 7.0) and stored at −40 °C until analysis. Control human plasma was a mixture of equal volume of plasma from five healthy volunteers and stored at −40 °C.

2.5. Analytical procedure

A working stock solution of biapenem was prepared daily at a concentration of 1 mg/mL in 50 mM MOPS buffer (pH 7.0) and dilutions of 0.16, 0.4, 2.0, 4.0, 20.0, 100.0 and 200.0 μg/mL were prepared in the same buffer. Control plasma (200 μL) was mixed with 50 μL of biapenem solutions. The final concentrations of biapenem corresponded to 0.04, 0.1, 0.5, 1.0, 5.0, 25.0 and 50.0 μg/mL in plasma, because clinical samples also included 50 μL of 50 mM MOPS buffer (pH 7.0). Samples were then mixed with 200 μL of 1 M MOPS buffer (pH 7.0) and transferred to a Nanosep® 10K Centrifugal Filter Device. Clinical plasma samples that were mixed with the same volume of 1 M MOPS buffer (pH 7.0) and stored at −40 °C, were thawed to room temperature. A 400-μL aliquot was mixed with 50 μL of 50 mM MOPS buffer (pH 7.0), and transferred to a Nanosep® 10K Centrifugal Filter Device. The devices were centrifuged at 12,000 × *g* for 10 min at room temperature. Filtrate (20 μL) was injected into the HPLC system for analysis.

2.6. Method validation

Plasma standard samples (0.1, 0.5, 1.0, 5.0, 25.0 and 50.0 μg/mL) were prepared in sextuplicate or duplicate, and analyzed on 5 separate days during method validation. The calibration curves were not weighted. Intra- and inter-assay precision and accuracy were determined from these data. The limits of detection (LOD) and of quantitation (LOQ) of biapenem were determined from the peak and standard deviation of the noise level, S_N. The LOD and LOQ were defined as the sample concentration of biapenem that resulted in peak heights of 3- and 10-fold the S_N, respectively, and validation was established at the LOQ (intra-assay, $n = 6$).

2.7. Recovery

The recovery of biapenem by ultrafiltration was determined by comparing the peak areas from plasma standards with those from biapenem standards that were similarly prepared except water replaced control plasma and they were not filtered, as follows. Control plasma (200 μL) and control plasma diluted 1:2 or 1:4 with water were mixed with 50 μL biapenem at 25 μg/mL (final concentration) and 200 μL of 1 M MOPS buffer (pH 7.0) and then transferred to a Nanosep® 10K Centrifugal Filter Device. The devices were centrifuged at 12,000 × *g* for 10 min at room temperature. The controls for recovery evaluation included 200 μL of water, 50 μL of biapenem and 200 μL of 1 M MOPS buffer (pH 7.0) without ultrafiltration.

2.8. Validation and recovery test after plasma protein precipitation using ammonium sulfate

Control plasma (200 μL) was mixed with 50 μL of biapenem (final concentration of biapenem, 1.0, 5.0 and 50.0 μg/mL in plasma), 200 μL of 1 M MOPS buffer (pH 7.0) and 600 μL of 30% ammonium sulfate and then vortex mixed, and centrifuged at 12,000 × *g* for 3 min at room temperature. Supernatant (20 μL) was applied to HPLC. For the recovery test, controls were processed in the same way plasma samples, except they included water instead of plasma and were not separated by centrifugation.

2.9. Stability

We examined the stability of biapenem in plasma. Control plasma (1.95 ml) was spiked with 50 μL of stock solution to contain 1.0, 5.0 and 25.0 μg/mL of biapenem. An equal volume of 1 M MOPS buffer (pH 7.0) was added, mixed, separated into 1 mL aliquots and stored at −40 °C. The biapenem concentration was determined at 0, 6, 15 and 30 days ($n = 4$).

2.10. Application to pharmacokinetic studies in patients

Bacteria constitute an important cause of infection in bone marrow transplants and in neutropenic patients with cancer, and are also an important cause of morbidity [12,13]. Pediatric leukemia or other cancer patients aged 6–16 years of age were infused with 300 mg of biapenem over 1 h. Plasma concentrations of biapenem were measured at 0, 1, 3, 5 and 7 h after starting the infusion. Changes in the plasma biapenem concentration were fitted to a two-compartment model and analyzed with the nonlinear least-squares computer program (MULTI) [14].

3. Results

3.1. Typical chromatograms

Fig. 2 shows chromatograms typical of blank, spiked plasma and patient samples. The chromatographic conditions were as described in Section 2.3. Interfering peaks were not evident and the retention time for biapenem was 3.8 min.

3.2. Limits of detection and quantitation

The LOD and LOQ from the ultrafiltration methods were 0.01 and around 0.04 μg/mL (C.V.: 4.16%, accuracy: 118.4%, intra-assay, $n = 6$), respectively, using a 20 μL injection volume. These values from ammonium sulfate precipitation were 0.4 and >1.5 μg/mL (C.V.: 6.84%, accuracy: 147.5%, intra-assay, $n = 6$), respectively, using the same injection volume.

Table 1
Validation of this method and ammonium sulfate plasma protein precipitation method

Concentration added (μg/mL)	Concentration found (mean ± S.D.) (μg/mL)	C.V. (%)	Accuracy (%)
This method			
Intra-assay ($n = 6$)			
0.04	0.047 ± 0.002	4.16	118.4
0.1	0.112 ± 0.001	0.76	111.5
0.5	0.545 ± 0.008	1.47	109.0
1.0	1.071 ± 0.018	1.71	107.1
5.0	4.867 ± 0.042	0.87	97.3
25.0	25.45 ± 0.437	1.72	101.8
50.0	49.79 ± 1.037	2.08	99.6
Inter-assay ($n = 5$)			
0.1	0.112 ± 0.003	2.77	112.3
0.5	0.518 ± 0.038	7.31	103.6
1.0	1.052 ± 0.034	3.24	105.2
5.0	5.041 ± 0.151	2.99	100.8
25.0	25.31 ± 0.142	0.56	101.2
50.0	49.84 ± 0.077	0.15	99.7
Ammonium sulfate plasma protein precipitation method			
Intra-assay ($n = 6$)			
1.0	2.419 ± 0.232	9.59	241.9
5.0	6.771 ± 0.234	3.46	135.4
50.0	49.79 ± 1.366	2.74	99.6

S.D.: standard deviation. C.V. (%): coefficient of variation.

3.3. Method validation

The linearity of the ultrafiltration method was good between 0.04 and 50 μg/mL ($r^2 = 0.9999$ (mean) $\pm 9.3 \times 10^{-5}$ (S.D.) ($n = 5$)). Table 1 summarizes the reproducibility and accuracy at each calibration standard. The method was compared with ammonium sulfate precipitation using intra-assay validation of latter (Table 1).

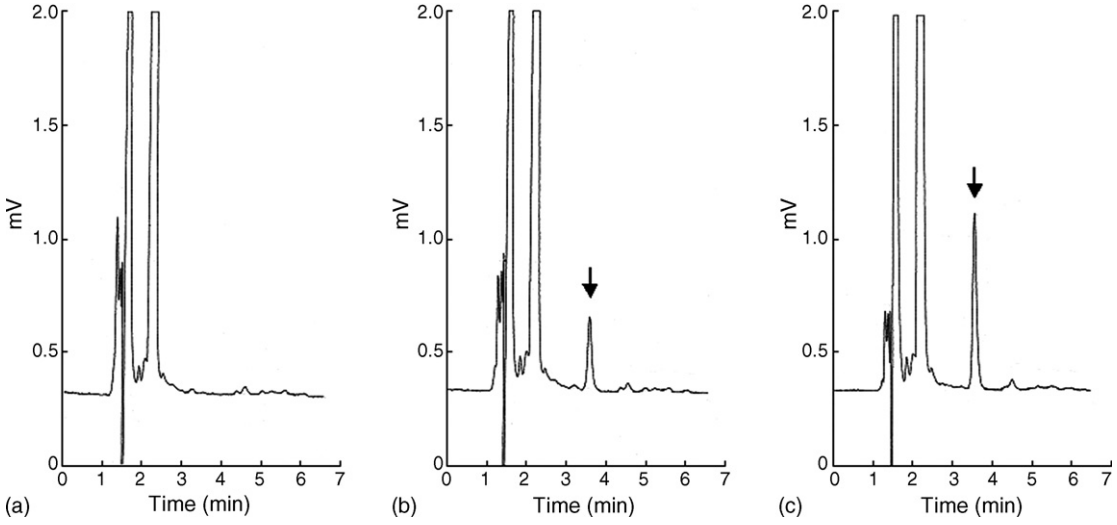

Fig. 2. Typical chromatograms. (a) Blank control plasma. (b) Control plasma spiked with 0.5 μg/mL biapenem. (c) Patient plasma sample (1.2 μg/mL). Arrows indicate biapenem peaks.

Table 2
Recovery study

Concentration of biapenem (μg/mL)	Recovery ($n=6$) (%)
0.1	93.4 ± 0.8
0.5	93.6 ± 1.4
1.0	96.5 ± 1.6
5.0	95.8 ± 0.8
25.0	97.1 ± 1.7
50.0	97.4 ± 2.0

3.4. Recovery

Table 2 shows the results of the recovery test. The plasma concentration affected recovery at 25 μg/mL of biapenem as follows: 95.1 ± 3.4% (S.D.) ($n=6$) and 100.3 ± 3.4% (S.D.) ($n=6$) for control plasma diluted 1:2 and 1:4, respectively. In addition, the recovery of 25 μg/mL of biapenem from control plasma was not affected by standing the mixture for up to 1 h at room temperature (data not shown). The recovery of 5 and 50 μg/mL of biapenem using ammonium sulfate precipitation was 45.5 ± 1.7% (S.D.) ($n=6$) and 33.5 ± 1.0% (S.D.) ($n=6$), respectively.

3.5. Stability

The stability of the plasma samples at 1.0, 5.0 and 25.0 μg/mL of biapenem was examined using an equal volume of 1 M MOPS buffer (pH 7.0) as a stabilizer after storage at −40 °C. The concentrations at 30 days were 103.2 ± 6.9% (S.D.) ($n=4$), 98.2 ± 2.5% (S.D.) ($n=4$) and 99.1 ± 0.9% (S.D.) ($n=4$) of the initial concentration at 1.0, 5.0 and 25.0 μg/mL, respectively.

3.6. Application to pharmacokinetic studies in patients

Fig. 3 shows the results from four pharmacokinetic studies of pediatric patients aged 6–16 years with leukemia. They were all infused with 300 mg of biapenem over 1 h and then the plasma concentrations of biapenem were measured (see Section 2). Changes in the plasma biapenem concentration were fitted to the two-compartment model (Fig. 3). The time course profile of the plasma biapenem concentration at the same dosage regimen widely differed among the four individuals, and might depend not only on body weight but also on renal function (manuscript in preparation).

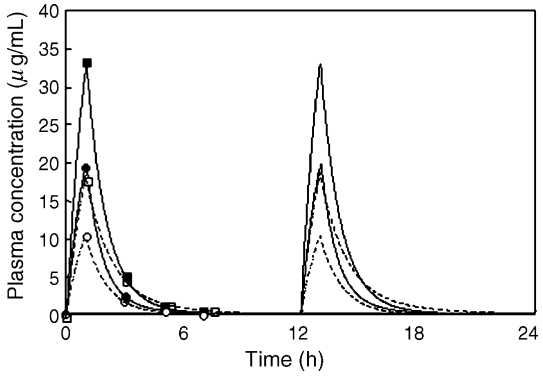

Fig. 3. Time course of plasma biapenem concentrations in four patients infused with 300 mg biapenem for 1 h every 12 h. Symbols, measured data; lines, change in plasma biapenem concentration fitted to two-compartment model. Solid squares and solid line, 6-year-old patient weighing 20 kg. Solid circles and solid line, 13-year-old patient weighing 45 kg. Open squares and dotted line, 16-year-old patient weighing 37 kg. Open circles and dotted line, 13-year-old patient weighing 46 kg.

4. Discussion

The LOD and LOQ values using our method were more than 40-fold lower than those obtained using ammonium sulfate precipitation. The LOQ of our method was around 0.04 μg/mL (C.V., 4.16%; accuracy, 118.4%; $n=6$, intra-assay). The intra-day assay precision was <2.08% C.V. and accuracy was within 97.3% and 111.5% at over 0.1 μg/mL (Table 1). The inter-day assay precision was <7.31% C.V. and accuracy was within 99.7% and 112.3%, at over 0.1 μg/mL (Table 1). These values are sufficient for clinical measurements. The low accuracy (241.9%) and reproducibility (9.59%) values of the precipitation method at 1.0 μg/mL (Table 1) were due to plasma peaks. Ultrafiltration was superior to ammonium sulfate precipitation in terms of LOD, LOQ, precision and accuracy, and no internal standard was required.

Biapenem bound with plasma protein cannot be separated by ultrafiltration. The plasma protein binding ratio of biapenem is small and the ratio is 6.6 ± 2.8% at 10 μg/mL, and 4.3 ± 1.8% at 50 μg/mL (ultrafiltration and radioimmunoassay data from Meiji Seika Kaisha Ltd.). Therefore, biapenem binding to plasma proteins might minimally influence the ultrafiltration method. To confirm this, we examined the influence of a variation in the plasma protein content upon recovery using control plasma and control plasma diluted 1:2 and 1:4 with water. Recovery tended to increase at higher concentrations of biapenem (Table 2) and lower concentrations of plasma. These conditions might reduce the amount of binding between biapenem and plasma protein. However, the recovery values were 93.4–97.4% at several concentration of biapenem in control plasma (Table 2). Under clinical conditions, the protein content of plasma might fall to about 50% at most. The recovery was 95.1% in control plasma diluted 1:2 at 25 μg/mL of biapenem (see Section 3), and 97.1% in control plasma (Table 2). Recovery of biapenem at 25 μg/mL was not affected by standing at room temperature for up to 1 h after mixing. Therefore, the variation in recovery appears to be within the error range. We concluded that recovery using the ultrafiltration method is about 95% and that the concentration of biapenem and variations in human plasma protein content have minimal effects on recovery, probably due to the plasma protein binding ratio. The low recovery of biapenem (45.5% at 5 μg/mL and 33.5% at 25 μg/mL of biapenem) obtained after protein precipitation using ammonium sulfate might be due to coprecipitation with plasma protein, which might also explain the low reproducibility.

Biapenem is unstable in plasma. Yanagi et al. [6] reported that biapenem in two-fold diluted solutions of plasma with 1 M MOPS buffer (pH 7.0) or 50 mM MOPS buffer (pH 7.0)/5% ethylene glycol mixture (1:1) were stable for 4 days at −20 °C

and 53 days at −80 °C. Our stability studies show that biapenem in plasma was stable for over 30 days when stabilized with an equal volume of 1 M MOPS buffer (pH 7.0) and stored at −40 °C. In our clinical study, all plasma samples of biapenem were processed within 7 days.

In conclusion, we developed a simple method using ultrafiltration for removing plasma protein so that biapenem levels can be determined in human plasma. After a 10 min centrifugation, filtrates can be applied to HPLC, which requires only a few minutes. Thus, this method is rapid enough to monitor the plasma biapenem concentration in real time.

Pharmacokinetic studies are presently in progress. The efficiency of biapenem is thought to be closely correlated with time above MIC. According to our method for calculating this value (% in 24 h) from the time course profile of the plasma biapenem concentration (not shown in detail), it could be used to determine individual dosing regimens. Patient factors that affect pharmacokinetic profiles were investigated and we concluded that not only body weight but also renal function is important. The simple and rapid determination described here should be suitable for measuring plasma biapenem concentrations in pharmacokinetic studies.

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