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1 Development of a novel multi-penicillin assay and assessment of the impact of analyte
2 degradation: lessons for scavenged sampling in antimicrobial pharmacokinetic study design
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15 Running Head: Challenges of beta-lactam scavenged sampling.

16 Abstract

17 Penicillins are widely used to treat infections in children, however the evidence is continuing to
18 evolve in defining optimal dosing. Modern paediatric pharmacokinetic study protocols frequently
19 favour opportunistic, "scavenged" sampling. This study aimed to develop a small volume single
20 assay for five major penicillins and to assess the influence of sample degradation on inferences
21 made using pharmacokinetic modelling, to investigate the suitability of scavenged sampling
22 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. All penicillins were present in the same sample over the time of the storage. 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

46 changes related to growth and organ maturation, and also due to pathophysiology, especially in
47 critical illness. Selecting the best antimicrobial dose to use in children is challenging because of
48 this extensive variability in patients' pharmacokinetics.(5) As a result, a drug's PK profile can be
49 unpredictable,(6) which in the case of antibiotics can lead to subtherapeutic concentrations(7) –
50 with associated treatment failure, and the possible emergence of antimicrobial resistance
51 (AMR).(8) Identifying the optimum dosing regimens is thus key to improving therapeutic
52 outcomes, reducing toxicity(9) and in limiting the development of AMR. Given the wide use of
53 beta-lactam antibiotics in clinical practice, the knowledge of PK variability has led researchers to
54 question whether there is a potential role for beta-lactam therapeutic drug monitoring (TDM) to
55 help individualise dosing strategies in an intensive care unit (ICU) setting.(6)
56 Penicillins are unstable β-lactam antimicrobials and spontaneous degradation of these drugs
57 occurs. Diverse degradation pathways caused by the β-lactam group are common to all penicillins,
58 yet they vary with storage conditions resulting in different degradation products.(10) One of the
59 main challenges when determining the pharmacokinetics of these drugs is how to maintain their
60 stability in biological samples under various storage conditions. The instability of penicillins,
61 which is already well established, can result from the β-lactam ring opening in acidic and basic
62 conditions, enzymatic (hydrolysis and aminolysis) degradation and degradation by the presence of
63 metal ions and from temperature changes(10). Therefore, detailed stability (ST%) studies during
64 bioanalytical assay validation are crucial. The most important stability from the clinical study
65 perspective is the short term stability, taking into account possible delays and interferences during
66 the sampling and sample handling in the hospital (both at the bedside and also when transporting
67 the sample from the patient to the laboratory).

68 Importantly, opportunistic or so-called "scavenged" sampling techniques are being used
69 increasingly in paediatric PK studies as a sparse sampling methodology.(11, 12) These approaches
70 mean that the blood samples from patients are initially processed in the same way as routine blood
71 samples required for clinical care, before being processed and stored specifically for antibiotic
72 quantification, the results of which are then used for pharmacokinetic analysis. The use of
73 scavenged sampling itself, which incorporates processing delays into the standard operating
74 procedures, thus might influence the PK analysis results in the case of chemically unstable drugs.
75 Understanding the impact of such delays on both laboratory and pharmacometric analysis is
76 particularly important as opportunistic, scavenged sampling methods are now being further
77 advocated specifically for neonatal pharmacokinetic studies (13).
78 Numerous simultaneous bioanalytical methods have been developed over the years for beta-lactam
79 antibiotics (14-22), with evidence of increased interest recently (16, 18, 20). The number of
80 simultaneously determined drugs varies (maximum 21 (17)) and the required sample volumes
81 range from as little as 20 µL(21) and 50 µL (14, 16, 18, 19, 22) up to 1 mL(17).
82 The aim of this laboratory study was to develop and validate a bioanalytical method for measuring
83 penicillins in small-volume plasma samples from paediatric and neonatal patients, in order to use
84 the data for population PK modelling and dose optimisation studies.(2, 23) The influence of sample
85 degradation on measured concentrations was then studied using pharmacokinetic modelling in
86 order to evaluate the suitability of scavenged sampling strategies for the penicillins as chemically
87 unstable drugs. The penicillins studied included amoxicillin, ampicillin, benzylpenicillin
88 (penicillin G), piperacillin and flucloxacillin (Figure 1). The bioanalytical methods were developed
89 for the Neonatal and Paediatric Pharmacokinetics of Antimicrobials study (NAPPA: EudraCT
90 2013-002366-40, NCT01975493).(24)

91

92

93 *****Figure 1 goes here*****

94

95 ## Results

96 ***LC-MS method development***

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

101 ***Figure 2 goes here***

102

103 ***Method validation and selectivity***

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

107 ***Lower limit of quantification (LLOQ)***

108 For all analytes the LLOQ was 0.1 mg/L. The within-day accuracy for amoxicillin ranged from
109 98-106%, for ampicillin from 105-110%, for penicillin G from 105-108%, for piperacillin from
110 97-106% and for flucloxacillin from 96-105% at the LLOQ level. Within-day precision for
111 amoxicillin ranged from 2.5-5.4%, for ampicillin from 2-3.5%, for penicillin G from 4.6-10.6%,
112 for piperacillin from 2.9-4.2% and for flucloxacillin from 4.3-11.7% at the LLOQ level.

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

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

115 Nine calibration concentration levels (0.1, 0.5, 1, 5, 25, 60, 100, 150 and 200 mg/L) were used to
116 compose the matrix matched calibration lines, in addition to the calibrators the blank sample
117 (processed using internal standard) and the double blank sample (processed without internal

118 standard) were analyzed. All samples were analysed in duplicates. Back calculated concentrations
119 using linear regression fitting with $1/x^2$ weighting (Table 1) ranged from 96-110% for all analytes
120 in all concentration levels from 0.1 mg/L to 200 mg/L.
121 *****Table 1 goes here*****
122 Carry-over (presented in supplementary data file) was considered acceptable for all analytes and
123 the IS. Matrix effects (presented in supplementary data file) and the calibration (sTable 1) were
124 evaluated for all analytes. Matrix effects ranged from 96 to 107.6 % for all penicillins.
125 Accuracy and precision was tested in four different concentrations (in 5 replicates): 0.1 mg/L (as
126 LLOQ), 0.5 mg/L as low concentration, 50 mg/L as medium concentration and 150 mg/L as high
127 concentration.
128 Within-run and between-run assay accuracies for all analytes ranged from 1.4% to 10.5% at LLOQ
129 concentration and from 0.3% to 8.8% for the low, medium and high concentrations, respectively
130 (Table 2 and 3). Within-run assay precisions for all analytes ranged from 2.0 to 6.1% at LLOQ
131 concentration and from 2.0% to 5.4% for low, medium and high concentrations (Table 2).
132 Between-run assay precision for all analytes ranged from 1.6 to7.3% at LLOQ concentration and
133 from 1.3% to 7.4% for low, medium and high concentrations (Table 3).
134 *****Table 2 goes here*****
135 *****Table 3 goes here*****
136
137
138 ***Stability***
139 ***Short term stability of analytes in blood plasma at room temperature.***

140 Short term stability data of the penicillin-containing plasma samples when stored at room
141 temperature (23 ± 2˚C) for 24 h indicated degradation of flucloxacillin, piperacillin and penicillin
142 G in the plasma samples (at low, medium and high concentrations), since only 40-63%, 52-64%
143 and 66-70%, respectively, of the drug was detectable after applying room temperature as a stress
144 condition. Ampicillin and amoxicillin however had slightly better stability on the bench-top, with
145 89-96% and 71-89%, respectively, of the drugs detectable after 24 h at room temperature.
146 In addition, short term stability was tested over a 4 h period at room temperature, which also
147 indicated compound degradation: flucloxacillin, piperacillin and penicillin G maintained 68-80%,
148 83-89% and 89-95% of their original concentration. Ampicillin and amoxicillin both remained in
149 the range of 95-98% and 96-99% after 4 h at the room temperature.
150 All penicillins indicated good stability at room temperature over 1 h remaining in the range of 98-
151 103% from the original concentration.
152 ***Autosampler stability***
153 Rapid degradation of ampicillin occurred in the samples kept in the cooled (+10 ˚C)
154 autosampler for 24 h: only 35-57% of the original drug concentration remained in the samples.
155 However, all other penicillins maintained 85-99% of their original content. In order to improve the
156 autosampler stability, rapid analysis of beta-lactams is required. Therefore, ampicillin stability was
157 tested over shorter time-periods. Within a 10-hour period, approximately 92-96% of ampicillin
158 original concentration remained in the samples stored in the autosampler in five replicates.
159
160 *****Figure 3 goes here*****
161 ***Freeze and thaw stability***

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

166 ***Long term stability***

167 Long term stability of analytes in the plasma samples was evaluated over 6 month time-period at-
168 80˚C. All penicillins remained within the range of 95-104% of their original content at low,
169 medium and high concentrations (tested in five aliquots of the same sample, i.e. n=5).

170

171 ***PK modelling and simulations results***

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

175 *****Table 4 goes here*****

176

177 Discussion

178 A simultaneous method for measuring amoxicillin, ampicillin, benzylpenicillin, piperacillin and
179 flucloxacillin was developed. To the best of our knowledge, the combination of these five
180 penicillins using sample volumes as low as 50 µL in a single LC-MS assay has not previously been
181 reported, although other simultaneous beta-lactam LC-MS assays have been described(15, 17, 21,
182 22). As the associated clinical study (NAPPA) was investigating penicillin pharmacokinetics in
183 neonatal and paediatric patients, the main challenge of the method development was the restricted
184 sample volume in this patient population. Knowing the stability issues of beta-lactam antibiotics,
185 sample preparation was done rapidly using protein precipitation and dilution afterwards to avoid
186 unnecessary contamination of the LC-MS system from the high content of sample matrix.

187 Using data from the stability studies during method development, the impact of analyte
188 degradation was assessed, and we have shown, using piperacillin as an example drug, that
189 inferences made during PK modelling may be biased if analytes have degraded within clinical
190 samples. As scavenged sampling methods are increasingly recommended for neonatal
191 pharmacokinetic studies, these findings are of clear significance and serve as an important
192 reminder of tailoring scavenged sampling protocols in future paediatric pharmacokinetic studies
193 according to each specific analyte's stability. Furthermore, given the growing interest in the use of

194 TDM for beta lactams(6) to optimize pharmacotherapy in clinical practice these findings have
195 further implications for TDM sampling protocols.
196 Rapid and simultaneous multi-drug assays are the key for successful TDM services which need to
197 be delivered in a timely manner.(22) This novel assay, which has both good accuracy and good
198 precision, also benefits from rapid sample preparation and decreased matrix effects compared to
199 previous assays, (15) even when more complex and cleaner sample preparation was used. The
200 lower matrix effects obtained were most likely due to the appropriate dilution during sample
201 preparation.
202 One of the key validation parameters for beta-lactams is stability, both short- and long-term.
203 Despite previous recognition of both the instability of beta-lactams at room temperature and the
204 importance of pre-analytical stability(27), there is a persisting lack of stability data in published
205 assay validations.(17, 22) Our results indicated more rapid degradation in plasma samples with
206 EDTA for piperacillin and amoxicillin than reported in the literature previously for lithium-
207 heparinized tubes and tubes without the gel separator.(27) Another study(15) indicated slightly
208 better stability over 4 h at room temperature for penicillin G, piperacillin and flucloxacillin without
209 mentioning the anticoagulant used for plasma. Importantly, beyond assay validation, we then
210 sought to interrogate the chemical stability data to understand in a quantifiable way its potential
211 impact on the output from pharmacokinetic modelling, which when used for model-based dosing
212 recommendations could have direct clinical implications in future.
213 From the results of the pharmacokinetic modelling, it can be seen that, in the absence of sample
214 degradation, the estimated CL was close to the simulated value, and whilst CL did increase with
215 increasing processing time, it was not until samples were left for 24 hours that degradation started
216 to falsely show nonlinearity. It should be noted however that the nonlinear CL reported by

217 Landersdorfer et al(26) included data on urinary piperacillin. Since we did not test piperacillin
218 stability in urine, it is still possible that more rapid urinary piperacillin degradation coupled to
219 longer times between sample collection inherent in urinary pharmacokinetic studies may enhance
220 an apparent nonlinear pharmacokinetic effect.

221 In the assessment of scavenged sampling, the estimates for CL and V were 11.3 L/h and 12.3 L,
222 when sample degradation was assumed not to have occurred. Whilst for a more complex 2-
223 compartment pharmacokinetic model, scavenged sampling has been shown to bias parameter
224 estimates and should therefore generally not be preferred over optimally timed samples(28), this
225 result indicates that for this simple 1-compartment model the scavenged design could potentially
226 work if samples were processed immediately. It should be noted that truly immediate processing
227 is rarely possible in a clinical environment, however, when it comes to so-called 'scavenged'
228 samples. Further, when degradation was allowed by having samples processed between 4 and 24
229 hour post collection (representing lengths of delays that can occur when samples are scavenged
230 from the laboratory), the CL and V were 14.3 L/h and 15.4 L respectively. This represents a 30%
231 and 28% bias in CL and V which could potentially cause unnecessarily high doses to be
232 recommended on the basis of pharmacometric (PK model-based) analyses. Indeed, the authors of
233 the scavenged sampling study(11) acknowledge this potential problem, and further penicillin
234 studies using scavenged samples require accurate recording of sample processing times so that
235 degradation can be accounted for during pharmacokinetic modelling. Nonetheless, for future beta-
236 lactam TDM methods, we have demonstrated the acceptability of storage for up to one hour at
237 room temperature for plasma samples collected from patients, which is very promising, and this
238 should make the TDM standard operating procedures both realistic and acceptable in the context
239 of a busy clinical setting.

240 To our knowledge this is the first study to use data obtained during bioanalytical method validation
241 to demonstrate how the use of scavenged sampling methods could impact significantly upon the
242 results of pharmacokinetic modelling. This is relevant when dealing with unstable analytes present
243 in patient samples destined for drug quantification assays, whether these be samples for paediatric
244 pharmacokinetic research using opportunistic sampling strategies, or samples from ICU patients
245 intended for TDM. These findings underscore the importance of detailed evaluation of the stability
246 of beta-lactam antibiotics in specific matrices during the bioanalytical method validation. This is
247 not only important for planning the laboratory workflow, but also should be considered all the way
248 from bedside to bench, with consideration of sample collection methods, transportation and
249 storage. For researchers developing dosing guidance using pharmacokinetic analyses of such data,
250 the instability of these analytes can cause significant bias in the prediction of pharmacokinetic
251 parameters. With the implementation of novel TDM initiatives based on individualized
252 pharmacokinetic profiles and forecasting, this could lead to suboptimal dosing recommendations,
253 which could adversely affect clinical outcomes. However, when carefully evaluated, the instability
254 can be accounted for when creating pharmacokinetic models. This work highlights the importance
255 of continued close collaboration between bioanalytical chemists, pharmacometricians, and
256 clinicians when developing novel TDM protocols. For future work we would recommend that the
257 importance of strict sampling handling procedures be incorporated into all standard operating
258 procedures for beta-lactam TDM methods, to ensure that our knowledge of beta-lactam stability is
259 fully embedded in forthcoming innovative dose individualization strategies. A potentially simple
260 way to overcome these challenges is through the use of point-of-care methods for beta-lactam
261 quantification at the bedside, which if economically viable, would present an ideal solution.

262

263 # Materials and methods

264 ***Instrumentation***

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

272

273 ***Chemicals***

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

281

282 ***Sample preparation***

283 Plasma samples were kept at –80°C for storage. For analysis they were removed from the freezer
284 and kept at room temperature for thawing. Once at room temperature, matrix matched calibrators
285 and quality control samples were mixed on an IKA Vibrax 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 C, 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.

307 Optimised parameters for ESI and MS were used with capillary voltage of 0.8 kV, cone voltage
308 31 V, source temperature 120°C, desolvation gas temperature 350°C and desolvation gas flow rate
309 800 L/h and cone gas flow rate 30 L/h.

310 ***Stability experiments***

311 Short term stability data of the penicillin-containing plasma samples were obtained by storage of
312 quality control samples (at low, medium and high concentrations) at room temperature (23 ± 2˚C)
313 for 4 h and 24 h and comparing the results with the analysis performed at the starting time (time 0
314 h) of the analysis. The freeze-thaw stability of penicillins was evaluated in low and high
315 concentration plasma samples (each in 5 replicates) after 3 freeze-thaw cycles. Plasma samples
316 were kept frozen at -80˚C and thawed, keeping them at the room temperature (23±2 C) for 1 h.
317 Thereafter, samples were refrozen and thawed again after 24 h. Long term stability of analytes in
318 the plasma samples was evaluated for the storage over 6 month time-period at -80˚C (each tested
319 in 5 aliquots of the sample plasma sample, i.e. n=5). Autosampler stability at the temperature of
320 +10 ˚C was evaluated over 24 h.

321 Each time freshly prepared calibration solutions were measured and the concentration in quality
322 control samples was calculated. Stability of the analyte was evaluated as following:

323 $$ST\% = \frac{c_0}{c_t},$$

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

327

328 ***Pharmacokinetic modelling and simulations***

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

335 To investigate whether more rapid sample degradation at lower concentrations could account for
336 observed nonlinear pharmacokinetics, concentration-time data were simulated using NONMEM
337 version 7.3. The 1500 mg and 3000 mg doses described by Landersdorfer *et al* 2002(26) were
338 used. This study estimated a 3-compartment model using NONMEM version 7.3(30) and found
339 nonlinear clearance with 3000 mg yielding a value of 11.0 L/h whereas 1500 mg yielded a CL of
340 13.5 L/h. In this study the renal component of CL, estimated by urinary piperacillin excretion,
341 decreased 24% with a doubling of dose. Simulated data were adjusted according to the degradation
342 rate constants estimated above (with linear extrapolations made to account for changing rate
343 constant with concentration) to assume samples were left for 1, 2, 4, 8 and 24 hours post collection
344 before freezing, in keeping with known scavenged sampling protocols. Using this adjusted data,
345 CL was recalculated using noncompartmental pharmacokinetic estimation of $AUC_{(0-\infty)}$ (the area
346 under the plasma drug concentration-time curve). Under the hypothesis of linear CL, the PK
347 profiles for 4000, 1500, and 400 mg were also simulated and the above degradation adjustment
348 made to the simulated concentrations in order to assess whether more rapid degradation of lower
349 concentrations could yield apparent nonlinear CL upon re-estimation.

350 To investigate the effect of laboratory scavenged samples (any excess from routine clinical
351 samples assayed for piperacillin when reaching the lab), as reported by Cohen-Wolkowiez et

352 al(11), the above model was simplified to a one compartment structure with CL of 11 L/h and
353 volume of distribution (V) of 12 L. Fifty simulated subjects received 3000 mg 8 hourly over a 32-
354 hour interval (i.e. 4 dose intervals) and 4 random sample times within this interval were simulated
355 from a uniform distribution. The simplified model was used to simulate concentration-time profiles
356 assuming inter-individual variability on CL and V to be 30% and proportional residual variability
357 to be 10%. The simulated concentrations were then adjusted according to the procedure described
358 above to assume degradation. The time of processing for each sample was randomly generated
359 from a uniform distribution with an interval of 4-24 hours. Pharmacokinetic parameters were then
360 estimated from the adjusted and unadjusted datasets to assess potential bias caused by degradation.
361

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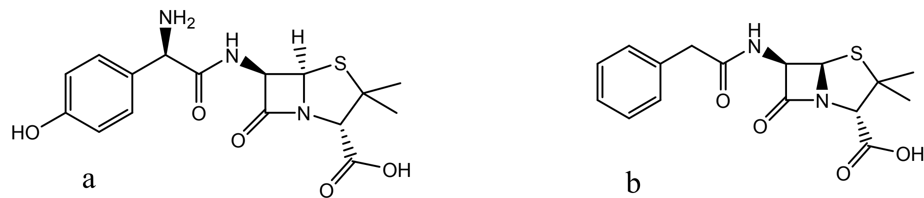

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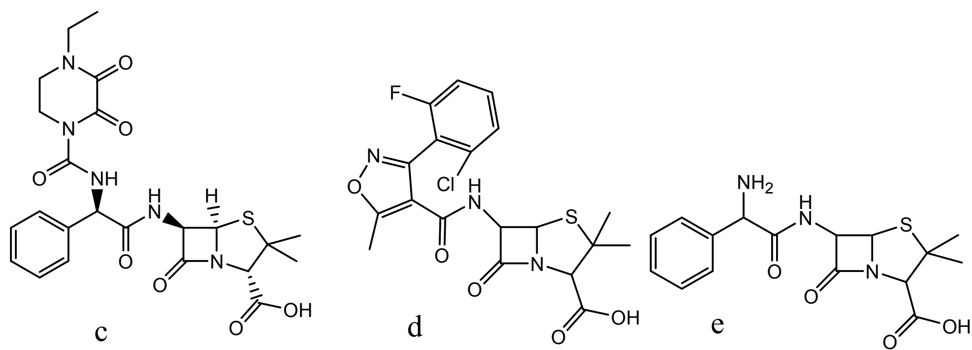

490 **Figure 1.** Chemical structures of amoxicillin (a), penicillin G (b), piperacillin (c), flucloxacillin

491 (d) and ampicillin (e).

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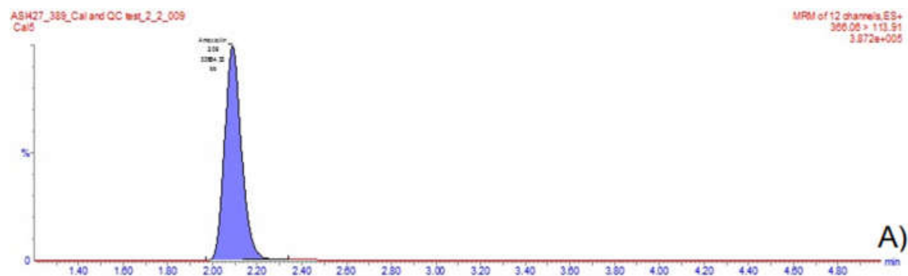
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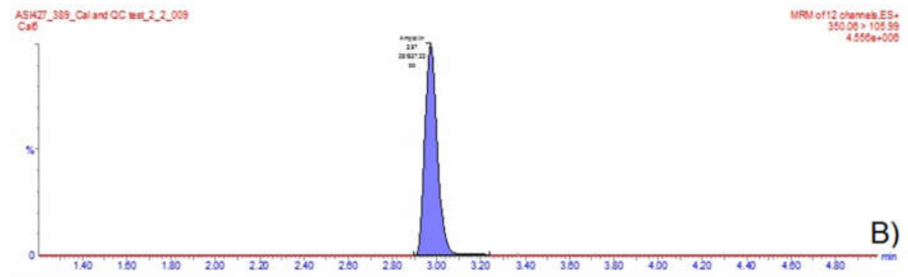
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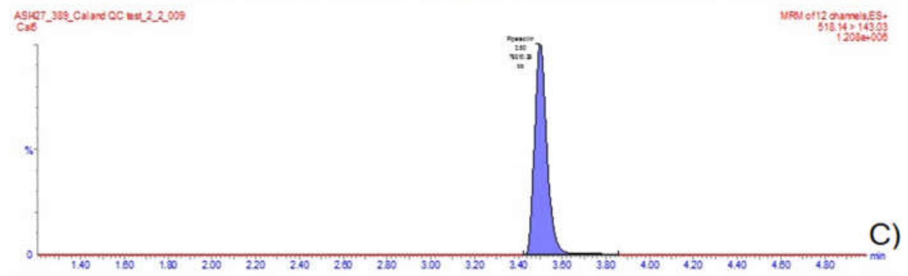
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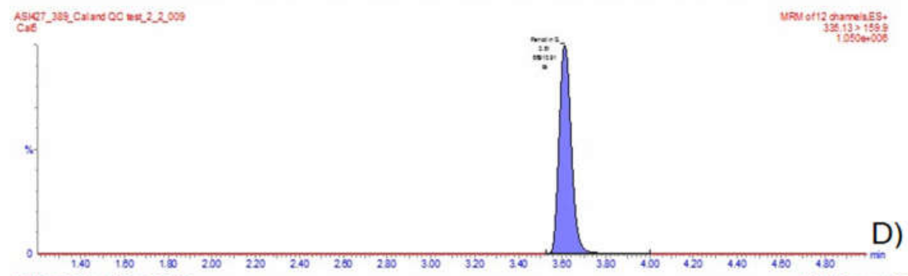
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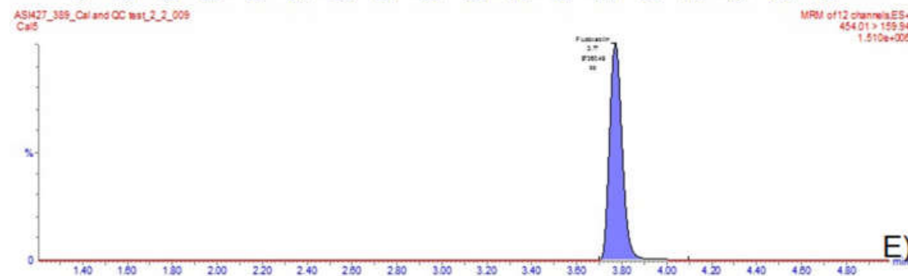
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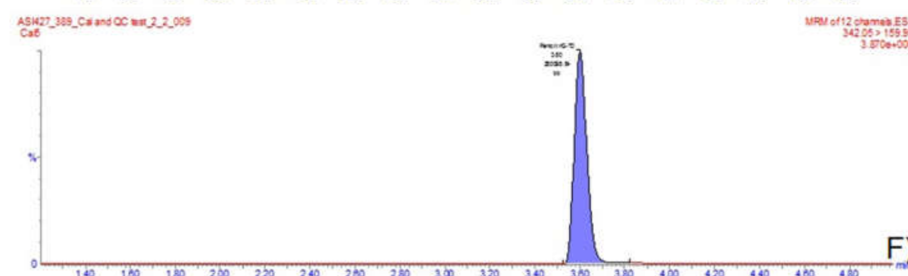
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494 **Figure 2.** Chromatograms in the plasma sample with concentration 25 mg/L for amoxicillin (A),
495 ampicillin (B), piperacillin (C), penicillin G (D), flucloxacillin (E) and penicillin G-D7, IS (F).
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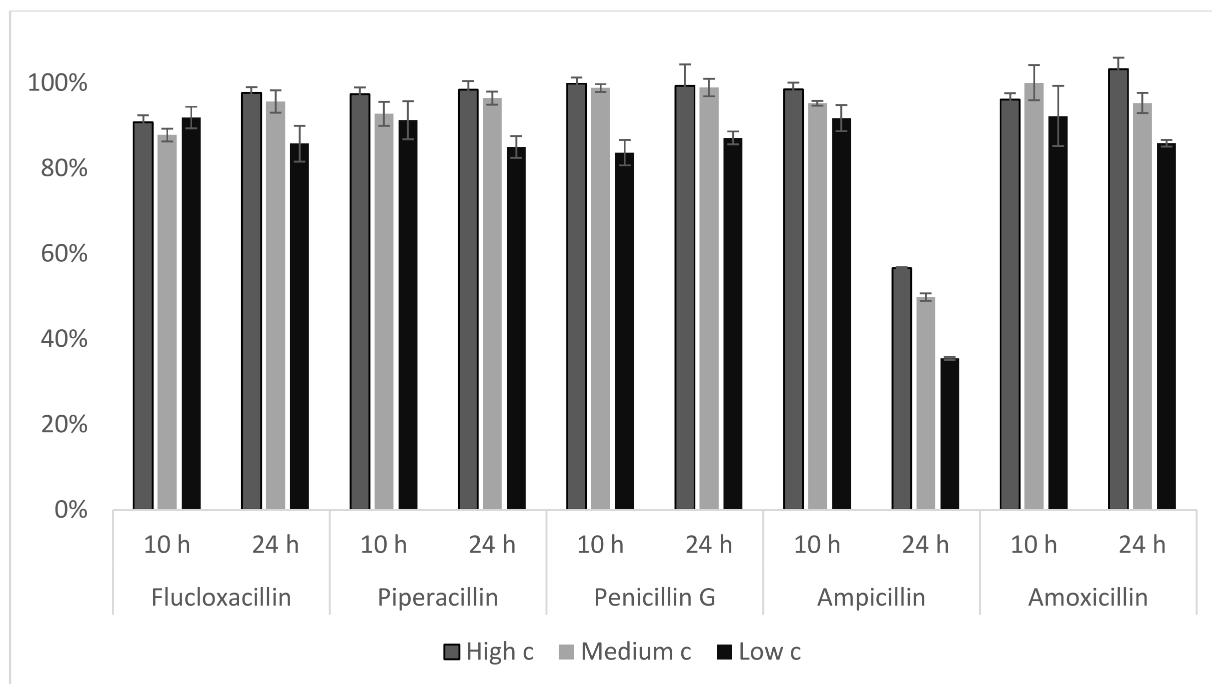

497

498 **Figure 3.** Average autosampler stability of penicillins over 10 and 24 h (n=5), error bars represent
499 the ±1 standard deviation.

500 **Table 1.** Calibration line parameters for all analytes.

Analyte	Slope	Intercept	Weighting	Coefficient of Determination (r2)
Amoxicillin	0.1120	0.0087	$1/x^2$	0.9997
Ampicillin	0.5005	0.0008	$1/x^2$	0.9998
Penicillin G	0.0241	0.0042	$1/x^2$	0.9996
Piperacillin	0.0447	0.0018	$1/x^2$	0.9999
Flucloxacillin	0.0192	0.0012	$1/x^2$	0.9992

501

502 **Table 2.** Within-run assay precision and accuracy (% mean deviation from the nominal
503 concentration) (n=5).

	Accuracy (%)				Precision (%)			
Analyte	LLOQ 0.1 mg/L	Low 0.5 mg/L	Medium 50 mg/L	High 150 mg/L	LLOQ 0.1 mg/L	Low 0.5 mg/L	Medium 50 mg/L	High 150 mg/L
Amoxicillin	-6.6	6.8	-3.3	-4.7	6.1	5.4	3.1	3.8
Ampicillin	5.1	5.6	0.3	-3.9	3.4	2.0	2.5	3.8
Penicillin G	4.7	8.8	1.5	6.6	2.0	5.0	3.7	3.3
Piperacillin	1.4	-2.2	2.4	-3.5	2.7	3.0	4.4	3.5
Flucloxacillin	2.1	5.2	1.2	-5.1	4.6	4.4	2.4	3.1

504

505

506 **Table 3.** Between-run assay precision and accuracy (n=3).

	Accuracy (%)				Precision (%)			
Analyte	LLOQ 0.1 mg/L	Low 0.5 mg/L	Medium 50 mg/L	High 150 mg/L	LLOQ 0.1 mg/L	Low 0.5 mg/L	Medium 50 mg/L	High 150 mg/L
Amoxicillin	-10.5	-0.7	1.8	1.2	7.3	5.1	5.6	2.5
Ampicillin	7.6	2.8	1.8	-1.4	2.3	5.3	5.6	1.3
Penicillin G	7.5	4.8	-2.6	-3.2	1.6	5.7	5.7	2.6
Piperacillin	1.8	5.7	1.5	-3.7	4.1	4.7	7.4	3.2
Flucloxacillin	2.2	5.7	1.5	-1.3	4.7	5.0	7.0	2.2

507

508

509 **Table 4.** Effect of sample degradation on potential conclusion of nonlinear piperacillin CL. A
510 simulated CL of 11.0 L/h was used for all doses (assumed linear pharmacokinetics).

511

Dose	CL (L/h) following varying delay in sample processing					
	0 h	1 h	2 h	4 h	8 h	24 h
4000 mg	10.9	11.1	11.2	11.6	12.3	15.5
3000 mg	10.9	11.1	11.3	11.7	12.6	16.4
1500 mg	10.9	11.2	11.4	11.9	12.9	18.1
400 mg	10.9	11.2	11.5	12.1	13.5	20.4

512