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Development and validation of a fast and uniform approach to quantify β-lactam antibiotics in human plasma by solid phase extraction-liquid chromatography–electrospray-tandem mass spectrometry

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ABSTRACT

Monitoring of plasma antibiotic concentrations is necessary for individualization of antimicrobial chemotherapy dosing in special patient populations. One of these special populations of interest are the post-bariatric surgery patients. Until today, little is known on the effect of this procedure on drug disposition and efficacy. Therefore, close monitoring of antimicrobial plasma concentrations in these patients is warranted. A fast and uniform ultra-high-performance liquid chromatography (UPLC) method with tandem mass spectrometric detection (MS/MS) has been developed and qualified for the simultaneous quantification of β-lactam antibiotics in human plasma. Compounds included in this multi-component analysis are: amoxicillin, ampicillin, phenoxymethylpenicillin, piperacillin, cefuroxime, cefadroxil, flucloxacillin, meropenem, cefepime, ceftazidime, tazobactam, linezolid and cefazolin. After spiking of five different stable isotope labelled internal standards, plasma samples were prepared for UPLC–MS/MS analysis by mixed-mode solid phase extraction. The developed method was proven to be free of (relative) matrix effects and proved to be reliable for the quantification of 12 out of 13 β-lactam antibiotics. As a proof of concept the method has been applied to plasma samples obtained from a healthy volunteer treated with amoxicillin. The analytical method is suitable for use in a therapeutic drug monitoring setting, providing the clinician with reliable measurements on β-lactam antibiotic plasma concentrations in a timely manner.

1. Introduction

Obesity is a worldwide problem, reaching epidemic proportions. According to the WHO in 2008, 1.5 billion adults were overweight and 500 million were obese [1]. Accordingly, the frequency of bariatric surgery, one of the most effective treatment options in terms of long-term weight loss and co-morbid medical conditions, is ever increasing. However, until today, the effect of these drastic surgical alterations of the gastrointestinal system on drug disposition remain unclear [2]. Changes in pharmacokinetic (PK) characteristics, for example in volume of distribution or in extent of absorption, may occur in this special patient population [3,4].

For most drugs, pharmacodynamic (PD) effects are easily measurable (eg. blood pressure, glycaemia etc.). These measurements provide the clinician with a tool to individualize drug therapy or to adjust drug dosing in special patient populations. However, for antibiotics (AB), no such easily measurable clinical endpoints are available. Nevertheless, for the majority of antibiotics, intermediate clinical endpoints, in terms of PK/PD indices, are defined to guide chemotherapeutic dosing. These PK/PD indices are not only correlated with therapeutic success, but show a negative correlation with the occurrence of antimicrobial resistance formation.

For the beta-lactam AB, the most frequently prescribed class of antibiotics (based on data of the Belgian national institute for health and disability insurance for the period 05/2008–05/2009), clinical efficacy is correlated with the time that the AB plasma concentration remains above the MIC [5]. Optimization of treatment should therefore be based on knowledge of the antibiotic plasma concentration as well as the results of the microbial susceptibility testing of the pathogen. On the one hand, prediction of antibiotic plasma concentrations, based on a thorough understanding of underlying AB pharmacokinetics, could prove useful in optimizing AB treatment. However, *a priori*, studies measuring AB plasma concentrations in different subjects are needed to develop PK models capable of making these predictions. On the other hand, therapeutic drug monitoring (TDM) programs could be set up to help the clinician in adjusting patient dosing to achieve therapeutic concentrations. In both cases, one relies heavily on accurate measurements of AB in human plasma samples to optimize clinical efficacy of AB treatment.

Various analytical methods for simultaneous quantification of β-lactam AB are described in literature, some claiming applicability in a therapeutic drug monitoring setting [6–10].

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Although MS/MS-detection is regarded superior over other detection-techniques, in terms of specificity, sensitivity and speed, few authors report TDM-assays based on this technique. Of all consulted reports on TDM-assays for β-lactam AB, only one is based on LC–MS/MS [8]. Others mainly rely on UV detection to quantify β-lactam AB in human plasma.

Besides specificity, speed is of the essence when determining patient plasma concentrations in a therapeutic drug monitoring setting. In order to provide quantitative results in a timely manner, chromatographic runtime as well as sample preparation time should be minimized. To the author's knowledge none of the previously published TDM-assays succeeded in reducing chromatographic run-time sufficiently. Apart from one author claiming chromatographic separation of eight β-lactam AB within 13 min [8], most published methods require up to 30 min of chromatographic run time. Generally, a minimum of 6 calibration standards and 6 quality control samples [11] are used to reliably quantify study samples. Although these recommendations were initially issued for the analysis of samples originating from clinical trials, given the lack of specific guidance on method validation in TDM assays, we adhere to these guidelines for the development of TDM capable assays. For the reported assays, given the FDA guidance, total chromatographic analysis would then add up to more than 6 h. Moreover, most of the reported TDM-assays were based on time-consuming low-throughput sample preparation steps. Use of laborious procedures, e.g. multiple centrifugation steps [7], or evaporation-to-dryness procedures [6,8] significantly lengthen the total analysis time.

To accommodate present and future needs for studies on β-lactam PK as well as therapeutic drug monitoring of β-lactam AB, we set out to develop a fast multi-drug assay. We focus on the development of a single analytical platform capable of addressing plasma samples containing a broad selection of different AB. In addition to some important penicillin and cephalosporin AB (respective molecular structures are given in Figs. 1 and 2), we choose to include the monobactam AB meropenem and doripenem, the β-lactamase inhibitor tazobactam and one AB not from the β-lactam group, linezolid (molecular structures of the latter four are shown in Fig. 3) The development of one single method for many different AB was favoured. Although these drugs are virtually never co-administered, it provides us, however, with one single analytical platform that can be used at any time, irrespective of which of the individual AB is in a particular instance the subject of a certain study or therapeutic follow-up. Additionally, sample preparation time as well as chromatographic run-time will be minimized in order to significantly reduce total analysis time, thus making it possible e.g. to provide the clinic with quantitative measurements in a timely manner.

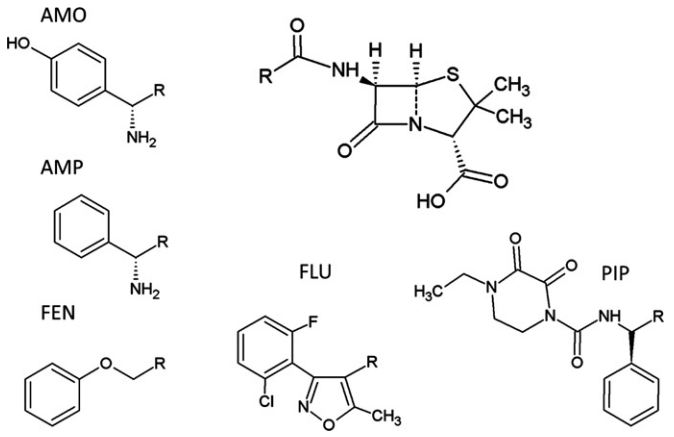

Fig. 1. Molecular structures of penicillin antibiotics: AMO, AMP, FEN, FLU and PIP.

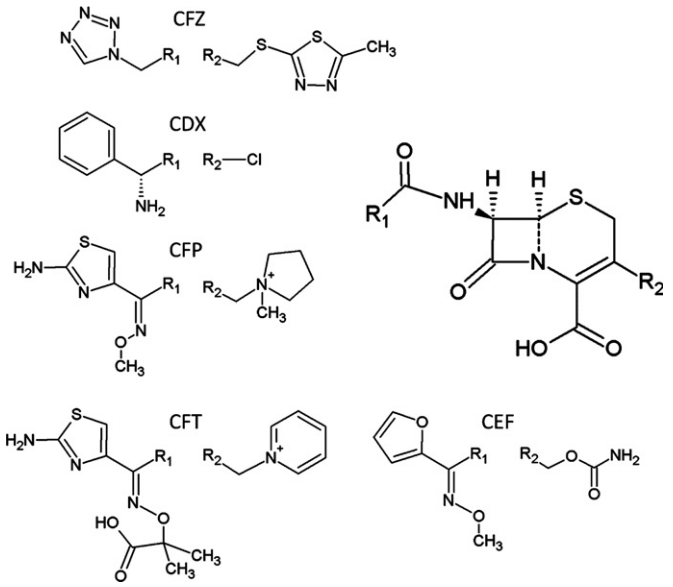

Fig. 2. Molecular structures of cephalosporin antibiotics: CDX, CEF, CFP, CFT and CFZ.

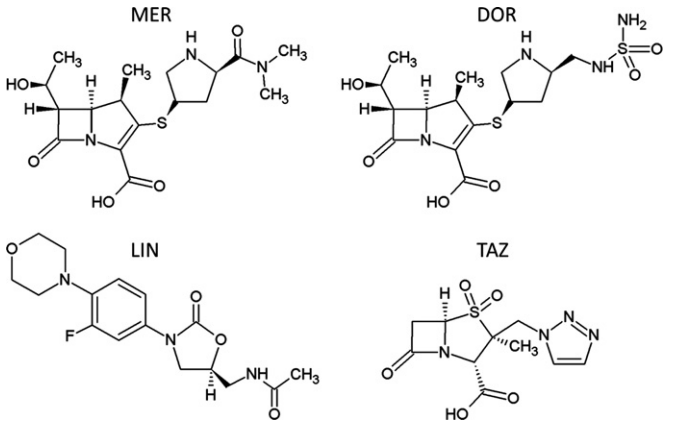

Fig. 3. Molecular structures of monobactam antibiotics: DOR and MER, β-lactamase inhibitor TAZ and oxazolidinone antibiotic LIN.

2. Material and methods

2.1. Reagents

Amoxicillin (AMO), Ampicillin (AMP), Phenoxymethylpenicillin (FEN), Piperacillin (PIP), Cefuroxime (CEF) and Cefadroxil (CDX) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Flucloxacillin (FLU), Meropenem (MER), Cefepime (CFP), and Ceftazidime (CFT) were purchased from the European directory for the quality of medicine (Strasbourg, France). Tazobactam (TAZ) was purchased from The United States Pharmacopeial Convention (Rockville, MD, USA). Linezolid (LIN), Cefazolin (CFZ), $^{2}H_{4}$-Amoxicillin, $^{2}H_{4}$-Cefadroxil, $^{2}H_{6}$-Meropenem and $^{2}H_{5}$-Phenoxymethylpenicillin were purchased from Toronto Research Chemicals (Ontario, Canada). $^{13}C,^{2}H_{3}$-Cefepime was purchased from AlsaChim (Illkirch, France). HPLC-grade water was prepared using a commercial water purification system (Millipore Synergy 185, Millipore, Billerica, MA, USA). All other reagents were of analytical grade. The Oasis® MCX 96-well μ-elution plate, as well as the Ostro® enhanced protein precipitation plate and the Oasis® 96-well sorbent selection plate (containing different mixed-mode solid phase sorbents: WCX, MAX, WAX and MCX) were from Waters (Milford, MA,USA). A vacuum

manifold (Waters) was used to force liquids through the Oasis®- and Ostro®-plate.

2.2. Instrumentation

Chromatographic separation was achieved on an Acquity UPLC system (Waters, Milford, MA) equipped with an Acquity HSS T3 column (50 mm × 2.1 mm, 1.7 μm particle size) and an Acquity BEH C18 guard-column (5 mm × 2.1 mm, 1.7 μm particle size) all from Waters (Milford, MA). The column was kept at 40 °C. An aliquot of 1 μl was injected into the mobile phase stream using full loop injection. The mobile phases consisted of a 1 mM CH_3COOH/CH_3COONH_4-buffer with 5% acetonitrile (MPA) and acetonitrile (MPB). Components were eluted using gradient-elution at a flow rate of 0.600 ml/min. From 0 to 1 min the mobile phase contained 100% of MPA. From 1 to 2 min the amount of MPB in the mobile phase was increased linearly to 21%. At 3 min, the system was switched to 99% MPB. Finally, at 4 min, the analytical column was re-equilibrated to initial gradient settings. Eluting compounds were detected using a Waters Quattro Ultima triple quadrupole system (Micromass Waters, Manchester, UK) equipped with an electrospray source (orthogonal Z-spray®) operated in positive (ESI+) or negative ionization (ESI−) mode. The MS/MS instrument was operated with a capillary voltage of 3.5 kV, source block temperature of 150 °C and cone voltage of 10 V. Nitrogen was used as desolvation gas and was heated to 400 °C and delivered to the source at 750 L/h. The system uses argon gas to induce fragmentation in the collision cell. The dwell time for each MRM transition was 50 ms and the interchannel and interscan delays were set to 20 ms. Details of the MRM-transitions for each compound are described in Table 1. Peak areas were integrated using MassLynx 4.1 software (Micromass Waters, Manchester, UK).

2.3. Stock solutions, standards and quality control samples

A first set of stock solutions were prepared for all components except AMO, AMP, CFT and TAZ. All stock solutions were prepared in water, except for LIN which was dissolved in DMSO. Due to their lower solubility, AMO, AMP, CFT and TAZ were dissolved directly into, and mixed with aliquots of the stock solutions of the other components rather than first preparing a separate stock solution. Details regarding stock solution/mix solution concentrations are found in Table 2. The concentrations of the different antibiotics in this mixture, containing all 13 components, were chosen proportionally to their expected maximum plasma concentrations in patients on a standard treatment regimen [12]. This resulting mix was further diluted with water to obtain working solutions. Blank human plasma (Red Cross, Ghent, Belgium) was spiked with the different working solutions to obtain calibrator samples containing all 13 components. In order to preserve the integrity of the plasma matrix, the plasma standards contained at most 5% (v/v) of working solution. A second independent set of stock solutions were prepared for all components. Apart from LIN, which was dissolved in DMSO, all stock solutions were prepared in water. These stock solutions were further diluted in water to obtain three working solutions. Again, concentrations were tuned to the appropriate measurement range for the particular AB. Plasma quality control samples (QC) were prepared by addition of working solution, containing a single component only, to appropriate amounts of blank human plasma. The final concentrations obtained in the calibrator and quality control samples as well as details regarding the construction of the calibration curves are summarized in Table 3. Internal standard stock solutions, prepared from the stable isotope labelled compounds, were prepared in water, except for $^2H_5$-Phenoxymethylpenicillin which was dissolved in 33% (v/v) acetonitrile. After mixing and further dilution of these stock solutions, an internal standard working solution was obtained. Details regarding IS stock and working solutions are given in Table 2. Stock solutions, internal standard stock solutions, plasma calibrator samples and quality control samples were stored in polypropylene tubes at −20 °C.

2.4. Solid phase extraction procedure

Frozen samples were allowed to thaw at room temperature and a rotatory mixer was used to ensure complete homogenization of the samples prior to analysis. Oasis® MCX μ-elution 96-well plates were conditioned and equilibrated using respectively

Table 1
Detection parameters for the different AB under study. Only one MRM transition (quantifier) was monitored for the stable isotope labelled internal standards, while for the analytes a quantifier and qualifier trace were monitored. Qualifier traces were used to calculate ion ratios for each studied AB (ratio of quantifier over qualifier). These ion-ratios were monitored during the analysis to confirm the identity of the measured AB.

Compound	Detection	MRM-transitions		CE (eV)
	Ionisation mode	Quantifier	Qualifier	
AMO	ESI −	364.2 > 222.6	364.2 > 319.9	6
AMP	ESI −	348.3 > 206.6	348.3 > 303.8	6
CDX	ESI +	364.2 > 207.7	364.2 > 346.6	8
CFZ	ESI +	455.2 > 322.6	455.2 > 294.7	10
CFP	ESI +	481.1 > 323.7	481.1 > 395.8	14
CFT	ESI +	547.1 > 468.1	547.1 > 396.0	12
CEF	ESI −	423.3 > 317.8	423.3 > 206.7	7
FLU	ESI −	452.1 > 310.6	452.1 > 407.9	6
LIN	ESI +	338.3 > 295.8	338.3 > 194.8	20
MER	ESI +	384.4 > 253.7	384.4 > 297.6	14
FEN	ESI −	349.1 > 207.8	349.1 > 304.8	6
PIP	ESI −	516.0 > 329.9	516.0 > 232.6	8
TAZ	ESI +	300.8 > 167.7	300.8 > 206.8	14
$^2H_4$-AMO	ESI −	368.3 > 227.2	–	8
$^2H_4$-CDX	ESI +	368.4 > 212.1	–	8
$^{13}H,^2H_3$-CFP	ESI +	485.5 > 327.6	–	14
$^2H_6$-MER	ESI +	390.4 > 259.8	–	14
$^2H_5$-FEN	ESI −	354.3 > 212.7	–	6

Table 2
Details on stock solution and mix working solution concentrations. All stock solutions were prepared in water, except for LIN, which was dissolved in DMSO. Mix working solutions were prepared in water. Internal standard working solution was prepared in water after appropriately diluting internal standard stock solutions.

Compound	Stock concentration (mg/ml)	Mix concentration (mg/ml)	IS working solution (μg/ml)
AMO	–	1.07	–
AMP	–	1.47	–
CDX	2.54	0.13	–
CFZ	76.66	1.53	–
CFP	21.53	0.45	–
CFT	–	1.91	–
CEF	25.24	1.21	–
FLU	10.63	0.19	–
LIN	4.16	0.13	–
MER	7.02	0.42	–
FEN	1.27	0.15	–
PIP	49.95	0.80	–
TAZ	–	0.59	–
$^2H_4$-AMO	1.02	–	12.20
$^2H_4$-CDX	0.59	–	2.40
$^{13}C,^2H_3$-CFP	0.96	–	5.80
$^2H_6$-MER	1.13	–	4.50
$^2H_5$-FEN	0.44	–	1.70

Table 3
Details on calibration curves and concentrations of plasma calibrator and QC samples. Plasma calibrator samples were constituted of a mixture of the 13 studied AB spiked to blank human plasma. QC samples contained a single AB in blank human plasma. Aliquots were stored in polypropylene vials at −20 °C.

Compound	Weighting factor			Plasma concentration (μg/ml)										
	Axis transformation	2nd order term	Internal standard	ST8	ST7	ST6	ST5	ST4	ST3	ST2	ST1	QC Low	QC Med	QC High
AMO	1/X	−	$^{2}H_4$-AMO	0.43	0.80	2.13	3.19	6.38	12.76	25.52	51.05	0.57	5.73	40.14
AMP	Log	+	$^{2}H_5$-FEN	0.58	1.09	2.92	4.38	8.76	17.51	35.02	70.05	0.83	8.28	57.95
CDX	Square root	+	$^{2}H_4$-CDX	0.05	0.09	0.25	0.38	0.75	1.51	3.02	6.04	0.07	0.69	4.83
CFZ	Log	+	$^{2}H_4$-CDX	0.61	1.14	3.04	4.56	9.13	18.25	36.50	73.01	0.78	7.82	54.76
CFP	Square root	+	$^{13}C,^{2}H_3$-CFP	0.18	0.34	0.90	1.35	2.69	5.38	10.77	21.53	1.11	4.46	17.84
CFT	1/X	+	$^{13}C,^{2}H_3$-CFP	0.76	1.42	3.78	5.68	11.35	22.70	45.40	90.81	3.61	14.43	57.71
CEF	Log	+	$^{2}H_5$-FEN	0.48	0.90	2.40	3.61	7.21	14.42	28.85	57.69	0.69	6.87	48.08
FLU	1/X	+	$^{2}H_5$-FEN	0.08	0.14	0.38	0.57	1.14	2.28	4.56	9.11	0.11	1.08	7.59
LIN	Log	+	$^{2}H_4$-CDX	0.05	0.09	0.25	0.37	0.74	1.48	2.97	5.94	0.07	0.71	4.95
MER	Square root	+	$^{2}H_6$-MER	0.17	0.31	0.84	1.25	2.51	5.01	10.03	20.06	1.04	4.18	16.71
FEN	1/X	+	$^{2}H_5$-FEN	0.06	0.11	0.30	0.45	0.91	1.82	3.64	7.28	0.09	0.87	6.07
PIP	1/X	+	$^{2}H_5$-FEN	0.32	0.59	1.59	2.38	4.76	9.51	19.03	38.06	0.42	4.25	29.73
TAZ	1/X	+	$^{2}H_4$-CDX	0.24	0.44	1.18	1.76	3.53	7.06	14.12	28.24	0.23	2.30	16.07

200 μL of methanol and 200 μL of water. 50 μL aliquots of plasma samples were pipetted into empty wells of a 96-well plate. Subsequently 50 μL of internal standard working solution was added. Prior to homogenization by repeated aspiration with a multichannel pipette, 400 μL of a 2.5% (v/v) H_3PO_4 solution was added to the wells. Of this mixture 200 μL was loaded onto the Oasis® μ-elution plate. 200 μL of a 2% (v/v) formic acid solution was used to wash the Oasis® plate prior to elution with 30 μL of a 5% (v/v) NH_4OH solution in acetonitrile/methanol (60:40). Finally, eluates were diluted with 90 μL of a 1 M CH_3COOH/CH_3COONH_4 buffer. After capping of the 96-well plate with a polypropylene cap mat, sample plates were placed in the autosampler at 5 °C until injection.

2.5. Relative matrix effect

For the assessment of the relative matrix effect, the previously described calibrator working solutions were used to prepare calibrator samples in 8 different lots of human plasma at three different concentration levels. The concentrations of the calibrator samples were chosen to reflect the concentrations of the different antibiotics at the upper limit of quantification (ULOQ), at the centre of the calibration curve and at 2 × the lower limit of quantification (LLOQ). All calibrator samples were analyzed in quadruplicate. To test for a statistically significant difference between analyte-to-internal standard peak area ratios obtained for different plasma lots, Anova analysis was performed using R® (R foundation for statistical computing, Vienna, Austria). Additionally, absolute matrix effect was calculated according to Matuszewski et al. [13].

2.6. Validation

The validation experiments were based on the "Guidance for Industry-Bioanalytical Method Validation" recommended by the Food and Drug Administration (FDA) of the United States. [11] Batches of quality control samples at three different concentration levels (Table 3) were prepared, aliquoted and stored at −20 °C. These samples were used throughout the validation experiments.

2.6.1. Calibration model selection and linearity evaluation

Calibrator working solutions were used to prepare calibrator samples at six different concentrations. All samples were analyzed in six-fold within the same run. Calibration model selection and linearity evaluation was performed according to Hartmann et al. [14].

2.6.2. Trueness and (intermediate) imprecision

Trueness was evaluated by analyzing QC samples in six-fold on the same day. It was calculated as the mean relative error of QC samples analyzed under repeatability conditions. Within-run imprecision was calculated as the coefficient of variation (CV%) of the concentration of the QC samples measured in six-fold on day 1. Between-run imprecision was evaluated by replicate measurements of QC samples on four different days (measurements in six-fold on day 1, triplicate measurements on days 2, 3 and 4). Between-run imprecision was calculated as the CV% of the average QC concentrations measured on four different days.

2.6.3. Lower limit of quantification (LLOQ)

The lower limit of quantification was defined as the concentration of the lowest calibrator sample which could be measured with an imprecision not exceeding 20% and a trueness between 80% and 120%.

2.6.4. Selectivity

Selectivity was assessed by examining peak interference (signal-to-noise ratio < 9) from eight independent sources of blank human plasma.

2.6.5. Stability

Stability of the β-lactam antibiotics was studied in different experimental settings. At first, antibiotic stock and internal standard stock solution stability was evaluated at −20 °C. Aliquots of internal standard stock solutions were kept at −20 °C and thawed at different time-points throughout an 86-day period. Stability data for the antibiotic stocks spanned a 35-day period. Samples were analyzed using UPLC-UV. Secondly, a short-term study was conducted to evaluate autosampler stability at 5 °C. At different time points throughout a 24 h study period, triplicate injections were made from a vial kept in the autosampler compartment at 5 °C. Analyte-to-internal standard peak area ratios were measured by UPLC–MS/MS. Finally, a preliminary study was set-up to evaluate antibiotic stability in plasma calibrator samples kept at −20 °C. In this study, calibrator samples were analyzed in triplicate before and after 4 days of storage at −20 °C. Analyte-to-internal standard peak area ratios were measured by UPLC–MS/MS. For the stock stability studies, linear regression models of the peak areas versus time were constructed. From these models, regression coefficients were

estimated and used to calculate the time point at which 15% of the initial concentration had degraded. In the autosampler stability and the calibrator stability experiment, statistical significance testing was performed at each time-point. A two-sided one-sample t-test at the 5% level of significance was used to assess whether the obtained measurement was significantly different from the initial measurement.

3. Results and discussion

3.1. Method development

3.1.1. Chromatography and mass spectrometric detection

Prior to the optimization of the chromatographic separation, preliminary precursor and product-ion mass spectra were obtained for the different antibiotics in both negative and positive ionization mode. The choice of ionization mode was based on a comparison of the obtained sensitivity between either modes for each compound. Seven out of 13 components were detected in the negative ionization mode, while the other components proved more suitable for positive ionization electrospray. Details of ionization mode are presented in Table 1. Rather than focusing on accomplishing baseline separation for all adjacent peak pairs, we set out to maximize chromatographic resolution for most compounds, whilst minimizing overall run time. The limited retention of β-lactam antibiotics on conventional reversed-phase C_{18} columns was previously described by Ohmori et al. [8] Similar to the experiments described by these authors, we compared chromatographic separation and capacity factors between two different analytical columns. Both columns were chosen based on their compatibility with high aqueous content mobile phases. Initial experiments were carried out on a hybrid-particle column (Waters BEH C_{18} UPLC column; 50 mm × 2.1 mm × 1.7 μm particles) but provided insufficient resolution. In contrast, better overall resolution was seen in experiments conducted with the second column (Waters HSS T3 UPLC column, 50 mm × 2.1 mm × 1.7 μm particles). After optimization of mobile phase flow rate and gradient elution parameters, we achieved full chromatographic separation for all components detected in ESI− within 4 min. On the other hand, of all components measured in ESI+, two pairs were not baseline separated. A chromatogram of a calibrator sample is presented in Fig. 4. Finally, once all mobile phase constituents were selected, MS/MS detection parameters were optimized by post-column infusion of antibiotic stock solutions into the mobile phase stream. Optimized parameters as well as details on selected MRM transitions are given in Table 1.

3.1.2. Extraction procedure

3.1.2.1. Protein precipitation. Two different protein precipitation protocols were evaluated in terms of recovery and matrix effect. Procedure 1 consisted of precipitating plasma samples using cold acetonitrile. After centrifugation and transfer of the supernatant

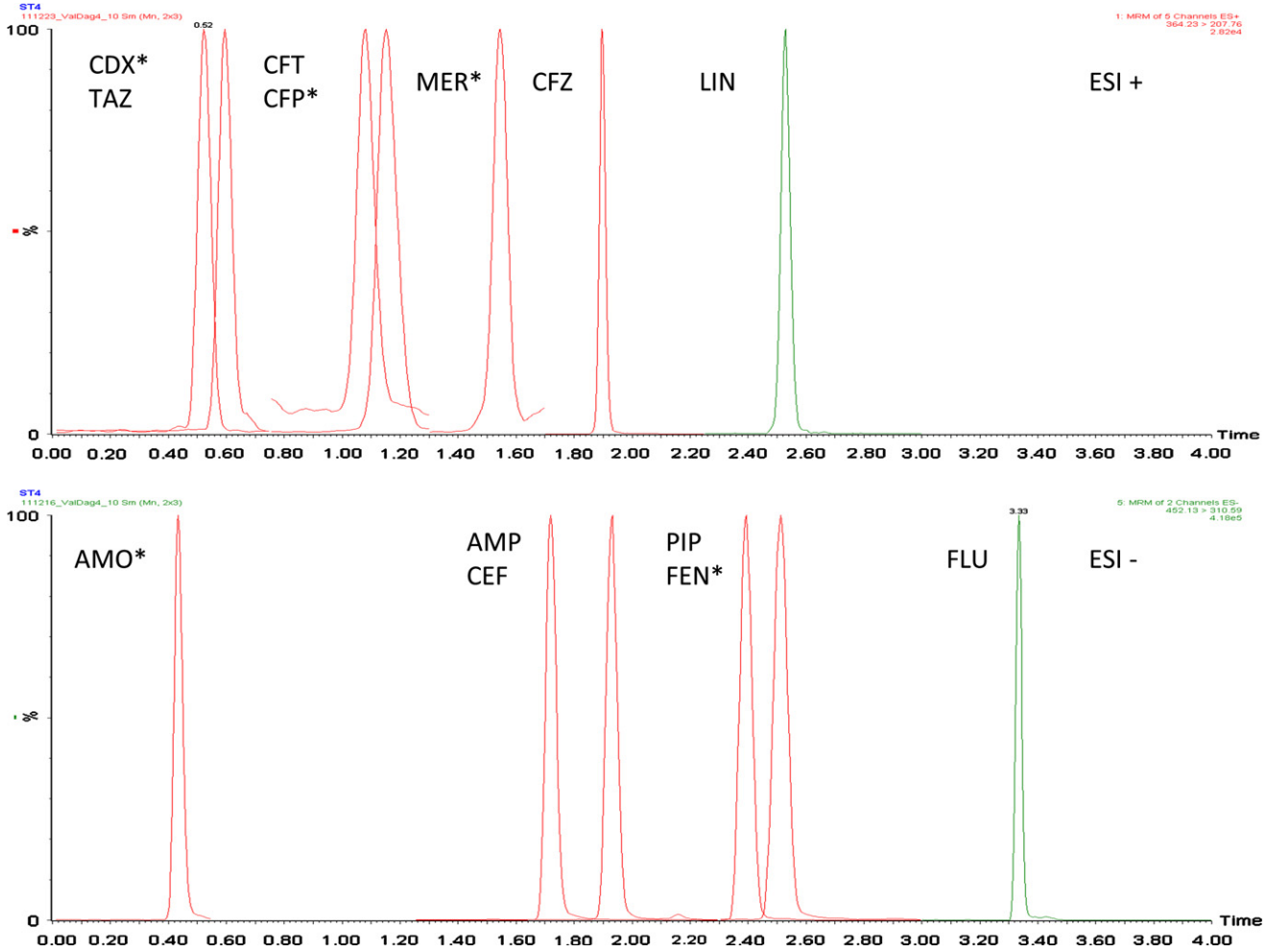

Fig. 4. Chromatogram of a calibrator sample. Concentrations of the different antibiotics in the calibrator sample according to Table 3. An asterisk denotes compounds for which a stabile isotope labelled internal standard was included. Analytical conditions as described under 2.2 Instrumentation.

to a glass tube, the samples were evaporated to dryness under a stream of nitrogen gas at 30 °C. Prior to injection onto the chromatographic system, samples were reconstituted in a 5% (v/v) solution of acetonitrile in water. In procedure 2 we used a solution of 1% (v/v) formic acid in acetonitrile to precipitate plasma proteins. Subsequently, samples were filtered through an Ostro® plate to remove lipoproteins (Ostro® plates contain a reversed phase type sorbent embedded in the protein filter material) and precipitated proteins. Samples were then evaporated to dryness under a stream of nitrogen gas at 30 °C. Finally, the obtained dry residue was dissolved in a 5% (v/v) solution of acetonitrile in water before being injected onto the analytical column. Recovery and matrix effect were calculated at 3 different concentration levels according to Matuszewski et al. [13]. All samples were analyzed in quadruplicate. Results of the analysis for the recovery experiment are summarized in Table 4. No evidence is found for an overall difference in recovery between both procedures. Although recovery seems less variable for protocol 2 (illustrated by the lower standard error (SE)), the apparent lack of recovery for MER limits the suitability of this sample clean-up procedure for our set of compounds. Moreover, the results of the matrix effect experiment, as shown in Fig. 5, reveal significant ion suppression for several compounds in both procedures. Based on these observations, we concluded that other sample preparation techniques, providing a reasonable recovery for all compounds as well as a less pronounced matrix effect, should be explored.

3.1.2.2. Mixed-mode solid phase extraction. The 13 β-lactam antibiotics studied greatly differ in their physical/chemical properties. Our selection contains acidic compounds (FLU, PIP, FEN, TAZ, CEF, CFZ), neutrals (LIN), as well as several zwitterionic compounds (AMO, AMP, CDX, CFP, CFT, MER) (Molecular structures are depicted in Figs. 1–3). Therefore, ordinary solid phase extraction, solely relying on reversed phase retention, would be of little practical use in this case. However, providing an additional ion-exchange retention mechanism, mixed-mode solid phase extraction should provide sufficient retention for sample clean-up. A screening experiment was set-up to evaluate suitability of this mixed-mode solid phase extraction for our set of compounds. Antibiotics from a spiked plasma sample were extracted on 4 different kinds of solid phase material (MCX, MAX, WAX and WCX; all Waters Oasis® sorbents) using 2 different protocols, as suggested by the solid phase extraction plates manufacturer. Afterwards, recovery-values were calculated

Table 4
Recovery for protein precipitation protocol 1 and 2. Mean Recovery and standard error (SE) were calculated on quadruplicate analysis at 3 different concentration levels. ($n=12$ for both mean recovery and standard error).

Compound	Concentrations (μg/ml)	Protocol 1		Protocol 2	
		Mean recovery (%)	SE (%)	Mean recovery (%)	SE (%)
AMO	0.25–5.90–46.48	56.7	3.1	49.4	1.5
AMP	0.42–10.03–60.98	58.1	2.0	58.4	1.6
CDX	0.03–0.82–6.54	65.7	6.3	49.2	1.8
CFZ	0.39–9.36–74.91	81.7	6.2	59.1	1.1
CFP	0.11–2.70–21.6	45.6	5.4	27.6	1.3
CFT	0.41–9.85–73.35	28.6	3.8	22.4	2.0
CEF	0.31–7.50–60.04	74.2	3.9	68.4	1.2
FLU	0.05–1.16–9.27	57.5	2.6	56.9	1.6
LIN	0.03–0.82–6.55	73.7	2.6	61.9	0.7
MER	0.08–1.90–15.19	78.1	4.8	0.9	0.2
FEN	0.04–0.85–6.8	49.9	3.8	57.0	2.5
PIP	0.21–5.02–40.16	61.0	2.8	55.5	1.6
TAZ	0.11–2.66–15.5	54.4	2.0	73.7	2.2

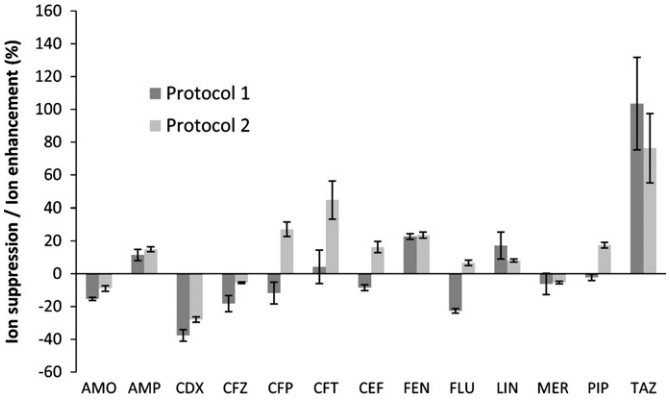

Fig. 5. Matrix effect for protein precipitation protocol 1 and 2. The height of the bars represents the mean matrix effect for 4 replications at 3 different concentration levels. Error bars represent ±1 standard error of the mean, ($n=12$).

Table 5
Recovery for the final mixed-mode solid phase extraction protocol using the MCX sorbent with a single elution step, ($n=3$).

Compound	Concentration (μg/ml)	Mean recovery (SE) (%)
AMO	44.30	69.4 (2.0)
AMP	59.20	84.8 (2.6)
CDX	6.25	96.1 (4.1)
CFZ	75.40	82.1 (2.0)
CFP	21.50	80.8 (2.0)
CFT	73.20	74.8 (2.0)
CEF	63.05	72.9 (2.3)
FLU	6.65	73.9 (2.8)
LIN	8.90	52.4 (0.6)
MER	6.10	91.5 (2.4)
FEN	15.15	52.2 (1.1)
PIP	39.95	67.4 (3.5)
TAZ	22.75	12.7 (1.0)

and compared for the different types of mixed-mode SPE materials (data not shown). The manufacturer's standard protocol dictates differential elution using two different solvents, allowing separation of neutral molecules from (strong) acidic or (strong) basic compounds. Comparing the 4 SPE materials, it was clearly seen that overall recovery was highest for the MCX sorbent. However, none of both elution steps provided adequate recovery for all compounds. Consequently, the protocol was amended to accommodate elution of all compounds, at the same time aiming for a single elution step. A 5% (v/v) NH_4OH solution in a 60:40 mixture of acetonitrile and methanol was finally chosen. Ohmori et al. [8] evaluated the use of an Oasis® HLB sorbent (general SPE sorbent, lacking the ion-exchange capabilities) to extract eight β-lactam AB from plasma. They reported an absolute recovery of MER of approximately 70% due to the highly hydrophilic nature of the compound. Our results clearly demonstrate that the additional ion-exchange capabilities of the mixed-mode SPE sorbents significantly improves the recovery of hydrophilic compounds (average absolute recovery of MER ± SE: 91.5 ± 2.4). In parallel to the recovery assessment for this approach, also matrix effect was evaluated. Results are given in Table 5 and Fig. 6. Despite the low recovery noted for TAZ, on average recovery was equally as good or better than using protein precipitation protocol 1 (average recovery SPE and protein precipitation are respectively 75% and 64%). Moreover, in contradiction to the ion suppression noted in the matrix effect experiment under 3.1.2.1, no significant ion suppression was observed after mixed-mode solid phase extraction. These observations are in agreement with those made by Ohmori et al. [8]. The authors demonstrated a lack of

significant ion-suppression after SPE using a generic Oasis® HLB sorbent for sample clean-up. Similar to our protocol, the protocol of Ohmori et al. consisted of a single wash step (using an ammoniumformate solution) to rinse off interfering endogenous substrates. This observation, together with our results, suggest that a single wash step SPE protocol (as opposed to the general two-wash-step protocols reported in literature) might be sufficient to overcome significant matrix-effects. The elution step of our final MCX-sorbent SPE protocol thus comprises elution with a 5% (v/v) NH_4OH solution in acetonitrile/ methanol 60:40 (v/v). This strong alkaline solution does not allow direct injection of the eluate onto the analytical column. Therefore, an additional dilution-neutralization step is added. Compatibility with the mobile phase and autosampler stability was evaluated for three different solutions: a more concentrated mobile phase buffer (ammoniumacetate), a phosphate buffer and an acetic acid solution. The 1 M ammoniumacetate buffer (pH 4.76) on the one hand was chosen because of mobile phase compatibility, the 1 M phosphate buffer (pH 7.20) and the 1.3% (v/v) acetic acid solution on the other hand were chosen to evaluate whether β-lactam AB autosampler stability would improve if the pH of the eluate was adjusted to neutral (pH 7.20). Because of lack of evidence for improved stability for either the phosphate buffer- or the acetic acid solution-neutralized eluate (data not shown), we finally chose a 1 M CH_3COOH/CH_3COONH_4 buffer (pH 4.76) for dilution of the elute prior to injection onto the analytical column.

3.1.3. Relative matrix effect

Generally, absolute and relative matrix effect experiments are performed as part of the formal qualification/validation experiments. However, in our laboratory, we opt to perform these experiments prior to qualification/validation experiments. In our opinion, the extent of (relative) matrix effect is a very important performance characteristic of a LC–MS based analytical method. Furthermore, rather than focussing on developing an analytical methodology free of absolute matrix effect, we direct our efforts towards developing robust methods free of relative matrix effect. Therefore, a thorough study on the presence/absence of relative matrix effect is conducted prior to looking into other performance characteristics (e.g. precision, trueness). As a consequence, if a significant relative matrix effect is found, the sample preparation process is re-evaluated and the relative matrix effect experiment is repeated.

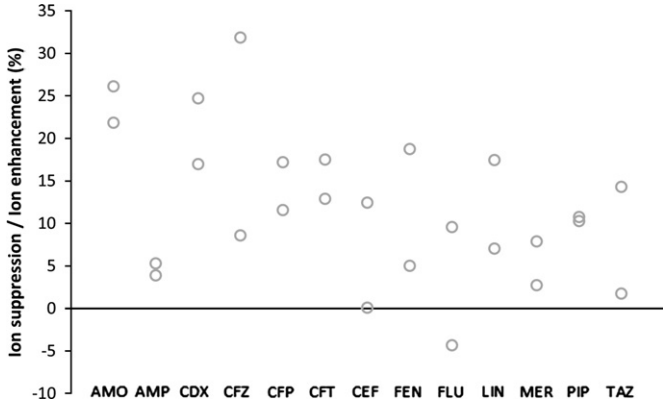

Fig. 6. Matrix effect for MCX sorbent with single elution step, (n=2).

For each compound, on every concentration level, an Anova-analysis was performed. Resulting p-values for the different Anova-tests performed are given in Table 6. The relative matrix effect (in these experiments represented by the between-group variability) was calculated on the quadruplicate measurements of compounds in eight different types of plasma. However, for LIN, at the high concentration level, only data from seven different types of plasma was used due to the presence of an outlier. To our opinion, the design of the experiment was not compromised by leaving out one of the plasma types for the LIN high concentration samples. Overall, we can state that at the 5% level of significance, no statistically significant evidence is found in the data to suspect the presence of a relative matrix effect.

In terms of absolute matrix effect, no significant ion-suppression was observed. Absolute matrix effect ranged from 3% to 24%. As stated by De Bock et al. [15], the absence of an absolute matrix effect is not a prerequisite for a reliable bioanalytical method. However, the lack of variability in matrix effects between individual subjects (hence relative matrix effect) is indispensable in the development of reliable bioanalytical methods. Although relative matrix effects may influence the quantification of analytes using mass-spectrometry based techniques it is rarely formally tested [8] using plasma/serum from different subjects. Using different plasma lots, we extensively demonstrated our method to be free of a relative matrix effect, so no further adaptations were made to our final sample preparation protocol, despite of the documented absolute matrix effect for both AMO and CDX.

3.2. Validation

3.2.1. Calibration model selection and linearity evaluation

After a visual/statistical check for outliers different weighting factors/axis transformations were applied to the linear regression model and tests for homoscedasticity were performed. Subsequently, mean weighted residual plots were inspected to detect deviations from linearity. If necessary, a second order term was added to the linear regression model. Prior to starting the validation experiments, an experiment was set-up to evaluate the trueness and precision of the determination of single QC samples using the above mentioned calibration curves. It was noted that although trueness and precision were acceptable for the mid-range and low quality control levels, the high level quality control samples could not be measured accurately (data not shown). Upon investigation of these experiments, we found that the recovery for our calibration standards (mix of 13 β-lactam antibiotics) significantly differed from the recovery for the quality control standards (single antibiotic compound in plasma). Owing to the high concentration of the different compounds present in the plasma calibrator samples (concentrations up to 160 μg/ml), saturation of the binding sites of the mixed-mode

Table 6
Resulting p-values for the different Anova-test performed on the analyte-to-internal standard peak area ratios for the different lots of plasma. ($Df_{Between}$=7, Df_{Within}=24) Responses in eight different types of plasma were evaluated. All within-plasma type samples were analysed in quadruplicate. For the ULOQ level for LIN the results for 1 plasma type (n=4) were omitted prior to the Anova analysis.

Level	AMO	FLU	AMP	CEF	FEN	PIP	CDX	CFZ	CFT	LIN	MER	CFP	TAZ
2 × LLOQ	0.907	0.977	0.743	0.743	0.948	0.756	0.917	0.326	0.529	0.821	0.864	0.891	0.988
Mid-range	0.277	0.281	0.270	0.780	0.505	0.758	0.611	0.459	0.056	0.972	0.375	0.379	0.729
ULOQ	0.975	0.614	0.402	0.819	0.868	0.938	0.102	0.753	0.232	0.223	0.543	0.512	0.882

SPE might have taken place. After reduction of the analytical range of our method and addition of an extra dilution step prior to loading of the samples onto the mixed-mode SPE plates, the statistically different recovery for the QC samples versus the calibrator samples disappeared. Final plasma calibrator sample concentrations, plasma quality control sample concentrations and details regarding the construction of the calibration curve are provided in Table 3.

3.2.2. Trueness and (intermediate) precision

Between-day imprecision, within-day imprecision (both defined by CV% for replicate analyses) and trueness were evaluated (Table 7). For eight out of the 13 β-lactam AB studied the performance characteristics of our analytical method fell well within the specifications dictated by the FDA-guidance on bioanalytical method validation [11]. In contradiction to these results, our validation experiments revealed a significant bias for CFP. Calculated trueness for the low, medium and high QC samples were respectively 104%, 125% and 131%. Additional experiments (data not shown) revealed a difference in CFP extraction recovery between the calibrator samples and the QC samples. Similar to the experiments described under 3.2.1., we hypothesised that the difference in total AB concentration between the calibrator samples (mix of all AB) and the QC samples (single AB in plasma) might be causing this differential recovery. Given that this difference in recovery was only observed for CFP, the addition of another dilution step prior to sample preparation was considered unfavourable. We acknowledge that a bias as severe as this observed for our CFP assay might compromise future study results. To overcome this differential extraction recovery, a potential solution might be the replacement of the currently used internal standard ($^{13}C,^{2}H_3$-Cefepime) with an internal standard which might better correct for the different extraction behaviour of CFP in the calibrator versus quality control samples (e.g. pure ^{13}C-CFP analogue).

Upon reviewing the results of the validation experiments, for some compounds, we found some measures of imprecision and trueness that did not fully comply with the specifications proposed by the FDA guidance on method validation [11]. For FLU the measures for trueness for the low and medium level are respectively 122% and 117%. For CEF, PIP and TAZ for the medium QC level and for FLU for the high QC level, we failed to demonstrate the imprecision to be smaller than 15%. Nevertheless, in all cases imprecision was limited to approximately 20%, which equals the maximum amount of imprecision allowed at the low end of the analytical range.

With respect to the observed variation in AB plasma concentrations in e.g. the critically ill [16], a subpopulation for whom our assay might be used to guide drug dosing, the additional error introduced by our assay by not complying with the FDA specifications, is considered negligible. On the other hand, when performing PK studies e.g. to compare bioavailability between different formulations, this additional measurement error might (although to our opinion this is very unlikely) impact statistical power. Prior to conducting these studies, a formal assessment of the impact of the method's performance characteristics on the study outcome is deemed necessary, although this equally holds for every other assay used in a particular PK study.

3.2.3. Lower limit of quantification

In accordance with the criteria described in Section 2, the lowest standard on the calibration curve for each compound in our selection was accepted as the limit of quantification.

3.2.4. Selectivity

No interferences were observed at the retention times of the analytes and the IS when analyzing blank human plasma from 8 independent sources (data not shown).

3.2.5. Stability

Table 8 summarizes the results of the different stability studies. For some compounds (AMO, AMP, CFT and TAZ) no stock stability study was performed. This is simply because in our routine practice no individual solutions of these compounds are made. Apart from limited stock stability for MER and CFT, overall stock stability at −20 °C is acceptable (overall median=35 days). Autosampler stability is limited for MER, LIN and CFZ, with time upon which significant degradation occurs respectively being 6, 11 and 11 h. All other compounds remained stable in the autosampler compartment at 5 °C up until 20 h. Of all compounds present within the plasma calibrator samples, only 5 showed no statistical evidence of degradation after storage at −20 °C for 4 days. Highly significant differences are noted for AMO, AMP, CFP, CFT, LIN and MER. For the latter one, we estimated the degree of degradation as high as 30%. The main reason for setting-up this stability experiment was to assess whether plasma calibrator samples, once made, could be used for quantitation purposes during an entire week. From these results we concluded that calibrator samples should be made ex-tempore. Nevertheless, stock stability data clearly indicate that within a relatively short time-frame (1 month) plasma calibrator samples may be prepared from stored stock solutions.

Table 7
Results for the analysis of validation samples for the different antibiotics under study.

Compound	Nominal concentration (μg/ml)	Within-day assay ($n=6$)		Between-day assays ($n=4$)
		Trueness (%)	Imprecision (RSD%)	Imprecision (RSD%)
Amoxicillin	0.57–5.74–40.14	103.1–98.4–102.2	8.2–8.1–9.2	2.5–9.7–5.4
Ampicillin	0.83–8.28–57.95	92.5–110.3–108.6	18.2–12.4–15.1	10.7–5.9–6.9
Cefadroxil	0.07–0.69–4.83	117.6–104.1–109.9	19.8–4.5–5.7	9.9–8.8–7.3
Cefazolin	0.78–7.82–54.76	112.6–109.2–100.3	20.3–10–10.8	4.4–10.5–5.5
Cefepime	1.12–4.49–17.94	103.8–125.1–131.2	14.9–7.3–12.5	1.6–5.7–10.3
Ceftazidime	3.61–14.43–57.71	96–93.6–88.7	6.6–11.4–7.8	1.7–9.2–7.5
Cefuroxime	0.69–6.87–48.08	93.1–102.9–98.7	18.8–17.6–15.9	12.5–7.9–11.2
Flucloxacillin	0.11–1.09–7.59	122–116.5–92	18–10.2–8.2	12.8–7.7–20.8
Linezolid	0.07–0.71–4.95	93.5–92.6–90.3	10.6–5.9–11.2	0.8–3.8–8.1
Meropenem	1.05–4.18–16.71	100.7–106.1–105.6	6.6–8.2–5.8	7.5–2.1–4.6
Phenoxymethylpenicillin	0.09–0.87–6.07	99.1–96.8–101.6	12.2–9.7–11.4	4.9–12.5–4
Piperacillin	0.42–4.25–29.73	105.2–95.5–96	17.6–17.7–6.9	5.4–4.9–15.5
Tazobactam	0.23–2.3–16.07	107.1–96–100.8	16–5.9–11.7	10.9*–16.4*–9.7*

* The results from the analysis of the medium QC samples of day 2 were excluded from the final trueness and imprecision calculations.

Table 8
Results for the different stability experiments performed according to experimental conditions as described in Section 3.2.4. The minus sign indicates that stability was not assessed for a certain compound.

Compound	Stock stability (days)	Autosampler stability (h)	Calibrator stability (*p*-value t-test)
AMO	–	20	< 0.01
AMP	–	20	< 0.01
CDX	35	20	0.77
CFZ	35	11	< 0.05
CFP	35	20	< 0.01
CFT	–	20	< 0.01
CEF	35	20	0.31
FEN	35	20	0.94
FLU	35	20	0.17
LIN	35	11	< 0.01
MER	19	6	< 0.01
PIP	35	20	0.80
TAZ	–	20	< 0.05
$^{2}H_4$-AMO	54	–	–
$^{2}H_4$-CDX	82	–	–
$^{13}C,^{2}H_3$-CFP	78	–	–
$^{2}H_6$-MER	12	–	–
$^{2}H_5$-FEN	86	–	–

4. Conclusion

This study presents the development and validation of a multi-drug assay to be used in pharmacokinetics studies as well as TDM-programs. Sufficient evidence is provided to gain insights in the performance of our multi-component method. Nevertheless, prior to the application of the analytical method some additional study-specific experiments have to be performed. On one hand, we did not include a formal selectivity study. On the other hand, additional information is to be gathered on the plasma stability of the different compounds. Selectivity studies as well as stability studies should be performed in a manner consistent with the future use of the method and are therefore study specific. For example, stability after storage of plasma samples is known to be dependent on type of sample tube used for storage or on the kind of anticoagulant added prior to blood collection. [17] Similarly, selectivity studies should account for, among other things, co-medication which is expected to be present in patient samples. In the case of TDM-assays, drugs frequently prescribed in the institution where the TDM-program is run should be included in the study.

Through the use of an ultra-high performance liquid chromatography instrument, we minimized chromatographic runtime, resulting in a cycle-time (time between sequential injections) of 5 min. Furthermore, the use of a 96-well SPE plate in combination with a multi-channel pipette allowed us to minimize time spent on sample preparation. If our method were to be applied in a therapeutic drug monitoring setting, time between sampling and reporting of plasma concentrations could be as little as 1.5 h (analysis of calibrator samples and quality control samples included), demonstrating its suitability as, among other things, a potential multi-drug TDM assay. Moreover, the analytical method, as presented in this paper, can be used in the bio-analytical support of pharmacokinetic studies.

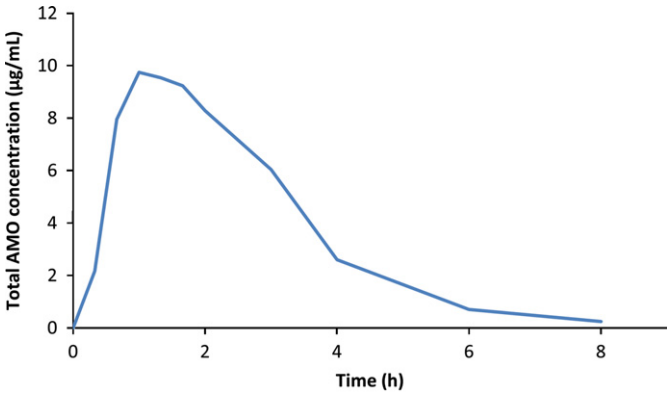

Fig. 7. Plasmaconcentration–time profile in a healthy volunteer after administration of a 500 mg oral dose of amoxicillin.

As a concluding proof-of-concept, we used our multi-drug assay to analyse samples from a healthy volunteer given a standard 500 mg oral dose of Amoxicillin. The full pharmacokinetic results from this study are beyond the scope of this publication. However, in Fig. 7, the pharmacokinetic profile of amoxicillin after a standard 500 mg oral dose in one healthy volunteer is given, illustrating the practical applicability of our analytical methodology.

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