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Simultaneous determination of eight β-lactam antibiotics in human serum by liquid chromatography–tandem mass spectrometry

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ABSTRACT

A liquid chromatography–tandem mass spectrometry (LC–MS/MS) method was developed for the simultaneous determination of eight β-lactam antibiotics, including ampicillin, cefazolin, cefepime, cefmetazole, cefotaxime, doripenem, meropenem, and piperacillin, in human serum. Sample specimens were subjected to solid phase extraction (SPE) using Waters Oasis® HLB cartridges (30 mg). Chromatographic separation was performed with a high-resolution octadecyl silica column compatible with hydrophilic compounds, using a gradient of 10 mM aqueous ammonium formate containing 0.1% formic acid-methanol. Antibiotics were detected by a triple quadrupole mass spectrometer (MS/MS) with electrospray ionization and quantified by the multiple reaction monitoring mode. A total run time of 13 min was applied. Linearity in the calibration was obtained over a range of 0.1–50 μg/mL of the β-lactam antibiotics, except for doripenem. The lower limit of quantification was 0.005–0.5 μg/mL, using 50 μL serum. The recovery rate exceeded 80.2% for these analytes, except for doripenem (49.1%) and meropenem (62.3%). The present method is applicable to routine therapeutic monitoring of β-lactam antibiotics in clinical practice.

1. Introduction

β-Lactam antibiotics are the most frequently prescribed antimicrobial agents for the treatment of bacterial infections. The time above the minimum inhibitory concentration (T > MIC) is one of the most important parameters that correlates with the therapeutic efficacy of β-lactam antibiotics. It is generally accepted that a bacteriostatic effect is observed when T > MIC is maintained for 30% of the dosing interval for penicillins, 40% for cephalosporins and 20% for carbapenems, whereas the maximum bactericidal effect is reached when T > MIC exceeds for 50% of the interval for penicillins, 60–70% for cephalosporins and 40% for carbapenems [1–4]. These findings led to the optimization of dosage regimens to increase clinical efficacy and to reduce the generation of resistant mutants [5–8]. Therefore, monitoring of serum concentrations of antibiotics in reference to the MIC of the micro-organism causing infection is important in individualizing antimicrobial therapy.

Numerous HPLC–UV methods have been reported for the analysis β-lactam antibiotics in several biological fluids, including human plasma [8–10]. Because of the inherent low sensitivity, UV methods required a large sample volume.

LC–MS/MS has led to a major breakthrough in the field of quantitative bioanalysis due to its inherent specificity, sensitivity, and speed. Thus, LC–MS/MS is regarded as the preferred technique of quantification small molecule drugs and their metabolites in biological matrices such as plasma, blood, serum, urine, and tissues [11]. For example, LC–MS/MS for the quantification of β-lactam antibiotics has been reported to include clavulanic acid in porcine tissues [12], multiple β-lactam antibiotics in the bovine kidney [13,14], five β-lactam antibiotics in raw milk [15], cefepime in mouse plasma [16] and amoxicillin in human plasma [17]. LC–MS/MS has long been used in food safety to measure antibiotics, and the LC and MS conditions can be translated for clinical use, even if the matrix is different. However, there have been few articles on the simultaneous determination of penicillin, cephem and carbapenem β-lactam compounds in human specimens.

In the present study, an accurate and sensitive LC–MS/MS method was established to simultaneously determine β-lactam antibiotics in human serum. In patients admitted to the intensive care unit (ICU), particularly those with sepsis, serum drug concentrations tend to be reduced due to the increased volume of distribution (Vd) and cardiac output [4,18,19]. Therefore, the

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Table 1
Parameters of the LC–MS/MS method for 8 β-lactam antibiotics and its internal standard.

Compounds	MRM transition (*m/z*)	Ionization mode	Cone voltage (V)	Collision energy (eV)
Cefepime	480.9 > 85.9	ESP+	24	14
Doripenem	420.9 > 273.9	ESP+	24	18
Meropenem	384.0 > 141.0	ESP+	26	18
Cefmetazole	471.8 > 355.7	ESP+	24	10
Cefotaxime	455.8 > 395.7	ESP+	24	10
Ampicillin	349.9 > 105.9	ESP+	22	14
Cefazolin	454.8 > 322.9	ESP+	20	10
Piperacillin	517.9 > 143.1	ESP+	28	22
Ethylparaben (IS)	165.0 > 91.8	ESP−	32	24

present method was applied to therapeutic drug monitoring in patients admitted to the ICU.

2. Experimental

2.1. Chemicals and materials

Antibiotics were obtained from the following pharmaceutical companies: cefazolin from Astellas Pharma Inc. (Tokyo, Japan), cefepime from Bristol-Myers Squibb (Tokyo), cefmetazole from Daiichi Sankyo Co. (Tokyo), meropenem from Dainippon Sumitomo Parma Co. (Osaka, Japan), ampicillin from Meiji Seika Kaisha (Tokyo), cefotaxime from Nichi-Iko Pharmaceutical Co. (Toyama, Japan), doripenem from Shionogi & Co. (Osaka), and piperacillin from Taisho Toyama Pharmaceutical Co. (Tokyo). Formic acid was purchased from Wako Pure Chemical Industries (Osaka). Ethylparaben (internal standard, IS) and ammonium formate were obtained from Nacalai Tesuque (Kyoto, Japan). HPLC grade methanol was obtained from Kishida Chemical (Osaka). Water was purified using a Milli-Q system (Millipore, Bedford, MA, USA). All reagents were analytical reagent grade.

2.2. Stock solutions

Stock solutions of antibiotics (5 mg/mL) and IS solution (1 μg/mL) were prepared by dissolving powdered antibiotics in water and IS in methanol. They were stored at −70 °C until use. For the experiment they were dissolved in drug-free human serum to obtain concentrations of 0.05, 0.1, 0.5, 1, 5, 10, 25, and 50 μg/mL. Quality control (QC) samples at 0.1, 1, 10 and 50 μg/mL were prepared by adding appropriate volumes of the respective stock solutions to drug-free human serum. The prepared serum standards and quality control standards were transferred into 15 mL polypropylene tubes and stored at −70 °C until analysis.

2.3. Sample preparation

The solid phase extraction (SPE) method was used. Briefly 50 μL aliquots of serum were spiked with 20 μL IS solution (10 μg/mL in methanol) and 280 μL of 10 mM ammonium formate, and vortex mixed for 20 s. Samples were then loaded onto an Oasis® HLB SPE cartridge (30 mg; Waters Co., Milford, USA), which was preconditioned with methanol (1 mL), followed by water (1 mL). The cartridge was rinsed with 10 mM ammonium formate (1 mL) and then eluted with methanol (1 mL). The eluate was evaporated under nitrogen at 50 °C. The residue was reconstituted in a mixture of 200 μL of 10 mM ammonium formate and methanol (95:5), and it was transferred to a low volume sampling vial. Blood samples were collected into test tubes and centrifuged at 5000 rpm for 10 min at 4 °C. The serum was frozen at −70 °C in micro tubes until analysis.

2.4. LC–MS/MS method

LC–MS/MS analyses were performed using a Waters 2695 HPLC system (Milford, MA, USA) coupled with a Micromass Quattromicro™ API triple quadrupole mass spectrometer (Manchester, UK), which was operated using electrospray ionization in positive and negative ion mode (ESI+ and ESI−) with multiple reaction monitoring (MRM).

Separation was performed on a Unison UK-C18 (3 μm reversed-phase porous ODS, 50 mm × 2 mm I.D., Imtakt, Kyoto). Chromatographic separations were carried out under gradient conditions, in which the mobile phase consisted of solvent A (10 mM aqueous ammonium formate containing 0.1% formic acid) and solvent B (methanol containing 0.1% formic acid). The gradient was as follows: the ratio of A to B was initially 95:5 at time 0 to 0.5 min, then the ratio was increased by a linear gradient from A:B 95:5 to A:B 30:70 at time 0.5 to 4.5 min, and finally the ratio was 30:70 at time 4.5 to 8.0 min. The flow rate was set at 0.3 mL/min. The column and auto sampler tray temperature were 30 and 5 °C, respectively. A 20 μL aliquot of each sample was injected and the LC effluent was directed to the ESI source without splitting.

The MS/MS instrument was operated with a capillary voltage of 3.5 kV, source block temperature of 120 °C and desolvatation gas (nitrogen) heated to 400 °C and delivered at 600 L/h. Collision cell pressure was 3.0×10^{-3} Torr of the indicated argon pressure. The dwell time for each transition was 50 ms and the interchannel delay was 20 ms. Sensitivity of MRM was optimized by infusing a mixture of antibiotics containing 1 μg/mL each in 10 mM ammonium formate mixture and methanol (1:1) with QuanLynx™ (Micromass, Manchester, UK). The optimized MRM, cone voltage and collision energy of each analyte are summarized in Table 1. Peak areas for all components were automatically integrated using Masslynx™ NT 4.0 software (Micromass).

2.5. Validation of the LC–MS/MS method

Validation samples were prepared and analyzed to evaluate the intra-day and inter-day accuracy and precision of the analytical method for human serum. Six replicates of each of the validation concentration sera (0.1, 1, 10 and 50 μg/mL) were analyzed along with one set of standard samples every day for 6 days. The limit of detection (LOD) was determined from a signal to noise ratio (S/N) of 3 and the lower limit of quantification (LOQ) from S/N of 10.

2.6. Recovery and matrix effect

Recovery and the matrix effect were assessed by six replicates of spiked human serum at 0.1, 1 and 10 μg/mL of the analytes, according to Matuszewski et al. [20]. Relative recovery was expressed as the ratio of the peak area of the analyte spiked before extraction to the peak area of an equivalent concentration analyte in the same matrix spiked after extraction. Absolute recovery was expressed as

Table 2
LOD, LOQ and linear regression data from calibration curves.

Compounds	LOD (μg/mL)	LOQ (μg/mL)	Linearity range (μg/mL)	Correlation coefficient (r^2)
Cefepime	0.05	0.01	0.1–50	0.99
Doripenem	0.10	0.50	0.5–50	0.99
Meropenem	0.05	0.10	0.1–50	0.99
Cefmetazole	0.01	0.05	0.1–50	0.99
Cefotaxime	0.05	0.10	0.1–50	1.00
Ampicillin	0.01	0.05	0.1–50	0.99
Cefazolin	0.05	0.10	0.1–50	1.00
Piperacillin	0.001	0.005	0.1–50	1.00

the ratio of the peak area of the analyte spiked before extraction to the peak area of authentic solution at the same concentration. The matrix effect was assessed by comparing the peak area of the analyte spiked post-extraction to the peak area of an equivalent concentration of authentic solution.

2.7. Application

This method was used for the determination of β-lactam antibiotics in serum concentrations from patients. The present study was carried out in accordance with the Declaration of Helsinki by the Ethics Committee of Gifu Graduate School of Medicine (approval No. 22–61 of the institutional review board).

Table 3
Relative recovery (RR), absolute recovery (AR) and matrix effect (ME) for the assay of 8 β-lactam antibiotics in six different lots of human serum ($n = 6$).

Compounds	Nominal concentrations (μg/mL)	RR (%)	AR (%)	ME (%)
Cefepime	0.1	98.6	100.4	1.8
	1	87.6	88.5	1.0
	10	86.7	89.0	2.6
Doripenem	0.1			
	1	51.7	53.0	2.5
	10	49.1	50.6	2.9
Meropenem	0.1	72.3	73.0	1.1
	1	62.3	63.7	2.3
	10	75.7	76.6	1.3
Cefmetazole	0.1	93.8	100.1	6.7
	1	90.1	93.0	3.3
	10	93.5	91.7	-1.9
Cefotaxime	0.1	95.3	104.9	10.0
	1	87.2	92.7	6.2
	10	87.3	88.7	1.6
Ampicillin	0.1	94.3	101.8	8.0
	1	87.8	91.8	4.5
	10	88.1	89.1	1.2
Cefazolin	0.1	84.2	91.4	8.6
	1	80.2	83.7	4.3
	10	85.0	85.5	0.5
Piperacillin	0.1	98.4	95.7	-2.8
	1	90.5	95.4	5.4
	10	90.5	91.9	1.5

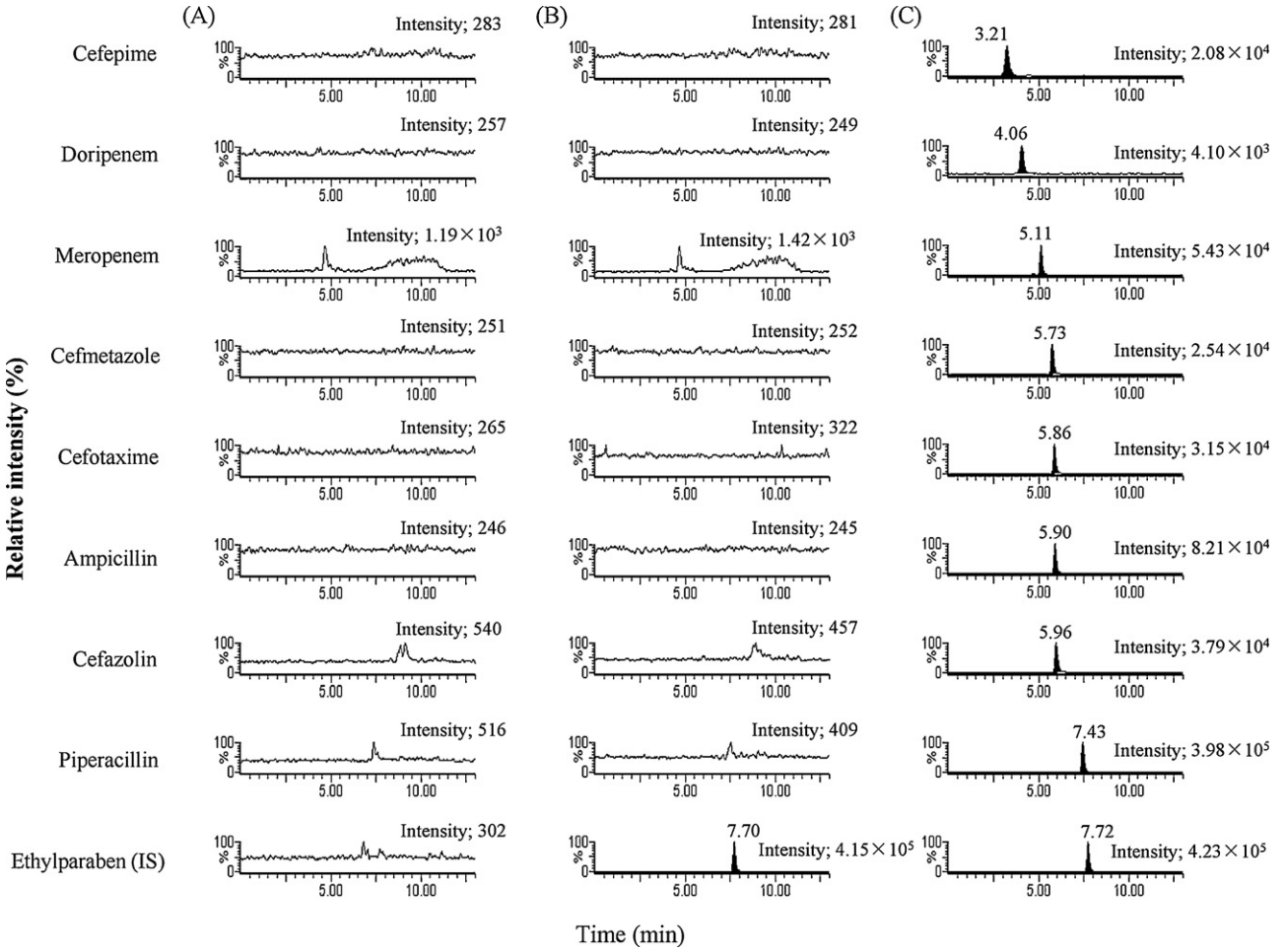

Fig. 1. MRM chromatograms obtained from blank human serum (A), human serum spiked with IS (B) and human serum spiked with 1 μg/mL β-lactam antibiotics and IS (C).

3. Results and discussion

3.1. Chromatography and mass spectrometry

β-Lactam antibiotics reveal varied chromatographic behaviors because of their wide differences in polarity. These differences in polarity limit the number of compounds that can be successfully separated by chromatography. Furthermore, since several antibiotics reveal hydrophilic behavior, they have very limited retention on conventional reversed-phase C_{18} columns. In order to separate the analytes from matrix interference, the retention of hydrophilic compounds should be improved. Recently, a high-resolution C_{18} column, Unison-UK C18, compatible with aqueous compounds, has been reported for polar molecules in biological matrix [21,22]. Cadenza CD-C18 is a conventional reversed-phase C_{18} column. In the initial stage of the present study, the ability to separate β-lactam antibiotics was compared between the Unison-UK C18 (3 μm reversed-phase porous ODS, 50 mm × 2 mm I.D.; Imtakt, Kyoto) and the Cadenza CD-C18 (3 μm reversed-phase porous ODS, 50 mm × 2 mm I.D.; Imtakt). The Unison-UK C18 was found to be superior to the Cadenza CD-C18 in the retention of the aqueous compound (data not shown).

Desolvation temperature, collision energy and argon gas flow were determined when the maximum response of the product ion was observed. Furthermore, the following mobile phases were tested: 10 mM aqueous ammonium formate (containing 0.1% formic acid, pH 3.2)/methanol and 10 mM ammonium formate/methanol. Satisfactory results in terms of peak intensity and peak shape were obtained under gradient conditions with the acidic mobile phase.

Fig. 1 shows representative MRM chromatograms obtained from human serum spiked with eight β-lactam antibiotics and IS. All compounds were well separated within 13 min, and no endogenous interference was found during analysis of the blank serum.

In the present study, several compounds, including zonisamide, primidone, and ethylparaben, were tested for suitability as IS. As a result, ethylparaben, a compound with antiseptic action, was found to be the most suitable among the analytes tested. Stable isotope-labeled compounds are desirable as IS when analysis is performed by ESI–MS; however, ethylparaben had no issues in the present study, because ethylparaben as IS was stably detected in all measurements.

3.2. Limit of detection, limit of quantification and linearity

The LOD, LOQ and linearity of β-lactam antibiotics in human serum are summarized in Table 2. The LOD and LOQ of each compound ranged from 0.001 to 0.1 μg/mL and 0.005 to 0.5 μg/mL, using 50 μL serum, respectively. Thus, as low as 50 μL aliquots of serum specimens were enough to determine β-lactam antibiotics. Denooz and Charlier [8] reported that the LOQs of their method were 0.5 μg/mL for piperacillin and meropenem, and 1.0 μg/mL for cefepim when using 500 μL plasma specimens. The sensitivity of our method was highly improved over conventional LC methods.

The linearity of the calibration was satisfactory over the concentration range of 0.1–50 μg/mL for all the compounds except for doripenem (0.5–50 μg/mL).

3.3. Recovery and matrix effect

The matrix effects in quantitative LC–MS/MS are complex, particularly when analyzing multiple compounds in the same bioanalytical run [23–28]. Recovery and the matrix effect were assessed by comparing the chromatograms of six different lots of

Table 4
Intra-, inter-day precision and accuracy for assay of 8 β-lactam antibiotics in human serum (n = 6).

Compounds	Intra-day											
	0.1 μg/mL			1 μg/mL			10 μg/mL			50 μg/mL		
	Mean ± S.D. (μg/mL)	Precison (%)	Accuracy (%)	Mean ± S.D. (μg/mL)	Precison (%)	Accuracy (%)	Mean ± S.D. (μg/mL)	Precison (%)	Accuracy (%)	Mean ± S.D. (μg/mL)	Precison (%)	Accuracy (%)
Cefepime	0.10 ± 0.009	9.2	99.9	0.9 ± 0.03	3.5	94.2	9.6 ± 0.3	3.1	96.3	50.5 ± 1.8	3.6	101.0
Doripenem		No Data		1.0 ± 0.05	5.3	99.9	9.1 ± 0.5	5.2	91.5	49.2 ± 2.0	4.2	98.4
Meropenem	0.10 ± 0.009	9.4	95.2	1.0 ± 0.02	2.4	95.2	10.6 ± 0.4	4.1	106.2	49.2 ± 3.3	6.7	98.3
Cefmetazole	0.09 ± 0.012	13.0	91.8	0.9 ± 0.04	4.9	88.7	9.6 ± 0.2	1.9	95.7	48.4 ± 1.8	3.7	96.7
Cefotaxime	0.09 ± 0.013	14.3	90.1	0.9 ± 0.05	4.9	93.5	9.8 ± 0.2	2.4	97.8	50.7 ± 1.0	2.0	101.4
Ampicillin	0.09 ± 0.010	10.7	92.7	1.0 ± 0.05	4.8	95.6	9.8 ± 0.2	1.6	97.8	51.3 ± 0.6	1.1	102.7
Cefazolin	0.09 ± 0.010	11.2	86.4	0.9 ± 0.05	4.9	93.5	9.8 ± 0.3	2.7	97.9	51.0 ± 1.5	2.8	102.0
Piperacillin	0.10 ± 0.011	11.1	97.2	0.9 ± 0.05	5.1	91.7	10.6 ± 0.2	2.1	106.3	49.1 ± 0.9	1.8	98.1
Compounds	Inter-day											
	0.1 μg/mL			1 μg/mL			10 μg/mL			50 μg/mL		
	Mean ± S.D. (μg/mL)	Precison (%)	Accuracy (%)	Mean ± S.D. (μg/mL)	Precison (%)	Accuracy (%)	Mean ± S.D. (μg/mL)	Precison (%)	Accuracy (%)	Mean ± S.D. (μg/mL)	Precison (%)	Accuracy (%)
Cefepime	0.10 ± 0.012	12.6	95.9	0.9 ± 0.06	6.9	92.2	11.1 ± 0.9	8.3	110.7	48.5 ± 3.9	8.1	96.9
Doripenem		No Data		1.1 ± 0.13	11.6	111.3	10.2 ± 1.5	14.3	101.8	47.1 ± 7.0	14.8	94.2
Meropenem	0.11 ± 0.016	14.6	107.6	1.1 ± 0.14	13.7	105.4	10.1 ± 1.0	10.1	101.5	46.6 ± 4.8	10.4	93.2
Cefmetazole	0.11 ± 0.008	7.6	107.1	0.9 ± 0.13	14.7	90.0	11.1 ± 1.1	10.1	111.0	48.1 ± 5.4	11.3	96.1
Cefotaxime	0.10 ± 0.004	4.5	95.2	1.1 ± 0.12	11.6	105.4	11.2 ± 1.0	8.9	112.3	49.2 ± 1.8	3.7	98.3
Ampicillin	0.11 ± 0.006	5.7	110.9	1.0 ± 0.04	4.5	98.7	10.8 ± 1.1	10.5	107.6	49.7 ± 3.5	7.1	99.5
Cefazolin	0.11 ± 0.015	13.8	106.2	1.0 ± 0.14	13.6	100.7	10.7 ± 1.5	14.0	107.3	49.8 ± 2.7	5.4	99.6
Piperacillin	0.10 ± 0.006	6.3	101.2	1.0 ± 0.08	7.8	101.4	10.9 ± 0.8	7.7	109.3	49.4 ± 3.3	6.8	98.7

Table 5
Trough concentration (C_{min}) of β-lactam antibiotics from patients treated with intravenous infusion for 1 h.

Compounds	Daily dose (g)	Dosage interval	Number of samples	Median (range) concentrations (μg/mL)	
Cefepime	6	2 g per 8 h	6	29.7	(16.9–35.3)
Meropenem	2	1 g per 12 h	7	9.8	(6.5–14.4)
	3	1 g per 8 h	4	6.3	(3.4–15.1)
Cefmetazole	2	1 g per 12 h	1	1.9	(1.9–1.9)
Ampicillin	8	2 g per 6 h	5	3.8	(1.4–26.9)
Cefazolin	6	2 g per 8 h	8	10.9	(6.6–15.9)
Piperacillin	8	2 g per 6 h	1	64.3	(64.3–64.3)
	16	4 g per 6 h	13	18.0	(3.8–62.4)

blank human serum with the corresponding spiked serum. There were no significant interfering substances in human serum during the course of analysis. Table 3 summarizes the data for relative recovery, absolute recovery, and matrix effects. Relative recoveries ranged from 80.2 to 98.6%, while absolute recoveries from 83.7 to 104.9% for analytes, excluding doripenem and meropenem, in which the absolute recoveries for doripenem and meropenem were 50.6–53.0% and 63.7–76.6%, respectively. Since doripenem and meropenem are highly hydrophilic compounds, the low retention of these antibiotics in the Oasis® HLB may reduce extraction recovery. On the other hand, matrix effects ranged from −2.8 to +10.0% for all analytes.

3.4. Accuracy and precision

The accuracy and precision of 8 β-lactam antibiotics were calculated from six replicates analyses of the quality control serum samples at four concentrations. Table 4 summarizes the intra- and inter-day precision for all β-lactam antibiotics. The intra-day precision ranged from 1.1 to 14.3%, and the mean accuracy values ranged from 86.4 to 106.3%. The inter-day precision and mean accuracy ranged from 3.7 to 14.8% and from 90.0 to 112.3%, respectively. Accuracies for all compounds were within the quantitative bioanalytical method validation guidelines set by the FDA, i.e. 85–115%, and the precision was within 15% [29].

3.5. Application

Serum samples were taken from patients admitted to the ICU to estimate trough concentration of antibiotics (C_{min}). Table 5 summarizes the C_{min} of β-lactam antibiotics in patients treated with intermittent administration. In the present study, the median (range) piperacillin C_{min} was 18.0 (3.8–62.4) μg/mL, which was comparable to the data reported by Conil et al. [30], in which the median value (range) of the C_{min} of piperacillin was 11.9 (<1–156.3) μg/mL with wide variations among patients receiving intravenous infusion of 4 g piperacillin three to four times a day.

Although the present method is used routinely, these findings suggest that our method is a promising tool for the therapeutic monitoring of β-lactam antibiotics, such as ampicillin, cefazolin, cefepime, cefmetazole, meropenem, and piperacillin, in the clinical setting.

4. Conclusion

We developed a rapid and sensitive LC–MS/MS method for the simultaneous determination of β-lactam antibiotics in human serum. This method was highly sensitive and required as low as 50 μL sample, and was successfully applied to the measurement of β-lactam antibiotics in patients admitted to the ICU.

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