Waters™

Nota de aplicación

Analysis of Antibiotics in Plasma for Clinical Research

Stephen Balloch, Lisa J. Calton, Gareth Hammond

Waters Corporation

For research use only. Not for use in diagnostic procedures.

Abstract

This application note describes a clinical research method using protein precipitation of a plasma sample with internal standards.

Benefits

- Low volume, simple sample preparation
- One preparation method for the quantification of 16 antibiotics that cover a wide range of polarities

Introduction

A reliable, clinical research method for the analysis of a large number of antibiotics in a single sample may play a role in understanding the pharmacokinetic and pharmacodynamic effects of their administration, as such behavior, is not currently well-understood.

Here we describe a clinical research method using protein precipitation of a plasma sample with internal standards. Chromatographic elution was completed within 5 minutes, with the panel analyzed in two runs, using

a Waters ACQUITY UPLC BEH C_{18} Column on a Waters ACQUITY UPLC I-Class followed by detection on a Xevo TQD Mass Spectrometer utilizing polarity switching (Figure 1).

Figure 1. The Waters ACQUITY UPLC I-Class and Xevo TQD Mass Spectrometer.

Experimental

Sample Preparation

Plasma calibrators and quality control materials were prepared in-house using pooled human plasma supplied by BioIVT (West Sussex, UK). Concentrated stock solutions were prepared from certified powders and solutions

supplied by Cambridge Bioscience (Cambridgeshire, UK), Fisher (Loughborough, UK), Merck Life Science (Dorset, UK), and Toronto Research Chemicals (Ontario, Canada). Stable labelled internal standards were supplied by Alsachim (Strasbourg, France), Cambridge Bioscience (Cambridgeshire, UK), and Toronto Research Chemicals (Ontario, Canada). The calibration and QC concentrations (at low, medium, and high concentrations) are detailed in Table 1.

Analyte	Calibration range (µg/mL)	QC concentrations (µg/mL)
Ampicillin	0.5–50	1.25, 3.75, 30
Azithromycin	0.005–0.5	0.0125, 0.0375. 0.3
Cefazolin	1–100	2.5, 7.5, 60
Cefepime	1–100	2.5, 7.5, 60
Cefotaxime	0.5–50	1.25, 3.75, 30
Ceftazidime	1–100	2.5, 7.5, 60
Chloramphenicol	0.5–50	1.25, 3.75, 30
Ciprofloxacin	0.1–10	0.25, 0.75, 6
Clindamycin	0.1–10	0.25, 0.75, 6
Daptomycin	2–200	5, 15, 120
Flucloxacillin	1–100	2.5, 7.5, 60
Linezolid	0.5–50	1.25, 3.75, 30
Meropenem	1–100	2.5, 7.5, 60
Piperacillin	2–200	5, 15, 120
Sulbactam	1–100	2.5, 7.5, 60
Tazobactam	0.5–50	1.25, 3.75, 30

Table 1. Calibrator and QC concentrations.

Sample Extraction

To 50 µL of sample in a microcentrifuge tube, 150 µL of internal standard in methanol was added, the concentrations of internal standards are detailed in Table 2.

Internal Standard	Concentration (µg/mL)
Ampicillin-$^{2}H_{5}$	5
Azithromycin-$^{2}H_{3}$	0.01
Cefazolin-$^{13}C_{2}{}^{15}N$	3
Cefepime-$^{2}H_{3}$	3
Cefotaxime-$^{2}H_{3}$	1.25
Ceftazidime-$^{2}H_{5}$	3
Chloramphenicol-$^{2}H_{5}$	1.67
Ciprofloxacin-$^{2}H_{8}$	1
Clindamycin-$^{13}C_{2}H_{3}$	0.2
Daptomycin-$^{2}H_{5}$	15
Flucloxacillin-$^{13}C_{4}$	3
Linezolid-$^{2}H_{3}$	6.5
Meropenem-$^{2}H_{6}$	6.5
Piperacillin-$^{2}H_{5}$	2.5
Sulbactam-$^{2}H_{5}$	3
Tazobactam-$^{13}C_{2}{}^{15}N_{3}$	1.67

Table 2. Internal standard concentrations.

Tubes were placed on a multi-tube vortex mixer at 2500 rpm for 30 seconds, then centrifuged for 2 minutes at 16,100 g. 100 µL of supernatant was transferred to a 1 mL 96-well plate and 300 µL water containing 1% formic acid added. The plate was then centrifuged at 4,696 g for 2 minutes prior to analysis. The extracts were analyzed in two runs (Set 1 and Set 2). Meropenem samples should be analyzed first, as the extracts are unstable. It is recommended the analysis is completed within 8 hours.

UPLC Conditions

System:	ACQUITY UPLC I-Class with FTN
Needle:	30 µL
Column:	ACQUITY UPLC BEH C_{18} Column; 1.7 µm, 2.1 x 100 mm (p/n: 186002352)
Mobile phase A:	Water + 0.1% ammonia
Mobile phase B:	Methanol
Needle wash solvent:	80% Aqueous methanol + 0.1% formic acid
Purge solvent:	10% Aqueous methanol
Seal wash:	20% Aqueous methanol
Column temp:	60 °C (precolumn heater active)
Injection volume:	2 µL*/10µL**
Flow rate:	0.50 mL/min
Gradient elution:	Table 3
Run time:	5.0 minutes (5.5 minutes injection-to-injection)

** Set 1: Cefazolin, cefepime, ciprofloxacin, clindamycin, flucloxacillin, linezolid, meropenem, and piperacillin.*

*** Set 2: Ampicillin, azithromycin, cefotaxime, ceftazidime, chloramphenicol, daptomycin, sulbactam, and tazobactam.*

Gradient Elution

Time (minutes)	% Mobile phase A	% Mobile phase B	Curve
Initial	90	10	Initial
3.00	0	100	6
4.00	0	100	6
4.10	90	10	6
5.00	90	10	6

Table 3. Chromatographic elution timetable.

MS Conditions

System:	Xevo TQD
Resolution:	MS1 (0.7 FWHM) MS2 (0.7 FWHM)
Acquisition mode:	Multiple Reaction Monitoring (MRM) (see Table 4 for details)
Polarity:	ESI positive ionization/ESI negative ionization (ESI +/ESI -)
Capillary:	3.0 kV (ESI+)/3.0 (ESI-)

Source temperature:	150 °C
Desolvation temperature:	500 °C
Cone gas:	100 L/hr
Inter-scan delay:	0.003 seconds
Polarity/mode switch inter-scan delay:	0.020 seconds
Inter-channel delay:	0.003 seconds

Data Management

Software:	MassLynx v4.2 with TargetLynx Application Manager

Method Conditions

Set 1

Function (acquisition time)	Analyte	Polarity	Precursor ion (*m/z*)	Product ion (*m/z*)	Cone voltage (V)	Collision energy (eV)	Dwell time (ms)
1 (0.5–1.2)	Ciprofloxacin (Quan)	ESI+	332.1	245.1	40	26	0.015
	Ciprofloxacin (Qual)	ESI+	332.1	203.1	40	30	0.015
	Ciprofloxacin-$^{2}H_{6}$	ESI+	340.1	249.1	40	26	0.015
	Meropenem (Quan)	ESI+	384.1	141.1	30	18	0.015
	Meropenem (Qual)	ESI+	384.1	114.1	30	32	0.015
	Meropenem-$^{2}H_{6}$	ESI+	390.1	147.1	30	18	0.015
	Cefazolin (Quan)	ESI+	454.9	156.1	24	18	0.015
	Cefazolin (Qual)	ESI+	454.9	124.1	24	32	0.015
	Cefazolin-$^{13}C_{2}{}^{15}N$	ESI+	457.9	156.1	24	18	0.015
	Cefepime (Quan)	ESI+	481.1	167.1	30	24	0.015
	Cefepime (Qual)	ESI+	481.1	156.1	30	24	0.015
	Cefepime-$^{13}C^{2}H_{3}$	ESI+	485.1	167.1	30	24	0.015
2 (1.2–2.2)	Linezolid (Quan)	ESI+	338.1	235.1	46	22	0.020
	Linezolid (Qual)	ESI+	338.1	148.1	46	42	0.020
	Linezolid-$^{2}H_{3}$	ESI+	341.1	236.1	46	22	0.020
	Flucloxacillin (Quan)	ESI+	454.1	196.1	24	28	0.020
	Flucloxacillin (Qual)	ESI+	454.1	238.1	24	16	0.020
	Flucloxacillin-$^{13}C_{4}$	ESI+	458.1	198.1	24	28	0.020
	Piperacillin (Quan)	ESI+	518.1	359.1	26	8	0.020
	Piperacillin (Qual)	ESI+	518.1	302.1	26	8	0.020
	Piperacillin-$^{2}H_{5}$	ESI+	523.1	364.1	26	8	0.020
3 (2.7–3.5)	Clindamycin (Quan)	ESI+	425.1	83.1	45	66	0.050
	Clindamycin (Qual)	ESI+	425.1	377.1	45	18	0.050
	Clindamycin-$^{13}C^{2}H_{3}$	ESI+	429.1	87.1	45	66	0.050

Set 2:

Function (acquisition time)	Analyte	Polarity	Precursor ion (*m/z*)	Product ion (*m/z*)	Cone voltage (V)	Collision energy (eV)	Dwell time (ms)
1 (0.4–0.75)	Tazobactam (Quan)	ESI+	301.1	99.1	34	28	0.010
	Tazobactam (Qual)	ESI+	301.1	168.1	34	16	0.010
	Tazobactam-$^{13}C_{2}{}^{15}N_{3}$	ESI+	306.1	99.1	34	28	0.010
	Ceftazidime (Quan)	ESI+	547.1	468.1	30	12	0.010
	Ceftazidime (Qual)	ESI+	547.1	167.1	30	26	0.010
	Ceftazidime-$^{2}H_{5}$	ESI+	552.1	468.1	30	12	0.010
2 (0.4–0.8)	Sulbactam (Quan)	ESI–	232.1	140.1	24	12	0.010
	Sulbactam (Qual)	ESI–	232.1	188.1	24	12	0.010
	Sulbactam-$^{2}H_{5}$	ESI–	237.1	145.1	24	12	0.010
3 (0.75–1.35)	Ampicillin (Quan)	ESI+	350.1	160.1	34	12	0.010
	Ampicillin (Qual)	ESI+	350.0	114.1	34	30	0.010
	Ampicillin-$^{2}H_{5}$	ESI+	355.1	160.1	34	12	0.010
	Cefotaxime (Quan)	ESI+	456.1	112.1	32	30	0.050
	Cefotaxime (Qual)	ESI+	456.1	125.1	32	24	0.050
	Cefotaxime-$^{2}H_{3}$	ESI+	459.1	112.1	32	30	0.050
4 (1.35–2.0)	Daptomycin (Quan)*	ESI+	810.8	313.1	40	38	0.060
	Daptomycin (Qual)*	ESI+	810.8	187.1	40	46	0.060
	Daptomycin-$^{2}H_{5}$ *	ESI+	812.8	316.1	40	38	0.060
5 (1.5–2.0)	Chloramphenicol (Quan)	ESI–	320.9	151.9	26	18	0.035
	Chloramphenicol (Qual)	ESI–	320.9	256.9	26	12	0.035
	Chloramphenicol-$^{2}H_{5}$	ESI–	325.9	155.9	26	18	0.035
6 (3.1–3.8)	Azithromycin (Quan)	ESI+	749.5	158.1	60	45	0.060
	Azithromycin (Qual)	ESI+	749.5	591.4	60	40	0.060
	Azithromycin-$^{2}H_{3}$	ESI+	752.5	158.1	60	45	0.060

Table 4. Guideline MRM parameters for analytes and internal standards used in this study.

*Note: Daptomycin has a molecular weight of 1620.7 g/mol, the doubly charged $[M+2H]^{2+}$ ion is most abundant.

Results and Discussion

No system carryover was observed following analysis of plasma samples containing antibiotics at the corresponding highest calibrator concentration for each analyte shown in Table 1.

Figure 2 shows an example chromatogram for the analysis of the 16 antibiotics.

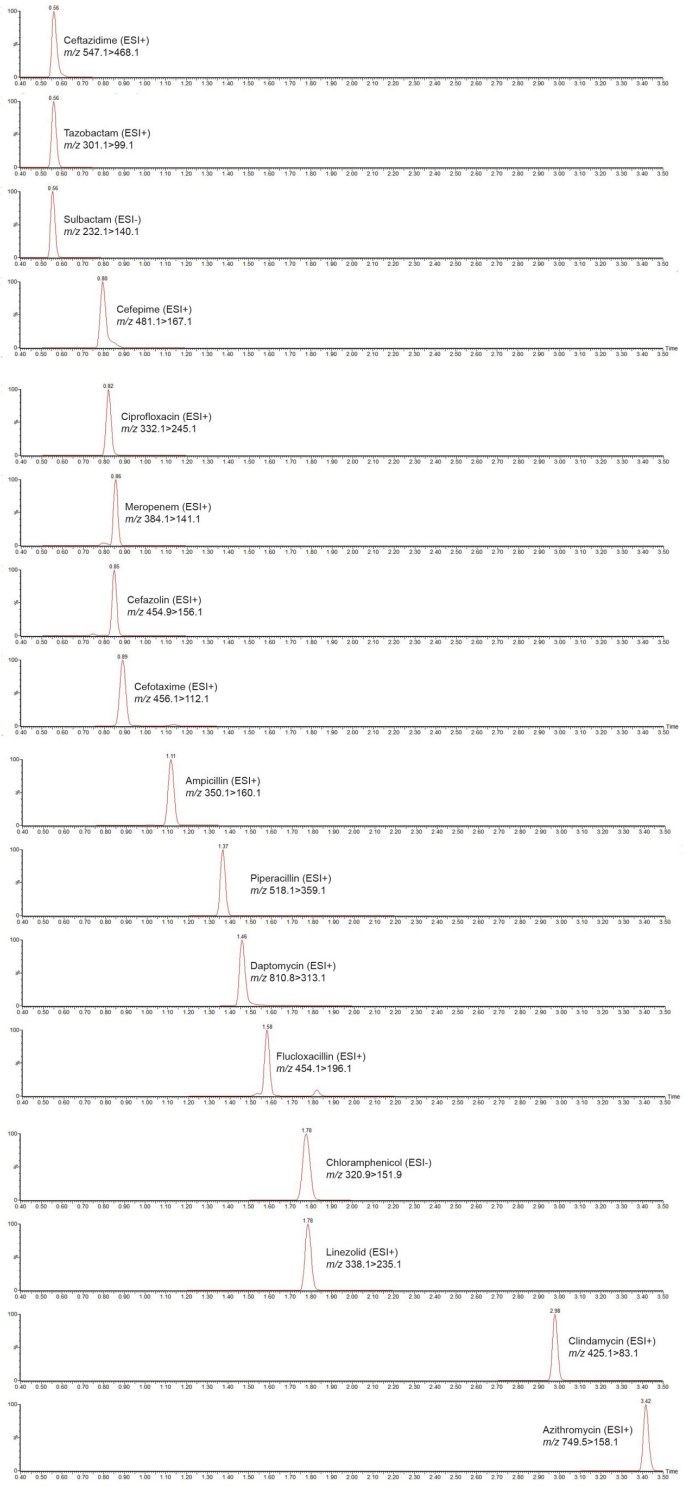

Figure 2. Chromatogram showing the analysis of antibiotics using the ACQUITY UPLC I-Class/Xevo TQD IVD System.

Analytical sensitivity was assessed by extracting and quantifying 10 replicates of low concentration samples prepared in plasma over five days (n=50). Investigations indicated the method would allow for precise quantification (≤20% CV, ≤15% bias) at the concentrations shown in Table 5.

Analyte	Analytical sensitivity (µg/mL)	Precision (%CV)	Bias (%)
Ampicillin	0.375	13.9	–13.2
Azithromycin	0.00375	15.4	–9.1
Cefazolin	1.00	18.8	–2.9
Cefepime	0.900	18.9	2.6
Cefotaxime	0.375	10.2	–14.4
Ceftazidime	0.750	16.4	–8.5
Chloramphenicol	0.375	9.6	–4.8
Ciprofloxacin	0.0750	18.5	–12.0
Clindamycin	0.0750	17.8	–4.0
Daptomycin	1.50	10.2	–12.3
Flucloxacillin	1.00	16.4	–14.4
Linezolid	0.375	16.6	–1.1
Meropenem	0.900	18.2	–14.1
Piperacillin	0.500	17.0	7.9
Sulbactam	0.750	18.1	–5.1
Tazobactam	0.375	19.0	–1.2

Table 5. Analytical sensitivity summary.

Total precision was determined by extracting and quantifying five replicates of three concentrations of plasma pools over five separate days (n=25). Repeatability was assessed by analyzing five replicates at each QC level. Table 6 presents results of these experiments, where total precision and repeatability at the three concentrations assessed was ≤12.5 % RSD.

Analyte	Total QC precision (%RSD)			QC repeatability (%RSD)		
	Low	Mid	High	Low	Mid	High
Ampicillin	7.8	6.6	7.4	7.6	4.2	3.3
Azithromycin	8.3	5.5	3.6	2.6	2.1	1.6
Cefazolin	11.9	12.2	10.8	11.6	5.2	4.1
Cefepime	11.5	10.2	6.3	11.1	5.6	3.4
Cefotaxime	5.7	9.2	8.4	4.1	3.0	2.4
Ceftazidime	7.8	7.5	8.2	4.7	3.0	2.9
Chloramphenicol	10.9	5.7	8.6	3.9	4.2	3.1
Ciprofloxacin	12.5	11.6	8.5	6.1	5.8	3.6
Clindamycin	5.6	5.4	5.8	2.8	2.4	1.9
Daptomycin	6.4	7.6	7.5	4.3	2.1	1.2
Flucloxacillin	9.6	8.0	6.5	5.5	4.6	3.7
Linezolid	5.7	6.5	5.7	4.1	3.0	2.8
Meropenem	12.4	12.4	12.0	10.6	7.8	4.1
Piperacillin	4.6	6.9	9.3	3.6	2.8	1.5
Sulbactam	8.6	7.8	8.5	8.6	6.3	8.0
Tazobactam	11.2	8.4	10.4	8.1	6.7	7.9

Table 6. Total precision and repeatability performance.

The method was shown to be linear over the ranges shown in Table 1 for cefepime, daptomycin, piperacillin, and sulbactam when low and high pools were mixed in known ratios over the range. Ampicillin, azithromycin, cefazolin, cefotaxime, ceftazidime, chloramphenicol, ciprofloxacin, clindamycin, flucloxacillin, linezolid, and meropenem were determined to be quadratic fits over the ranges shown in Table 1.

Matrix effects were evaluated at low and high QC concentrations in plasma (n=6) taken as a percentage of extracted solvent samples spiked to equivalent concentrations. Calculation using analyte: internal standard response ratio indicated compensation for signal enhancement or suppression by the internal standard (Table 7).

Compound	Spiked concentration (µg/mL)	Matrix factor based on peak area mean (range)	Matrix factor based on response mean (range)
Ampicillin	1.25	0.93–0.99 (0.96)	0.96–1.03 (1.01)
	30	0.96–0.99 (0.97)	0.98–1.04 (1.01)
Azithromycin	0.0125	0.79–0.92 (0.86)	0.98–1.04 (1.01)
	0.3	0.77–0.90 (0.84)	1.00–1.04 (1.02)
Cefazolin	2.5	0.91–1.01 (0.96)	0.98–1.11 (1.03)
	60	0.95–0.99 (0.97)	0.99–1.06 (1.02)
Cefepime	2.5	0.94–1.25 (1.13)	0.99–1.11 (1.05)
	60	0.96–1.02 (1.00)	1.02–1.15 (1.06)
Cefotaxime	1.25	0.85–0.94 (0.91)	0.99–1.04 (1.01)
	30	0.93–1.00 (0.96)	0.96–1.07 (1.00)
Ceftazidime	2.5	0.59–0.71 (0.65)	1.00–1.05 (1.03)
	60	0.83–0.91 (0.86)	0.98–1.06 (1.03)
Chloramphenicol	1.25	0.92–0.96 (0.95)	0.97–1.05 (1.01)
	30	0.96–1.00 (0.98)	1.00–1.09 (1.04)
Ciprofloxacin	0.25	0.89–1.13 (0.98)	0.93–1.08 (1.00)
	6	0.94–1.01 (0.97)	1.01–1.13 (1.08)
Clindamycin	0.25	0.99–1.08 (1.03)	0.98–1.06 (1.01)
	6	1.04–1.07 (1.05)	1.01–1.05 (1.02)
Daptomycin	5	1.17–1.46 (1.35)	0.96–1.06 (1.03)
	120	1.00–1.11 (1.08)	0.99–1.08 (1.03)
Flucloxacillin	2.5	0.95–1.05 (1.00)	0.96–1.10 (1.03)
	60	0.98–1.03 (0.99)	0.99–1.09 (1.02)
Linezolid	1.25	0.92–1.04 (0.98)	0.92–1.03 (0.97)
	30	0.98–1.02 (1.00)	0.96–1.06 (1.00)
Meropenem	2.5	0.92–1.06 (0.98)	0.97–1.01 (1.00)
	60	0.94–1.01 (0.98)	1.02–1.05 (1.03)
Piperacillin	5	0.98–1.01 (1.00)	0.99–1.06 (1.03)
	120	0.98–1.02 (1.00)	1.00–1.10 (1.04)
Sulbactam	2.5	0.42–0.77 (0.53)	0.93–1.13 (1.04)
	60	0.70–0.94 (0.78)	0.98–1.08 (1.04)
Tazobactam	1.25	0.70–0.81 (0.75)	0.99–1.14 (1.07)
	30	0.90–0.99 (0.94)	0.96–1.17 (1.04)

Table 7. Matrix factor summary.

Potential interference from endogenous compounds (albumin, bilirubin, cholesterol, creatinine, triglycerides, and uric acid) spiked at high concentrations was assessed by determining the recovery (n=3) from low and high pooled plasma samples (QC1 and QC3 concentrations). Recoveries ranged from 85.7–115.5%.

Conclusion

The developed method for clinical research demonstrates the capabilities of the sample preparation and UPLC-

MS/MS system to quantify 16 antibiotics in plasma, using one sample preparation procedure analysed in two runs. The method demonstrated no system carryover over the test range and matrix effects observed for each analyte were compensated for very effectively using the chosen stable labeled internal standards.

Featured Products

- ACQUITY UPLC I-Class / Xevo TQ-XS IVD System < https://www.waters.com/waters/nav.htm?cid=135034342>
- MassLynx MS Software <https://www.waters.com/513662>
- TargetLynx <https://www.waters.com/513791>

720007388, October 2021