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FAST AND RELIABLE HPLC METHOD FOR DETERMINATION OF CEFUROXIME IN HUMAN SERUM: APPLICATION TO OPTIMIZATION OF DOSING REGIMEN IN PATIENTS WITH LOWER RESPIRATORY TRACT INFECTION

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Abstract: A rapid and inexpensive high-performance liquid chromatography method with UV detection for determination of cefuroxime (CFU) in small human serum samples was developed and validated. In this method, serum samples were spiked with an internal standard and proteins were precipitated by 0.4 M perchloric acid. Separation was carried out on an RP-18 column with a mobile phase composed of 20 mM potassium dihydrogen phosphate buffer and methanol (85 : 15; v/v), pH 4.5. In order to assess the usefulness of newly developed method in CFU dosage design, CFU concentrations in serum from 6 patients with lower respiratory tract infections ranging in age from 43 to 91 years were determined. The antibiotic was administered intravenously at a dose of 1500 mg every 8 hours for 10-14 days. Pharmacokinetic analysis and simulations were performed using Phoenix WinNonlin. Dosage optimization was based on pharmacokinetic-pharmacodynamic (PK/PD) indices. The lower limit of quantification of the assay was 0.25 µg/mL and the calibration curve was linear at the concentration range from 0.25 to 300 µg/mL. The method was characterized by an excellent precision (≤ 6.4%) and accuracy (≤ 9.0%). Recoveries ranged from 92% to 96%. CFU in serum samples was stable when stored at -30°C for at least 10 days, at room temperature (+22°C) for up to 6 h, and during three freeze–thaw cycles, when stored at -30°C and thawed to room temperature. Pharmacokinetic analysis showed significant differences in pharmacokinetic parameters of CFU in the studied patients: volume of distribution was from 8.9 to 20.6 L, terminal elimination half-life from 1.3 to 5.3 h, and total body clearance from 31 to 232 mL/min. In the elderly patients studied dosage optimization was required. These results suggest that our simple and rapid HPLC method may be useful to monitor serum CFU concentrations in patients on standard dosages and to support determination of CFU dosage regimens based on the PK/PD indices.

Keywords: cefuroxime; HPLC/UV, PK/PD indices, computer simulations, elderly

The beta-lactam antibiotics constitute the most important family of antimicrobial agents, in terms of both the large number of compounds available and the prescription volume. Until recently, beta-lactams were not considered strong candidates for therapeutic drug monitoring (TDM), as they are assumed to have a wide therapeutic index and the most frequent adverse effects involve non-dose-related allergic reactions. But concepts are changing, and several studies demonstrate the advantage of TDM for these antibiotics (1).

Clinicians and pharmacologists agree that certain adverse events like encephalopathy, seizure, and pseudolithiasis occur when their doses are excessive. Various infectious diseases, e.g., endocarditis, meningitis, and osteomyelitis require administration of high-dose antibiotics *via* intermittent or continuous infusion (2). Knowledge of the antibiotic plasma concentrations combined with bacterial susceptibility evaluated in terms of the minimum inhibitory concentration (MIC) would enable treatment efficacy to be optimized by limiting the risk of dose related adverse effects and avoiding suboptimal concentrations (3).

Cefuroxime sodium (CFU) is a semisynthetic, broad-spectrum, second-generation cephalosporin antibiotic agent for parenteral administration. It is resistant to destruction by beta-lactamases produced

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by staphylococci and most Gram-negative aerobic bacteria and is active against many bacteria resistant to cephalothin. CFU is the most active of the cephalosporins against gonococci and *Haemophilus influenzae*, particularly against beta-lactamase producing strains. Given by intravenous or intramuscular injection CFU is effective against a wide variety of infections caused by Gram-positive or Gram-negative aerobes, but has no effect against infections caused by *Pseudomonas aeruginosa* or *Bacteroides fragilis*. In addition, it is of value in the treatment of respiratory infections due to *Haemophilus influenzae* and *Streptocococcus pneumoniae* and is useful against cephalosporin-resistant *Klebsiella spp.* and *Enterobacter* infections. It is an alternative to spectinomycin for the treatment of beta-lactamase producing *Neisseria gonorrhoeae* infections. Moreover, this antibiotic is generally well tolerated and does not appear to be nephrotoxic when given alone at usual dosages. CFU has been widely used for the treatment of patients having infections of soft tissues, respiratory tract, urinary tract, genital tract, central nervous system, and bone and joint tissues (4, 5).

Pharmacokinetics of CFU has been examined both in healthy subjects and in patients with infections or renal failure. The volume of distribution of this drug ranges from 12 to 18 liters and, at therapeutic concentrations, 30% of this drug is bound to plasma proteins. Renal excretion accounts for 96%, whereas the biological half-life is from 1.4 to 1.8 h in normal subjects and increases to approximately 20 h in anuric patients (6-10) The pharmacokinetic parameters are highly variable between individuals, therefore determination of CFU serum concentrations is necessary to ensure the effectiveness of treatment. Inter-patient differences in pharmacokinetics and the frequently associated toxicity of CFU after prolonged use makes therapeutic drug monitoring strongly recommended. Therefore, a simple, fast, inexpensive, and reliable analytical method for the determination of CFU in body fluids is needed.
Increased knowledge of the pharmacokinetic/pharmacodynamic (PK/PD) properties of antibiotics is useful for optimising dosage regimens. The bactericidal action of CFU, similarly to all β-lactam antibiotics depends on the time at which blood concentrations will be greater than the MIC of bacteria causing infection (TC > MIC) (11-13). These drugs have relatively slow bactericidal action and no or short post-antibiotic effects (PAEs) (14, 15). Experimental studies have shown that for β-lactam antibiotics this time value in moderate infections should be at least 70% and in severe infections 100% of the dosing interval (16-18). Literature data indicate significant changes in the processes of elimination of CFU observed especially in geriatric patients that manifest mainly by decreased clearance of the drug. As a result, with the usual dosage of CFU in this group of patients significant changes in the values of the parameter TC > MIC are observed that affect the effectiveness of treatment and create a need for individualized dosing of the drug, e.g., based on the measured concentration of this drug in blood (16-20).

The analytical method used to monitor the concentration of the drug in biological material should have an excellent accuracy, sensitivity, precision, and selectivity. Moreover, it should be fast, inexpensive, require small sample volumes of biological material as well as sample preparation time should be minimized (21).

Up to date, several analytical methods for monitoring of plasma CFU levels have been reported. Most of these assays were based on the use of microbiological techniques (8, 22) or high performance liquid chromatography (HPLC) coupled with UV-detection (23-28). Among the currently available bio-analytical techniques, liquid chromatography coupled with mass spectrometry (LC–MS) and tandem mass spectrometry (LC–MS/MS) has been emerged as the preeminent analytical tool for quantification of small molecular weight drugs in biological matrix (29-32). Different extraction procedures including solid phase extraction (23, 25, 29, 30), ultrafiltration (34), protein precipitation combined with back-extraction (33) or protein precipitation followed by supernatant evaporation (24, 28) have been used for cleanup and enrichment of plasma samples. However, all of these methods have weaknesses, e.g., although the absolute recoveries obtained with solid phase extraction were reasonably good, the main problems encountered with this procedure was the high plasma volume of at least 0.5 to 1 mL required, multi-step purification and evaporation as well as a relatively high cost (23, 25, 29). Ultrafiltration is another effective extraction method providing good recoveries and the measure of the free fraction of the drug but it is quite expensive (34). The mass spectrometric detection had the advantage of being a fast, specific, and sensitive technique for the simultaneous determination of many β-lactams (29-32) but it could not be implemented in all laboratories due to the high cost of analyses and the special equipment required. It is substantially more sensitive than known HPLC-UV methods as it allows for CFU quantification in the range 0.025-50.0 μg/mL using only 100 μL of plas-

ma (32). Still, much less expensive HPLC-UV methods enable sufficient for the pharmacokinetic studies of CFU administered at standard doses sensitivity, i.e., lower limit of quantification (LLOQ = 0.1- 0.2 μg/mL) (24-26).

An analytical method suitable for TDM must be fast and robust, and it should provide results to the clinical ward within a few hours. Most of the reported CFU assays were based on time-consuming low-throughput sample preparation steps. They include laborious procedures, e.g., multiple centrifugation steps (24, 28) or evaporation-to-dryness procedures (23, 25, 29) that significantly lengthen the total analysis time. Therefore, there is a need to develop a fast, simple, inexpensive, and reliable analytical method for CFU determination to be used for TDM purposes.

The aim of this work was to develop a simple, precise, rapid, and low-cost HPLC method for determination of CFU in human serum and to validate this method according to the US Food and Drug Administration and European Medicines Agency guidelines (35, 36). In addition, this method was used to determine CFU serum levels in patients hospitalized in the Department of Internal Medicine and Gerontology, Jagiellonian University Medical College due to bacterial infections in order to adjust the dosage of this drug so as to optimize the time above MIC and minimize the risk of dose related adverse effects.

MATERIALS AND METHODS

Chemicals and reagents

Cefuroxime sodium salt and cefotaxime sodium salt (internal standard, IS) were supplied by Sigma-Aldrich (Steinheim, Germany). Other drugs used in this study included ciprofloxacin hydrochloride, gentamicin sulfate, amikacin sulfate, acetaminophen, amiodarone, digoxin, disopyramide, amphotericin B, flunitrazepam, ertapenem, propranolol, salicylic acid, and theophylline and were also from Sigma-Aldrich, Germany. Methanol for chromatography LiChrosolv® was obtained from Merck (Darmstadt, Germany). Analytical-grade concentrated orthophosphoric acid (85%) and potassium dihydrogen phosphate were purchased from Fluka (Germany). Drug free human serum was received from a healthy volunteer.

Apparatus and chromatographic conditions

The HPLC/UV system (Thermo Separation Products, San Jose, CA, USA) consisted of a P100 pump, a Rheodyne injector 7125 (Rheodyne, Cotati, CA, USA) with a 20 μL loop, a UV100 variable-wavelenghth UV/VIS detector, and a SP4400 (ChromJet) integrator. The analysis was performed on a LiChrospher 100RP-18 (125 mm x 4 mm i.d., 5 μm particle size) column coupled with a LiChroCART® guard column (4.0 × 4.0 mm, 5 μm) (Merck, Darmstadt, Germany). The mobile phase consisted of a mixture of methanol : 20 mM potassium dihydrogen phosphate buffer (15 : 85; v/v). It was prepared with 2.7218 g potassium dihydrogen phosphate dissolved in 1000 mL of water. The pH was adjusted to 4.5 with concentrated orthophosphoric acid. The mobile phase was prepared daily, filtered through a 0.45 μm membrane filter (Supelco, Germany) and degassed before use. CFU was eluted isocratically at room temperature with a flow rate of 1.2 mL/min. The UV detection wavelength for CFU and IS was 275 nm. The sample injection volume was 20 μL and the chromatographic run time was 10 min.

Preparation of solutions

A stock solutions of CFU (2 mg/mL) and IS (1 mg/mL) were prepared by dissolving 21.0865 mg cefuroxime sodium salt and 10,4830 mg cefotaxime sodium salt (IS) in 10 mL deionized water and stored at -30°C. The stock solution was diluted with water to obtain working solutions of 0.25; 0.5; 5; 25; 50, 10, 200, and 300 μg/mL for serum calibration samples.

Quality control (QC) samples were prepared by adding an appropriate volume of the working solutions to drug free serum to obtain a final concentrations of 0.25, 1, 150, and 300 μg/mL (LOQ – limit of quantification, low, medium, and high QC, respectively). QC samples were divided into 500 μL aliquots and stored at -30°C.

Sample preparation

In order to isolate CFU from serum, to 100 μL of serum samples containing this antobiotic 10 μL of IS (100 μg/mL) was added. Then, the samples were vortex-mixed for 10 s and deproteinized with 0.4 M $HClO_4$ (200 μL). The solution was vortexed for 15 s and centrifuged for 5 min at 12 000 rpm at +4°C (EBA 12R, Hettich, Germany). Finally, 20 μL of clear supernatant of each sample was injected into the HPLC system. All samples were prepared in duplicate.

Validation

The method was validated according to the procedures and acceptance criteria recommended for bioanalytical method validation for pharmacoki-

netic studies (35, 36). The method was then validated regarding selectivity, specificity, linearity, accuracy and precision, limit of detection, limit of quantification, recovery, and stability.

Selectivity and specificity

The selectivity were measured by analyzing six different drug-free serum samples applying the developed analytical procedure. The retention times of endogenous compounds were compared with that obtained for CFU.

Specificity was assessed using different serum samples containing 10 drugs frequently prescribed in the elderly patients that were analyzed to investigate potential interferences. The drugs were acetaminophen, amiodarone, digoxin, disopyramide, flunitrazepam, amphotericin B, ertapenem, propranolol, salicylic acid, and theophylline. Due to the fact that several patients received CFU in combination with other anti-infective drugs, such as gentamicin, amikacin, and ciprofloxacin, potential interferences of CFU with these drugs were also investigated.

Linearity

Linearity study was performed by analysis of standards (from 0.25 to 300 μg/mL) in sixplicate. Calculations were made using the ratio of the observed peak areas of CFU and IS. Linear regression analysis of the calibration data was performed using the equation: $y = ax + b$, where y is the peak area ratio, x is serum concentration of CFU, and a and b are two constants. Unknown concentrations were computed from the linear regression equation of the peak area ratio against CFU concentrations. Slopes, intercepts, and coefficient determination (R^2) were obtained by the linear regression analysis.

Precision and accuracy

Accuracy was determined by the mean of the measured QC concentration relative to the theoretical value and was reported in percentage. Precision was defined as the ratio of the standard deviation of the observed QC concentration to the mean observed QC concentration. The intra-day and the inter-day precision and accuracy for serum QCs were calculated for three concentrations (low – 1 μg/mL, medium – 150 μg/mL, and high – 300 μg/mL) analyzed six times on the same day, while inter-day study was performed by assaying the same three QC concentrations once-a-day on 5 separate days. Precision was reported as the coefficient of variation (CV) and calculated according to the equation: (S.D./mean)×100 and accuracy as the bias (RSD) and it was calculated as follows: [(measured concentration - theoretical concentration)/(theoretical concentration)] × 100. Acceptance criteria for accuracy and precision were: bias within ±15% (except ± 20 % for the LOQ QC) and CV lower than 15% (except 20 % for the LOQ QC).

Limit of detection and limit of quantitation

The limit of detection (LOD) was determined based on the signal-to-noise ratio of 4 : 1. It was experimentally determined by analyzing serum samples spiked with CFU at 0.1, 0.25, 0.5, 1.0, 2.0, and 2.5 μg/mL concentrations. The lower LOQ was chosen as the concentration which provided measurements with a precision and accuracy within the recommended ± 20% from their nominal values (36).

Recovery

CFU recovery was quantified at concentration levels corresponding to the lowest (1 μg/mL), medium (150 μg/mL), and highest (300 μg/mL) standard concentrations analyzed in sixplicate. Absolute recovery of CFU from serum was estimated by the peak area integrated for CFU in serum assayed according to the procedure described above *versus* the peak area integrated for CFU after direct injection of the same concentration in purified water expressed as percentage.

Cefuroxime stability

Stability of CFU in human serum was evaluated by assaying samples that had been stored at +22, +4, and -30°C for 10 days. Low (1 μg/mL), medium (150 μg/mL), and high (300 μg/mL) QC concentrations were prepared in triplicate from a freshly made stock solution in human serum and were investigated under different conditions. Bench-top stability of QC samples was assessed after exposure of the serum samples to room temperature (+22˚C) for 6 h, which exceeds the residence time of the sample processing procedures. The freeze-thaw stability was performed after freezing (at -30°C) and thawing the QC samples at room temperature for three cycles. In addition, the stability of CFU and IS stock solutions were evaluated by testing their validity at room temperature. The stock solutions studied were freshly prepared and their aliquots were kept at room temperature up to 6 h (stability samples). After this period of time, they were diluted with water to the concentration of 1, 150, and 300 μg/mL and the mean peak areas were compared with those of freshly prepared stock solutions. All data were compared with the results obtained from freshly prepared and analyzed QC samples using the formula: [stability (%)

= (stored QC concentration/freshly prepared QC concentration)·100]. The samples were considered stable in human serum at each concentration if the deviation from the mean calculated concentration of stability quality control samples was within ± 10% (35, 36).

Patients

The developed method was applied to the quantitative analysis of CFU in serum of 6 patients (4 females, 2 men) ranging in age from 41 to 91 years with lower respiratory tract infections, hospitalized in the Department of Internal Medicine and Gerontology, Jagiellonian University Medical College. Patients were treated with CFU (Zinacef, GlaxoSmithKline, UK) administered intravenously at a dose of 1500 mg every 8 h for 10-14 days. All CFU treated patients were diagnosed with bronchopneumonia or lobar pneumonia and underwent routine examinations. Moreover, X-ray of the chest and the basic laboratory tests were performed. Determinations were made by standardized biochemical methods.

Serum creatinine was used to calculate creatinine clearance (CL_{cr}) according to equation:

$$CL_{cr} = \{F_x [140 - \text{age (years)}] \cdot \text{weight (kg)}\} / S_{cr}\ (\mu M/L)\ [mL/min] \quad (1)$$

where F_x is 1.04 for women and 1.23 for men, and S_{cr} is serum creatinine concentration (37).

On the basis of the interview which was carried out in the entire group of patients, it was found that 2 of them had type 2 diabetes. The demographic data of the study patients are presented in Table 1.

Material for microbiological testing was isolated from blood cultures. Microbiological examination included assessment and identification of the isolated pathogen and determination of antibiotic susceptibility (MIC). The sensitivity to CFU was measured by E-test kits (AB Biodisk, Solna, Sweden) in accordance with the package insert. MICs of CFU for isolated pathogens were determined by the clinical microbiology laboratory at the institution where the study was performed. The results of the microbiological tests are shown in Table 6.

Blood samples were collected from the forearm vein at steady state (3-4 days of treatment) before the next dose, just after administration, followed by 0.5, 1.5, 5.5, and 7 h after dosing. Blood was allowed to clot for 15-20 min and centrifuged at 1000 × g for 10 min. Serum samples were immediately transported on ice to the laboratory and stored at -30°C until analysis. In the day of analysis they were thawed and followed the procedure described above. The study protocol was approved by the Bioethics Committee of the Jagiellonian University.

Pharmacokinetic analysis and simulations

The pharmacokinetic parameters of CFU were estimated by both model-independent and model-dependent methods using Phoenix WinNonlin v. 6.3 (Pharsight Corp., Mountain View, CA, USA). In the non-compartmental approach, the terminal slope (l_z) was calculated from the terminal portion of the log concentration *vs*. time profile by linear regression and the terminal elimination half-life ($t_{0.5\lambda z}$) was calculated as $0.693/\lambda_z$. The area under serum concentration–time curve ($AUC_{0-\tau}$) and the area under the first moment of serum concentration–time curve ($AUMC_{0-\tau}$) over one dosing interval were calculated using the linear trapezoidal rule. The mean residence time ($MRT_{0-\tau}$) was calculated as $AUMC_{0-\tau}/AUC_{0-\tau}$, the total body clearance (CL_T) was calculated according to the equation: $CL_T = dose/AUC_{0-\tau}$, and the steady-state volume of distribution (Vd_{ss}) was calculated as $MRT_{iv} \times CL_T$. The two-compartment pharmacokinetic model was used to estimate pharmacokinetic parameters of CFU that were subsequently used to predict steady-state concentration-time profiles for each patient at different dosing schemes. Goodness of fit was evaluated by the standard procedures (38). Simulated concentrations

Table 1. Characteristics of patients with lower respiratory tract infections treated with CFU.

Patient no.	Age (yr)	Wt (kg)	Height (cm)	Sex (F - female, M - male)	Serum creatinine (μM/L)	CL_{cr} (mL/min)
1.	43	52	164	F	72	73
2.	43	131	178	M	89	176
3.	69	70	164	F	121	43
4.	66	71	178	M	106	49
5.	86	50	158	F	92	30.5
6.	91	58	166	F	77	37

were corrected for 33% protein binding of CFU (39).

Determination of predictive PK/PD index

The percentage of time that unbound CFU concentrations exceeded the MIC (*% f* TC > MIC*)* was determined according to the equation:

$$\% fTC > MIC = \ln[Dose \cdot f/(V_d \cdot MIC)] \cdot [t_{0.5}/0.693] \cdot [100/DI] \quad (2)$$

where, f is the fraction of unbound drug, V_d is volume of distribution (L), $t_{0.5}$ is elimination half-life (h), DI is dosing interval (h), and MIC is the lowest concentration of an antibiotic that completely inhibits the growth of a microorganism (µg/mL).

The percentage of time during which free CFU concentrations were higher than MIC (*% f*TC > MIC) was calculated for each patient. The pharmacodynamic goal for CFU was the target *%f*TC > MIC of unbound drug equal to at least 70% of the dosing interval. All calculations were performed by Microsoft Excel® program.

Table 2. Within- and between-day precision (CV) and accuracy (RSD) of the HPLC method for the determination of CFU in human serum.

Theoretical concentration of CFU [µg/mL]	Intra-day (n = 6)			Inter-day (n = 6)		
	Mean (SD) measured concentration [µg/mL]	CV [%]	RSD [%]	Mean (SD) measured concentration [µg/mL]	CV [%]	RSD [%]
1	0.93 ± 0.039	4.19	-7.00	0.91 0.058	6.37	-9.00
150	148.38 ± 2.06	1.39	-1.08	148.25 2.99	2.01	-1.17
300	301.53 ± 3.30	1.09	0.51	302.83 7.13	2.35	0.94

Table 3. Mean ± SD recoveries (%) of CFU (n = 6).

Concentration of CFU	1 µg/mL	150 µg/mL	300 µg/mL
Mean ± SD	94.70 ± 6.70	92.37 ± 2.03	96.04 ± 7.92

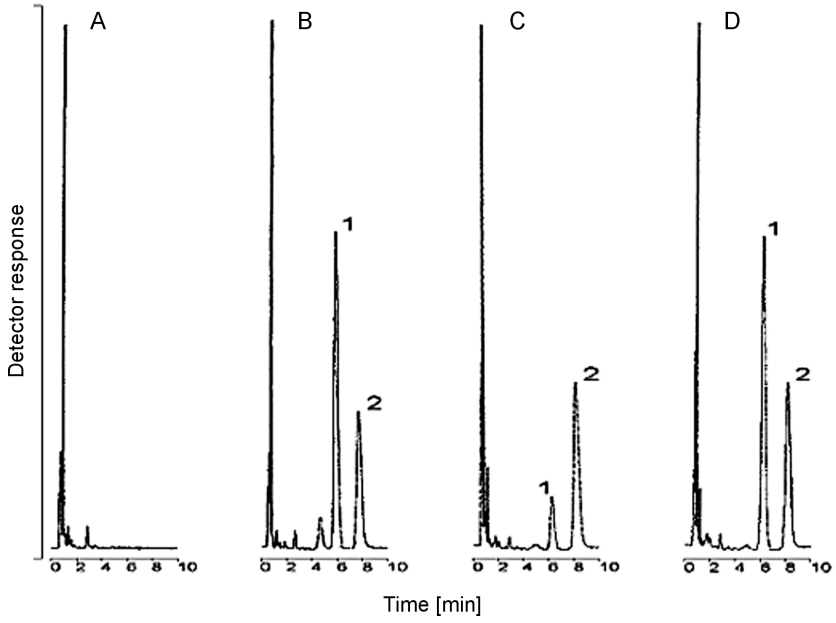

Figure 1. Chromatograms of control serum (A), serum containing CFU at a concentration of 200 µg/mL and IS at a concentration of 100 µg/mL (B), serum of a patient treated with CFU at a dose of 1500 mg iv every 8 hours taken just before the next dose (C), and serum of a patient treated with CFU at a dose of 1500 mg iv every 8 hours taken immediately after administration of the next dose (D). Peaks: 1 = CFU, 2 = IS

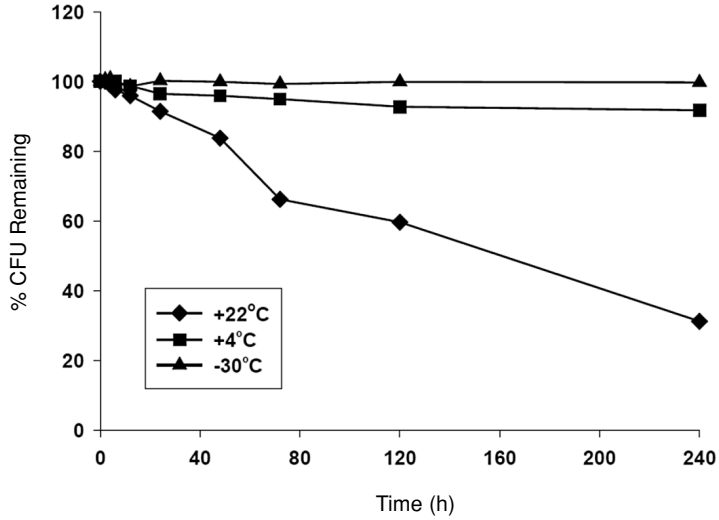

Figure 2. Long-term stability of CFU at a concentration of 150 µg/mL in human serum at 22°C (room temperature), +4°C, and -30°C (n = 3)

RESULTS

Method validation

Representative chromatograms of blank serum, serum sample spiked with 200 µg/mL of CFU, and serum samples from patients treated with CFU are shown in Figure 1. The average retention times were 6.15 ± 0.36 min and 8.63 ± 0.29 min for CFU and IS, respectively.

The method was found to be selective as no interference was found with endogenous compounds in five different blank sera. In addition, the method was specific as no interference was found with other drugs (acetaminophen, amiodarone, digoxin, disopyramide, flunitrazepam, amphotericin B, ertapenem, propranolol, salicylic acid, and theophylline), including other anti-infective agents (gentamicin, amikacin, and ciprofloxacin) that may be taken concomitantly in this patient population.

The calibration curves for CFU were linear in the concentration range evaluated (0.25–300 µg/mL human serum). The representative linear equation was $y = 0.0078x + 0.00048$, with a coefficient determination ($R^2 = 0.9993 - 0.9997$) highly significant for the method.

The LOD of the assay was 0.1 µg/mL, whereas LOQ was 0.25 µg/mL with the relative error not exceeding 10%. Acceptance criteria for accuracy and precision were met in all cases. Intra-day accuracy ranged from -7.0 to 0.51% and inter-day accuracy ranged from -9.00 to 0.94%. Intra- and inter-day precisions were less than 4.2 and 6.4%, respectively (Table 2).

The mean recovery of CFU calculated for three standard concentrations was 94.37 ± 1.9% (Table 3). The mean recovery of IS was 91.33 ± 6.1%.

The stability of CFU in human serum was inspected during all the storage steps (i.e., at +22°C, +4°C, and -30°C). The stability of CFU in human serum was satisfactory up to 240 h of storage at -30°C and up to 72 h at +4°C, whereas an important degradation occurred after 24 h of storage at room temperature (22°C). The results of long-term stability studies for the concentration of 150 µg/mL as an example are presented in Figure 2. The stability results summarized in Table 4 showed that CFU spiked into human serum was stable for at least 6 h at room temperature, and during three freeze–thaw cycles, when stored at around -30°C and thawed to room temperature. The stock solution of CFU and IS were also stable at room temperature for at least 6 h.

To assess the usefulness of the developed method, serum samples from 6 patients with lower respiratory tract infections receiving intravenous dose of 1500 mg CFU every 8 h were analyzed. Figure 3 illustrates individual profiles of CFU concentrations determined at steady state (3-4 day of treatment) before the next dose, just after drug administration, followed by sampling at 0.5, 1.5, 5.5, and 7 h after dosing.

Pharmacokinetic parameters of CFU estimated using non-compartmental analysis by means of Phoenix WinNonlin v. 6.3 are shown in Table 5.

Table 6 shows the times at which free serum concentrations of CFU were greater than MIC (f·TC > MIC) of the pathogen causing the infection expressed

in percentages of dosing interval (%t) and in hours and, where necessary, new dosing intervals (DI) were proposed and corresponding f·TC > MIC values were calculated. The calculations were performed for each patient using Equation 2. Based on the pharmacokinetic parameters estimated using a two-compartment pharmacokinetic model computer simulations were carried out in order to confirm the appropriateness of the new dosing intervals presented in Table 6 (last column). The results of these simulations together with the MIC values of the bacterial strains isolated from blood of patients are presented in Figure 4.

DISCUSSION

The knowledge of pharmacokinetics and pharmacodynamics of an antibiotic is the basis for its dose optimization in order to increase efficacy of therapy and minimize the risk of side effects. In the case of drugs with a low therapeutic index (e.g., aminoglycoside antibiotics) individualization of dosing plays an important role due to their toxicity, while in the case of less toxic drugs (e.g., β-lactam antibiotics) because of possible underdosing and thus, the danger of an increased resistance of bacterial strains. The pharmacokinetic and pharmacodynamic criteria allow predicting the likelihood of a therapeutic effect. Therapeutic drug monitoring is particularly desirable in patients having a large deviation of pharmacokinetic parameters from the population mean values as a result of pathophysiological (renal failure, liver, cardiovascular system) or physiological (age, gender, pregnancy) factors (19, 20).

Advancing age may influence the pharmacokinetics of antibiotics in several ways. The renal elim-

Table 4. Stability data for CFU in human serum/deionized water under various conditions (n = 4).

	Nominal concentration [µg/mL]	Average measured concentration [µg/mL]	SD	CV [%]	Remaining [%] ± SD
Three freeze/thaw cycles (-30°C)	1	0.981	0.012	1.18	98.10 ± 1.15
	150	152.83	2.45	1.60	101.39 ± 1.63
	300	301.47	3.65	1.21	100.49 ± 1.21
Bench-top stability for 6 h in human serum (22°C)	1	0.989	0.063	6.33	98.99 ± 6.26
	150	142.52	1.83	1.29	95.01 ± 1.22
	300	309.36	4.43	1.43	103.12 ± 1.48
Bench-top stability for 6 h in deionized water (22°C)	1	0.977	0.011	1.09	97.69 ± 1.06
	150	143.31	3.52	2.45	95.54 ± 2.34
	300	289.49	3.89	1.34	96.49 ± 1.29

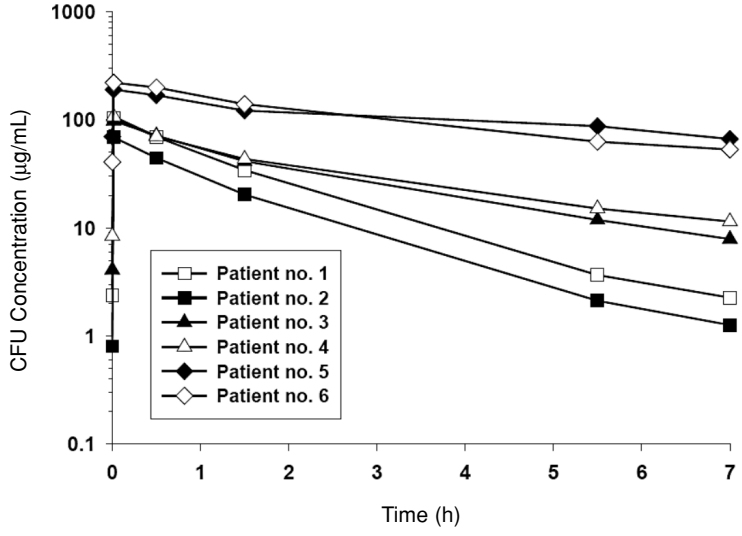

Figure 3. Individual steady-state CFU serum concentration versus time plots of 6 patients receiving this drug intravenously at a dose of 1500 mg every 8 hours

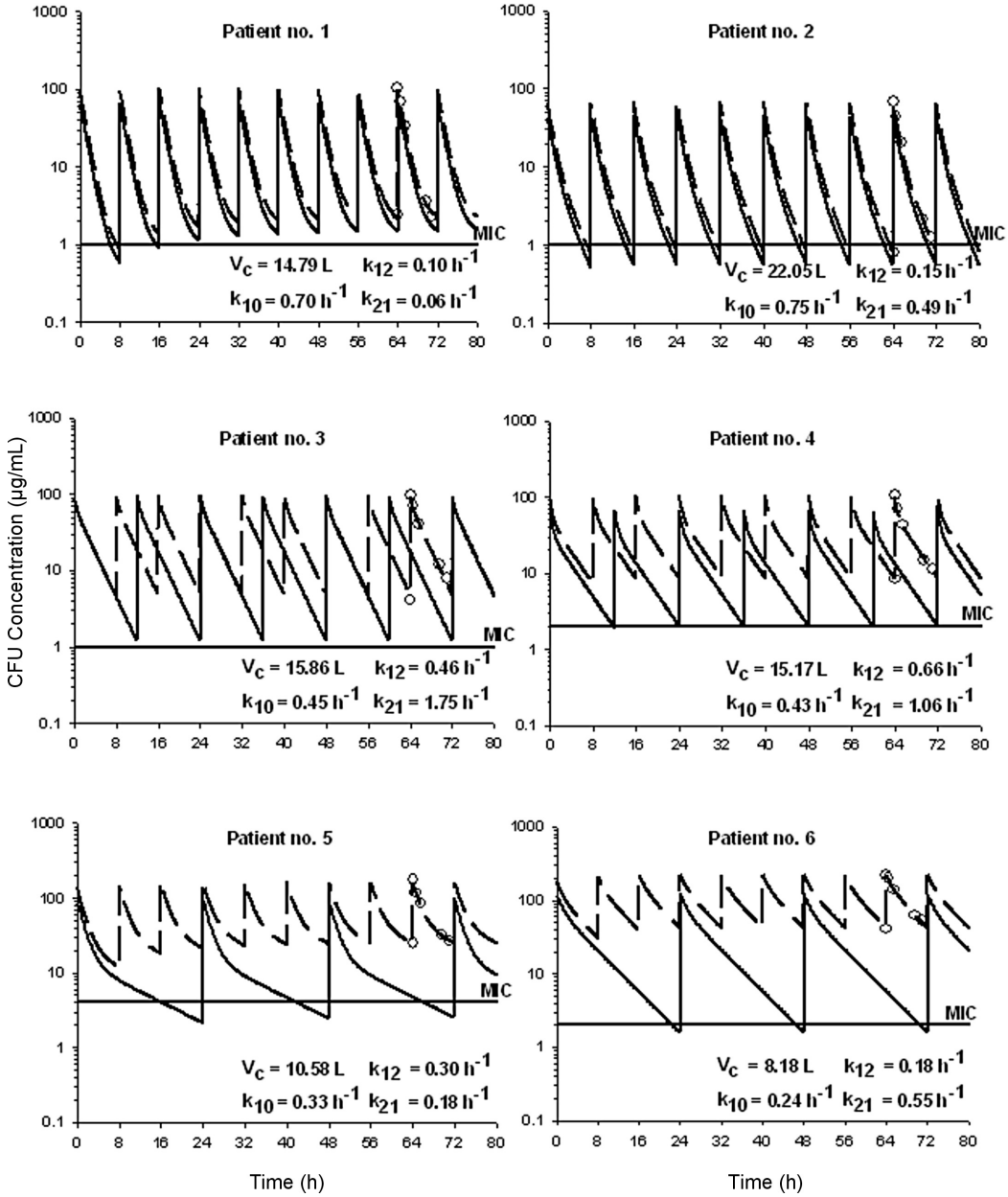

Figure 4. Observed (•) and predicted (- -) total serum concentrations of CFU in 6 patients relative to the MIC values of pathogens that caused infections in these patients. Solid lines represent simulated concentrations of unbound CFU in serum. Simulations were performed based on the individual pharmacokinetic parameters and dosing intervals proposed in Table 6 (last column)

ination of a number of compounds has been shown to decline in parallel with the age-associated reduction in renal function. In turn, the evidence of changes in distribution, metabolism, and biliary excretion in the elderly is less conclusive. Despite a number of known age-associated alterations in gastrointestinal function, no significant change in the absolute bioavailability of antibiotics has been reported (40). Antibiotic treatment of elderly patients is difficult because of the progressive and age-related changes that occur in the body of the patient for example such as: multiple organ dysfunction, an increased body fat, or a reduction in total body water. Moreover, in parallel with the dis-

comfort from aging, chronic diseases, such as cardiovascular disease, osteoarthritis, and diabetes are more common in this population, which explains why most of the elderly are treated with polytherapy. All of these factors can significantly affect the pharmacokinetics and therapeutic efficacy of antimicrobial drugs and create the need for individualization of dosage as well as continuous monitoring of therapeutic effects in the elderly patients (41).

The most crucial step in optimization of drug therapy is the development of a suitable analytical method that allows for precise and accurate determination of drug concentration in biological fluids. Literature data indicate that among methods available for the determination of CFU in serum, HPLC technique is the most frequently used (24-28).

The aim of the study was to develop a simple, rapid, and low-cost assay for determination of CFU in human serum using HPLC method coupled with ultraviolet detection and to validate this method according to the FDA and EMA guidelines (35, 36). This method was applied for determination of CFU serum concentrations in infected patients in order to obtain pharmacokinetic profiles of this antibiotic at steady state. Furthermore, this method can be applied in the clinical field to adjust the doses of CFU based on the PK/PD indices. The advantage of our method over those previously published lies in the simple preparation of the samples. When developing the method, the use of acetonitrile, methanol, and 0.4 M perchloric acid for protein precipitation was evaluated. The best recoveries were obtained using perchloric acid (data not shown). Compared to other procedures reported in the literature, our method is cost-effective (23, 25, 29, 30). The relatively low price of a single column and reagents are important for pharmacokinetic and clinical laboratory investigations. In the developed analytical method only 100 µL of serum was used. Small volume of a sample is very important for the pharmacokinetic studies, particularly in children and geriatric and critically ill patients. The methods presented in literature usually require 0.5-1 mL of serum (23-25). In addition, in our method a total run time

Table 5. Individual pharmacokinetic parameters of CFU (1500 mg *iv* every 8 h) estimated in patients with lower respiratory tract infections using non-compartmental analysis.

Patient no.	$AUC_{0-\tau}$ [mg · h/mL]	$MRT_{0-\tau}$ [h]	Vd_{ss} [L]	CL_{CFU} [mL/min]	λ_z [h^{-1}]	$t_{0.5\lambda z}$ [h]
1.	174.20	1.439	12.275	142.20	0.551	1.25
2.	106.92	1.482	20.582	231.54	0.491	1.41
3.	217.82	2.715	18.124	111.25	0.286	2.42
4.	235.93	3.363	20.489	101.55	0.238	2.91
5.	765.79	2.854	8.901	19.65	0.131	5.30
6.	759.57	4.962	9.217	30.96	0.176	3.94
Mean ± SD	376.705 ± 275.931	2.802 ± 1.197	14.930 ± 4.987	106.192 ± 70.952	0.312 ± 0.156	2.872 ± 1.415

$AUC_{0-\tau}$ = area under the serum concentration-time curve of the 8 h dosing interval; $MRT_{0-\tau}$ = mean residence time; Vd_{ss} = volume of distribution at steady-state; CL = total body clearance; λ_z = terminal slope $t_{0.5/\lambda z}$ = terminal half-life.

Table 6. Microbiological data of the patients receiving CFU at a dose of 1500 mg iv every 8 h and calculated PK/PD indices.

Patient no.	Bacteria strain	MIC [µg/mL]	% fTC > MIC [%τ]	fTC > MIC [h]	New DI/ %fTC > MIC
1.	*S. aureus*	1	99.32	7.95	-
2.	*S. pneumoniae*	1	98.89	7.91	-
3.	*M. catarrhalis*	1	175.28	14.02	12 h/116.85
4.	*S. pneumoniae*	2	167.95	13.44	12 h/111.97
5.	*H. influenzae*	4	319.34	25.55	24 h/106.45
6.	*S. aureus*	2	284.17	22.73	24 h/94.72

DI - dosing interval

is < 10 min. The retention times of CFU in serum/plasma described by other authors were 10 or 20 min (23, 24).

The blank serum showed no interference of endogenous substances with CFU and IS. Potentially co-administered drugs had retention times different than CFU or were not extracted and detected when using our method. This points to the possibility of monitoring the concentrations of CFU using the developed analytical method in patients treated with combination of CFU and other drugs concomitantly used especially in the elderly patients. Calibration range was based on CFU concentrations observed in human serum at steady-state when CFU was administered at a dose of 1500 mg *iv* every 8 h. The complete recovery of CFU from serum (approximately 95%) reported for this method provides evidence that this simple extraction procedure is sufficient for therapeutic drug monitoring. Moreover, the developed method was characterized by good inter- and intra-day accuracy and precision. The obtained LOQ of 0.25 µg/mL from only 100 mL of serum samples was sufficient for clinical applications, as reported minimal concentrations were higher than 0.5 µg/mL at recommended CFU dosages (24, 29, 34). Moreover, according to the European Committee on Antimicrobial Susceptibility Testing (42), clinical breakpoints of CFU ranging from 0.5 to 8.0 µg/mL are greater than our LOQ, allowing clinical applications of this method.

The stabilities of CFU were investigated at three concentrations of QC samples (low, medium, and high concentrations) to cover expected conditions during analysis, storage, and processing of all samples, which include the stability data from various conditions like bench-top, freeze/thaw, and long-term storage. CFU was considered stable in serum if at least 90% of antibiotic was retained at the end of the study period (35, 36). Long-term stability tests showed that concentrations of the CFU in human serum remained stable at -30°C for at least 240 h (all biases were comprised between -8.7 and 7.8%). No trend towards degradation of CFU was found in relation to freeze-thaw cycles, as average stability results ranged from 98.1% (1.2%) to 101.4% (1.6%) in serum. Storing QC samples at room temperature for 6 h had no influence on CFU quantification. Mean CFU concentrations ranged from 95.0% (1.2%) to 103.1% (1.5%) for human serum. Stock solutions were stable for at least 6 h at room temperature because no significant degradation was found between solutions stored at 22°C for 6 h and solutions freshly prepared. Consequently, blood samples should be transported to the laboratory preferably on ice, immediately centrifuged, and serum samples may be stored at -30°C for at least 240 h after collection.

The newly developed method was used for determination of CFU concentrations in six patients receiving CFU intravenously at a dose of 1500 mg every 8 h for 10-14 days. Basic pharmacokinetic parameters of the drug were calculated using non-compartmental analysis. The values of volume of distribution of CFU estimated in the study group varied and ranged from 8.9 to 20.6 L. These significant differences in this parameter could be due to pathological changes, mainly in the cardiovascular system and associated diseases, such as type II diabetes or hypertension (1, 5, 41). Values of CFU clearance in the study group was 106.19 ± 70.95 mL/min and in 4 out of 6 patients was almost 2-3 times lower than those in the average population (CL_{CFU} = 230-172 mL/min) indicating a significant weakening of the function of kidneys. This observation may be further confirmed by the diversity in the biological half-life, which in the our study group ranged from 1.3 to 5.3 h and in 4 patients of advanced age it was more than twice higher than the value reported in subjects with normal renal function (8-10). Similar observations were made in 44 patients at the age of 44–96 years treated with ciprofloxacin at a dose of 200 mg every 12 h as a constant rate infusion over 0.5 h (43).

Pharmacokinetic-pharmacodynamic (PK/PD) indices were used to predict the efficacy of treatment and to optimize the dosage of the antibiotic. To this end, the pharmacokinetic study results were combined with the microbiological data. The PK/PD parameter of cephalosporins is the time at which the concentration of antibiotic is greater than the MIC of micro-organism (f·TC > MIC) and it is often expressed as a percentage of the dosing interval. It is recommended that the optimum value of this time for cephalosporins in patients with infections should be at least 70% of the dosing interval (11-20).

Microbiological analysis revealed that in studied patients, isolates were represented by 4 Gram-positive and 2 Gram-negative strains. Gram-positive pathogens were *S. aureus* and *S. pneumoniae*, while *H. influenza* and *M. catharralis* were identified Gram-negative pathogens. The MIC of CFU for sensitive isolates ranged from 1 to 2 µg/mL (mean 1.5 µg/mL) and from 1 to 4 µg/mL (mean 2.5 µg/mL), respectively, for Gram-positive and Gram-negative strains (for details, see Table 6). The analysis of the obtained values of f·TC>MIC indicated that in the case of only two younger patients CFU dosage

(1500 mg *iv* every 8 h) for infections of *S. aureus* (MIC = 1 μg/mL) and *S. pneumoniae* (MIC = 1 μg/mL) assured effectiveness of the treatment as the value of this time is optimal and amounted to 99.3% (7.95 h) and 98.9% (7.91 h) of the dosing interval. In the case of four patients infected with *M. catarrhalis* (MIC = 1 μg/mL), *S. pneumonia* (MIC = 2 μg/mL), *H. influenzae* (MIC = 4 μg/mL), and *S. aureus* (MIC = 2 μg/mL), who were treated with a dose of 1500 mg CFU every 8 h, free CFU concentrations in serum of these patients significantly exceeded determined MICs and maintained above the MIC values for 14 – 25.6 h. Such large values of f·TC > MIC in these patients suggest that a reduction of the dose used or dosing interval prolongation is required. The latter solution seems to be more convenient for both the patient and the medical staff. The correctness of new dosing intervals proposed based on Eq. 2 were further confirmed by computer simulations. Stimulations of unbound CFU concentrations indicated that two patients with moderate renal impairment (CLcr = 40-50 mL/min) should be treated with CFU given at a dose of 1500 mg *iv* every 12 hours. In turn, in two patients with severe renal failure (CLcr = 30-37 mL/min), the dose of 1500 mg *iv* CFU every 24 h was recommended. Based on the simulations performed (Fig. 4) it seems that CFU dosage of 1500 mg and extended dosing interval (12 h or 24 h) ensure optimal PK/PD parameters values with respect to the pathogens that cause infections, reducing the risk of toxicity and costs of treatment.

CONCLUSION

In this report, an assay for measuring the concentrations of CFU in low-volume serum samples is described. This method appears to be suitable for use in the clinical laboratory for CFU monitoring and PK/PD based dosage adjustment. It can be routinely used in clinical diagnostic laboratories where a basic HPLC/UV system is available. The method is fast, simple, reliable, and reproducible. The lower limits of quantification allow the measurement of CFU concentrations in serum down to the MIC values reported for most relevant Gram-negative pathogens. The large differences in pharmacokinetic parameters observed in the studied patients indicate a need to consider both pharmacokinetic and pharmacodynamic criteria during antibiotic therapy. The presented approach can be useful for optimizing antimicrobial therapy with CFU leading to an increased efficacy and safety and thus, contributing to the cost savings of treatment with this antibiotic.

Conflict of interest

None of the authors declared conflict of interest.

REFERENCES

1. Roberts J.A., Ulldemolins M., Roberts M.S., McWhinney B., Ungerer J. et al.: Int. J. Antimicrob. Agents 36, 332 (2010).
2. Verdier M.C., Tribut O., Tattevin P., Le Tulzo Y., Michelet C. et al.: Antimicrob. Agents Chemother. 55, 4873 (2011).
3. Li R.C., Zhu M., Schentag J.J.: Clin. Pharmacokin. 37, 1 (1999).
4. Perry C.M., Brogden R.N.: Drugs 52, 125 (1996).
5. Brogden R.N., Heel R.C., Speight T.M., Avery G.S.: Drugs 17, 233 (1979).
6. Broekhuysen J., Deger F., Douchamps J., Freschi E., Mal N.: Br. J. Clin. Pharmacol. 12, 801 (1981).
7. Havard C.W., Bax R.P., Samanta T.C., Pearson R.M., Brumfitt W.: Thorax. 35, 379 (1980).
8. Bundtzen R.W., Toothaker R.D., Nielson O.S., Madsen P.O., Welling P.G.: Antimicrob. Agents Chemiother. 19, 443 (1981).
9. Brogard J.M., Koperschmitt J., Pinget M., Arnaud J.P., Lavillaureix J.: Proc. R. Soc. Med. 70, 42 (1977).
10. Foord R.D.: Antimicrob. Agents Chemother. 9, 741 (1976).
11. Sánchez-Navarro A., Sanchez R.: Clin. Pharmacokinet. 37, 289 (1999).
12. Craig W.A.: Infect. Dis. Clin. 17, 479 (2003).
13. Craig W.A.: Clin. Infect. Dis. 26, 1 (1998).
14. Zhanel G.G., Craig W.A.: Clin. Pharmacokinet. 27, 377 (1994).
15. Zhanel G.G., Hoban D.J., Harding G.K.: Ann. Pharmacother. 25, 153 (1991).
16. Craig W.A.: Diagn. Microbiol. Infect. Dis. 22, 89 (1995).
17. Drusano G.L.: Nat. Rev. Microbiol. 2, 289 (2004).
18. Cars O.: Diagn. Microbiol. Infect. Dis. 27, 29 (1997).
19. Czock D., Giehl M., Keller F.: Clin. Pharmacokinet. 38, 367 (2000).
20. Hyatt J.M., McKinnon P.S., Zimmer G.S., Schentag J.J.: Clin. Pharmacokinet. 28, 143 (1995).
21. Boguslaski R.C., Burd J.F.: Am. J. Med. Technol. 49, 551 (1983).
22. Garton A.M., Rennie R.P., Gilpin J., Marrelli M., Shafran S.D.: J. Antimicrob. Chemother. 40, 903 (1997).

23. Denooz R., Charlier C.: J. Chromatogr. B 864, 161 (2008).
24. Szlagowska A., Kaza M., Rudzki P.J.: Acta Pol. Pharm. 67, 677 (2010).
25. Piva G., Farin D., Gozlan I., Kitzes-Cohen R.: Chromatographia. 51, 154 (2000).
26. Rosseel M.T., Peleman R., VanHoorebeke H., Pauwels R.A.: J. Chromatogr. B 21, 438 (1997).
27. Al-Saida M.S., Al-Khamisa K.I., Niazya E.M., El-Sayeda Y.M., Al-Rashooda K.A. et al.: Biopharm. Drug. Disp. 21, 205 (2000).
28. Wolff F., Deprez G., Seyler L., Taccone F., Hites M., et al.: Talanta 103, 153 (2013).
29. Partani P., Gurule S., Khuroo A., Monif T., Bhardwaj S.: J. Chromatogr. B 878, 428 (2010).
30. Colin P., Bock L.D., Tjollyn H.: Talanta 103, 285 (2013).
31. Hu X., Huang M., Liu J., Chen J., Shentu J.: Adv. Pharmacol. Sci. 2014, 1 (2014).
32. Viberg A., Sandström M., Jansson B.: Rapid Commun. Mass Spectrom. 18, 707 (2004).
33. Bulitta J.B., Landersdorfer C.B., Kinzig M., Holzgrabe U., Sorgel F:. Antimicrob. Agents Chemother. 53, 3462 (2009).
34. Nerli B., Romanini D., Picü G.: Chem. Biol. Interact. 104, 179 (1997).
35. European Medicines Agency. Guideline on Bioanalytical Method Validation. (EMEA/CHMP/EWP/192217/2009 Rev. 1 Corr. 2**). 21 July 2011.
36. U.S. Department of Health and Human Services. Food and Drug Administration. Center for Drug Evaluation and Research (CDER). Center for Veterinary Medicine (CVM). Guidance for Industry. Bioanalytical Method Validation. May 2001.
37. Marx G.M., Blake G.M., Galani E., Steer C.B., Harper S.E. et al.: Ann. Oncol. 15, 291 (2004).
38. Gabrielsson J., Weiner D.: in The Pharmacokinetic and Pharmacodynamic Data Analysis: Concepts and Applications, 2nd edn., Rowland M., Tozer T.N. Eds., Swedish Pharmaceutical Press, Stockholm 1997.
39. Thummel K.E., Shen D.D.: in Goodman& Gilman's, The Pharmacological Basic of Therapeutics, 10th edn., Hardman J.G., Limbird L.L., Gilman A.G. Eds. p. 1938, McGraw-Hill, New York 2001.
40. Ljungberg B., Nilsson-Ehle I.: Rev. Infect. Dis. 9, 250 (1987).
41. Cunha B.A.: Clin. Microbiol. Infect. 7, 581 (2001).
42. http://www.eucast.org/ (accessed on 3.11. 2016).
43. Cios A., Wyska E., Szymura-Oleksiak J., Grodzicki T.: Exp. Gerontol. 57, 107 (2014).

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