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Quantitative Analysis of β-Lactam Antibiotics in Human Plasma by High Sensitivity LC/MS/MS Method

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Daryl Kim Hor Hee[1]; Zhi Wei Edwin Ting[2]; Lawrence Soon-U Lee[1]; Jie Xing[2]; Kelvin Loh Shun Cheng[3]; ZHAOQI ZHAN[2]

[1] Clinical Analysis Centre, Department of Medicine Research Laboratories, National University of Singapore;
[2] Shimadzu (Asia Pacific) Pte Ltd, Singapore;
[3] ITP student from NTU, Singapore

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Introduction

The β-lactam type antibiotics are used in the treatment of various bacterial infections in human over decades. One of the consequences of continuous uses of antibiotics is the progressive development of drug resistance of bacteria in human [1]. Therapeutic Drug Monitoring (TDM) aims at obtaining pharmacokinetic pattern of an antibiotic in patient to develop personalized medicine treatment. Conventional TDM methods such as immunoassays are well-established. However, one of the drawbacks of immunoassays is lack of specificity due to cross-reactivity with metabolites, which may give false positives [2,3]. Recently, LC/MS/MS has been used for fast and direct measurement of β-lactam antibiotics such as amoxicillin [4] and piperacillin, etc. [5,6] in human plasma. In this application news, a fast LC/MS/MS method with a simple sample pre-treatment procedure for quantitative analysis of five β-lactam antibiotics, meropenem (MER), tazobactam (TAZ), piperacillin (PIP), cefepime (CEF) and ceftazidime (CFT) is described. A small injection volume of sample of this MRM-based method is required only, which minimizes the contamination of sample matrix, as such, reducing the cleaning and maintenance time of the interface of LC/MS/MS in clinical research work

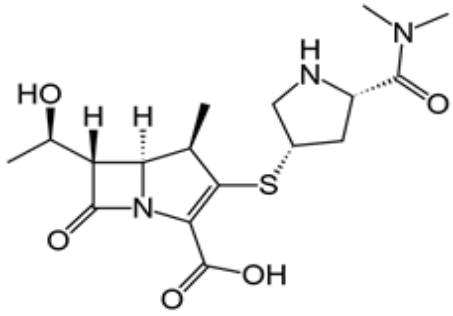

Figure 1: Structure of meropenem (MER) with a β-lactam ring.

Experimental

Sample preparation and analytical conditions

Five antibiotics used in this study are meropenem (MER), tazobactam (TAZ), piperacillin (PIP), cefepime (CEF) and ceftazidime (CFT). The compounds and four stable isotope-labelled meropenem-d6, piperacillin-d5, cefepime-cd3 and ceftazidime-d6 as internal standards were purchased from certified suppliers. Pool human plasma was obtained from i-DNA Biotechnology Pte Ltd and used as matrix. The sample pre-treatment and spiked sample preparation procedure are illustrated in Figure 1. A simple protein crash method was applied by adding ACN:MeOH (1:1) to plasma in a ratio of 3:1, followed by vortex and centrifuge. A calibration series of spiked standard samples were prepared: 20, 40, 80, 200, 400, 2000 and 4000 ng/mL in plasma. The concentrations of internal standards were 200 ng/mL or 800 ng/mL in these calibrants. A LCMS-8060, a triple quadrupole LC/MS/MS system with heated ESI was employed in this work. The analytical conditions and instrumental parameters are compiled into Table 1.

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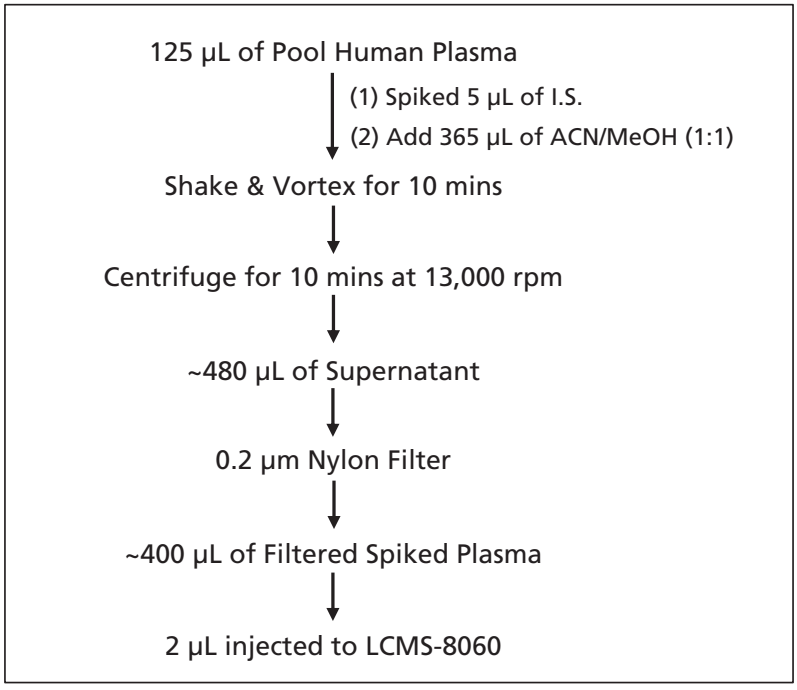

Figure 2: Procedure of protein crash and spiked-sample preparation

Table 1: Analytical conditions and parameters on LCMS-8060

Column	: Kinetex 1.7µ C18 100A (100 mmL x 2.10mm I.D.)
Mobile Phase	: A: Water with 0.1% FA
	B: Acetonitrile with 0.1% FA
Elution Program	: Gradient elution (5.5 minutes)
	B: 5% (0 to 0.2 min) → 90% (3.5 to 4.0 min) → 5% (4.1 to 5.5 min)
Flow Rate	: 0.5 mL/min
Oven Temp.	: 40°C
Injection	: 2 µL
Interface	: ESI (heated)
MS Mode	: MRM, Positive
Block Temp.	: 400°C
DL Temp.	: 250°C
Interface Temp.	: 300°C
CID Gas	: Ar, 270 kPa
Nebulizing Gas	: N_2, 3.0 L/min
Drying Gas	: N_2, 5.0 L/min
Heating Gas	: Zero Air, 15L/min

Results and Discussion

Fast MRM-based method for five β-lactam antibiotics

Table 2 shows the summarized results of optimized MRM transitions and parameters of the five β-lactam antibiotics studied and four stable isotope-labelled internal standards. Two MRM transitions were selected for each compound, with one as the quantitation ion and the other for confirmation.

Furthermore, a fast gradient elution MRM method was established with a total run time of 5 minutes. The MRM chromatograms of a mixed standard sample in plasma are shown in Figure 3. Due to lack of stable isotope-labelled tazobactam, MER-d6 was also used as the internal standard for tazobactam (TAZ) in this work.

Table 2: MRM transitions and parameters of five β-lactam antibiotics and internal standards on LCMS-8060

Compd.	Formula	E. Mass	MRM (m/z)	CE (V)	Int (%)
Tazobactam (TAZ)	$C_{10}H_{12}N_4O_5S$	300.05	301.1>168.2	-15	100
			301.1>122.1	-22	92
Cefepime (CEF)	$C_{19}H_{24}N_6O_5S_2$	480.13	481.1>86.2	-15	100
			481.1>396.0	-13	63
Meropenem (MER)	$C_{17}H_{25}N_3O_5S$	383.15	384.1>68.1	-41	100
			384.1>141.1	-16	64
Ceftazidime (CFT)	$C_{22}H_{22}N_6O_7S_2$	546.10	547.1>468.0	-13	100
			547.1>396.1	-19	42
Piperacillin (PIP)	$C_{23}H_{27}N_5O_7S$	517.16	518.2>143.1	-21	100
			518.2>160.1	-15	25
CEF-cd3	$C_{18}{}^{13}CH_{21}D_3N_6O_5S_2$	484.13	485.2>86.1	-16	100
			485.2>400.1	-13	66
MER-d6	$C_{17}H_{19}D_6N_3O_5S$	389.15	390.2>147.2	-18	100
			390.2>114.1	-27	74
CFT-d6	$C_{22}H_{16}D_6N_3O_7S_2$	552.10	553.1>474.0	-16	100
			553.1>319.1	-20	59
PIP-d5	$C_{23}H_{22}D_5N_5O_7S$	522.16	523.1>148.1	-21	100
			523.1>160.1	-14	23

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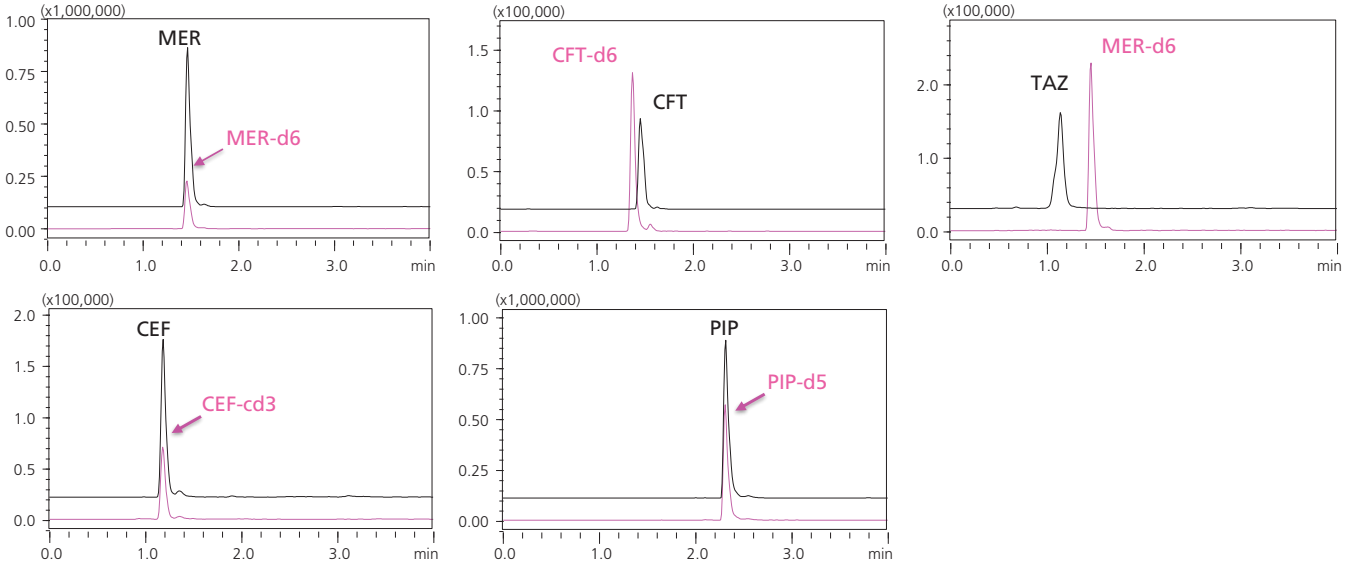

Figure 3: MRM chromatograms of five β-lactam antibiotics each (400 ng/mL) with internal standards in plasma on LCMS-8060

Calibration curves with IS

As shown in Figure 4, linear calibration curves with IS method were constructed using the standard samples prepared by pre-spiked in plasma matrix. The method parameters are summarized in Table 3. It can be seen that good linearity with R2 greater than 0.997 was obtained for the five compounds in the range from 20 ng/mL to 4000 ng/mL in plasma.

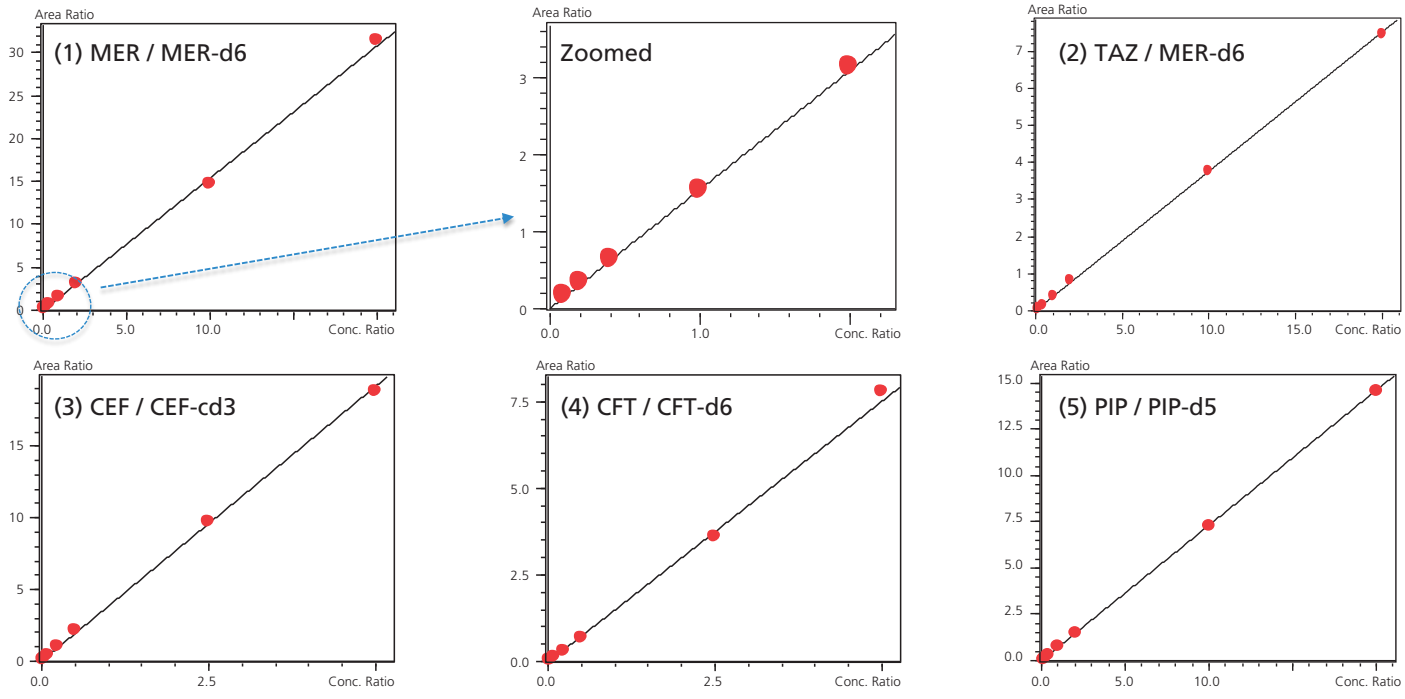

Figure 4: Calibration curves of five β-lactam antibiotics with stable isotope labelled internal standards in human plasma on LCMS-8060. Details of the calibration information are shown in Table 3.

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Table 3: MRM quantitation method of five β-lactam antibiotics with internal standards on LCMS-8060

Compd.	RT (min)	qMRM (m/z)	IS	IS (ng/mL)	Range (ng/mL)	R^2
TAZ	1.18	301.1 > 168.1	MER-d6	200	20~4000	0.9993
CEF	1.21	481.1 > 86.1	CEF-cd3	800	20~4000	0.9991
MER	1.47	384.1 > 68.1	MER-d6	200	20~4000	0.9989
CFT	1.47	547.1 > 468.0	CFT-d6	800	20~4000	0.9971
PIP	2.33	518.2 > 143.1	PIP-d5	200	20~4000	0.9999

Evaluation of method performance

Accuracy of the quantitation method was evaluated with pre-spiked standard samples at all concentration levels with duplicate injections. The results are shown in Table 4, which indicate that reliable quantitation accuracy was obtained, except CFT at 20 ng/mL, due to employing IS method.

Repeatability of the method on LCMS-8060 was evaluated with pre-spiked samples, post-spiked samples and mixed standards in solvent at low, middle and high concentration levels. The %RSD results of pre- and post-spiked sample are shown in Table 5. The results indicate excellent repeatability achieved, which is believed to be due to employing IS method and the excellent operation stability of the LCMS-8060 system.

Table 4: Results of accuracy (%, n=5) of five β-lactam antibiotics with IS in plasma samples on LCMS-8060

Compd.	Accuracy (%)						
	20 ng/mL	40 ng/mL	80 ng/mL	200 ng/mL	400 ng/mL	2000 ng/mL	4000 ng/mL
TAZ	91	97	100	106	107	101	99
CEF	87	97	102	105	109	101	98
MER	97	102	102	99	102	95	102
CFT	126	103	93	87	91	96	104
PIP	99	100	97	103	102	100	100

Table 5: Repeatability (RSD %, n=5) of five β-lactam antibiotics with IS in plasma samples on LCMS-8060

Compd.	At 40 ug/mL		At 200 ng/mL		At 2000 ng/mL	
	Post-spiked	Pre-spiked	Post-spiked	Pre-spiked	Post-spiked	Pre-spiked
TAZ	4.1	1.8	4.0	4.2	4.9	3.9
CEF	5.1	5.0	4.2	2.0	1.9	3.8
MER	4.2	4.5	1.2	1.1	1.0	2.0
CFT	6.4	5.4	7.2	7.2	4.9	5.1
PIP	5.1	3.8	5.3	3.6	3.6	1.9

Recovery of the sample pre-treatment method was evaluated based on the peak area ratios of pre-spiked samples and post-spiked samples at all concentration levels. The results shown in Table 6 indicate excellent recovery were obtained.

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Matrix effect of the method was determined by the peak area ratios of spiked samples and mixed standards in pure solvent at all concentration levels. The results are shown in Table 7. It can be seen that strong matrix effect occurred for CFT (33%~40%) and TAZ (128%~149%). This could be due to interference from plasma, which causes ion suppression and ion amplification. By further dilution of 2.5 times of the plasma samples with pure water before injection into LCMSMS, the matrix effects of CFT and TAZ were improved significantly to 62%~85% and 95%~109%, respectively.

Table 6: Recovery (%) of five β-lactam antibiotics in plasma samples by protein crash pre-treatment

Compd.	Recovery (%)						
	20 ng/mL	40 ng/mL	80 ng/mL	200 ng/mL	400 ng/mL	2000 ng/mL	4000 ng/mL
TAZ	86	89	90	94	97	92	97
CEF	94	87	87	88	90	88	89
MER	103	104	98	102	102	97	103
CFT	113	94	93	97	103	100	101
PIP	104	106	98	105	103	98	102

Table 7: Results of matrix effect (%) of five β-lactam antibiotics in plasma samples on LCMS-8060

Compd.	Matrix effect (%)						
	20 ng/mL	40 ng/mL	80 ng/mL	200 ng/mL	400 ng/mL	2000 ng/mL	4000 ng/mL
TAZ*	149	140	145	139	137	134	128
CEF	118	115	115	120	117	123	117
MER	93	93	101	98	99	99	103
CFT*	33	38	37	39	40	36	34
PIP	96	94	102	99	99	101	97

*Note: the matrix effect was improved significantly by diluting the plasma sample with pure water before injection

Specificity of the method for detection and confirmation of the five β-lactam antibiotics is demonstrated in Figure 5. In addition, the confirmation criteria include the MRM transitions, the ratios with reference MRM transitions (variation < 30%) as well as retention time (shift < 5%).

Limit of quantitation (LOQ) of the method was estimated from the chromatograms of the lowest level spiked sample (20 ng/mL). Based on S/N = 10, the estimated LOQ of the method are 5.8, 6.0, 1.9, 2.9 and 0.7 ng/mL for TAZ, CEF, MER, CFT and PIP, respectively.

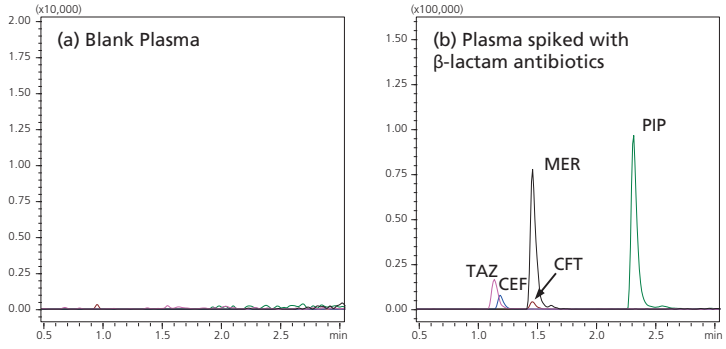

Figure 5: MRM chromatograms of blank plasma and plasma spiked with five β-lactam antibiotics (40 ng/mL)

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Conclusions

A fast MRM-based method for quantitation of five β-lactam antibiotics tazobactam, cefepime, meropenem, ceftazidime and piperacillin in human plasma was developed on LCMS-8060. A simple sample pre-treatment with protein crash by organic solvent was applied and a small injection volume of 2 µL was required due to the high sensitivity of the LCMS-8060 employed. The method performance was evaluated on the linearity, accuracy, repeatability, recovery, matrix effect, specificity and limit of quantitation (LOQ). The estimated LOQs of the method for the five antibiotics are in the range from 0.7 ng/mL to 6.0 ng/mL with an injection volume of 2 uL.

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