Eurachem Workshop „Validation of targeted and non-targeted methods of analysis", Tartu, 20th–21th May 2019

Quantification of beta-lactam antibiotics in human plasma by HPLC-MS/MS method. Validation study.

Maria Kulp, Maria Kuhtinskaja, Kristiina Leiman, Merike Vaher

Tallinn University of Technology, Faculty of Science, Department of Chemistry and Biotechnology, Ehitajate tee 5, 19086 Tallinn, Estonia
maria.kulp@taltech.ee

INTRODUCTION

β-Lactam antibiotics are the cornerstone of antibacterial treatment and frequently prescribed drugs, especially in the intensive care units (ICU) of hospitals. Optimization of antibiotic dosing is a key intervention to improve clinical outcomes. Therapeutic drug monitoring (TDM) is a commonly used dosing strategy to optimize exposure and thereby minimize toxicity and maximize the efficacy. Currently, TDM of β-lactam antibiotics is rarely performed, due to poor availability in clinical practice.

The aim of this paper is to describe a newly developed and validated HPLC–MS method for the determination of ampicillin, amoxicillin, cefepime, ceftazidime, imipenem, meropenem and piperacillin in human plasma. The combination of antibiotics selected in this study is aimed at those commonly used in severely ill patients in the ICU of North Estonia Medical Centre.

EXPERIMENTAL

Chemicals, reagents and standards

Antibiotic standards (ACS grade) (Figure 1), internal standard (IS) flucloaxcillin and acetonitrile (ACN) (HPLC grade) were purchased from Sigma-Aldrich. Milli-Q water was used for preparations of the solutions.

Calibrator and quality control solutions

Antibiotic stock solutions 1000 µg/mL were prepared in MilliQ water and stored at -80°C. Working standards and quality controls (QC) were prepared in three different matrices used in validation process: in MilliQ water (1), standards spiked into the pooled plasma before (3) and after (2) extraction in the range of 0,5-100 µg/mL, depending on the matrix. The QC solutions were prepared in a similar way from antibiotic standards with different LOT from those that were used for the calibrations.

Chromatographic conditions

Chromatographic separation was carried out on an Agilent 1200 Infitity system (Agilent Technologies, Santa Clara, CA) equipped with an Agilent Poroshell 120 EC-C18 (100 mm × 4.6 mm i.d.) with 2.7 µm particle size, operating at a temperature 40°C. A binary mobile phase with a gradient elution was used. Mobile phase A was MilliQ water/0.1% formic acid and mobile phase B was ACN/0.1% formic acid. The flow rate was set for 0.7 mL/min. The elution gradient was as follows: 0% B–30% B in 5 min, 95% B in 10 min and then 5 min at 95% B and afterwards the analytical column was re-equilibrated to initial gradient settings. The sample injection volume was 3 µL.

Mass spectrometry

MSD Trap XCT mass spectrometer (Agilent Technologies, USA), with an ESI+ ion source was used for experiments. The MS/MS instrument operated with a capillary voltage of 5 kV. Used nebulizer gas (N_2) T=350°C at velocity 7 L/min. Helium was used to induce fragmentation in the collision cell. Details of the MRM- transitions for each compound are described in Table 1. Data analysis was performed using Agilent ChemStation software.

Table 1. MRM transition parameters for analytes under study

Compound	ESI mode	Quantifier	Qualifier
IMP	ESI+	300.2→227.0	300.2→272.0
CFZ	ESI+	547.1→468.1	547.1→396.0
AMX	ESI+	366.2→113.9	366.2→349.0
CEF	ESI+	481.1→323.7	481.1→395.8
MER	ESI+	384.4→253.7	384.4→297.6
AMP	ESI+	350.2→106.0	350.4→160.0
CIL	ESI+	359.3→202.3	359.3→342.3
PIP	ESI+	518.0→331.8	518.0→234.6
FLU (IS)	ESI+	454.3→312.6	454.3→409.9
TAZ	ESI+	300.8→167.7	300.8→206.8

Human plasma collection and preparation

Whole blood samples were donated by ten healthy people from the staff personnel of the North Estonia Medical Centre. Separated plasma proteins were precipitated with ice-cold ACN (1:1) followed by sample centrifugation (14000 rpm, 5 min, +4°C). Supernatant was diluted with MilliQ water (1:2), spiked with IS (2 ng/mL) and filtered through 0.22 µm.

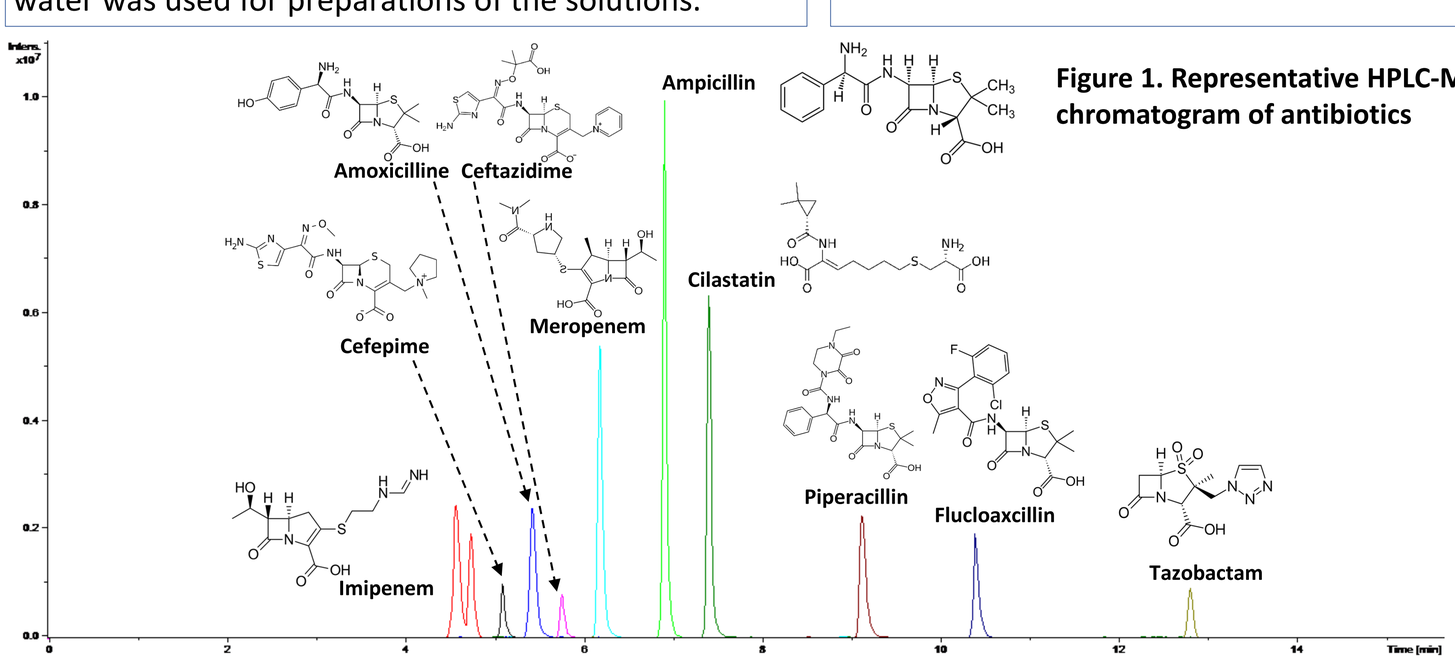

Figure 1. Representative HPLC-MS/MS chromatogram of antibiotics

Table 2. Analytical reliability parameters of the method

Analyte	Intermediate precision RSD (%)			Accuracy RSD (%)		
	QC-L	QC-M	QC-H	QC-L	QC-M	QC-H
IMP	16.1	5.2	7.3	18.3	5.6	10.2
CEF	6.8	5.5	4.3	7.5	5.4	8.6
AMX	4.2	5.6	5.8	6.2	6.0	6.5
CFZ	4.3	2.5	4.0	4.5	5.4	7.6
MER	7.7	6.8	8.2	3.9	6.2	6.9
AMP	8.2	4.6	4.2	7.3	8.1	7.6
PIP	8.2	3.6	5.8	6.2	5.9	6.8

VALIDATION RESULTS

The developed procedure was validated according to the EMA Guidelines [1].

For **selectivity** assessment, ten independent blank and spiked blank plasmas fortified with IS were analysed by developed HPLC-MS method. Due to the selectivity of MS/MS-detection the absence of the endogenous signal contributions for any analyte of interest was confirmed according to EMA criteria: response was less than 20% of the LOQ for the analyte and 5% for the IS.

Table 3 depicts the results of the method validation regarding the **linearity** of the working range, **instrumental quantitation limit** (IQL) **and limit of quantitation of the procedure** (LOQ), extraction **recoveries** (R) and **matrix effects** (ME). The plots of the peak area ratios (analyte to IS) versus the concentrations of the standards in water (1), in prepared plasma (2) and in plasma with spiked standards before extraction (3) exhibited adequate linearities for the studied analytes, all with acceptable statistical parameters: the regression errors (RE) were <10% and probability (*P*) associated with *F* statistic for a on-sided distribution with *k-2* (MS_{LOF}) and *N-k* (MS_{ME}) degrees of freedom: *P*>0.05 (lack-of-fit ANOVA), where k=6, N=18.

Matrix effects were assessed by the slope ratios of the calibration curves (2) (in the presence of matrix) and (1) (in the absence of matrix) according to: $ME(\%) = \frac{b_2}{b_1} \times 100$

Significant ion enhancement effect (about 50%) was observed for IMP and ion suppression effect for CEF due to the presence of interfering components in the matrix. When quantifying β-lactam antibiotics the presence of matrix effects can be taken into account by using the calibration curves in the matrix.

The extraction **recoveries** from the sample matrix were assessed by the slope ratios of the calibration curves (3) and (2): $R(\%) = \frac{b_3}{b_2} \times 100$

The **intermediate precision** and the **accuracy** were estimated by using six fortified matrix samples at three concentration levels –low (L), medium (M) and high (H) (Table 2). Precision and accuracy values were within the acceptance value (RSD ≤ 15%), with the exception of the IMP (16.1 and 18.3%), both for only the QC-L samples.

The blank samples that were injected immediately after the analysis of high-level samples containing 100 µg/mL of each analyte showed no evidence of **carry-over** (the signals were below the LOD for all analytes).

Table 3. Linearity, LOQ, matrix effect and recovery.

Analyte	Regression (1) (0.5-20 µg/ml)		Regression (2) (0.5-20 µg/ml)		Regression (3) (3-100 µg/ml)		IQL/LOQ (µg/ml)	ME (%)	R (%)
	P	RE, %	P	RE, %	P	RE, %			
IMP	0.55	7.8	0.48	8.7	0.56	8.3	0.2/1.1	150	95
CEF	0.98	6.5	0.84	6.8	0.75	6.8	0.2/1.2	47	110
AMX	0.34	5.8	0.42	6.2	0.52	6.3	0.1/0.8	102	98
CFZ	0.56	4.6	0.64	9.1	0.62	9.3	0.2/1.1	119	97
MER	0.75	3.8	0.68	5.6	0.52	5.8	0.1/0.7	115	102
AMP	0.53	4.3	0.51	5.3	0.41	8.4	0.2/1.2	110	107
PIP	0.68	5.9	0.78	6.4	0.65	9.5	0.2/1.1	103	87

CONCLUSIONS

We validated simple and rapid HPLC-MS/MS assay for the simultaneous quantification of seven (potentially ten) frequently used β-lactam antibiotics. The method is accurate, reproducible and is successfully being used in TDM studies in the ICU of North Estonia Medical Centre.

[1] European Medicines Agency, Guideline on bioanalytical method validation, (EMEA/CHMP/EWP/192217/2009 Rev.1 Corr.2**) (2005).