Development of a novel multi-penicillin assay and assessment of the impact of analyte degradation: lessons for scavenged sampling in antimicrobial pharmacokinetic study design

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Running Head: Challenges of beta-lactam scavenged sampling.

Abstract

Penicillins are widely used to treat infections in children, however the evidence is continuing to evolve in defining optimal dosing. Modern paediatric pharmacokinetic study protocols frequently favour opportunistic, “scavenged” sampling. This study aimed to develop a small volume single assay for five major penicillins and to assess the influence of sample degradation on inferences made using pharmacokinetic modelling, to investigate the suitability of scavenged sampling strategies.
Using a rapid ultra-high performance liquid chromatographic-tandem mass spectrometric method, an assay for five penicillins (amoxicillin, ampicillin, benzylpenicillin, piperacillin and flucloxacillin) in blood plasma was developed and validated. Penicillin stabilities were evaluated under different conditions. Using these data, the impact of drug degradation on inferences made during pharmacokinetic modelling was evaluated.

All evaluated penicillins indicated good stability at room temperature (23 ± 2°C) over 1 hour remaining in the range of 98-103% of the original concentration. More rapid analyte degradation had already occurred after 4 hours with stability ranging from 68% to 99%. Stability over longer periods declined: degradation of up to 60% was observed with delayed sample processing of up to 24 hours. Modelling showed that analyte degradation can lead to a 30% and 28% bias in clearance and volume of distribution, respectively, and falsely show nonlinearity in clearance.

Five common penicillins can now be measured in a single low volume blood sample. Beta-lactam chemical instability in plasma can cause misleading pharmacokinetic modelling results, which could impact upon model-based dosing recommendations and the forthcoming era of beta-lactam therapeutic drug monitoring.

**Introduction**

Penicillins have been widely used in both children(1) and adults for over 50 years and arguably remain the most important group of antibiotics. There is still considerable variation in the rate of antimicrobial prescribing in different countries(2) and also the doses used.(3) Even for very common antibiotics, such as the penicillins, there remains a marked lack of information about optimal dosing.(4) Pharmacokinetic (PK) variability in children can arise from the physiological changes related to growth and organ maturation, and also due to pathophysiology, especially in
critical illness. Selecting the best antimicrobial dose to use in children is challenging because of this extensive variability in patients’ pharmacokinetics. As a result, a drug’s PK profile can be unpredictable, which in the case of antibiotics can lead to subtherapeutic concentrations – with associated treatment failure, and the possible emergence of antimicrobial resistance (AMR). Identifying the optimum dosing regimens is thus key to improving therapeutic outcomes, reducing toxicity and in limiting the development of AMR. Given the wide use of beta-lactam antibiotics in clinical practice, the knowledge of PK variability has led researchers to question whether there is a potential role for beta-lactam therapeutic drug monitoring (TDM) to help individualise dosing strategies in an intensive care unit (ICU) setting.

Penicillins are unstable β-lactam antimicrobials and spontaneous degradation of these drugs occurs. Diverse degradation pathways caused by the β-lactam group are common to all penicillins, yet they vary with storage conditions resulting in different degradation products. One of the main challenges when determining the pharmacokinetics of these drugs is how to maintain their stability in biological samples under various storage conditions. The instability of penicillins, which is already well established, can result from the β-lactam ring opening in acidic and basic conditions, enzymatic (hydrolysis and aminolysis) degradation and degradation by the presence of metal ions and from temperature changes. Therefore, detailed stability (ST%) studies during bioanalytical assay validation are crucial. The most important stability from the clinical study perspective is the short term stability, taking into account possible delays and interferences during the sampling and sample handling in the hospital (both at the bedside and also when transporting the sample from the patient to the laboratory).

Importantly, opportunistic or so-called “scavenged” sampling techniques are being used increasingly in paediatric PK studies as a sparse sampling methodology. These approaches
mean that the blood samples from patients are initially processed in the same way as routine blood samples required for clinical care, before being processed and stored specifically for antibiotic quantification, the results of which are then used for pharmacokinetic analysis. The use of scavenged sampling itself, which incorporates processing delays into the standard operating procedures, thus might influence the PK analysis results in the case of chemically unstable drugs. Understanding the impact of such delays on both laboratory and pharmacometric analysis is particularly important as opportunistic, scavenged sampling methods are now being further advocated specifically for neonatal pharmacokinetic studies (13).

Numerous simultaneous bioanalytical methods have been developed over the years for beta-lactam antibiotics (14-22), with evidence of increased interest recently (16, 18, 20). The number of simultaneously determined drugs varies (maximum 21 (17)) and the required sample volumes range from as little as 20 µL(21) and 50 µL (14, 16, 18, 19, 22) up to 1 mL(17).

The aim of this laboratory study was to develop and validate a bioanalytical method for measuring penicillins in small-volume plasma samples from paediatric and neonatal patients, in order to use the data for population PK modelling and dose optimisation studies.(2, 23) The influence of sample degradation on measured concentrations was then studied using pharmacokinetic modelling in order to evaluate the suitability of scavenged sampling strategies for the penicillins as chemically unstable drugs. The penicillins studied included amoxicillin, ampicillin, benzylpenicillin (penicillin G), piperacillin and flucloxacillin (Figure 1). The bioanalytical methods were developed for the Neonatal and Paediatric Pharmacokinetics of Antimicrobials study (NAPPA: EudraCT 2013-002366-40, NCT01975493).(24)

***Figure 1 goes here***
Results

**LC-MS method development**

The LC-MS assay was developed to simultaneously quantify concentrations of 5 major penicillins in blood plasma. Chromatographic separation was achieved within 4 minutes for all analytes (representative chromatogram from the sample with concentration 25 mg/L, Figure 2): amoxicillin, ampicillin, benzylpenicillin, piperacillin and flucloxacillin.

***Figure 2 goes here***

**Method validation and selectivity**

The method was fully validated according the European Medicines Agency (2011) Guideline on bioanalytical method validation(25). Selectivity was evaluated analysing blood plasma samples from six different sources.

**Lower limit of quantification (LLOQ)**

For all analytes the LLOQ was 0.1 mg/L. The within-day accuracy for amoxicillin ranged from 98-106%, for ampicillin from 105-110%, for penicillin G from 105-108%, for piperacillin from 97-106% and for flucloxacillin from 96-105% at the LLOQ level. Within-day precision for amoxicillin ranged from 2.5-5.4%, for ampicillin from 2-3.5%, for penicillin G from 4.6-10.6%, for piperacillin from 2.9-4.2% and for flucloxacillin from 4.3-11.7% at the LLOQ level.

Signal to noise ratio remained higher than 5 for all analytes, ranging from 89 to 251.

**Calibration, carry-over, matrix effects, accuracy and precision**

Nine calibration concentration levels (0.1, 0.5, 1, 5, 25, 60, 100, 150 and 200 mg/L) were used to compose the matrix matched calibration lines, in addition to the calibrators the blank sample (processed using internal standard) and the double blank sample (processed without internal
standard) were analyzed. All samples were analysed in duplicates. Back calculated concentrations using linear regression fitting with $1/x^2$ weighting (Table 1) ranged from 96-110% for all analytes in all concentration levels from 0.1 mg/L to 200 mg/L.

***Table 1 goes here***

Carry-over (presented in supplementary data file) was considered acceptable for all analytes and the IS. Matrix effects (presented in supplementary data file) and the calibration (sTable 1) were evaluated for all analytes. Matrix effects ranged from 96 to 107.6 % for all penicillins. Accuracy and precision was tested in four different concentrations (in 5 replicates): 0.1 mg/L (as LLOQ), 0.5 mg/L as low concentration, 50 mg/L as medium concentration and 150 mg/L as high concentration.

Within-run and between-run assay accuracies for all analytes ranged from 1.4% to 10.5% at LLOQ concentration and from 0.3% to 8.8% for the low, medium and high concentrations, respectively (Table 2 and 3). Within-run assay precisions for all analytes ranged from 2.0 to 6.1% at LLOQ concentration and from 2.0% to 5.4% for low, medium and high concentrations (Table 2). Between-run assay precision for all analytes ranged from 1.6 to 7.3% at LLOQ concentration and from 1.3% to 7.4% for low, medium and high concentrations (Table 3).

***Table 2 goes here***

***Table 3 goes here***

**Stability**

*Short term stability of analytes in blood plasma at room temperature.*
Short term stability data of the penicillin-containing plasma samples when stored at room temperature (23 ± 2°C) for 24 h indicated degradation of flucloxacillin, piperacillin and penicillin G in the plasma samples (at low, medium and high concentrations), since only 40-63%, 52-64% and 66-70%, respectively, of the drug was detectable after applying room temperature as a stress condition. Ampicillin and amoxicillin however had slightly better stability on the bench-top, with 89-96% and 71-89%, respectively, of the drugs detectable after 24 h at room temperature.

In addition, short term stability was tested over a 4 h period at room temperature, which also indicated compound degradation: flucloxacillin, piperacillin and penicillin G maintained 68-80%, 83-89% and 89-95% of their original concentration. Ampicillin and amoxicillin both remained in the range of 95-98% and 96-99% after 4 h at the room temperature.

All penicillins indicated good stability at room temperature over 1 h remaining in the range of 98-103% from the original concentration.

**Autosampler stability**

Rapid degradation of ampicillin occurred in the samples kept in the cooled (±10 °C) autosampler for 24 h: only 35-57% of the original drug concentration remained in the samples. However, all other penicillins maintained 85-99% of their original content. In order to improve the autosampler stability, rapid analysis of beta-lactams is required. Therefore, ampicillin stability was tested over shorter time-periods. Within a 10-hour period, approximately 92-96% of ampicillin original concentration remained in the samples stored in the autosampler in five replicates.

***Figure 3 goes here***

**Freeze and thaw stability**
The freeze-thaw stability of ampicillin also indicated the degradation of the compound in plasma samples, since 82-99% of the original content remained to the plasma samples after 3 freeze-thaw cycles, while the other penicillins maintained approximately 98-100% of their original content in the plasma samples.

**Long term stability**

Long term stability of analytes in the plasma samples was evaluated over 6 month time-period at -80°C. All penicillins remained within the range of 95-104% of their original content at low, medium and high concentrations (tested in five aliquots of the same sample, i.e. n=5).
**PK modelling and simulations results**

Table 4 gives the estimated piperacillin CL at different dose levels assuming varying levels of degradation using the doses reported by Landersdorfer et al(26) (3000 mg and 1500 mg), the current usual dose (4000 mg) and 400 mg for illustration of a 10-fold range.

***Table 4 goes here***

**Discussion**

A simultaneous method for measuring amoxicillin, ampicillin, benzylpenicillin, piperacillin and flucloxacillin was developed. To the best of our knowledge, the combination of these five penicillins using sample volumes as low as 50 µL in a single LC-MS assay has not previously been reported, although other simultaneous beta-lactam LC-MS assays have been described(15, 17, 21, 22). As the associated clinical study (NAPPA) was investigating penicillin pharmacokinetics in neonatal and paediatric patients, the main challenge of the method development was the restricted sample volume in this patient population. Knowing the stability issues of beta-lactam antibiotics, sample preparation was done rapidly using protein precipitation and dilution afterwards to avoid unnecessary contamination of the LC-MS system from the high content of sample matrix. Using data from the stability studies during method development, the impact of analyte degradation was assessed, and we have shown, using piperacillin as an example drug, that inferences made during PK modelling may be biased if analytes have degraded within clinical samples. As scavenged sampling methods are increasingly recommended for neonatal pharmacokinetic studies, these findings are of clear significance and serve as an important reminder of tailoring scavenged sampling protocols in future paediatric pharmacokinetic studies according to each specific analyte’s stability. Furthermore, given the growing interest in the use of
TDM for beta lactams(6) to optimize pharmacotherapy in clinical practice these findings have further implications for TDM sampling protocols.

Rapid and simultaneous multi-drug assays are the key for successful TDM services which need to be delivered in a timely manner.(22) This novel assay, which has both good accuracy and good precision, also benefits from rapid sample preparation and decreased matrix effects compared to previous assays, (15) even when more complex and cleaner sample preparation was used. The lower matrix effects obtained were most likely due to the appropriate dilution during sample preparation.

One of the key validation parameters for beta-lactams is stability, both short- and long-term. Despite previous recognition of both the instability of beta-lactams at room temperature and the importance of pre-analytical stability(27), there is a persisting lack of stability data in published assay validations.(17, 22) Our results indicated more rapid degradation in plasma samples with EDTA for piperacillin and amoxicillin than reported in the literature previously for lithium-heparinized tubes and tubes without the gel separator.(27) Another study(15) indicated slightly better stability over 4 h at room temperature for penicillin G, piperacillin and flucloxacillin without mentioning the anticoagulant used for plasma. Importantly, beyond assay validation, we then sought to interrogate the chemical stability data to understand in a quantifiable way its potential impact on the output from pharmacokinetic modelling, which when used for model-based dosing recommendations could have direct clinical implications in future.

From the results of the pharmacokinetic modelling, it can be seen that, in the absence of sample degradation, the estimated CL was close to the simulated value, and whilst CL did increase with increasing processing time, it was not until samples were left for 24 hours that degradation started to falsely show nonlinearity. It should be noted however that the nonlinear CL reported by
Landersdorfer et al (26) included data on urinary piperacillin. Since we did not test piperacillin stability in urine, it is still possible that more rapid urinary piperacillin degradation coupled to longer times between sample collection inherent in urinary pharmacokinetic studies may enhance an apparent nonlinear pharmacokinetic effect.

In the assessment of scavenged sampling, the estimates for CL and V were 11.3 L/h and 12.3 L, when sample degradation was assumed not to have occurred. Whilst for a more complex 2-compartment pharmacokinetic model, scavenged sampling has been shown to bias parameter estimates and should therefore generally not be preferred over optimally timed samples (28), this result indicates that for this simple 1-compartment model the scavenged design could potentially work if samples were processed immediately. It should be noted that truly immediate processing is rarely possible in a clinical environment, however, when it comes to so-called ‘scavenged’ samples. Further, when degradation was allowed by having samples processed between 4 and 24 hour post collection (representing lengths of delays that can occur when samples are scavenged from the laboratory), the CL and V were 14.3 L/h and 15.4 L respectively. This represents a 30% and 28% bias in CL and V which could potentially cause unnecessarily high doses to be recommended on the basis of pharmacometric (PK model-based) analyses. Indeed, the authors of the scavenged sampling study (11) acknowledge this potential problem, and further penicillin studies using scavenged samples require accurate recording of sample processing times so that degradation can be accounted for during pharmacokinetic modelling. Nonetheless, for future beta-lactam TDM methods, we have demonstrated the acceptability of storage for up to one hour at room temperature for plasma samples collected from patients, which is very promising, and this should make the TDM standard operating procedures both realistic and acceptable in the context of a busy clinical setting.
To our knowledge this is the first study to use data obtained during bioanalytical method validation to demonstrate how the use of scavenged sampling methods could impact significantly upon the results of pharmacokinetic modelling. This is relevant when dealing with unstable analytes present in patient samples destined for drug quantification assays, whether these be samples for paediatric pharmacokinetic research using opportunistic sampling strategies, or samples from ICU patients intended for TDM. These findings underscore the importance of detailed evaluation of the stability of beta-lactam antibiotics in specific matrices during the bioanalytical method validation. This is not only important for planning the laboratory workflow, but also should be considered all the way from bedside to bench, with consideration of sample collection methods, transportation and storage. For researchers developing dosing guidance using pharmacokinetic analyses of such data, the instability of these analytes can cause significant bias in the prediction of pharmacokinetic parameters. With the implementation of novel TDM initiatives based on individualized pharmacokinetic profiles and forecasting, this could lead to suboptimal dosing recommendations, which could adversely affect clinical outcomes. However, when carefully evaluated, the instability can be accounted for when creating pharmacokinetic models. This work highlights the importance of continued close collaboration between bioanalytical chemists, pharmacometricians, and clinicians when developing novel TDM protocols. For future work we would recommend that the importance of strict sampling handling procedures be incorporated into all standard operating procedures for beta-lactam TDM methods, to ensure that our knowledge of beta-lactam stability is fully embedded in forthcoming innovative dose individualization strategies. A potentially simple way to overcome these challenges is through the use of point-of-care methods for beta-lactam quantification at the bedside, which if economically viable, would present an ideal solution.
Materials and methods

Instrumentation

Chromatographic separation and mass spectrometric detection of five analytes were carried out using Waters Acquity UPLC (Ultra High Performance Liquid Chromatography) system equipped with Waters TQ Detector (Waters, Milford, USA). The UPLC system consists of a binary solvent manager, a sample manager and a column thermostat. ESI-MS detection was carried out in positive ion detection mode. The UPLC-MS/MS instrument was controlled by Waters MassLynx software version 4.1 (Waters, Milford, USA). Data analysis was carried out using TargetLynx software version 4.1 (Waters, Milford, USA).

Chemicals

Pharmaceuticals – ampicillin, amoxicillin, flucloxacillin, penicillin G, piperacillin and an internal standard (IS), penicillin G-D7 N-ethylpiperidinium salt and LC-MS grade methanol and formic acid were purchased from Sigma (St. Louis, MO, USA). Acetonitrile was obtained from Rathburn Chemicals Ltd (Walkerburn, Scotland). Water was purified (18.2 MΩ*cm at 25 °C and a TOC (total organic carbon) value below 3 ppb) in house using a Millipore Advantage A10 system from Millipore (Bedford, USA). Blood plasma with EDTA was obtained from the Biological Specialties Corporation (Colmar, PA, USA).

Sample preparation

Plasma samples were kept at –80°C for storage. For analysis they were removed from the freezer and kept at room temperature for thawing. Once at room temperature, matrix matched calibrators and quality control samples were mixed on an IKA Vibra VXR mixer (Esslab, Essex, England).
For 50 µL of each calibrator and quality control sample, 200 µL of internal standard (penicillin G-D7) in acetonitrile (stored at -20 ºC, prepared once every two weeks) was added. Samples were mixed on the IKA Vibrax VXR for 5 minutes and centrifuged at 13500 x g for 5 minutes. 1000 µL of de-ionized water was added to 100 µL of supernatant and then the samples were vortexed for 10 seconds. 10 µL of the sample was injected into the LC-MS/MS (liquid chromatography with tandem mass spectrometry) system.

**Liquid chromatography-mass spectrometry**

For the chromatographic separation of penicillins, 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in methanol (mobile phase B) with gradient elution and a reversed phase analytical column (50mm x 2.1mm; 1.7 µm Acquity UPLC BEH C18) was used. Separation was obtained using the gradient program starting from 5% of mobile phase B for the first 1.2 minutes (directed to the waste), mobile phase B content was raised to 100% over 2.3 minutes and kept at 100% for 1 minute, thereafter lowered again to 5% over 0.2 minutes and kept at 5% of mobile phase B for 1.8 minutes. Eluent flow rate was 0.25 mL/min. Electrospray (ESI) interface was in use for the mass-spectrometric detection in the positive multiple reaction monitoring (MRM) mode for detection of penicillins. Triple quadrupole detector transitions m/z 335 [M+1] -> m/z 160; 176 (for penicillin G); m/z 350 [M+1] -> m/z 160; 106 (for ampicillin); m/z 366 [M+1] -> m/z 114; 208 (for amoxicillin); m/z 518 [M+1] -> m/z 143; 160 (for piperacillin); m/z 454 [M+1] -> m/z 160; 295 (for flucloxacillin), and m/z 342 [M+1] -> m/z 160 (for penicillin G-D7, IS) were used for quantification and qualification.
Optimised parameters for ESI and MS were used with capillary voltage of 0.8 kV, cone voltage 31 V, source temperature 120°C, desolvation gas temperature 350°C and desolvation gas flow rate 800 L/h and cone gas flow rate 30 L/h.

**Stability experiments**

Short term stability data of the penicillin-containing plasma samples were obtained by storage of quality control samples (at low, medium and high concentrations) at room temperature (23 ± 2°C) for 4 h and 24 h and comparing the results with the analysis performed at the starting time (time 0 h) of the analysis. The freeze-thaw stability of penicillins was evaluated in low and high concentration plasma samples (each in 5 replicates) after 3 freeze-thaw cycles. Plasma samples were kept frozen at -80°C and thawed, keeping them at the room temperature (23±2°C) for 1 h. Thereafter, samples were refrozen and thawed again after 24 h. Long term stability of analytes in the plasma samples was evaluated for the storage over 6 month time-period at -80°C (each tested in 5 aliquots of the sample plasma sample, i.e. n=5). Autosampler stability at the temperature of +10 °C was evaluated over 24 h.

Each time freshly prepared calibration solutions were measured and the concentration in quality control samples was calculated. All penicillins were present in the same sample over the time of the storage. Stability of the analyte was evaluated as following:

\[
ST\% = \frac{c_0}{c_t},
\]

where \(c_0\) is the initial concentration, determined without introducing any extra pauses in the analysis process; \(c_t\) is the concentration obtained when analysis is carried out with making a pause with duration \(t\) in the analysis.

**Pharmacokinetic modelling and simulations**
To assess the impact of drug degradation on inferences made during pharmacokinetic modelling, data from the stability tests was used. The slope of the log concentration with time was estimated for each initial concentration of each drug using linear regression in the statistical software R version 3.1.0(29). Piperacillin was chosen as the model drug to assess the impact of sample degradation on two aspects of pharmacokinetic modeling results: reported nonlinearity in clearance (CL) and the utility of laboratory scavenged samples.

To investigate whether more rapid sample degradation at lower concentrations could account for observed nonlinear pharmacokinetics, concentration-time data were simulated using NONMEM version 7.3. The 1500 mg and 3000 mg doses described by Landersdorfer et al 2002(26) were used. This study estimated a 3-compartment model using NONMEM version 7.3(30) and found nonlinear clearance with 3000 mg yielding a value of 11.0 L/h whereas 1500 mg yielded a CL of 13.5 L/h. In this study the renal component of CL, estimated by urinary piperacillin excretion, decreased 24% with a doubling of dose. Simulated data were adjusted according to the degradation rate constants estimated above (with linear extrapolations made to account for changing rate constant with concentration) to assume samples were left for 1, 2, 4, 8 and 24 hours post collection before freezing, in keeping with known scavenged sampling protocols. Using this adjusted data, CL was recalculated using noncompartmental pharmacokinetic estimation of AUC$_{(0-\infty)}$ (the area under the plasma drug concentration-time curve). Under the hypothesis of linear CL, the PK profiles for 4000, 1500, and 400 mg were also simulated and the above degradation adjustment made to the simulated concentrations in order to assess whether more rapid degradation of lower concentrations could yield apparent nonlinear CL upon re-estimation.

To investigate the effect of laboratory scavenged samples (any excess from routine clinical samples assayed for piperacillin when reaching the lab), as reported by Cohen-Wolkowiez et
al(11), the above model was simplified to a one compartment structure with CL of 11 L/h and volume of distribution (V) of 12 L. Fifty simulated subjects received 3000 mg 8 hourly over a 32-hour interval (i.e. 4 dose intervals) and 4 random sample times within this interval were simulated from a uniform distribution. The simplified model was used to simulate concentration-time profiles assuming inter-individual variability on CL and V to be 30% and proportional residual variability to be 10%. The simulated concentrations were then adjusted according to the procedure described above to assume degradation. The time of processing for each sample was randomly generated from a uniform distribution with an interval of 4-24 hours. Pharmacokinetic parameters were then estimated from the adjusted and unadjusted datasets to assess potential bias caused by degradation.

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