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Rapid quantification of six β-lactams to optimize dosage regimens in severely septic patients

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ABSTRACT

A fast analytical procedure was developed for the simultaneous quantification of cefepime (CEF), meropenem (MEM), ceftazidime (CZA), cefuroxime (CFX), aztreonam (AZT), and piperacillin (PIP) in serum of intensive care patients. The β-lactam pharmacokinetic parameters can be altered in severe sepsis due to changes in the distribution, the metabolism and the elimination process. Therapeutic drug monitoring (TDM) of β-lactams is therefore recommended in critically ill patients. The plasma samples were spiked with cefoperazone as internal standard and proteins were precipitated with methanol. The different β-lactams were separated with high performance liquid chromatography within 18 min, and quantified by UV spectrophotometry with a diode array detector. The method was validated by means of the accuracy profile approach based on β expectation tolerance intervals. The acceptance limits were settled at ±30% according to the regulatory requirements. Assay validation demonstrated good performance for all β-lactams analyzed in terms of trueness, repeatability, linearity and intermediate precision over the range of 2–200 μg/mL. The simple extraction procedure provides respective absolute and relative recoveries ranging from 70% to 86% and from 66% to 89% for all the β-lactams analyzed. Few interferences were observed and the method was easily applicable to TDM in intensive care patients. The quantification of β-lactams should allow for antibiotic regimen adjustment in critically ill patients.

1. Introduction

The β-lactams are hydrophilic compounds weakly bound to plasma proteins, with a low volume of distribution, that are excreted by the kidneys [1]. Their pharmacokinetics (PK) can be described by a two-compartment open model. After intravenous administration of a single dose, β-lactam concentrations decline rapidly with a distribution half-life ranging from 0.18 to 0.20 h [2,3]. Then, plasma concentrations decrease more slowly and the elimination rate becomes monoexponential. Different PK parameters (volume of distribution, total body clearance, first-order elimination rate constant: k_e) can be calculated during the monoexponential decline of plasma concentrations according to the model initially described by Sawchuk and Zaske [4].

Severe sepsis or septic shock alters the β-lactam PK principally through increases in apparent volume of distribution due to changes in body compartments (increased capillary permeability, fluid resuscitation) and increased cardiac output [5,6]. The metabolism and elimination process can also be affected in critically ill patients. They may present hepatic and/or renal failure [7]. Other patients may present augmented renal clearance, where creatinine clearances are increased despite normal serum creatinine levels [8]. Because it is currently impossible to predict PK parameters in critically ill patients, and because there is great inter- and intra-variability of these parameters [7,9,10], standard dosage regimens are often insufficient to achieve optimal serum concentrations. Therapeutic drug monitoring for antibiotics is therefore recommended for such patients to optimize efficacy of antibiotics, to reduce selection of resistant mutants, and to reduce potential concentration related adverse effects [11]. The best relevant parameter indicating β-lactam efficacy is the time plasma antibiotic levels remain above the minimum inhibitory concentration (MIC) of the given pathogen [12,13]. The maximal bactericidal effect is reached when β-lactam concentrations remain above the MIC of the given pathogen during 40%, 50% and 60–70% of the dose interval for carbapenems, penicillins and cephalosporins, respectively [14–16]. Depending on the type of pathogen, clinical breakpoints have been defined by the European

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Committee on antimicrobial susceptibility testing (EUCAST) [17]. Different in vitro [18,19] and animal studies [20] have shown that β-lactams bactericidal activity reaches a maximal value at four or five times the MIC. It is therefore interesting to report time spent by the β-lactam concentrations above a level of four times the breakpoint to achieve optimal bactericidal activity. For *Pseudomonas aeruginosa*, breakpoints defined by EUCAST are 16 μg/mL for aztreonam (AZT), 2 μg/mL for meropenem (MEM), 8 μg/mL for cefepime (CEF) and ceftazidime (CZA) and 16 μg/mL for piperacillin (PIP) associated to tazobactam [23]. For *Enterobacteriaceae*, a breakpoint of 8 μg/mL was chosen as the target MIC for cefuroxime (CFX). *Pseudomonas aeruginosa* and *Enterobacteriaceae* are the most frequent and most resistant gram negative pathogens encountered in intensive care unit patients (ICU).

Various methods to quantify β-lactams in human plasma or biological fluids using high performance liquid chromatography with UV (LC-UV) or mass-spectrometry detection have been developed and validated. They employed different extraction processes including solid phase extraction [21,22], ultrafiltration [23] and methanol/acetonitrile precipitation [24,25]. Although the absolute recoveries obtained with solid phase extraction were reasonably good, ranging from 57.4% to 83.4% [21] and from 66.4% to 102% [22] depending on the β-lactam analyzed, the main problem encountered with this procedure was the high plasma volume required and the cost. Ultrafiltration was another effective extraction method providing good recoveries and the measure of the free fraction of the drug but it was very expensive [23]. The mass spectrometric detection had the advantage of being a fast, specific and sensitive technique for the simultaneous determination of many β-lactams [26], but it could not be implemented in all laboratories due to the high cost and the special equipment required.

The main objective of our study was to optimize and validate a simple LC-UV method allowing the simultaneous quantification of six antibiotics currently used in our intensive care units: CEF, MEM, CZA, CFX, AZT and PIP. The extraction process employed was a methanol precipitation. The quantification of the β-lactams analyzed was performed by UV detection at three different wavelengths.

The criteria of analytical performance (trueness, repeatability, intermediate precision and limit of quantification) were assessed by means of a recent validation strategy based on accuracy profiles with β-expectation tolerance intervals [27–30]. The second objective was to apply this technique to routine monitoring of β-lactam levels in intensive care patients.

2. Methods and material

2.1. Reagents and chemicals

CEF and AZT were purchased from Bristol-Myers (Braine-l'Alleud, Belgium), MEM from AstraZeneca (Brussels, Belgium), CFX and CZA from GlaxoSmithKline (Wavre, Belgium), PIP from Wyeth Pharmaceuticals (Louvain-la-Neuve, Belgium) and Cefoperazone was obtained from Fluka. Acetonitrile (ACN) and methanol (MeOH) were purchased from Biosolve (Valkenswaard, The Netherlands), sodium dihydrogenophosphate (NaH2PO4.H2O) from VWR. All reagents were analytical grade. Ultra-pure water was obtained by means of a Milli-Q water purification system (Millipore, Brussels, Belgium).

A pool of human drug-free serum was prepared with blood from volunteers having given their informed consent in accordance with the Ethics Committee of Erasme Hospital (reference number: P2011/028).

2.2. Chromatographic system

The separation and quantification of β-lactams were conducted using an Agilent 1200 HPLC system (Agilent Technologies, Diegem, Belgium) equipped with a quaternary pump, a degassing line, a thermostated autosampler, a column oven and a photodiode array detector. Samples were thermostated at 4 °C. The HPLC separation was performed at 35 °C using an YMC ODS AQ (AIT, France) column (250 mm × 4.6 mm) packed with 5 μm diameter particles and protected with a Phenomenex C18 guard column (4 mm × 3 mm). The volume of injection was 40 μL. The mobile phase consisting in acetonitrile and phosphate buffer 0.05 M, pH 3.8 was delivered at a flow rate of 1.2 mL/min according to the following gradient: at 0 min, the mobile phase contained 8% acetonitrile; from 0 to 7.0 min, the percentage of acetonitrile linearly increased to 10.3%; from 7.0 to 7.1 min, the percentage of acetonitrile increased to 22% and was maintained at this percentage until 12.0 min; from 12.0 to 12.1 min, the percentage of acetonitrile increased to 30%; from 12.1 to 15.0 min, the percentage of acetonitrile increased to 35% and was maintained until 18.0 min; from 18.0 to 18.1 min, the percentage of acetonitrile returned to the initial conditions and was maintained at 8% until 21.0 min. The column eluent was monitored spectrophotometrically in the 200–380 nm range using the Agilent 1200 photodiode array detector. On the basis of the absorbance spectra of each β-lactam, AZT and PIP were monitored at 240 nm while CEF, CZA, CFX and cefoperazone were measured at 260 nm and MEM at 300 nm. The purity of each peak was confirmed for all samples by comparison of spectra with a library built in the ChemStation program (Agilent Technologies, Diegem, Belgium) from pure substances.

2.3. Preparation of stock solutions, calibration and validation standards

Standard stock solutions of CEF, MEM, CZA, CFX, AZT, PIP and cefoperazone used as internal standard (IS) were prepared by dissolving respective powdered antibiotics in purified water at a final concentration of 40 mg/mL. All the stock solutions (standards and IS) were stored at −80 °C and were stable at this temperature for at least 12 months.

Calibration (CS) and validation (VS) standards were prepared by diluting the stock solutions in a pool of drug-free sera at final concentrations of 2, 5, 20, 50, 100 and 200 μg/mL.

In routine analysis, quality control samples were prepared by diluting stock solutions in a pool of drug-free sera to achieve final antibiotic concentrations of 10 μg/mL and 75 μg/mL, respectively.

2.4. Sample preparation

Blood samples were collected without any anticoagulant, immediately chilled and centrifuged at 4000 rpm for 10 min. Sera were frozen at −80 °C until analysis. Fifty microliters of the IS working solution (200 μg/mL) were added to 200 μL of sample. Controls, CSs and VSs were treated using the same pre-treatment protocol as the samples. Serum protein precipitation was performed by adding 400 μL of methanol. The mixture was vortex-mixed for 30 s and centrifuged at 4 °C for 10 min at 4000 rpm. The solution was then evaporated with a SpeedVac SVC 100 centrifugal evaporator (Savant, Belgium) and the residue was reconstituted with 200 μL of phosphate buffer 0.05 M pH of 2.5. The resulting solutions were transferred into HPLC microvials and 40 μL were injected in the HPLC system.

2.5. Specificity

Interference experiments were run accordingly to the National Committee for Clinical Laboratory Standards (NCCLS) recommendations [31] with regard to antibiotics frequently co-administered with the six β-lactams analyzed: vancomycin, azithromycin, tobramycin, amikacin, clarithromycin, moxifloxacin, ciprofloxacin, colimycin and ampicillin.

An aqueous solution of the potential interfering substance was added to CSs containing β-lactams at 20 μg/mL and 100 μg/mL to yield a final concentration in the clinical range. The added volume of interfering compound solution never exceeded 10% of total volume to minimize the dilution of the CSs. Vancomycin, azithromycin, clarithromycin, moxifloxacin, ciprofloxacin and ampicillin were tested at a final concentration of 20 μg/mL. Interferences from tobramycin, amikacin and colimycin were assessed at final concentrations of 2 μg/mL and 2000 I.E./mL, respectively. Paired CSs containing the same dilution with Milli-Q water were analyzed simultaneously. All samples were analyzed in duplicate to minimize random error. The difference between mean concentrations measured in paired samples was defined as the observed error. The decision of acceptability was made by comparing the observed error with the acceptable error according to the Clinical Laboratory Improvement Amendments (CLIA) proficiency testing criteria for acceptable performance (i.e. for toxicology analyses: 25%).

2.6. Relative and absolute recoveries

The relative recoveries (%) of β-lactams were calculated in triplicate at two concentration levels (5 and 200 μg/mL) by dividing the analyte peak height ratio obtained from extracted spiked sera with those obtained after extraction of water spiked at the same concentration.

The absolute recoveries (%) of β-lactams were assessed in triplicate at two concentration levels (5 and 200 μg/mL) by dividing the ratios of analyte peak height of calibration standards subjected to methanol precipitation compared with spiked aqueous solutions at the same levels not subjected to the extraction procedure. Absolute recoveries were determined by testing methanol versus acetonitrile as solvent for protein precipitation.

2.7. Method validation

A novel validation strategy using accuracy profiles was used to evaluate the reliability of the results according to the Société Française des Sciences et Techniques Pharmaceutiques validation guidelines [27]. This predictive methodology is based on defining a two-sided β-expectation tolerance interval in which each future result will fall with a defined probability (β). In our study, β-expectation tolerance limit was fixed at 95% and the acceptability limit at ±30% according to the regulatory requirement [32]. Calibration standards (CSs) were assessed at six different levels (2, 5, 20, 50, 100 and 200 μg/mL), three times during three consecutive days. The validation standards (VSs) were analyzed independently at six different levels in quadruplicate during three consecutive days. The relationship between detector response (CSs/IS peak height ratio) and concentration of each β-lactam was assessed. Several mathematical models (linear, weighted linear $1/X$, quadratic, weighted quadratic $1/X$) were used for evaluating the response function. The concentrations of VSs were calculated using the calibration lines. The tolerance interval was computed for each VSs level using its bias and estimated standard deviation. The accuracy profiles obtained between the upper and lower tolerance limits were plotted in order to select the most appropriate calibration model for each β-lactam. The method was considered valid as long as the β expectation tolerance interval profile remained within the acceptability limits for each concentration. Validation parameters including linearity, precision, accuracy and limits of quantification were investigated according to this predictive methodology [27].

2.8. Application to pharmacokinetic studies

Therapeutic drug monitoring for CEF, MEM, CZA and PIP was performed in septic patients. Samples were drawn before administration (t_0) and at least 60 min after the end of a 30 min infusion (t_2). The exact sampling time and the dosage administered of the antibiotic were recorded. The drug concentration was measured in both samples. As sampling was performed during the elimination phase, the following equation [$\ln C_t: -k_e.t+\ln C_0$] was used to estimate the serum concentration of the drug at one given time [33]. On the basis of the dose interval, the time (in h or %) spent above four times the clinical breakpoint was estimated.

This pharmacokinetics model was validated in 32 ICU patients receiving standard dosage regimens of β-lactams adapted to renal function calculated with the Cockroft-gault equation [7]. Four patients received 2 g of CEF two times daily (BID), 14 patients received 1 g of MEM three times daily, 3 patients received 2 g of CZA two times daily (BID), 11 patients received 4 g of PIP four times daily. Blood samples were drawn before dosing and 1, 2, and 5 h after the end of antibiotic infusion. An additional sample was collected between the sixth and eighth hours following the IV administration for CZA, CEF and MEM. The $t_{1/2}$ calculated as $0.693/k_e$ and the time (in %) spent above four times the clinical breakpoint were estimated for each patient. These pharmacokinetics data calculated on the basis of four (for PIP) or five blood samples drawn were compared with those calculated using two blood samples.

3. Results

3.1. Chromatographic conditions

The absence of compounds co-eluting with CEF, MEM, CZA, CFX, AZT, PIP and cefoperazone in the pool of human drug-free serum was monitored chromatographically.

Fig. 1 illustrate chromatograms of an extracted blank serum sample (a) and an extracted calibration standard with a final antibiotic concentration of 50 μg/mL (b). Under the conditions described, the peaks corresponding to each β-lactam and IS were resolved with retention times of 6.22, 7.31, 8.68, 9.38, 12.03, 13.71 and 16.90 min for CEF, MEM, CZA, CFX, AZT, IS, and PIP, respectively.

3.2. Specificity

None of the nine antibiotics (vancomycin, azithromycin, tobramycin, amikacin, clarithromycin, moxifloxacin, ciprofloxacin, colimycin, ampicillin) co-administered with the β-lactams interfered with the chromatographic quantification of CEF, MEM, CZA, CFX, AZT and PIP. The difference between the CSs at 20 and 100 μg/mL and the ones spiked with potential interfering substances never exceeded the acceptable error (Data not shown).

3.3. Extraction procedure

Absolute and relative recoveries obtained for each β-lactam are summarized in Table 1. They were well reproducible at the concentrations tested (5 and 200 μg/mL) and ranged from 70% to 86% and from 66% to 89%, respectively. The mean (±SD) absolute

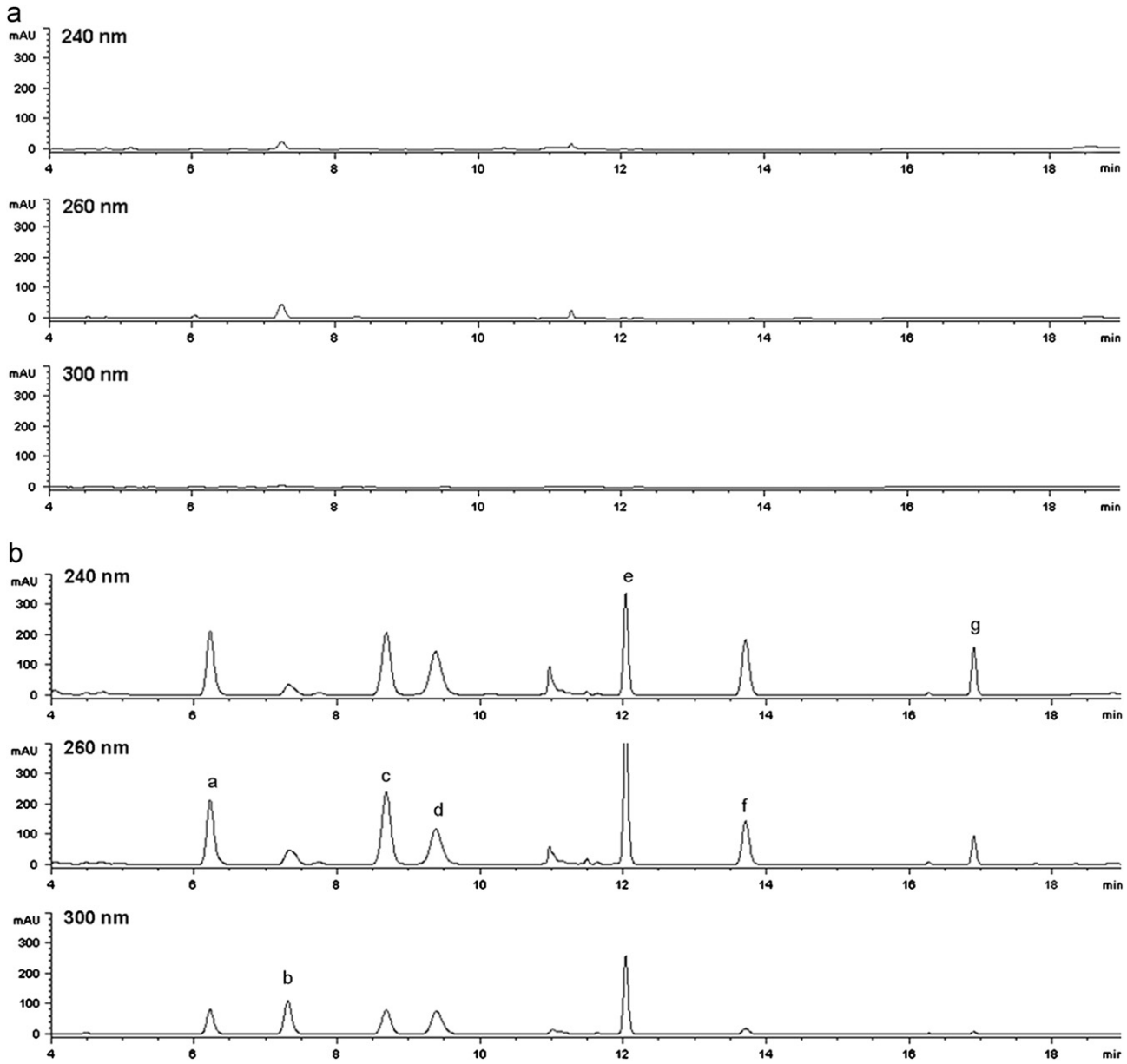

Fig. 1. Chomatograms of an extracted blank serum sample (a) and an extracted calibration standard containing a final antibiotic concentration of 50 μg/mL (b). Cefepime (a), meropenem (b), ceftazidime (c), cefuroxime (d), aztreonam (e), cefoperazone (f) and piperacillin (g) were separated within 18 min and monitored at three different wavelengths (240, 260 and 300 nm) with a photodiode array detector.

Table 1
Means of absolute (AR) and relative (RR) recovery (standard deviation) obtained for the six β-lactam antibiotics analyzed at two different concentrations (5 and 200 μg/mL).

β-lactam antibiotic	Nominal concentration (μg/mL)	AR (%) – SD	RR (%) – SD
Cefepime	5	73 – 5	66–5
	200	70 – 3	67 – 5
Meropenem	5	73 – 6	89 – 7
	200	71 – 5	84–8
Ceftazidime	5	76 – 4	73–5
	200	72 – 2	70 – 5
Aztreonam	5	76 – 3	76–4
	200	76 – 3	72 – 6
Cefuroxime	5	86 – 5	85–4
	200	84 – 3	76–5
Piperacillin	5	82 – 4	87 – 4
	200	77 – 2	75 – 5

recovery obtained for the IS analyzed in triplicate was 87% (± 2.7). The absolute recoveries obtained with acetonitrile versus methanol precipitation were quite similar, except for MEM for which slightly lower recoveries were obtained (Data not shown).

3.4. Method validation

Accuracy profiles were plotted to select the most suitable regression model for the intended use of the analytical method. For each β-lactam, the best results were obtained with a $1/X$ weighted quadratic regression. Accuracy profiles obtained for each antibiotic analyzed are shown in Fig. 2 with acceptance limits set at ± 30%. The β-expectation tolerance interval profiles remained within the acceptance limits for each β-lactam studied in the 2–200 μg/mL concentration range.

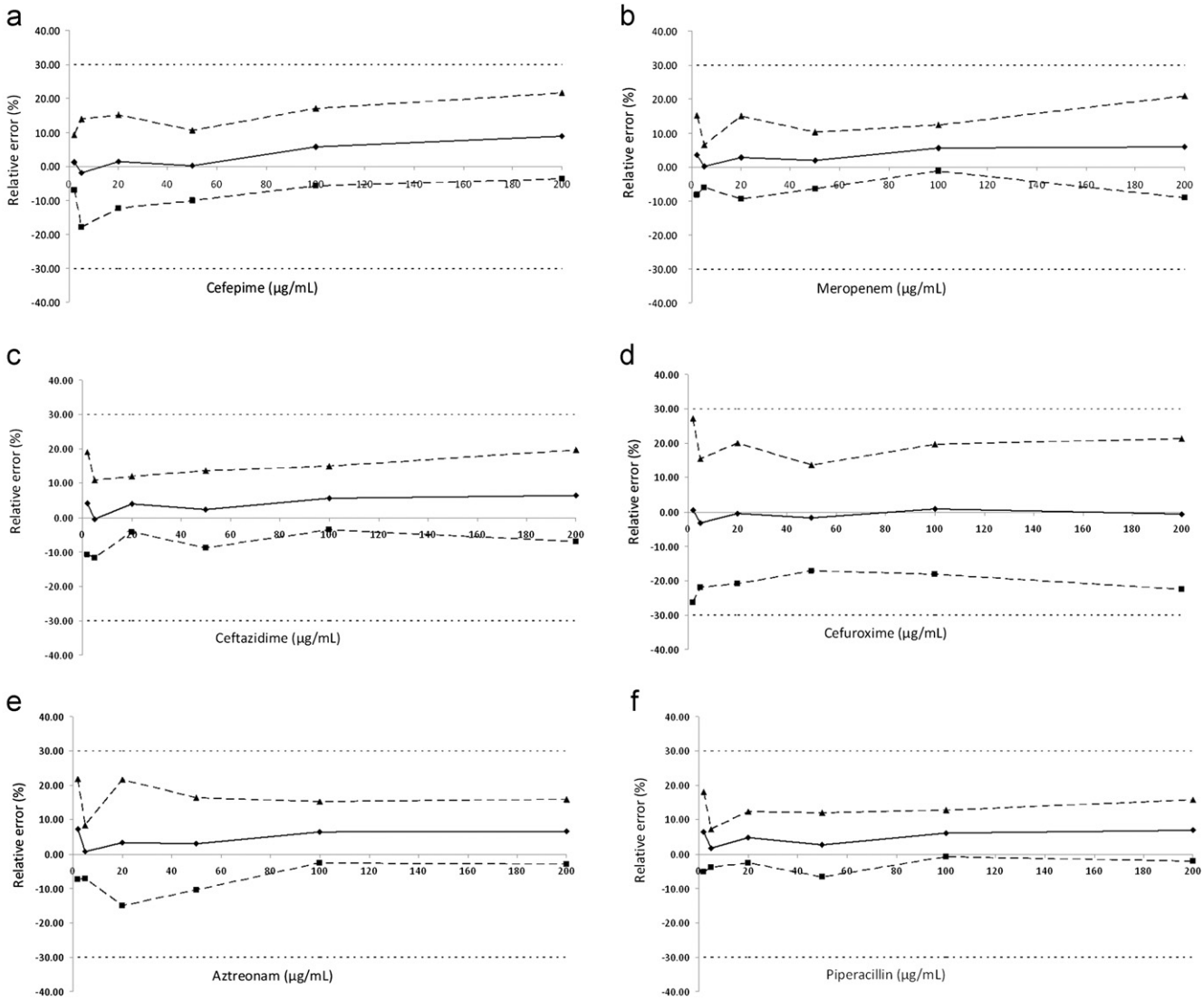

Fig. 2. Accuracy profiles obtained for CEF (a), MEM (b), CZA (c), CFX (d), AZT (e) and PIP (f). The acceptance limits (dotted lines) have been set at ±30%. The plain line represents the relative bias and the dashed lines are the β expectation tolerance limits.

3.4.1. Trueness

The relative bias was less than 9% for all levels of each β-lactam quantified in the validated concentration range (2–200 µg/mL) (Table 2).

3.4.2. Linearity

The slope (95% confidence intervals) of the relationship obtained between calculated and introduced concentrations was reported for each β-lactam in Table 2.

3.4.3. Precision

Precision is estimated using the coefficient of variation for repeatability and the intermediate precision at each concentration level of validation standards. The coefficient of variation for repeatability or intermediate precision estimated at six levels of concentration for each β-lactam never exceeded 11% in the validated concentration range of concentration (2–200 µg/mL).

3.4.4. Accuracy

The respective accuracy profiles obtained for each β-lactam did not exceed the acceptance limits (30%) in the range 2–200 µg/mL. The HPLC method used was therefore accurate over this range.

3.4.5. Limits of quantification

The lower and upper limits of quantification for each β-lactams analyzed were 2 and 200 µg/mL, respectively.

3.5. Pharmacokinetic studies in ICU patients

The elimination half-life and the time (in %) spent above four times the clinical breakpoint calculated on the basis of four (PIP) or five concentrations (CEF, MEM, CZA) were comparable with those obtained from two blood samples drawn during the elimination phase. Results are summarized in Table 3. The median elimination half-life calculated for the 32 ICU patients were systematically higher than those published for healthy volunteers (i.e. 1.6 h for CZA, 0.8 h for PIP, 0.9 h for CEF and 1 h for MEM) [2,34–36] and ranged from 4.9 to 9.2 h, 1.7 to 6.4 h, 3.7 to 7.4 h, and 2.6 to 9.6 h for CZA, PIP, CEF and MEM, respectively.

The time (in %) the serum concentration of the antibiotic spent above four times the clinical breakpoint was similar when estimated on the basis of two or four/five samples drawn during the elimination process. The pharmacokinetic profiles of CEF, MEM, CZA and PIP obtained for respectively 4, 14, 3 and 11 ICU patients are depicted in Fig. 3.

Table 2
Validation criteria obtained with the accuracy profiles (relative bias, repeatability, intermediate precision, β-expectation intervals and linearity).

Validation criteria	Cefepime	Meropenem	Ceftazidime	Aztreonam	Cefuroxime	Piperacillin
Response function	Weighted (1/*X*) quadratic regression					
Trueness	**Mean concentration (μg/mL)/relative bias (%)**					
Concentration: 2 μg/mL	2.02/1.16	2.07/3.48	2.08/4.19	2.14/7.24	2.01/0.50	2.13/6.41
Concentration: 5 μg/mL	4.90/−1.96	5.01/0.19	4.98/−0.47	5.03/0.68	4.84/−3.19	5.08/1.64
Concentration: 20 μg/mL	20.29/1.43	20.56/2.81	20.79/3.94	20.67/3.36	19.9/−0.48	20.97/4.87
Concentration: 50 μg/mL	50.11/0.21	50.55/1.90	51.23/2.45	51.53/3.06	49.15/−1.71	51.34/2.68
Concentration: 100 μg/mL	105.7/5.7	105.54/5.54	105.72/5.72	106.43/6.43	100.87/0.87	106.06/6.06
Concentration: 200 μg/mL	217.84/8.92	211.75/5.88	212.85/6.42	213.16/6.58	198.82/−0.59	213.83/6.92
Precision	**CV repeatability (%)/ CV intermediate precision (%)**					
Concentration: 2 μg/mL	2.96/2.96	3.17/4.80	3.25/6.03	3.23/5.90	2.07/10.55	2.61/4.72
Concentration: 5 μg/mL	4.01/6.49	2.45/2.67	4.95/4.95	2.25/3.21	2.94/7.47	2.31/2.41
Concentration: 20 μg/mL	4.31/5.73	3.89/5.07	2.93/3.42	2.87/7.31	4.42/8.27	2.62/3.14
Concentration: 50 μg/mL	4.65/4.65	3.71/3.71	3.83/4.71	4.07/5.58	2.22/6.10	3.17/3.91
Concentration: 100 μg/mL	4.93/4.96	3.20/3.02	3.18/3.90	4.34/3.97	2.43/7.51	2.85/2.93
Concentration: 200 μg/mL	4.58/5.33	3.95/6.14	3.57/5.46	3.36/3.96	6.45/9.05	3.62/3.84
Accuracy	**Lower β−expectation limit (%)/Upper β−expectation limit (%)**					
Concentration: 2 μg/mL	−6.96/9.28	−8.20/15.15	−10.71/19.09	−7.31/21.8	−26.21/27.21	−5.23/18.06
Concentration: 5 μg/mL	−17.85/13.94	−6.03/6.41	−11.76/10.83	−7.08/8.44	−21.90/15.51	−3.91/7.21
Concentration: 20 μg/mL	−12.34/15.21	−9.35/14.97	−4.14/12.01	−14.96/21.68	−20.92/19.97	−2.55/12.30
Concentration: 50 μg/mL	−10.16/10.59	−6.48/10.27	−8.76/13.66	−10.39/16.50	−17.02/13.60	−6.63/11.98
Concentration: 100 μg/mL	−5.67/17.07	−1.27/12.35	−3.56/15.01	−2.46/15.33	−18.03/19.77	−0.69/12.80
Concentration: 200 μg/mL	−3.66/21.5	−9.11/20.86	−6.88/19.72	−2.79/15.95	−22.48/21.30	−2.01/15.84
Linearity	**Slope (95% confidence intervals)**					
	1.092 (1.074−1.109)	1.061(1.044−1.078)	1.066(1.051−1.082)	1.068(1.055−1.081)	0.9964(0.971−1.022)	1.071(1.059−1.083)

Table 3
Elimination half-life and time (%) spent above four times the clinical breakpoint estimated on the basis of four/five blood samples versus two blood samples drawn during the elimination process.

Patients	β-lactam administered	Dosage (g/d)	PK parameters estimated with 4/5 samples drawn after the onset of infusion		PK parameters estimated with two samples drawn after the onset of infusion	
			$t_{1/2}$ (h)	$t > 4 \times$ MIC (%)	$t_{1/2}$ (h)	$t > 4 \times$ MIC (%)
1	CEF	2 × 2	7.4	0.0	5.1	0.0
2	CEF	2 × 2	3.7	22.4	7.7	24.3
3	CEF	2 × 2	5.0	1.0	3.5	0.0
4	CEF	2 × 2	7.1	43.0	5.2	39.0
5	MEM	1 × 3	9.6	100.0	11.1	100.0
6	MEM	1 × 3	3.1	59.3	3.2	59.6
7	MEM	1 × 3	3.8	48.4	4.2	40.0
8	MEM	1 × 3	3.3	98.9	3.4	99.4
9	MEM	1 × 3	4.9	100.0	5.4	100.0
10	MEM	1 × 3	3.7	90.3	4.1	93.7
11	MEM	1 × 3	4.3	75.0	5.0	78.1
12	MEM	1 × 3	5.2	82.0	7.1	93.7
13	MEM	1 × 3	4.8	100.0	7.2	100.0
14	MEM	1 × 3	4.3	100.0	5.0	100.0
15	MEM	1 × 3	6.9	100.0	6.9	100.0
16	MEM	1 × 3	7.9	100.0	8.8	100.0
17	MEM	1 × 3	2.6	81.7	2.7	81.5
18	MEM	1 × 3	7.1	100.0	7.3	100.0
19	CAZ	2 × 2	4.9	78.2	5.1	81.7
20	CAZ	2 × 2	7.1	100.0	10.1	100.0
21	CAZ	2 × 2	9.2	99.7	11.4	100.0
22	PIP	4 × 4	1.7	38.0	1.3	43.0
23	PIP	4 × 4	4.6	100.0	5.8	100.0
24	PIP	4 × 4	3.9	95.0	4.2	96.0
25	PIP	4 × 4	2.1	25.5	2.3	20.5
26	PIP	4 × 4	3.3	7.2	3.8	0.0
27	PIP	4 × 4	4.1	100.0	4.1	100.0
28	PIP	4 × 4	6.4	100.0	4.8	100.0
29	PIP	4 × 4	4.2	88.6	4.0	88.6
30	PIP	4 × 4	3.8	100.0	4.1	100.0
31	PIP	4 × 4	6.3	86.7	6.6	82.7
32	PIP	4 × 4	4.3	100.0	4.7	100.0

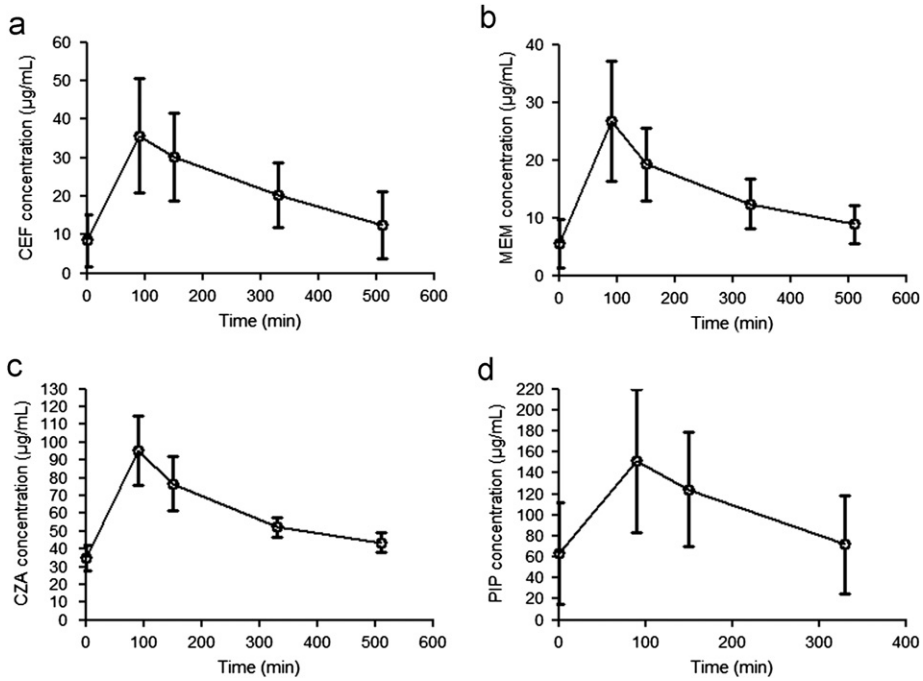

Fig. 3. Mean (± standard error of the mean) β-lactam serum concentration-time curves of CEF (a), MEM (b), CZA (c) and PIP (d) after a 30 min infusion.

4. Discussion

We developed a LC-UV method for quantifying the total serum concentration of six β-lactams used in ICU. These β-lactams are compound with a high hydrophilicity. They are weakly bound to plasma proteins (17%, 7%, 10%, 56%, 30% and 20% for CEF, MEM, CZA, CFX, AZT and PIP, respectively) [37] and the measurement of free fraction is not needed.

Different sample preparation procedures were described in the literature including solid phase extraction [21,22], ultrafiltration [23] or solvent deproteination [24,25]. The process of solvent deproteination with methanol was chosen as it required a low sample volume and was methodologically simple.

Sample preparation was performed with methanol versus acetonitrile precipitation. The absolute recoveries obtained as well as the chromatogram of extracted blank sera were quite similar between the two kinds of procedures with a slight but significant increase of absolute recoveries obtained for MEM with the methanol precipitation. Since acetonitrile was currently much more expensive than methanol, the methanol protein precipitation was chosen offering an opportunity to reduce solvent cost while maintaining similar absolute recoveries.

The YMC-ODS AQ column allowed a prolonged retention of hydrophilic compounds with an improvement of the separation of the β-lactams. This C18 reversed phase column displays both a hydrophobic high carbon loading and a relatively hydrophobic surface allowing a better retention of polar organic compounds that tend to be unretained on ordinary C18 columns. The separation was performed at 35 °C to standardize the conditions without risking of degradation of thermolabile β-lactams.

All six β-lactams and IS were separated within 17 min and monitored at three different wavelengths (240, 260 and 300 nm) with a diode array UV detector allowing the confirmation of peak purity.

A recent validation strategy based on accuracy profiles with acceptance limits set at 30% was applied and demonstrated the reliability of the technique for quantifying CEF, MEM, CZA, CFX, AZT and PIP in the 2–200 µg/mL range. Criteria of analytical performance were not evaluated for lower concentrations as reporting values lower than 2 µg/mL provides few interest to monitor β-lactam levels in ICU patients. Indeed, therapeutic targets are 8 µg/mL for MEM, 32 µg/mL for CEF and CZA and 64 µg/mL for AZT and PIP.

The method presented some improvements compared with previously published methods. Denooz et al. used a solid phase extraction procedure requiring a sample volume of 500 µL and showing recoveries from 57 to 85%. Our extraction procedure was cheaper and simpler, required 200 µL only and gave absolute recoveries from 70 to 86%. Furthermore, six β-lactams were separated chromatographically instead of five and run times were significantly shorter (18 versus 23 min). [21].

Others antibiotics (vancomycin, azithromycin, tobramycin, amikacin, clarithromycin, moxifloxacin, ciprofloxacin, colimycin, ampicillin) commonly co-administered with β-lactams did not interfere in the assay.

The technique was successfully applied to clinical samples. Marked interindividual variations of β-lactam PK have been highlighted in ICU patients, emphasizing the importance of performing therapeutic drug monitoring [7,10]. Critical illness can modify β-lactam PK parameters through an increase in apparent volume of distribution and a decrease in the total clearance [1]. Moreover, continuous renal replacement therapies, frequently applied in severe sepsis, additionally makes β-lactam PKs unpredictable with a risk of emergence of resistant strains due to insufficient antibiotic concentrations [7]. Although it is well known that β-lactams efficacy is closely related to the time that concentrations remain above the MIC [38], there is still debate about the precise concentration target. Based on in vitro [18,19] and animal studies [20], it has been shown that β-lactam concentrations should be at least four or five times above the MIC during 40%, 50% and 60–70% of the dose interval for carbapenems, penicillins and cephalosporins, respectively to control serious infections [14–16]. By collecting two blood samples during the elimination phase, knowing the exact sampling time and dosage, the time spent above the clinical breakpoint could be easily estimated and the dose adjusted when needed.

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References

[1] R. Mehrotra, R. De Gaudio, M. Palazzo, Int. Care Med. 30 (2004) 2145–2156.
[2] D.K. Sommers, L. Walters, M. Van Wyk, S.M. Harding, AM. Paton, J. Ayrton, Antimicrob. Agents Chemother. 23 (1983) 892–896.
[3] H. Mattie, Clin. Pharmacokinet. 28 (1994) 99–106.
[4] R.J. Sawchuk, D.E. Zaske, J. Pharmacokinet. Biopharm. 4 (1976) 183–195.
[5] A. Bodenham, M.P. Shelly, G.R. Park, Clin. Pharmacokinet. 14 (1988) 347–373.
[6] P. De Paepe, F.M. Belpaire, W.A. Buylaert, Clin. Pharmacokinet. 41 (2002) 1135–1151.
[7] L. Seyler, F. Cotton, F.S. Taccone, D. De Backer, P. Macours, J.L. Vincent, F. Jacobs, Crit. Care 15 (2011) R137.
[8] A.A. Udy, J.A. Roberts, R.J. Boots, D.L. Paterson, J. Lipman, Clin. Pharmacokinet. 49 (2010) 1–16.
[9] T.M. Chapuis, E. Giannoni, P.A. Majcherczyk, R. Chioléro, M.D. Schaller, M.M. Berger, S. Bolay, L.A. Décosterd, D. Bugnon, P. Moreillon, Crit. Care 14 (2010) R51.
[10] F.S. Taccone, M. Hites, M. Beumier, S. Scolletta, F. Jacobs, Curr. Infect. Dis. Rep. 13 (2011) 406–415.
[11] F. Scaglione, J. Antimicrob. Agents 19 (2002) 349–353.
[12] J.W. Mouton, M.N. Dudley, O. Cars, H. Derendorf, GL. Drusano, J. Antimicrob. Chemother. 55 (2005) 601–607.
[13] J.D. Turnidge, Clin. Infect. Dis. 27 (1998) 10–22.
[14] G.L. Drusano, Nat. Rev. Micro. 2 (2004) 289–300.
[15] J.A. Roberts, J.D. Paratz, J. Lipman, Crit. Care Med. 36 (2008) 1663–1664.
[16] J.A. Roberts, J. Lipman, Crit. Care Med. 37 (2009) 840–851.
[17] European Committee on Antimicrobial Susceptibility Testing. C2011. Available from: 〈http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Disk_test_documents/EUCAST_breakpoints_v1.3_pdf〉.
[18] J.W. Mouton, J.G. den Hollander, Antimicrob. Agent Chemother. 38 (1994) 931–936.
[19] K.H. Lin, Y.C. Chuang, S.H. Lee, W.L. Yu, J. Microbiol. Immunol. Infect. 43 (2010) 317–322.
[20] B. Vogelman, S. Gudmundsson, J. Leggett, J. Turnidge, S. Ebert, WA Craig, J. Infect. Dis. 158 (1988) 831–847.
[21] R. Denooz, C. Charlier, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 864 (2008) 161–167.
[22] E. Nemutlu, S. Kir, D. Katlan, MS. Beksac, Talanta 80 (2009) 117–126.
[23] K. Ikeda, K. Ikawa, N. Morikawa, K. Kameda, N. Urakawa, H. Ohge, T. Sueda, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 867 (2008) 20–25.
[24] T. Legrand, S. Chhun, E. Rey, B. Blanchet, J.R. Zahar, F. Lanternier, G. Pons, V. Jullien, J. Chromatogr, B Analyt, Technol. Biomed. Life Sci. 875 (2008) 551–556.
[25] A.M. Brisson, J.B. Fourtillan, G. Berthon, J. Chromatogr. 233 (1982) 386–391.
[26] T. Ohmori, A. Suzuki, T. Niwa, H. Ushikoshi, K. Shirai, S. Yoshida, S. Ogura, Y. Itoh, J. Chromatogr, B Analyt, Technol. Biomed. Life Sci. 879 (2011) 1038–1042.
[27] P. Hubert, J.J. Nguyen-Huu, B. Boulanger, E. Chapuzet, P. Chiap, N. Cohen, P.A. Compagnon, W. Dewé, M. Feinberg, M. Lallier, M. Laurentie, N. Mercier, G. Muzard, C. Nivet, L. Valat, J. Pharm. Biomed. Anal. 36 (2004) 579–586.
[28] P. Hubert, J.J. Nguyen-Huu, B. Boulanger, E. Chapuzet, P. Chiap, N. Cohen, P.A. Compagnon, W. Dewé, M. Feinberg, M. Lallier, M. Laurentie, N. Mercier, G. Muzard, C. Nivet, L. Valat, E. Rozet, J. Pharm. Biomed. Anal. 45 (2007) 70–81.
[29] P. Hubert, J.J. Nguyen-Huu, B. Boulanger, E. Chapuzet, N. Cohen, P.A. Compagnon, W. Dewé, M. Feinberg, M. Laurentie, N. Mercier, G. Muzard, L. Valat, E. Rozet, J. Pharm. Biomed. Anal. 45 (2007) 82–96.
[30] P. Hubert, J.J. Nguyen-Huu, B. Boulanger, E. Chapuzet, N. Cohen, P.A. Compagnon, W. Dewé, M. Feinberg, M. Laurentie, N. Mercier, G. Muzard, L. Valat, E. Rozet, J. Pharm. Biomed. Anal. 48 (2008) 760–771.
[31] NCCLS Document EP7-P. Interference testing in clinical chemistry. Wayne, PA:NCCLS, 1986.
[32] C.T. Viswanathan, S. Bansal, B. Booth, A.J. DeStefano, M.J. Rose, J. Sailstad, V.P. Shah, J.P. Skelly, P.G. Swann, R. Weiner, Pharm. Res. 24 (2007) 1962–1973.
[33] T.P. Moyer, L.M. Shaw, Therapeutic drugs and their management, in: CA Burtis, ER Ashwood, DE. Bruns (Eds.), Tietz Textbook of Clinical Chemistry and Molecular Diagnostics, Elsevier Saunders, 2006.
[34] T.A. Tartaglione, L. Nye, N. Vishniavsky, W. Poynor, R.E. Polk, Clin. Pharm. 5 (1986) 911–916.
[35] K. Bächer, M. Schaeffer, H. Lode, C.E. Nord, K. Borner, P. Koeppe, J. Antimicrob. Chemother. 30 (1992) 365–375.
[36] J.W. Mouton, J.N. van den Anker, Clin. Pharmacokinet. 28 (1995) 275–286.
[37] A.P. MacGowan, R. Wise, J. Antimicrob. Chem. 48 (2001) 17–28.
[38] B. Vogelman, W.A. Craig, J. Pediatr. 108 (1986) 835–840.