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Short communication

Analysis of 12 beta-lactam antibiotics in human plasma by HPLC with ultraviolet detection

Brett C. McWhinney[a,*], Steven C. Wallis[b], Tara Hillister[a], Jason A. Roberts[b], Jeffrey Lipman[b], Jacobus P.J. Ungerer[a]

[a] Chemical Pathology, Pathology Queensland, Brisbane, Queensland, Australia
[b] Burns Trauma and Critical Care Research Centre, The University of Queensland, Brisbane, Queensland, Australia

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ABSTRACT

A simple and economical high performance liquid chromatography method was developed and validated for routine analysis of 12 Penicillin, Cephalosporin and Carbapenem antibiotics in 200 μL of human plasma. Antibiotics determined were Ceftazidime, Meropenem, Ceftriaxone, Ampicillin, Cefazolin, Ertapenem, Cephalothin, Benzylpenicillin, Flucloxacillin, Dicloxacillin, Piperacillin and Ticarcillin. There was a common sample preparation approach involving precipitation of proteins with acetonitrile and removal of lipid-soluble components by a chloroform wash. Separations were performed on a Waters X-bridge C18 column with, depending on analytes, one of three acetonitrile–phosphate buffer mobile phases. Detection was by UV at 210, 260 and 304 nm. Validation has demonstrated the method to be linear, accurate and precise. The method has been used in a pathology laboratory for therapeutic drug monitoring (TDM) of beta-lactams in critically ill patients.

1. Introduction

The beta-lactams are a major family of antibiotics that include the penicillins (e.g. Piperacillin), the carbapenems (e.g. Meropenem) and the Cephalosporins (e.g. Ceftazidime). The antimicrobial effect of the beta-lactams is related to the duration that the antibiotic concentration exceeds the minimum inhibitory concentration (MIC) of the pathogen [1]. The time spent above MIC (T > MIC) is the pharmacodynamic end-point that clinicians seek to maximise in order to ensure optimal therapy.

The beta-lactams are central to the treatment of sepsis and life-threatening infections in the Intensive Care Unit (ICU). There is potential for lower-than-expected concentrations of these drugs due to the unusual pharmacokinetics possible in the critically ill patient due to altered physiology and interventions like dialysis, which in turn can reduce T > MIC [2]. The consequences of insufficient antibiotic coverage are antibiotic resistance and treatment that fails to suppress or kill bacteria, which can increase length of stay, reduce the quality of recovery or even lead to death [3–6].

We have embarked on a pilot project for Therapeutic Drug Monitoring of selected beta-lactam antibiotics in the critically ill with the aim of determining its utility in the ICU. This endeavour requires the ability to reliably and quickly measure the concentrations of the antibiotics in patient plasma. The antibiotics in the TDM project are four cephalosporins (Ceftazidime, Ceftriaxone, Cefazolin, and Cephalothin), two carbapenems (Meropenem and Ertapenem), and six penicillins (Ampicillin, Benzylpenicillin, Flucloxacillin, Dicloxacillin, Piperacillin and Ticarcillin).

HPLC methodology for all these analytes exists in the literature, although too numerous to recite here; in recent years the bioanalysis of the penicillins and the Cephalosporins have been reviewed [7,8]. Whilst the patients in our project would usually be administered only one of the antibiotics at a time, and we are only interested in measuring one antibiotic in each sample, for the purposes of simplicity and efficiency we desired the minimum number of methods for measuring our multiple analytes. Both carbapenems have been measured simultaneously in plasma [9]. Three of the four Cephalosporins of interest were included in five beta-lactams simultaneously measured in plasma [10]. Multiple penicillins have been measured simultaneously in animal tissue for residue purposes [11,12]. However, there is no one method published suitable for the dozen antibiotics we are interested in, and so we developed the method presented here.

The method described here uses a common sample preparation method and stationary phase for all analytes. In order to maintain tolerable run times and peak shape over the entire range of analytes, analytes are grouped into three sets based on the isocratic mobile phase used (Table 1). Group 1A is for simultaneous

* Corresponding author at: Chemical Pathology, Level 3, Block 7, Royal Brisbane and Women's Hospital, QLD 4029, Australia. Tel.: +61 7 3636 0028; fax: +61 7 36363417.
E-mail address: brett_mcwhinney@health.qld.gov.au (B.C. McWhinney).

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Table 1
Chromatographic (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 (%)
Ceftazidime	1A	260	1.7	5–500	$y = 0.0030x + 0.0295$	0.9972	7.9
Meropenem	1A	304	2.4	5–250	$y = 0.0464x - 0.0135$	0.9953	1.4
Ceftriaxone	1A	260	4.6	5–500	$y = 0.0286x - 0.0118$	0.9997	2.1
Ampicillin	1A	210	5.7	5–500	$y = 0.0074x + 0.0022$	0.9926	3.6
Cefazolin	1B	260	3.7	5–500	$y = 0.0070x + 0.0007$	0.9890	6.7
Ertapenem	1B	304	4.4	5–250	$y = 0.0193x + 0.0005$	0.9952	4.1
Cephalothin	2	260	2.0	5–500	$y = 0.0642x + 0.0238$	0.9984	1.5
Piperacillin	2	210	2.6	10–1000	$y = 0.0088x + 0.0097$	0.9999	3.2
Benzylpenicillin	2	210	3.1	5–500	$y = 0.0074x + 0.0013$	0.9999	0.7
Flucloxacillin	2	210	15.3	5–500	$y = 0.0026x - 0.0004$	0.9950	2.5
Dicloxacillin	2	210	25.2	5–500	$y = 0.0018x - 0.0005$	0.9906	3.7
Ticarcillin	2	210	1.3	10–1000	$y = 0.0086x - 0.0087$	0.9995	1.4

analysis of Ceftazidime, Meropenem, Ceftriaxone and Ampicillin; Group 1B analytes are Cefazolin and Ertapenem; and Group 2 analytes are Cephalothin, Benzylpenicillin, Flucloxacillin, Dicloxacillin, Piperacillin and Ticarcillin.

This method possesses a number of advantageous features, notably in enabling access to a range of analyses with a simple and inexpensive platform. The analytical instrumentation is relatively commonplace compared to mass spectrometry detection or newer UPLC systems. Indeed, whilst the diode array detector used has advantages of peak purity and spectrum matching features, a single wavelength UV detector may be substituted if only a selection of analytes are of interest. The sample preparation is inexpensive, and requires only a small volume of plasma.

2. Experimental

2.1. Reagents and chemicals

Drug material was obtained as formulations for administration: Ceftazidime pentahydrate (Fortum, GlaxoSmithKline Australia), Meropenem trihydrate (Merrem IV, AstraZeneca), Ceftriaxone sodium (DBL Ceftriaxone for Injection, Hospira Australia), Ampicillin sodium (Ampicyn, Aspen Pharmacare Australia), Cefazolin Sodium (Kefzol, Aspen Pharmacare Australia), Ertapenem sodium (Invanz, Merck Sharp and Dohme (Aust.)), Cephalothin (Cephalothin sodium for injection, Mayne Pharma), Benzylpenicillin sodium (BenPen, CSL), Flucloxacillin sodium (Flucil, Aspen Pharmacare Australia), Dicloxacillin sodium (Diclocil Injection, Bristol-Myers Squibb Pharmaceuticals), Piperacillin sodium (DBL Piperacillin and Tazobactam Injection, Hospira Australia), Ticarcillin sodium (Timentin, GlaxoSmithKline Australia), and Cefotaxime (Cefotaxime sodium for injection, Mayne Pharma). Oxacillin sodium salt monohydrate was purchased from Sigma Chemical Company (St Louis, USA). Sodium dihydrogen orthophosphate and orthophosphoric acid were analytical grade, and acetonitrile and chloroform were HPLC grade. 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 two separate but similar Waters-brand systems. The primary system comprised an Alliance 2690 separations module, equipped with quaternary pump, on-line degasser and autosampler, and a 996 photodiode array ultraviolet detector. The secondary system was a 510 pump, a 717+ autosampler and a 996 PDA UV detector. Instrument control, data acquisition and data processing were achieved with Waters Empower software. The flow rate was 1.0 mL/min and the injection volume was 10 μL. The detector monitored wavelengths of 210, 260 and 304 nm depending on analytes (Table 1). The peak height of internal standard was obtained at the same wavelength as the analyte. Detector features of peak purity and spectrum matching were used.

Separations were performed at ambient temperature on a reverse phase Waters X-bridge C18 column (30 mm × 4.6 mm, 2.5 μm silica). One of three isocratic mobile phases (all acetonitrile–phosphate buffer combinations) was used depending on the group of analytes to be determined. Mobile phase for group 1A was acetonitrile (8%) and 50 mM phosphate buffer at pH 2.4 (92%); 1B was acetonitrile (12%) and 50 mM phosphate buffer at pH 2.4 (88%); and group 2 was acetonitrile (25%) and 100 mM phosphate buffer at pH 3.0 (75%). Mobile phases were recycled, i.e. the eluent from the detector was re-introduced back into the mobile phase reservoir. Typical back-pressure was approximately 700 psi.

2.3. Solutions

Analyte antibiotic formulations were dissolved in deionised water then combined to give two combined stocks: 5 μg/mL in Ceftazidime, Meropenem, Ceftriaxone, Ampicillin, Cefazolin and Ertapenem for group 1A and 1B; and 5 μg/mL in Flucloxacillin, Dicloxacillin, Benzylpenicillin and Cephalothin, and 10 μg/mL in Piperacillin and Ticarcillin for group 2. These combined stocks were diluted with blank plasma to give working standards which were stored at −70 °C for up to 6 months. The internal standard for group 1A and 1B was 500 μg/mL cefotaxime. The internal standard for group 2 was 250 μg/mL oxacillin.

Quality control specimens were prepared independently of standards in plasma at three concentration levels (Table 2). Aliquots of QC specimen were stored at −70 °C.

2.4. Sample preparation

Samples for analysis were split into groups 1 or 2 depending on the analyte to be determined, and matching standards, quality controls and internal standard working solutions were used. For each sample, standard and quality control, 200 μL of plasma was placed into a 1.8 mL polypropylene microfuge tube followed by 100 μL of internal standard. Acetonitrile (600 μL) was added to precipitate proteins and the tube vortex mixed for 30 s. Precipitated proteins were separated by centrifugation for 5 min at 14,000 × *g*. The supernatant was removed into a polypropylene tube and 600 μL of chloroform added. The tube was vortex mixed for 30 s to partition the acetonitrile and lipid-soluble plasma components into the chloroform organic phase, and centrifuged for 5 min at 1700 × *g* to separate the layers. An aliquot (100 μL) of the

Table 2
Accuracy and precision for low, medium and high concentration plasma quality controls measured within-day ($n = 10$) and between-day ($n = 10$).

Analyte	Within-day mean (μg/mL) (%CV)			Between-day mean (μg/mL) (%CV)		
	Low	Medium	High	Low	Medium	High
Ceftazidime	6.1 (2.8%)	28 (1.3%)	103 (1.0%)	5.1 (5.8%)	24 (5.1%)	102 (2.7%)
Meropenem	4.5 (2.6%)	24 (4.9%)	85 (1.9%)	4.9 (5.5%)	22 (3.1%)	100 (3.4%)
Ceftriaxone	4.6 (1.8%)	25 (1.6%)	100 (0.5%)	4.8 (3.6%)	24 (3.7%)	99 (2.6%)
Ampicillin	4.8 (1.8%)	26 (1.2%)	103 (0.7%)	5.0 (6.5%)	25 (5.5%)	103 (3.3%)
Cefazolin	5.6 (3.6%)	23 (2.1%)	102 (0.4%)	4.9 (4.8%)	23 (3.0%)	100 (3.6%)
Ertapenem	4.9 (2.1%)	23 (2.5%)	98 (1.2%)	5.1 (4.6%)	25 (4.0%)	102 (3.3%)
Cephalothin	5.3 (2.3%)	24 (1.5%)	96 (1.3%)	5.0 (3.4%)	24 (3.5%)	96 (2.2%)
Benzylpenicillin	5.4 (2.0%)	25 (1.7%)	102 (1.4%)	5.0 (3.7%)	25 (2.9%)	98 (1.6%)
Flucloxacillin	4.4 (1.8%)	24 (2.7%)	98 (1.1%)	4.6 (4.4%)	24 (3.1%)	97 (1.9%)
Dicloxacillin	4.6 (3.7%)	24 (1.4%)	97 (1.5%)	4.8 (4.5%)	24 (4.2%)	95 (3.3%)
Piperacillin	10.1 (2.4%)	45 (1.5%)	182 (1.7%)	10 (2.6%)	50 (2.3%)	194 (3.2%)
Ticarcillin	4.3 (3.0%)	52 (1.7%)	197 (1.2%)	5.0 (3.6%)	50 (3.1%)	188 (2.7%)

upper aqueous phase was transferred to an autosampler vial for 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 FDA's guidelines for bioanalytical method validation [13].

Linearity for each drug was tested by extracting plasma standards spiked at nominal concentrations of 5, 10, 25, 50, 100, 250 and 500 μg/mL (10, 25, 50, 100, 250, 500 and 1000 μg/mL for Piperacillin and Ticarcillin). The calibration line was generated by least squares linear regression of the peak height ratio (PHR) of analyte/internal standard against nominal concentration with a weighting of concentration^{-2}. The percentage deviation from nominal was back-calculated at each standard concentration, with ≤15% as acceptance criterion (≤20% at LOQ) for inclusion in the calibration curve.

The limit of quantification (LOQ) was validated by replicate analysis ($n = 10$) of plasma spiked at 5 μg/mL (10 μg/mL for Piperacillin and Ticarcillin), with precision ≤20% as acceptance criterion.

Precision and accuracy of the assay was assessed on both a within-day and a between-day basis by replicate ($n = 10$) analysis of plasma quality controls at low, medium and high concentration levels, being 5, 25 and 100 μg/mL (10, 50 and 200 μg/mL for Piperacillin and Ticarcillin, note the Ticarcillin low QC was made erroneously low and validation data is extrapolated below LOQ). Concentrations were back-calculated from calibration curves and the precision (%CV) and accuracy (percentage relative to nominal) calculated at each level. Within-day data was collected within a single run, whilst between-day data was collected over ten separate days.

The reproducibility of the assay was additionally investigated by reanalysis of incurred samples. Incurred samples were accumulated over a 4-week period and reanalysed at the conclusion of the period. The '4-6-20' rule was applied, where reproducibility was deemed acceptable if two-thirds (66%) of the samples reassayed to within 20% of their original result.

The stability of spiked calibrator samples held at −70 °C storage was tested by comparison of newly prepared samples with ones aged for 8 months, using five replicates at each of four concentrations.

The stability of the prepared samples in the autosampler was tested by comparing the results for a set of QCs injected after 24 h with those obtained on immediate injection, calculated from the original standard curve.

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

2.6. Application

This method has been in use in a pathology laboratory for therapeutic drug monitoring of beta-lactam antibiotics in critically ill patients. The accuracy and precision of quality controls during routine use has been recorded.

3. Results and discussion

3.1. Chromatography

There was adequate separation from interferences for each analyte under the specified mobile phase and wavelength conditions, as demonstrated in chromatograms for spiked and blank plasma for mobile phases 1A (Fig. 1), 1B (Fig. 2) and 2 (Fig. 3). Typical retention times are displayed in Table 1. Mobile phases 1A and 1B differ only in the amount of acetonitrile present (8% c.f. 12%); the greater percentage of organic in 1B was important to reduce run time and maintain peak shape. Mobile phase 2 (buffered at pH 3) was required for the penicillins as poor retention was observed at pH 2.4 (the pH in mobile phase 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 6-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

Calibration lines were adequately described by linear regression over the concentration range, although the range for Meropenem and Ertapenem was truncated to a maximum of 250 μg/mL (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). In all the standards there was only a single instance of a standard failing the acceptance criteria (i.e. >15% deviation from nominal) and being rejected from the calibration curve. For all analytes the mean absolute percentage deviation of standards was 5% or better, and in no case did an accepted standard deviate from nominal by more than 10%.

The precision of the LOQ standards were also acceptable (all within 8%).

The between- and within-day replicate analysis of quality controls demonstrated a high level of precision and accuracy of the method (Table 2). The within-day accuracy for the Ceftazidime low QC was particularly high, although this was not seen in the between-day data.

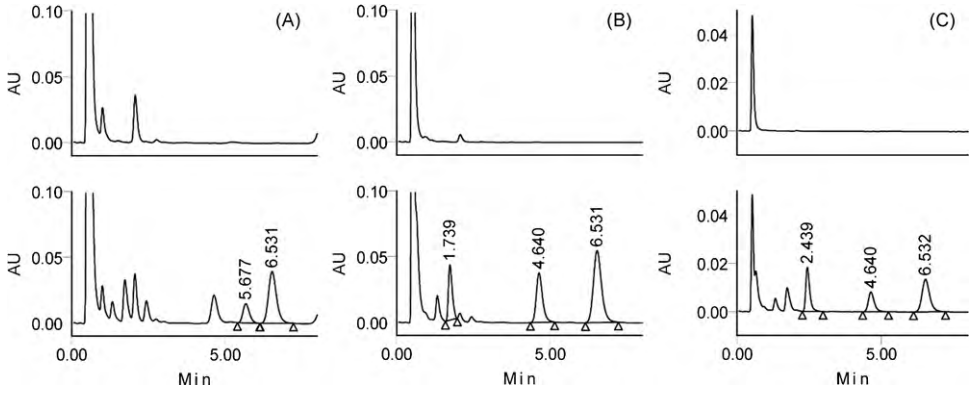

Fig. 1. Mobile phase 1A chromatograms (blank on top, spiked at 25 μg/mL on bottom): Detection at 210 nm for Ampicillin (5.7 min) (A); detection at 260 nm for Ceftazidime (1.7 min) and Ceftriaxone (4.6 min) (B); detection at 304 nm for meropenem (2.4 min) (C). Internal standard is Cefotaxime (6.5 min).

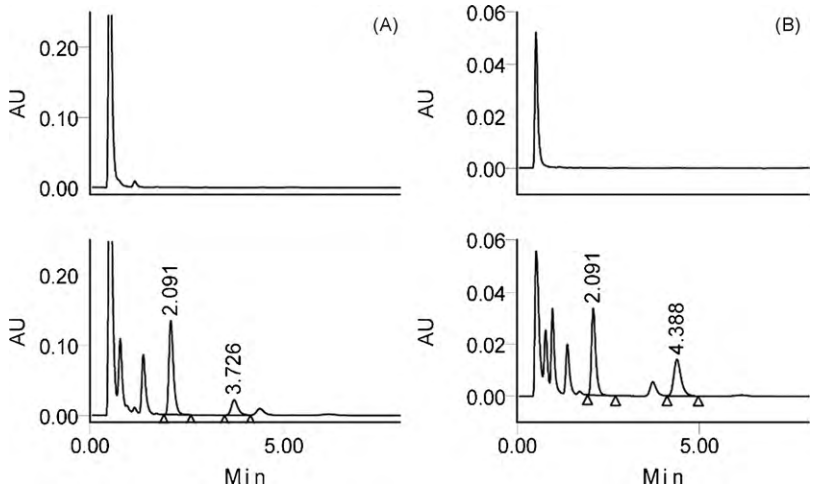

Fig. 2. Mobile phase 1B chromatograms (blank on top, spiked at 25 μg/mL on bottom): Detection at 260 nm for Cefazolin (3.7 min) (A); detection at 304 nm for Ertapenem (4.4 min) (B). Internal standard is Cefotaxime (2.1 min).

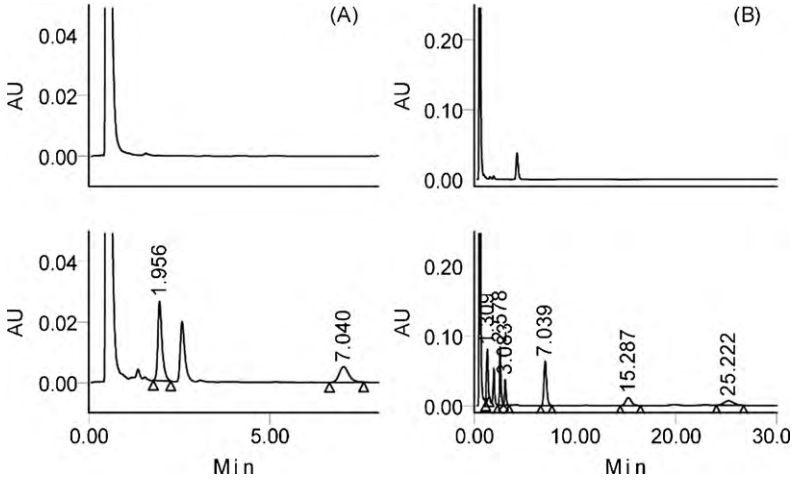

Fig. 3. Mobile phase 2 chromatograms (blank on top, spiked at 25 μg/mL (50 μg/mL for Piperacillin and Ticarcillin) on bottom): detection at 260 nm for Cephalothin (2.0 min) (A); detection at 210 nm for Ticarcillin (1.3 min), Piperacillin (2.6 min), Benzylpenicillin (3.1 min), Flucloxacillin (15.3 min) and Dicloxacillin (25.2 min) (B). Internal standard is Oxacillin (7.0 min).

For incurred sample reanalysis, not all analytes were present in the samples accumulated in the 4-week period. Although the number of reanalyses was relatively low ($n = 21$), all reanalyses were within 20% of the original. The analytes and their mean difference were: Ceftazidime 6% ($n = 1$), Meropenem 14% ($n = 6$), Cephazolin 1% ($n = 3$), Piperacillin 10% ($n = 5$), Benzylpenicillin 0% ($n = 3$) and Flucloxacillin 11% ($n = 4$).

The stability of the analytes in plasma after long term storage (8 months) at −70 °C was good. Averaged over all measurements

for each analyte, the difference between aged and newly prepared samples was no more than 6%.

The stability of the prepared samples in the autosampler, tested over a 24-h period – which exceeded the run time of the batch – was also acceptable, with a maximum difference of 6% seen for all analytes after 24 h aging at room temperature.

3.3. Application

This method has been used routinely over a 6-month period for in excess of 400 samples from critically ill patients. The method has performed solidly in the real-world pathology setting, providing the clinician with beta-lactam levels within 24 hours of sample collection. Over a 6-month period of operation the accuracy and precision of the method was within 6% ($n = 218$ sets of QC results, data not shown). This demonstrates the ruggedness of the method within the hands of four analytical staff and six preparations of mobile phase.

4. Conclusion

The method presented here is the first published to include all the 12 antimicrobials. It has the advantage of simplicity in sample preparation, chromatography and instrumentation, with the only variation in procedure being a selection of internal standard and mobile phase. The performance of the method has been proven by validation and extended use.

References

[1] W.A. Craig, Clin. Infect. Dis. 26 (1998) 1.
[2] J.A. Roberts, J. Lipman, Crit. Care Med. 37 (2009) 840.
[3] S. Harbarth, J. Garbino, J. Pugin, J.A. Romand, D. Lew, D. Pittet, Am. J. Med. 115 (2003) 529.
[4] J.A. Roberts, P. Kruger, D.L. Paterson, J. Lipman, Crit. Care Med. 36 (2008) 2433.
[5] M.H. Kollef, G. Sherman, S. Ward, V.J. Fraser, Chest 115 (1999) 462.
[6] J. Garnacho-Montero, J.L. Garcia-Garmendia, A. Barrero-Almodovar, F.J. Jimenez-Jimenez, C. Perez-Paredes, C. Ortiz-Leyba, Crit. Care Med. 31 (2003) 2742.
[7] V.F. Samanidou, E.N. Evaggelopoulou, I.N. Papadoyannis, J. Sep. Sci. 29 (2006) 1879.
[8] S.R. El-Shaboury, G.A. Saleh, F.A. Mohamed, A.H. Rageh, J. Pharm. Biomed. Anal. 45 (2007) 1.
[9] T. Legrand, S. Chhun, E. Rey, B. Blanchet, J.R. Zahar, F. Lanternier, G. Pons, V. Jullien, J. Chromatogr. B 875 (2008) 551.
[10] R. Denooz, C. Charlier, J. Chromatogr. B 864 (2008) 161.
[11] C.C. Hong, F. Kondo, J. Food Prot. 60 (1997) 1006.
[12] L.K. Sorensen, L.K. Snor, T. Elkaer, H. Hansen, J. Chromatogr. B 734 (1999) 307.
[13] Guidance for Industry, Bioanalytical Method Validation, US Department of Health and Human Services, Food and Drug Administration, 2001.