Journal of Chromatography B, 907 (2012) 178–184

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Short communication

A method for determining the free (unbound) concentration of ten beta-lactam antibiotics in human plasma using high performance liquid chromatography with ultraviolet detection

Scott E. Briscoe[a,*], Brett C. McWhinney[a], Jeffrey Lipman[b,c], Jason A. Roberts[b,c], Jacobus P.J. Ungerer[a]

[a] Chemical Pathology, Pathology Queensland, Brisbane, Queensland, Australia
[b] Royal Brisbane and Women's Hospital, Brisbane, Queensland, Australia
[c] Burns Trauma and Critical Care Research Centre, The University of Queensland, Brisbane, Queensland, Australia

ARTICLE INFO

Article history:
Received 10 June 2012
Accepted 8 September 2012
Available online 16 September 2012

Keywords:
Free
Unbound
Beta-lactam
Antibiotics
HPLC
TDM

ABSTRACT

With the clinical imperative to further research in the area of optimising antibiotic dosing in the intensive care setting, a simple high performance liquid chromatography method was developed and validated for routinely determining the free (unbound) concentration of ten beta-lactam antibiotics in 200 μL of human plasma. Antibiotics determined include three cephalosporins (ceftriaxone, cephazolin and cephalotin); two carbapenems (meropenem and ertapenem); and five penicillins (ampicillin, piperacillin, benzylpenicillin, flucloxacillin and dicloxacillin). There was a single common sample preparation method involving ultracentrifugation and stabilisation. Chromatography was performed on a Waters XBridge C18 column with, depending on analytes, one of four acetonitrile-phosphate buffered mobile phases. Peaks of interest were detected via ultraviolet absorbance at 210, 260 and 304 nm. The method has been validated and used in a pathology laboratory for therapeutic drug monitoring in critically ill patients. The significant variability in the level of protein binding that is common with antibiotics traditionally considered to have high protein binding (e.g. ceftriaxone, cephazolin, ertapenem, flucloxacillin and dicloxacillin) suggests that this assay should be preferred for measuring the pharmacologically active concentration of beta-lactam antibiotics in a therapeutic drug monitoring programme.

Crown Copyright © 2012 Published by Elsevier B.V. All rights reserved.

1. Introduction

The beta-lactams constitute the most important antibiotic family, both in terms of the large number of compounds available and in terms of prescription volume [1]. These drugs all share a common structure and mechanism of action, but have evolved into various classes with differing spectrums of antibiotic activity and unique qualities [2]. From a pharmacodynamic perspective, the beta-lactam family are categorised as 'time dependent' antibiotics where the pharmacokinetic/pharmacodynamic index that best correlates with bacterial killing is the time that concentrations are maintained above the minimum inhibitory concentration in a dosing interval [3–5].

It is well accepted that the pharmacological activity of antibiotics depends on the free, or unbound, concentration at the site of infection [6,7]. Given that previously published assays for beta-lactam therapeutic drug monitoring (TDM) measure total antibiotic concentrations [1,8], accurately estimating the unbound antibiotic concentration can be difficult, particularly in critically ill patients for highly protein bound antibiotics such as ceftriaxone, cephazolin, ertapenem, flucloxacillin and dicloxacillin [9,10]. For these drugs with protein binding >80%, small changes in protein binding can have a large effect on the unbound concentration [9].

Given the potential limitations of measuring total antibiotic concentrations, the aim of this paper is to describe a method to determine the free (unbound) concentration of ten beta-lactam antibiotics in human plasma using high performance liquid chromatography (HPLC) with ultraviolet (UV) detection. We also aim to describe our observations relating to the variability of unbound concentrations for some of the antibiotics analysed with this method.

2. Material and methods

The free (unbound) beta-lactam antibiotics which are determined via this method include three cephalosporins (ceftriaxone, cephazolin and cephalotin); two carbapenems (meropenem and ertapenem); and five penicillins (ampicillin, piperacillin,

* Corresponding author at: Chemical Pathology, Level 3, Block 7, Royal Brisbane and Women's Hospital, QLD 4029, Australia. Tel.: +61 7 3646 0024; fax: +61 7 3646 3417.
E-mail addresses: scott_briscoe@health.qld.gov.au, briscoe_scott@yahoo.com.au (S.E. Briscoe).

1570-0232/$ – see front matter. Crown Copyright © 2012 Published by Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jchromb.2012.09.016

benzylpenicillin, flucloxacillin and dicloxacillin). The method uses a common sample preparation and stationary phase for determination of all ten antibiotics. In order to maintain tolerable run times and peak shape, the antibiotics are grouped into four sets based on the isocratic mobile phase used for their simultaneous determination: group 1A is ampicillin, ceftriaxone and meropenem; group 1B is cephazolin and ertapenem; group 2A is piperacillin, benzylpenicillin and cephalotin; and group 2B is flucloxacillin and dicloxacillin.

2.1. Reagents and chemicals

Drug material was obtained as formulations for injection or infusion: ampicillin sodium (Ampicyn, Aspen Pharmacare Australia); ceftriaxone sodium (DBL Ceftriaxone Sodium for Injection, Hospira Australia); meropenem trihydrate (Merrem IV, AstraZeneca); cephazolin sodium (Kefzol, Aspen Pharmacare Australia); ertapenem sodium (Invanz, Merck Sharp and Dohme); piperacillin sodium (Tazopip Powder for Injection, Aspen Pharmacare Australia); benzylpenicillin sodium (BenPen, CSL Limited); cephalotin sodium (Keflin Neutral, Aspen Pharmacare Australia); flucloxacillin sodium (Flucil, Aspen Pharmacare Australia); and dicloxacillin sodium (Diclocil Injection, Bristol-Myers Squibb Pharmaceuticals). Acetonitrile (Merck, Darmstadt, Germany) was LiChrosolv HPLC grade. Sodium dihydrogen orthophosphate (Ajax Finechem, Sydney, Australia) and orthophosphoric acid 85% (Ajax Finechem, Sydney, Australia) were UNIVAR analytical grade. 2-(N-Morpholino)ethanesulphonic acid (MES) and its respective sodium salt (MES sodium salt) were purchased from Sigma (St. Louis, MO, USA). All water was deionised (18 MΩ resistivity). Pooled blank plasma was obtained from the hospital pathology service.

2.2. Chromatographic system

The method was developed, validated and operates on a 2690 Separations Module equipped with a quaternary pump, on-line degasser and autosampler (Waters, Milford, USA) coupled with a 996 Photodiode Array Detector (Waters, Milford, USA). Instrument control, data acquisition and data processing were carried out using Empower Pro V2 software (Waters, Milford, USA). Chromatographic separations were performed at ambient temperature on a reverse phase C18 2.5 μm 4.6 × 30 mm XBridge column (Waters, Milford, USA) using a 1.0 mL/min flow rate and 25 μL injection volume. A C18 4.0 × 3.0 mm SecurityGuard cartridge (Phenomenex, California, USA) was installed pre-column to extend the longevity of the analytical column.

One of four isocratic mobile phases was used depending on the analyte to be determined. Mobile phase 1A was acetonitrile (8%) and 50 mM phosphate buffer at pH 2.4 (92%) and was used for ampicillin, ceftriaxone and meropenem. Mobile phase 1B was acetonitrile (12%) and 50 mM phosphate buffer at pH 2.4 (88%) and was used for cephazolin and ertapenem. Mobile phase 2A was acetonitrile (25%) and 100 mM phosphate buffer at pH 3.0 (75%) and was used for piperacillin, benzylpenicillin and cephalotin. Mobile phase 2B was acetonitrile (40%) and 100 mM phosphate buffer at pH 3.0 (60%) and was used for flucloxacillin and dicloxacillin.

The peaks of interest were detected by UV absorbance at the wavelengths of: 210 nm for ampicillin, piperacillin, benzylpenicillin, flucloxacillin and dicloxacillin; 260 nm for ceftriaxone, cephazolin and cephalotin; and 304 nm for meropenem and ertapenem. Quantification was based on the peak height of each analyte and both peak identification and homogeneity were aided by utilising peak purity algorithms and 3D spectral library matching.

2.3. Solutions

Ampoules of the drug material were initially reconstituted in 5 mL of deionised water and then diluted to 10 mL with deionised water to give ten primary stocks ranging from 50 to 200 mg/mL for each analyte.

Primary stocks were diluted with deionised water to give four combined standards. Combined standard 1A consists of ampicillin, ceftriaxone and meropenem at 5 mg/mL. Combined standard 1B consists of cephazolin and ertapenem at 5 mg/mL. Combined standard 2A consists of piperacillin, benzylpenicillin and cephalotin at 5 mg/mL. Combined standard 2B consists of flucloxacillin and dicloxacillin at 5 mg/mL.

Four groups of three working standards were subsequently prepared in deionised water from each of the four respective combined standards. The antibiotic concentration at each level was 2.5 μg/mL, 10.0 μg/mL and 50.0 μg/mL. Working standards were stored in 300 μL aliquots at −70 °C for up to 12 months.

Quality control specimens were prepared independently of standards in blank plasma at a low and high concentration level of approximately 0.5 μg/mL and 5.0 μg/mL using protein binding data from the literature [9]. Aliquots (300 μL) of the quality control specimens were stored at −70 °C for up to 12 months.

A 1.0 M MES buffer (pH 6.6) was prepared by adding 5.58 g MES and 15.50 g MES sodium salt to a 100 mL volumetric flask and diluting with deionised water to the fiducial mark. This buffer acted as a stabilising agent and was a modification of the solution utilised by Musson et al. [11].

Mobile phase 1A was prepared by dissolving 7.8 g of sodium dihydrogen orthophosphate in 850 mL of deionised water, adding 80 mL of acetonitrile, adjusting the pH to 2.4 with orthophosphoric acid, then making the volume up to 1 L. Mobile phase 1B was prepared by dissolving 7.8 g of sodium dihydrogen orthophosphate in 800 mL of deionised water, adding 120 mL of acetonitrile, adjusting the pH to 2.4 with orthophosphoric acid, then making the volume up to 1 L. Mobile phase 2A was prepared by dissolving 15.6 g of sodium dihydrogen orthophosphate in 700 mL of deionised water, adding 250 mL of acetonitrile, adjusting the pH to 3.0 with orthophosphoric acid, then making the volume up to 1 L. Mobile phase 2B was prepared by dissolving 15.6 g of sodium dihydrogen orthophosphate in 550 mL of deionised water, adding 400 mL of acetonitrile, adjusting the pH to 3.0 with orthophosphoric acid, then making the volume up to 1 L. All mobile phases were degassed and filtered through a Durapore 0.45 μm HVLP filter (Millipore, North Ryde, Australia).

2.4. Sample preparation

For each standard (3), quality control (2) and patient sample, 200 μL of plasma was placed into a Amicon Ultra-0.5 mL 30,000 molecular weight cut-off centrifugal filter device (Millipore, Cork, Ireland) and centrifuged for 10 min at 16,200 × g. An aliquot (100 μL) of the ultrafiltrate was then transferred to an autosampler vial and vortex mixed for 30 s with 10 μL of 1.0 M MES buffer (pH 6.6) before proceeding with chromatographic analysis.

2.5. Validation of the method

The validation of the method was critical to ensure that the results were suitable for their intended purpose and was conducted with reference to the guidelines developed by the Food and Drug Administration for bioanalytical method validation [12].

Linearity for each antibiotic was tested by extracting aqueous standards spiked at nominal concentrations of 0.1, 0.5, 1.0, 2.5, 5.0, 10.0, 25.0 and 50.0 μg/mL. The calibration line was generated by least squares linear regression of the peak height of the analyte

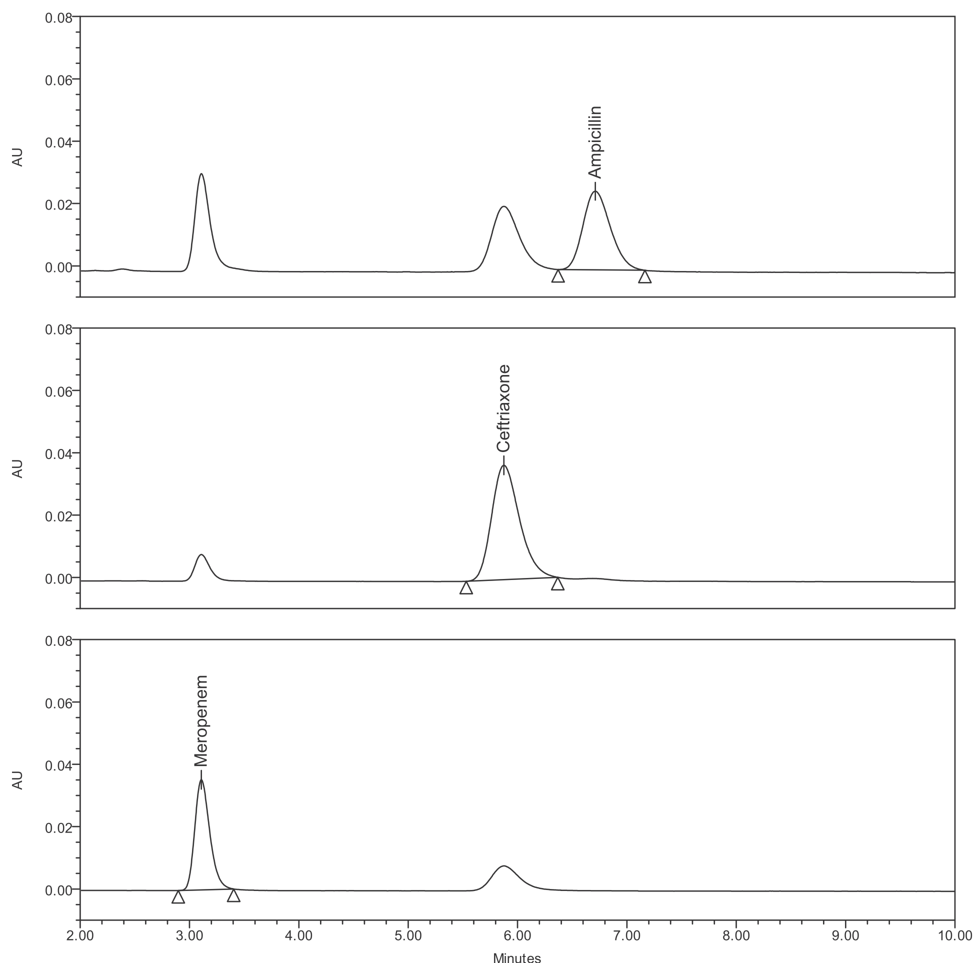


Fig. 1. Mobile phase 1A chromatograms: (top) detection at 210 nm for ampicillin (6.7 min) spiked at 10 μg/mL; (centre) detection at 260 nm for ceftriaxone (5.9 min) spiked at 10 μg/mL; (bottom) detection at 304 nm for meropenem (3.1 min) spiked at 10 μg/mL.

against nominal concentration. The percentage deviation from nominal was back-calculated at each standard concentration with ≤15% as acceptance criterion for inclusion in the calibration curve.

The limit of quantification (LOQ) was validated by replicate analysis ($n = 10$) of plasma spiked at 0.1 μg/mL, with precision ≤10% as the acceptance criterion.

Within-run and between-run precision of the assay was assessed by replicate analysis ($n = 10$) of the low and high plasma quality controls. Concentrations were determined from the calibration curves and the precision (%CV) calculated at each level. Within-run data was collected within a single run, whilst between-run data was collected over ten separate days.

The stability of the prepared samples in the autosampler was tested by comparing the results for the low and high plasma quality controls injected after 12 h with those obtained on immediate injection, calculated from the original standard curve.

Specificity of the assay was demonstrated by confirming chromatographically the absence of interfering peaks from co-medicated drugs.

3. Results

3.1. Chromatography

Figs. 1–4 depict the chromatography for each analyte under the specified mobile phase and wavelength conditions. Typical retention times are displayed in Table 1. Mobile phases 1A and 1B differ only in the amount of acetonitrile present (8% cf. 12%), as with mobile phases 2A and 2B (25% cf. 40%). The higher percentage of organic in both 1B and 2B was important to reduce run times and maintain peak shape. Buffering of mobile phases 2A and 2B at pH 3.0 was necessary for the penicillins, as poor retention was observed at pH 2.4 (the pH of mobile phases 1A and 1B). The choice of stationary phase was made on the basis of wanting a short run time with relatively high resolution.

In applying this method over a four month period to critically ill patients, for which the majority are co-medicated with at least one other drug, there have been no incidents of chromatographic interference with the analyte of interest.

3.2. Validation

All calibration lines were adequately described by linear regression over the concentration range (Table 1). Whilst r^2 is a common measure of goodness of fit, it can often be more informative when assessing the adherence of the standards to the calibration line to calculate the percentage deviation of the individual standards from their nominal concentration (i.e. accuracy). All standards passed the acceptance criterion (i.e. ≤15% deviation from nominal) and accordingly, none were rejected from the calibration curve. For all analytes the mean absolute percentage deviation of standards was 7.2% or better.

The average precision at the LOQ for all analytes was 5.6% and in no case did the precision fail the acceptance criterion of ≤10% (Table 1). There was sufficient sensitivity and absence of

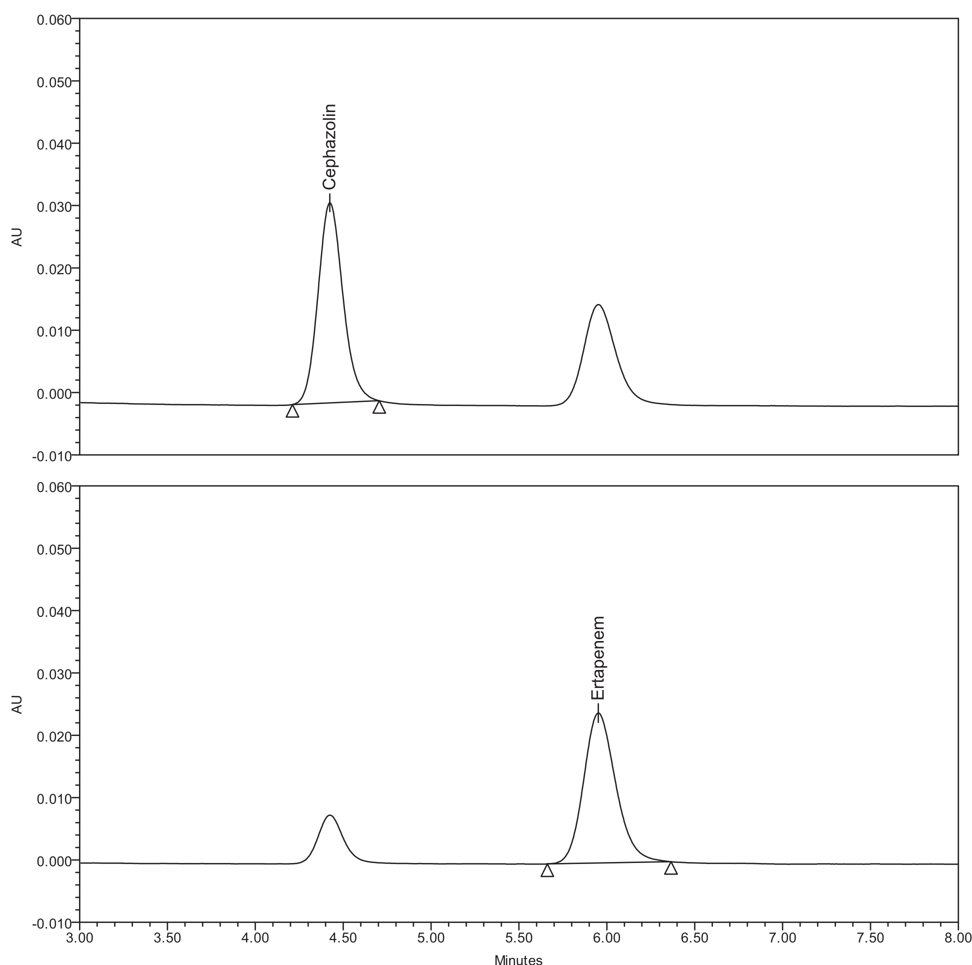


Fig. 2. Mobile phase 1B chromatograms: (top) detection at 260 nm for cephazolin (4.4 min) spiked at 10 μg/mL; (bottom) detection at 304 nm for ertapenem (6.0 min) spiked at 10 μg/mL.

interferences for exploring the potential of a lower LOQ (with precision ≤15% as the acceptance criterion) for all analytes, however our clients were satisfied with a 0.1 μg/mL cut-off.

The within-run and between-run replicate analysis of the low and high plasma quality controls is presented in Table 2.

After 12 h ageing at room temperature – a period which exceeded the run time of any batch by more than 10 h – the mean (range) percent difference between initial and aged low and high plasma quality controls for all analytes was 4.4 (0.0–10.7) and 4.4 (0.3–12.2) respectively.

3.3. Clinical application – variation in protein binding for ceftriaxone and piperacillin

Fig. 5 depicts the variation in data for the percentage free (unbound) of ceftriaxone and piperacillin that we have observed during our clinical application of this assay. This data shows that for a highly bound antibiotic (ceftriaxone) as well as a low-to-moderately bound antibiotic (piperacillin), predicting the level of protein binding accurately is difficult because of the inherent variability present.

Table 1
Chromatographic information (mobile phase, detector wavelength (λ) and retention time (t_R)) and linearity details (calibration range, equation, r^2 and precision at limit of quantitation (%CV at LOQ)).

Analyte	Mobile phase	λ (nm)	t_R (min)	Calibration range (μg/mL)	Equation	r^2	%CV at LOQ
Ampicillin	1A	210	6.7	0.1–50.0	$y = 1.0480x + 0.0873$	0.9995	3.1
Ceftriaxone	1A	260	5.9	0.1–50.0	$y = 0.9725x - 0.2058$	0.9996	5.1
Meropenem	1A	304	3.1	0.1–50.0	$y = 1.0069x - 0.0824$	0.9999	4.7
Cephazolin	1B	260	4.4	0.1–50.0	$y = 0.9933x - 0.0558$	1.0000	4.5
Ertapenem	1B	304	6.0	0.1–50.0	$y = 1.0425x + 0.1027$	0.9999	6.1
Piperacillin	2A	210	2.7	0.1–50.0	$y = 0.9669x - 0.1514$	0.9997	9.4
Benzylpenicillin	2A	210	3.2	0.1–50.0	$y = 0.9972x - 0.0243$	0.9999	5.0
Cephalotin	2A	260	2.1	0.1–50.0	$y = 0.9855x + 0.0288$	0.9999	3.9
Flucloxacillin	2B	210	1.9	0.1–50.0	$y = 0.9958x - 0.1837$	0.9999	5.4
Dicloxacillin	2B	210	2.4	0.1–50.0	$y = 0.9853x - 0.0721$	0.9996	9.1

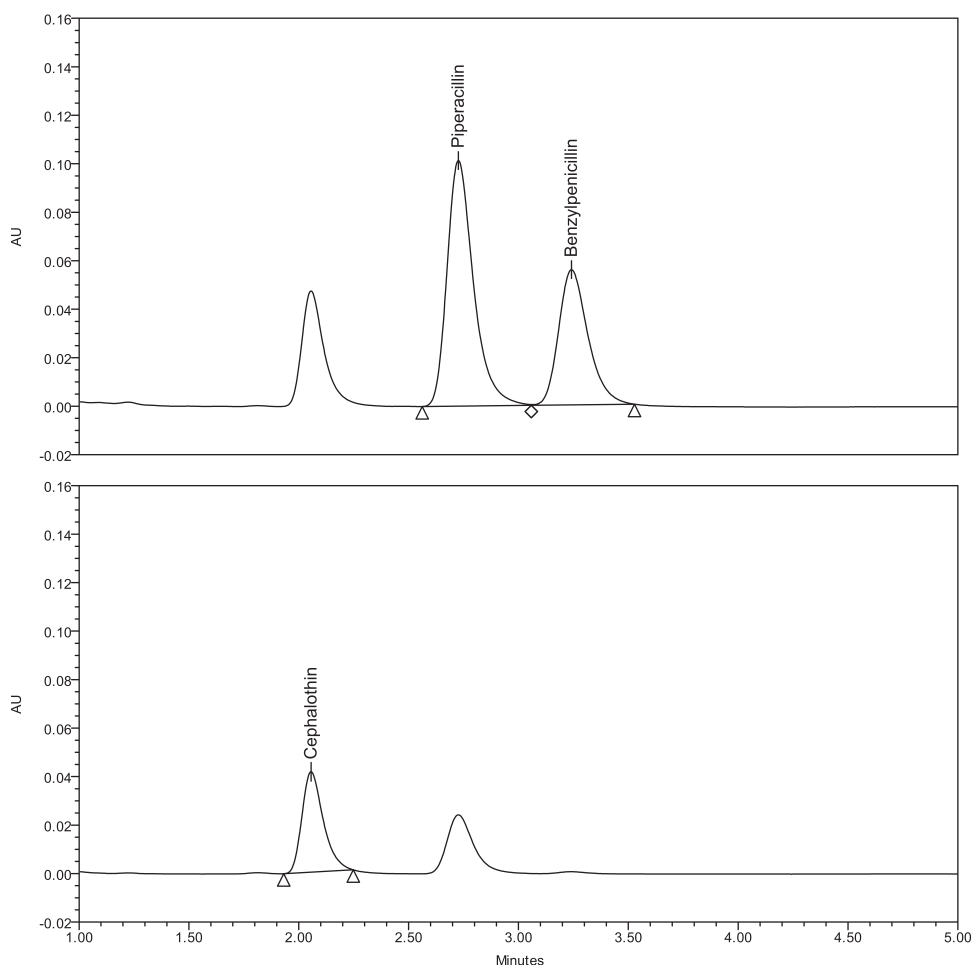


Fig. 3. Mobile phase 2A chromatograms: (top) detection at 210 nm for piperacillin (2.7 min) and benzylpenicillin (3.2 min) spiked at 10 μg/mL; (bottom) detection at 260 nm for cephalotin (2.1 min) spiked at 10 μg/mL.

4. Discussion

This method possesses a number of advantageous features for application within the laboratory. The most notable of these is the ability to access a range of analyses with a simple and inexpensive platform. The analytical instrumentation is relatively commonplace compared to mass spectrometry detection or newer ultra high performance liquid chromatography systems. Indeed, whilst the photodiode array detector used has advantages of peak purity and spectral matching features, a single wavelength UV detector may be substituted if only one, or a combination of the beta-lactam antibiotics are of interest. The mobile phase contains acetonitrile as the organic modifier, but the effluent can be recycled from the detector back into the reservoir. The sample preparation is simple, inexpensive and requires only a small volume of plasma.

The method has been used routinely over a 4 month period for in excess of 270 samples from critically ill patients. The method has performed solidly in a pathology laboratory, providing the clinician with free (unbound) beta-lactam levels within 24 h of sample collection (Table 3). This demonstrates the ruggedness of the method within the hands of four analytical staff and at least four preparations of mobile phase. Furthermore, our clinical data on the variability of protein binding suggest that where possible, direct determination of the free (unbound) fraction of antibiotics may be advantageous for optimising antibiotic dosing.

Table 2
Low and high concentration plasma quality controls measured within-run ($n = 10$) and between-run ($n = 10$).

Analyte	Within-run mean (μg/mL) (%CV)		Between-run mean (μg/mL) (%CV)	
	Low	High	Low	High
Ampicillin	0.58 (2.8)	5.98 (1.7)	0.62 (2.8)	5.68 (3.5)
Ceftriaxone	0.39 (2.4)	8.22 (3.2)	0.43 (2.4)	8.35 (4.5)
Meropenem	0.67 (2.6)	6.02 (2.6)	0.65 (2.6)	6.57 (4.2)
Cephazolin	0.42 (2.5)	7.48 (2.4)	0.37 (7.7)	7.49 (3.9)
Ertapenem	0.48 (3.2)	7.96 (2.5)	0.46 (9.1)	8.54 (7.0)
Piperacillin	0.33 (4.7)	6.21 (3.0)	0.33 (8.6)	6.99 (8.4)
Benzylpenicillin	0.57 (4.4)	7.46 (1.5)	0.62 (7.6)	8.07 (3.4)
Cephalotin	0.49 (3.1)	7.01 (1.6)	0.52 (7.3)	7.65 (4.5)
Flucloxacillin	0.22 (3.7)	6.37 (1.3)	0.19 (8.8)	6.84 (7.6)
Dicloxacillin	0.13 (5.7)	3.42 (5.6)	0.11 (8.3)	3.74 (8.3)

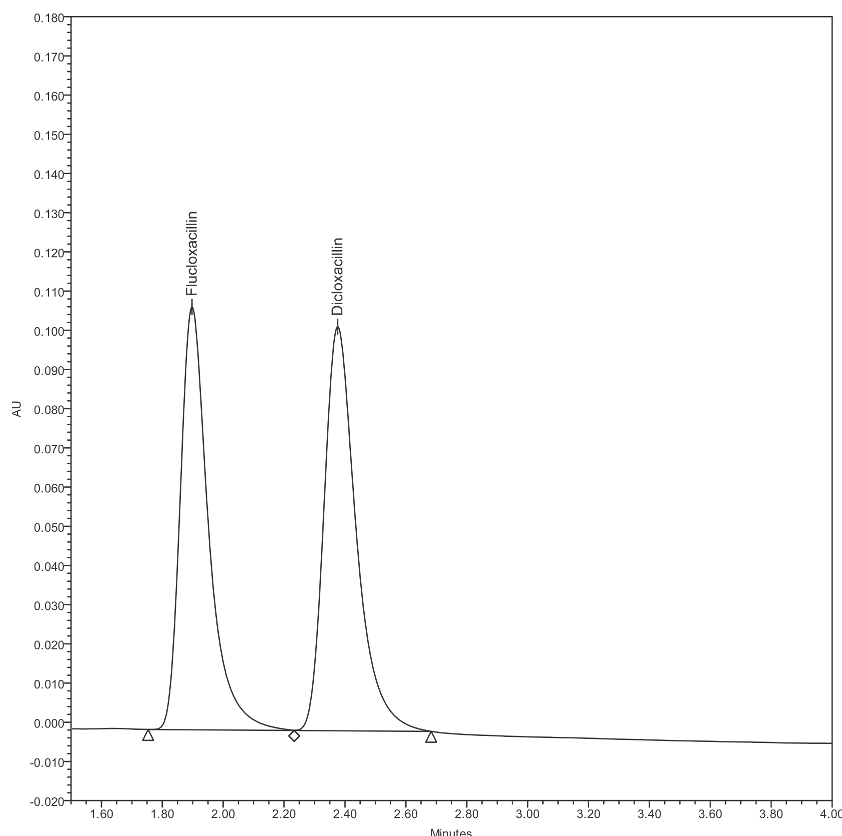
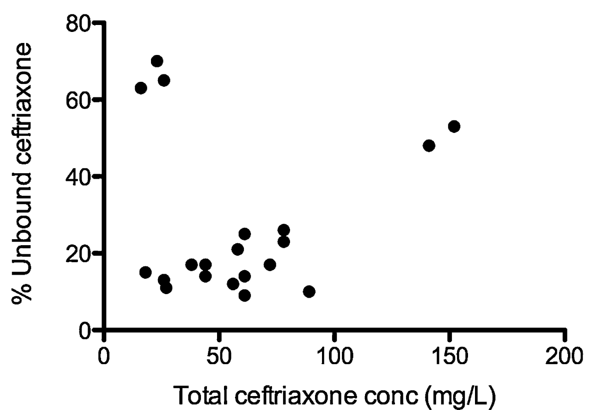


Fig. 4. Mobile phase 2B chromatogram: detection at 210 nm for flucloxacillin (1.9 min) and dicloxacillin (2.4 min) spiked at 10 μg/mL.



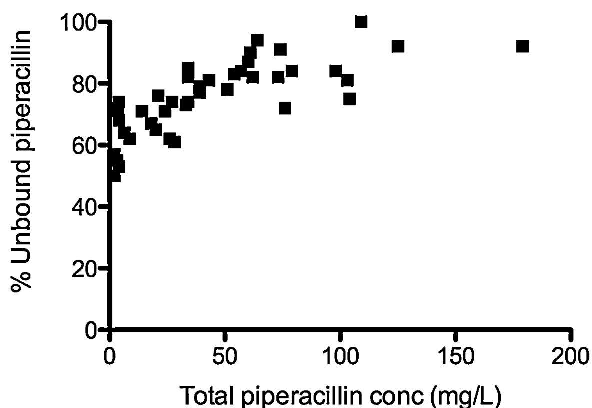


Fig. 5. The variability in protein binding for ceftriaxone (top) and piperacillin (bottom) from 20 and 43 critically ill patients respectively.

Table 3
Low and high concentration plasma quality controls measured over four months during routine therapeutic drug monitoring.

Analyte	*n*	Mean concentration (μg/mL) (%CV)	
		Low	High
Ampicillin	7	0.60 (5.1)	5.83 (3.7)
Ceftriaxone	6	0.40 (6.6)	8.28 (2.7)
Meropenem	10	0.66 (5.8)	6.48 (4.5)
Cephazolin	6	0.39 (8.1)	7.48 (5.7)
Ertapenem	5	0.47 (7.0)	8.25 (6.0)
Piperacillin	7	0.33 (6.7)	6.60 (6.0)
Benzylpenicillin	14	0.63 (6.4)	7.76 (4.4)
Cephalotin	2	0.51 (4.2)	7.33 (2.1)
Flucloxacillin	3	0.20 (2.8)	6.61 (2.9)
Dicloxacillin	3	0.12 (4.9)	3.58 (4.6)

5. Conclusion

To the best of our knowledge, the method presented here is the first published to determine the free (unbound) concentration of ten beta-lactam antibiotics in 10 min or under. It has the advantage of simplicity in sample preparation, chromatography and instrumentation, with the only variation in procedure being the selection of mobile phase. The performance of the method has been proven by validation and its application within a pathology laboratory. Given the variety of beta-lactams prescribed clinically, as well as the requirement for timely data when performing TDM, this method is highly advantageous from a laboratory and clinical perspective.

References

[1] M.C. Verdier, O. Tribut, P. Tattevin, Y. Le Tulzo, C. Michelet, D. Bentue-Ferrer, Antimicrob. Agents Chemother. 55 (2011) 4873–4879.

[2] S.I. Martin, K.M. Kaye, Infect. Dis. Clin. North Am. 18 (2004) 603–619.
[3] W.A. Craig, Clin. Infect. Dis. 26 (1998) 1–10.
[4] B.M. Patel, J. Paratz, N.C. See, M.J. Muller, M. Rudd, D. Paterson, S.E. Briscoe, J. Ungerer, B.C. McWhinney, J. Lipman, J.A. Roberts, Ther. Drug Monit. 34 (2012) 160–164.
[5] A.A. Udy, J.M. Varghese, M. Altukroni, S. Briscoe, B.C. McWhinney, J. Ungerer, J. Lipman, J.A. Roberts, Chest 142 (1) (2012) 30–39.
[6] J.A. Roberts, R. Norris, D.L. Paterson, J.H. Martin, Br. J. Clin. Pharmacol. 73 (2012) 27–36.
[7] U. Theuretzbacher, Curr. Opin. Pharmacol. 7 (2007) 498–504.
[8] B.C. McWhinney, S.C. Wallis, T. Hillister, J.A. Roberts, J. Lipman, J.P. Ungerer, J. Chromatogr. B 878 (2010) 2039–2043.
[9] M. Ulldemolins, J.A. Roberts, J. Rello, D.L. Paterson, J. Lipman, Clin. Pharmacokinet. 50 (2011) 99–110.
[10] M. Ulldemolins, J.A. Roberts, S.C. Wallis, J. Rello, J. Lipman, J. Antimicrob. Chemother. 65 (2010) 1771–1778.
[11] D.G. Musson, K.L. Birk, C.J. Kitchen, J. Zhang, J.Y. Hsieh, W. Fang, A.K. Majumdar, J.D. Rogers, J. Chromatogr. B 783 (2003) 1–9.
[12] US Food and Drug Administration, U.S.D.o.H.a.H. Services, 2001.