Microanalysis of beta-lactam antibiotics and vancomycin in plasma for pharmacokinetic studies in neonates

Running title: Microanalysis of antibiotics in neonatal plasma

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Abstract

Rational dosing of antibiotics in neonates should be based on pharmacokinetic (PK) parameters assessed in specific populations. PK studies in neonates are hampered by the limited total plasma volume, which restricts sample volume and sampling frequency. Available drug assay methods require large sample volumes, are labor-intensive or time-consuming. The objective of this study was to develop a rapid ultra-performance liquid chromatographic method with tandem mass spectrometry detection for simultaneous quantification of amoxicillin, meropenem, cefazolin, cefotaxime, deacetylcefotaxime, ceftriaxone and vancomycin in 50 μL of plasma. Cleanup consisted of protein precipitation with cold acetonitrile (1:4) and solvent evaporation before reversed phase chromatographic separation and detection using electrospray ionization tandem mass spectrometry. Standard curves were prepared over a large dynamic range with adequate limits of quantitation. Intra- and interrun accuracy and precision were within 100 ± 15% and 15%, respectively, with acceptable matrix effects. Coefficients of variation for matrix effects and recovery were less than 10% over six batches of plasma. Stability in plasma and aqueous stocks was generally sufficient, but stability of meropenem and ceftriaxone in extracts could limit autosampler capacity. Instrument runtime was approximately 3.50 min per sample. Method applicability was demonstrated with plasma samples from an ECMO-treated neonate. Different beta-lactam antibiotics can be added to this method with additional ion transitions. Using ultra-performance liquid chromatography mass spectrometry, this method allows simple and reliable quantification of multiple antibiotics in 50 μL of plasma for PK studies in neonates.
Successful drug therapy depends on the administration of the appropriate dose at the right dose interval, which implies an understanding of the drugs pharmacokinetic (PK) profile. This is particularly relevant in the treatment of neonatal and pediatric patients, considering potential variation in PK parameters due to an individual's developmental stage or specific morbidity. Therefore, PK parameters should be assessed in the specific populations to which the drugs are given to prevent either undertreatment or unnecessary toxicity. Unfortunately, pharmacokinetic studies in neonates are complicated by the limited amount of biological material (e.g. blood, plasma) available, which poses restrictions on sampling frequency and sample volume (20). Moreover, the number of patients participating in these studies is often low, especially when studying patients of narrowly defined age groups, with specific (co-) morbidity or during treatment with extracorporeal techniques. Ideally, analytes of interest should be quantitated simultaneously in as little sample as possible. This limits the burden on individual patients caused by sampling, while maintaining sufficient data points for reliable data analysis.

For years, drugs have been quantified via high-performance liquid chromatography with ultraviolet detection (HPLC-UV). Many of the published bio-analytical methods require sample volumes of 250 μL or more (5, 8, 11, 13, 16). Some are labor-intensive (5, 7, 11) or require long run-times (7, 11, 12), potentially leading to poor reproducibility for analytes that may decompose during analysis, such as certain β-lactam antibiotics. Analytes had to be chromatographically separated from the interfering endogenous and exogenous matrix components, and often an elaborate sample preparation was necessary to reach sufficient selectivity and specificity. Co-eluting components would often
interfere due to low specificity of UV-detection. With the advent of mass spectrometry (MS), selectivity greatly increased since specific analyte masses could be detected. This led to an even greater specificity when mass spectrometers were set up in sequence (tandem mass spectrometry, or MS/MS): now, not only a specific mass could be identified, but a specific fragmentation pattern could be monitored to differentiate between analytes with the same mass.

Equipment capable of ultra-performance liquid chromatography mass spectrometry (UPLC-MS/MS) has recently become available. With a smaller particle size and higher operating pressures compared to regular HPLC, UPLC provides a shorter runtime and sharper peak shape, which improves sensitivity and reduces potential interference by matrix components (6, 10, 17). UPLC combined with tandem mass spectrometry should therefore allow quantitative analysis of multiple analytes with minimal sample preparation and matrix effects.

Currently, clinical studies in the Sophia Children’s Hospital include pharmacokinetic evaluations of multiple antibiotics in patients receiving extracorporeal membrane oxygenation (ECMO) treatment. In order to facilitate these studies, a simple and reliable method was developed to simultaneously quantify amoxicillin, meropenem, cefazolin, cefotaxime, deacetylcefoxime, ceftriaxone and vancomycin in 50 µL of plasma. In this manuscript, the method is presented, validation results are reported and method applicability is demonstrated with data from an ECMO-treated patient.
Materials and methods

Reagents. LC-MS grade water, liquid chromatography grade methanol and acetonitrile were from Biosolve (Valkenswaard, Netherlands). Formic acid (FA, Sigma, Schnelldorf, Germany) was analytical grade. The following reference standards were purchased from Sigma (FA, Sigma, Schnelldorf, Germany): ceftriaxone (CRO), vancomycin (VAN), cefazolin (CFZ), cefotaxime (CTX) and oxacillin (OXA). Deacetylcefotaxime (DACT) was kindly provided by Sanofi-Aventis (Gouda, Netherlands). Meropenem (MEM) was from Molekula (Wimborne, United Kingdom) and amoxicillin (AMX) from Certa (Braine-l’Alleud, Belgium).

Quality control samples and standard solutions. Standard stock solutions containing β-lactam antibiotics were prepared by dissolving the required amount of antibiotic (calculated as free base) in 25 mL of water. Vancomycin solutions were prepared separately to prevent potential accelerated degradation of other antibiotics (3, 9). Varying quantities of stock solution were diluted with water, resulting in 8 working standards over the concentration range varying from the lower limit of quantitation (LLOQ) to the upper limit of quantitation (ULOQ). Calibration standards were prepared by diluting 1 part working standard with 9 parts human plasma. Quality control (QC) samples for intra- and interassay comparisons were similarly prepared using a separate stock solution and stored at -80°C; low (L), medium (M) and high (H) controls were prepared at concentrations of respectively 3-4 × LLOQ, 40% of ULOQ and 75% of ULOQ. A stock solution of the internal standard (IS) was prepared by dissolving 10 mg of oxacillin in 50 mL of water. Prior to analysis, 1 part stock solution was added to 99 parts chilled acetonitrile. This precipitant solution was freshly prepared before each analysis.
Sample preparation. To 50 μL of plasma, 200 μL of chilled acetonitrile containing IS was added. The sample was mixed (5°C, 1,250 rpm) for at least 15 min to complete protein precipitation. After centrifugation at 16,000 × g for 10 min, 200 μL of the supernatant was transferred to a clean vial. The solvent was evaporated to dryness at 40°C under nitrogen gas flow, after which the residue was reconstituted in 100 μL of 0.1% (v/v) aqueous formic acid and left to mix for 30 min (5°C, 1,250 rpm). When cloudy, samples were centrifuged again at 16,000 × g for 10 min. The supernatant was transferred to a polypropylene autosampler vial and stored at 5°C until analysis by UPLC-MS/MS.

UPLC-MS/MS conditions. The UPLC-MS/MS system consisted of a Waters Acquity Ultra Performance LC coupled to a Quattro Premier XE tandem quadrupole mass spectrometer (Waters Corp., Milford, MA, USA). The analytical column was an Acquity UPLC BEH C18 2.1 mm × 100 mm column with 1.7 μm particle size (Waters Ltd, Dublin, Ireland), to which a 0.2 μm pre-column filter unit was added. The mobile phase was a gradient of solution A (0.1% formic acid in water) and solution B (0.1% formic acid in methanol) with an initial composition of 20% B. Mobile phase composition changed linearly from 20% B at 0.5 min to 40% B at 1.0 min and onward to 100% B at 2.0 min. The composition was switched back to 20% B at 2.5 min and maintained until 3.0 min. The flow rate was 0.4 mL/min with a column temperature of 40°C. Of each sample, 10 μL was injected onto the column. Analytes were detected via MS with an electrospray ionization (ESI)-interface in positive multiple reaction monitoring (MRM)-mode. Optimized MRM-settings for the individual drugs, including cone voltage and collision energy, are listed in table 1. The acquisition settings were: capillary voltage = 3.4 kV; source temperature = 120°C; desolvation temperature = 300°C; desolvation gas flow = 500 L/hr; cone gas flow = 50 L/hr; dwell time= 80 ms.
**Data analysis.** Data were acquired using Masslynx V4.1 software and processed using Quanlynx V4.1 software (Waters Inc.). For all analytes except ceftriaxone, calibration curves were obtained by plotting the peak area ratios of drug vs internal standard against the theoretical concentration. Peak height was used for CRO based on superior reproducibility. Standard curves were constructed for each analyte over the calibration range via weighted least squares regression.

**Validation.** The method was validated based on FDA guidelines for bioanalytical method validation (2). Vancomycin was added at a later stage with a reduced validation procedure. The full validation procedure included the following parameters:

(i) **Specificity and selectivity.** Chromatograms from 3 aqueous calibration standards were compared to those of 6 batches of blank plasma and 10 patient samples before and after spiking. Ion traces of each analytes mass transition were checked for interferences at the respective retention times.

(ii) **Limit of quantification.** The lower limit of quantification (LLOQ) was defined as the lowest concentration that could be quantified with accuracy and precision ± 20%, as calculated from chromatograms of 6 independent samples.

(iii) **Standard curves.** Curves consisting of eight points were calculated by linear or polynomial regression, each point consisting of independent triplicate measurements. Best fit was selected after exploration of different regression models and weighting factors.

(iv) **Accuracy and precision.** Intra- and interrun accuracy and precision were calculated for the three quality controls with six duplicate measurements each, or with measurements on six experiments done on different days. Accuracy was defined as a percentual deviation from the theoretical concentration by quantifying QC samples on a
freshly prepared calibration curve. Precision was defined as the coefficient of variation

\[ CV = \frac{\text{standard deviation}}{\text{mean of 6 measurements}} \times 100\% \].

(v) Robustness. Variations in analytical conditions were mimicked based on observations of unexpected performance changes during method development: (a) signal intensity and 24 h autosampler stability of extracts (from low and high QC samples in duplicate) reconstituted in aqueous 0.1% FA were compared to those observed in samples reconstituted in water; (b) retention time and signal intensity in medium QC samples (in duplicate) at a column temp of 40°C were compared to those observed at a column temp of 30°C; and (c) signal intensity of medium QC samples (in duplicate) was compared to that observed in medium QC samples that were diluted pre-precipitation (1:9 with water) to see whether dilution improved analyte recovery.

(vi) Matrix effects. Plasma and eluent components in the ionization chamber cause batchspecific ion suppression or enhancement, leading to interpatient and intrapatient signal variability (4, 19). These matrix effects were evaluated in two ways. First, extracts of six batches of blank plasma were injected while analytes were continuously infused into the mass spectrometer. Ion traces were recorded for each compound over the entire runtime. Signal stability at the relevant retention time was visually assessed for each analyte over the six batches of blank plasma. Second, matrix effects were quantified as proposed by Matuszewski et al. (14). In short, chromatograms were recorded of plasma that was spiked pre-extraction, plasma spiked post-extraction and spiked aqueous eluent. In total, six batches of blank plasma were spiked with low and high concentrations of each analyte in duplicate. Recovery (RE) was defined as the relative signal of samples spiked post-extraction vs pre-extraction. Matrix effects (ME) were similarly defined as the relative signal of post-extraction spiked plasma samples vs spiked aqueous samples. Process efficiency (PE) was defined as the product of RE and
ME, i.e. the overall signal of spiked plasma vs an aqueous standard solution. Average values and coefficients of variation of RE, ME and PE were calculated over the six plasma batches.

(vii) Sample stability. Storage conditions and periods were chosen to mimic those at blood collection, during long-term storage of stock solutions and plasma, during freeze-thaw cycles, at table-top during processing and in the autosampler awaiting analysis.

QC samples were tested for stability over time (a) in aqueous stock solution and plasma at -80°C (¼ - 1 - 2 months), (b) in aqueous stock solutions and plasma at 5 and 20 °C and EDTA-decoagulated wholeblood at 5 °C (6 - 18 - 24 - 48 - 144 hr), (c) in extracts at -80, 5 and 20 °C (6 - 18 - 24 - 48 hr) and (d) in extracts after three freeze-thawcycles. Maximum storage periods were estimated, based on an allowed concentration drop of max 10%.

Measurement of plasma levels in a neonatal ECMO-patient. Patients receiving ECMO treatment were included after written parental consent. The study protocol was approved by the Institutional Ethics Committee. We present data from a term neonate with persistent pulmonary hypertension (PPHN) after meconium aspiration.

Antimicrobial treatment was given in accordance with the departmental protocol and included cefotaxime 50 mg/kg b.i.d. and amoxicillin 25 mg/kg q.i.d. for suspected sepsis and a single bolus injection of vancomycin 20 mg/kg in preparation of decannulation. In total, 11 samples were taken extracorporeally from a pre-oxygenator access point during the 84 hr ECMO-run. After this period, the patient was successfully decannulated and transferred to the referring NICU on conventional ventilation. Plasma levels of cefotaxime, deacetylcefotaxime, amoxicillin and vancomycin were simultaneously measured in 50 µL of plasma. Individual pharmacokinetic curves were constructed for CTX, AMX and VAN by fitting measured plasma levels to previously...
reported pharmacokinetic parameters using MW\Pharm software (MW\PHARM 3.58, Medware, The Netherlands). Cefotaxime was modelled on a one-compartment model derived from data in non-ECMO neonates (15) using iterative Bayesian fitting. Amoxicillin was modelled on a one-compartment model derived from non-ECMO neonates (18) using iterative Bayesian fitting. Vancomycin was modelled on a two-compartment model derived from ECMO-neonates (1) using non-Bayesian fitting.
Results

Selectivity was achieved by the independent separation mechanisms of chromatography and tandem mass spectrometry. None of the aqueous standards, plasma standards and spiked patient samples contained interfering components. Representative ion traces can be seen in fig 1. A minor interference in the CTX-iontrace was probably caused by $(m/z +1)$ isotopes of CFZ, but CTX and the interference were chromatographically separated with retention times of 1.67 vs 1.74 min respectively. An alternate mass transition for CFZ and CTX was tried but rejected based on loss of signal intensity. Standard curves were prepared with a weighting factor of $1/x$. Most of the standard curves were best described via non-linear regression. CFZ’s standard curve could be divided in a linear and a non-linear segment, resulting in a better fit. A linear calibration curve is generally more desirable and can be applied to samples with low to medium concentrations of CFZ. We tested whether high QC samples could be diluted tenfold with blank plasma before extraction. This led to a tenfold drop in concentration while maintaining accuracy and precision (results not shown), but led to a proportional increase in the LLOQ as well. Coefficients of determination ($R^2$) varied from 0.994 (VAN) up to 0.998 (CTX & DACT). See table 1 for the dynamic range of each analyte. The low end of the dynamic range was considered to be the LLOQ; accuracy and precision for all analytes were within $100 \pm 20\%$ and CV $< 20\%$, respectively. See table 2 for intrarun accuracy and precision. Interrun accuracy and precision were similar, with accuracy and precision of within $100 \pm 15\%$ and CV $< 15\%$ for all analytes.

Robustness. Reconstitution with water instead of 0.1% FA improved signal intensity of CFZ (+8%), DACT (+10%) and CRO (+300%); water had no effect on signal intensity of the other analytes. 24h degradation (autosampler, 5°C) however was considerably
worse in water for MEM (-67 vs -40%), AMX (-50 vs -7%), CTX (-45 vs -19%) and DACT (-38 vs -20%). A decrease in column temperature to 30°C led to longer retention times without a deterioration of signal intensity, peak shape and resolution. Tenfold sample dilution led to a correspondingly decreased signal intensity for all analytes except VAN; dilution of samples containing VAN likely improved sample clean-up resulting in relatively high signal intensity.

**Matrix effects.** Visual inspection of chromatograms of plasma injected during T-infusion revealed a signal loss of roughly 30-50% due to matrix components. Inter-plasmasample variability appeared small and there were no sudden signal losses or peaks around the respective retention times. ME, RE and PE were similar for low and high QC-samples, see fig. 2 for the high QC samples. PE varied between 20% (VAN) and 75% (CRO) with notable ME and RE for each analyte. CRO was the only analyte with ion enhancement due to matrix components, as opposed to the ion suppression seen with the other analytes. ME and RE coefficients of variation over the six different plasma batches were under 10% for each analyte.

**Sample stability.** Analytes were stable for at least 2 months in water and plasma at -80°C. Maximum storage periods of aqueous solutions, plasma, wholeblood and extracts (table 3) are based on a maximum degradation of 10%. After three freeze-thawcycles (n=2 for both low and high QC concentrations), average remaining concentrations in extracts were at least 90% of the initial concentration, except for MEM (79%).

**Measurement of plasma levels in a neonatal ECMO-patient.** Plasma levels of CTX, DACT, AMX and VAN were measured simultaneously in the 50 µL plasma samples and successfully fitted to the previously reported models. Fig. 3 contains individual concentration-time curves and measured concentrations. Individual parameters were as follows: elimination rate constant \((k_e) = 0.197 \text{ hr}^{-1}\) and volume of distribution \((V_d) = \)
0.912 L/kg for CTX; $k_d = 0.330 \text{ hr}^{-1}$ and $V_d = 0.713 \text{ L/kg}$ for AMX. Clearance = 7.63 L/hr/1.85m$^2$, $V_1 = 1.03 \text{ L/kg}$, $k_{12} = 1.5 \text{ hr}^{-1}$, $k_{21} = 2.634 \text{ hr}^{-1}$ for VAN.
Discussion

We report the development of a fast UPLC-MS/MS method with an analytical performance meeting FDA specifications. The required plasma volume is 50 µL, which is sufficient to allow reinjection. If the latter is not deemed necessary, the sample volume could probably even be reduced to 20 µL.

This method can be expanded for the quantification of other β-lactam antibiotics by scanning additional mass transitions. We have for instance tested ceftazidime and cefuroxime with sufficient retention, signal intensity and peak shape. From the presented group of antibiotics, only CRO had a potentially problematic peakshape, but accuracy and precision were adequate. We tested an adjusted gradient, but were unsuccessful in removing CRO tailing while maintaining resolution between CTX and the CFZ-isotope peak.

Limited availability of a reference standard complicated a full validation with respect to DACT-quantitation. Considering the structural, chromatographic and mass spectrometric similarities between DACT and CTX, we assumed that their analytical performances would be similar and limited the validation procedure to the critical aspects of matrix effects and sample stability.

Initially, this method was designed for beta-lactam antibiotics only. Vancomycin was later added with a reduced validation procedure to simultaneously quantify commonly used combinations of antibiotics. Since vancomycin stability has been demonstrated (13), we did not include stability testing.

Many reported LC-MS methods contain an elaborate clean-up procedure such as liquid-liquid extraction (LLE) or solid-phase extraction (SPE) to provide sufficient response without interfering matrix effects. Our method shows that a simple protein precipitation
with acetonitrile in combination with the narrow peakshape provided by UPLC-MS/MS can be used to quantify antibiotics with acceptable accuracy and precision, despite the presence of matrix effects. At least 80% of most analytes was recovered after protein precipitation. This could imply that either a small fraction of analyte was not displaced from protein binding sites, or that the analytes do not readily dissolve into the high-organic solvent. Despite incomplete recovery and matrix effects, method accuracy and precision comply with pre-defined specifications.

The limited stability of certain β-lactam antibiotics potentially limits analysis of large sample runs for the least stable antibiotics meropenem and ceftriaxone. Total analysis time is however less than 4 min per sample, which allows large sample runs for meropenem and ceftriaxone as well, provided that samples are processed and analyzed without delay.

We measured cefotaxime, deacetylcefotaxime, amoxicillin and vancomycin simultaneously in 50 µL samples taken from an ECMO-treated neonate. Compared to non-ECMO-treated neonates (15), cefotaxime showed similar clearance with a twofold increase in volume of distribution, which can be explained by the added volume of the ECMO-circuit and oedema. Amoxicillin clearance did not differ from the clearance found in non-ECMO-treated neonates (18); volume of distribution was also slightly increased but this may have been underestimated because of the few amoxicillin concentrations available from this particular patient directly following injection. Vancomycin clearance and volume of distribution were similar to those reported previously in ECMO-treated neonates (1), although clearance is higher in our patient.

With pharmacokinetic software, we were able to construct concentration-time curves and calculate individual pharmacokinetic parameters for this neonate using existing models. The high sampling frequency during classic pharmacokinetic studies can be
problematic in neonates. We expect to be able to compute population pharmacokinetic parameters for this specific population using sophisticated non-linear mixed effects modelling (NONMEM) software, combining sparse and randomly sampled concentration data from multiple patients. This complements the microanalysis method, making maximum use of as little and as few sample(s) as possible.

This UPLC-MS/MS method for quantification of amoxicillin, meropenem, cefazolin, cefotaxime, deacetylcefotaxime, ceftriaxone and vancomycin in 50 μL of plasma provides reliable concentration data. In combination with sophisticated pharmacokinetic modelling software, this enables efficient pharmacokinetic studies in neonates.
References


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4. **Careri, M., and A. Mangia.** 2006. Validation and qualification: the fitness for purpose of mass spectrometry-based analytical methods and analytical systems. Anal Bioanal Chem **386:**38-45.


<table>
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<th>Q1 (m/z)</th>
<th>Q3 (m/z)</th>
<th>CV (V)</th>
<th>CE (eV)</th>
<th>Dynamic Range (mg/L)</th>
<th>Linear Rt (min)</th>
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<td><strong>Glycopeptide</strong></td>
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<tr>
<td>VAN</td>
<td>725.3</td>
<td>143.8</td>
<td>25</td>
<td>25</td>
<td>0.7 - 70</td>
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<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>AMX</td>
<td>366.1</td>
<td>349.1</td>
<td>18</td>
<td>10</td>
<td>0.2 - 80</td>
<td>y</td>
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<td>CFZ</td>
<td>455.0</td>
<td>323.0</td>
<td>20</td>
<td>10</td>
<td>0.5 - 25</td>
<td>y</td>
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<tr>
<td>CRO</td>
<td>554.9</td>
<td>396.0</td>
<td>20</td>
<td>15</td>
<td>2 - 360</td>
<td>n</td>
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<tr>
<td>CTX</td>
<td>456.0</td>
<td>324.0</td>
<td>30</td>
<td>12</td>
<td>0.2 - 100</td>
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<td>MEM</td>
<td>384.2</td>
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<td>0.2 - 80</td>
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<tr>
<td>OXA</td>
<td>402.0</td>
<td>243.1</td>
<td>20</td>
<td>15</td>
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\^ a Q1, parent-ionmass; Q3, daughter-ionmass; CV, cone voltage; CE, collision energy
Table 2  Intrarun accuracy and precision (n = 6 for each concentration)

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<th>Precision</th>
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<tr>
<td></td>
<td>L</td>
<td>M</td>
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<tr>
<td>VAN</td>
<td>95.3%</td>
<td>103.6%</td>
</tr>
<tr>
<td>AMX</td>
<td>107.4%</td>
<td>101.2%</td>
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<td>104.8%</td>
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<td>CRO</td>
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<tr>
<td>CTX</td>
<td>107.7%</td>
<td>95.3%</td>
</tr>
<tr>
<td>DACT</td>
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<td>-</td>
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<tr>
<td>MEM</td>
<td>99.3%</td>
<td>93.0%</td>
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a not determined, assumed to be similar to CTX
Table 3  Maximum in-process and autosampler storage period (in hr)

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<tr>
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<th>stock</th>
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<th>wholeblood</th>
<th>extract</th>
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<tr>
<td></td>
<td>$5^\circ$C $^a$</td>
<td>$20^\circ$C $^b$</td>
<td>$5^\circ$C $^b$</td>
<td>$-80^\circ$C $^b$</td>
</tr>
<tr>
<td></td>
<td>(n=3)</td>
<td>(n=3)</td>
<td>(n=2)</td>
<td>(n=2)</td>
</tr>
<tr>
<td>AMX</td>
<td>144</td>
<td>48</td>
<td>24</td>
<td>48</td>
</tr>
<tr>
<td>CFZ</td>
<td>144</td>
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<td>OXA</td>
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$^a$ Maximum testing period was 144 hr

$^b$ Maximum testing period was 48 hr

$^c$ Corrected for CTX-degradation
Fig. 1 Representative chromatogram of a mixture of analytes in plasma with individual ion traces for each analyte. Most analytes have good peak shapes, with the exception of CTX (which has a peak at the retention time of CFZ) and CRO (which shows some tailing).

Fig. 2 Matrix effects (A), recovery (B) and overall process efficiency (C) of analytes in high QC samples. Values are averages with their corresponding 95%-confidence intervals. For vancomycin, only process efficiency was tested.

Fig. 3 Measured concentrations (open circles) and individually fitted curves (lines) for CTX (A), AMX (B) and VAN (C) in an ECMO-treated neonate. Deacetylcefotaxime-concentrations (closed circles) were not fitted.