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Short communication

Simultaneous determination of five β-lactam antibiotics (cefepim, ceftazidim, cefuroxim, meropenem and piperacillin) in human plasma by high-performance liquid chromatography with ultraviolet detection

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Abstract

Monitoring of plasma antibiotic drugs is useful for better clinical management in infected patients, particularly in intensive care units. A simple and sensitive high-performance liquid chromatography (HPLC) method with ultraviolet (UV) detection has been developed and validated for simultaneous quantification of five β-lactam antibiotics in human plasma: cefepim, ceftazidim, cefuroxim, meropenem and piperacillin. The plasma sample, after spiked with ceforanid as an internal standard (IS), was submitted to a solid-phase extraction (SPE) prior to HPLC analysis. A chromatographic separation was achieved on a C8 symmetry column with a mobile phase consisting of an acetonitrile and phosphate buffer (pH 7.4) mixture in a gradient mode. Detection was carried out at a wavelength between 200 and 400 nm. The method developed was linear over the concentration range of 2.5–60 μg/mL for each antibiotic in the plasma samples. Accuracy ranged from 93.2 to 107.1% and precision was between 0.9 and 12.2%. The method has been applied to plasma samples obtained from patients treated with β-lactam antibiotics and is appropriated for easy determination of plasma concentrations for therapeutic monitoring applications.

Keywords: β-Lactam antibiotics; Plasma; HPLC–UV

1. Introduction

The discovery of penicillin in the middle of the last century (1928) revolutionized the treatment of patients with infectious diseases. After the introduction of penicillin for clinical use, a large number of other antimicrobials were added to the antimicrobial arsenal: tetracyclines, cephalosporins, aminoglycosides. Eighty years later, β-lactam antibiotics are always one of the most frequently used antimicrobial agents, including penicillin derivates, cephalosporins, monobactams, carbapenems and β-lactamase inhibitors [1].

β-Lactams are time-dependent antibiotics, which implies that their activity is primarily related to the time during which their serum concentration remains above the minimal inhibition concentration (MIC) for the offending organism. This period above the MIC is the major parameter determining efficacy of β-lactam antibiotics. Many authors suggest that the time the concentration of the antibiotic remains above the MIC should be long, from 40 to 70% of the interval-time between doses [2]. Sometimes, a more long time, up to 100% of the dosing interval, may be needed in case of resistant organisms [3]. As announced in the scientific notices, the MIC for the described antibiotics are very different from one bacteria to another one, but are very often comprised between 0.5 and 4 μg/mL for the most sensitive germs. Determination of the serum concentration of antimicrobials and comparison with the MIC of the micro-organism causing the infection is now an effective tool in individualising antimicrobial therapy. These evaluations led to the optimisation of the dosage regimens which allow to increase clinical efficacy and to reduce the selection of resistant mutants [4–6].

The purpose of this study was to develop a reproducible, reliable, rapid and selective method for the determination

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of five β-lactam antibiotics in plasma for therapeutic drug monitoring. Various methods have already been developed to analyse antibiotics in biological fluids using different sample preparation procedures: precipitation, liquid- or solid-phase extraction, generally followed by liquid chromatography coupled to UV detection [7–15].

We introduced a simple and rapid assay method for the simultaneous determination of piperacillin (penicillin derivate), cefuroxim (second generation cephalosporin), ceftazidim (third generation cephalosporin), cefepim (fourth generation cephalosporin) and meropenem (carbapenem) in plasma. Compared to other LC methods using UV detection that have been published for β-lactams in biological samples, our technique is able to measure simultaneously these five different compounds under the same extraction and chromatographic conditions.

2. Experimental

2.1. Reagents and chemicals

Piperacillin was kindly provided by Wyeth Pharmaceuticals (Louvain-la-Neuve, Belgium). Ceftazidim and cefuroxim were generously supplied by GlaxoSmithKline (Erembodegem, Belgium), cefepim and ceforanid by Bristol-Myers (Braine-l'Alleud, Belgium) and meropenem by AstraZeneca (Destelbergen, Belgium). These products were obtained as certified reference compounds. Acetonitrile and methanol (MeOH) were purchased from Biosolve (Valkenswaard, The Netherlands), potassium dihydrogenophosphate and di-sodium hydrogenophosphate from Merck (Darmstadt, Germany). All reagents were at least of analytical grade. HPLC-grade water was produced by a laboratory MilliQ system (Millipore, Bruxelles, Belgium). Pooled blank plasma was purchased from the blood bank of the Hospital. End-capped C18 SPE cartridges (pore size: 60 Å; particle size: 45 μm; specific surface: 500 m^2/g; sorbent: 100 mg; volume: 1 mL; carbon content: 14%) were from Macherey-Nagel (Düren, Germany).

2.2. Chromatographic system

The development and validation work was carried out on a chromatographic system consisting of a Waters Alliance 2695 Separation Module, equipped with a quaternary, low-pressure mixing pump, a degassing line and a thermostated autosampler, connected with a Waters 2996 photodiode array detector (Zellik, Belgium). The Empower® software (Waters) was used to pilot the HPLC instrument and to process the data (area integration, calculation and plotting of chromatograms). Baselines were visually inspected and were manually adjusted when necessary.

HPLC separation was performed at 25 °C using a Symmetry® C8 analytical column (250 mm × 4.6 mm i.d.) packed with 5 μm diameter particles (Waters), equipped with a guard column (20 mm × 4.6 mm) containing identical packing material. The autosampler was programmed with an injection volume of 40 μL, a carousel temperature of 4 °C and a run time of 35 min.

The mobile phase consisted in acetonitrile (A) and a phosphate buffer (B). It was delivered at 1 mL/min, with the following step-wise gradient elution program: 5% A at time 0, held for 5 min; then the proportion of A was linearly increased to 50% in 20 min, held 1 min and finally decreased to 5% in 5 min. The UV–vis spectra were obtained in the range 200–400 nm.

2.3. Solutions

Stock standard solutions were prepared by dissolving antibiotics (20 mg) in 10 mL of ultrapure water to get a 2.0 mg/mL concentration for each component. Plasma calibration standards at 2.5, 5, 10, 20, 40 and 60 μg/mL concentration of each antibiotic, and plasma control samples at 15, 30 and 50 μg/mL were prepared in batches of 10 mL by adding appropriate volumes of the respective stock solutions to blank plasma. Calibration standards and control samples were stored as 500 μL aliquots in polypropylene Eppendorf tubes at −80 °C until use and thawed the day of analysis. The compound ceforanide was used as the internal standard at the concentration of 125 μg/mL in ultrapure water. This solution was stored at −80 °C.

Phosphate buffer solution was prepared by mixing 197 mL of a 1/15 M potassium dihydrogenophosphate solution (9.07 g dissolved in 1000.0 mL of ultrapure water) and 803 mL of a 1/15 M di-natrium hydrogenophosphate solution (11.87 g dissolved in 1000.0 mL of ultrapure water). The pH was adjusted to 7.4. The buffer solution was filtered through a 0.45-μm HV filter (Millipore) prior to use.

2.4. Sample preparation

Calibration curve standards, quality controls and patient samples were thawed and allowed to equilibrate at room temperature. The thawed samples were vortexed to ensure complete mixing of contents. The clean up procedure of the biological samples was performed by SPE using the 24 tubes vacuum manifold Macherey-Nagel (Düren, Germany). The C18 sep-pak cartridges (Waters) were conditioned with 3× 1 mL MeOH followed by 2× 1 mL phosphate buffer pH 7.4. Five hundred microliters of plasma was mixed in a polypropylene Eppendorf vial with 50 μL of I.S. solution. Then, 500 μL of the resulting solution were loaded on the cartridges and completely drawn through under light vacuum (typically 5 mm Hg). The cartridge was washed four times with 300 μL of phosphate buffer pH 7.4 solution and twice with 300 μL of 5% MeOH in phosphate buffer pH 7.4 solution. Antibiotics were desorbed three times with 500 μL of MeOH. The eluted solutions were evaporated under a nitrogen stream at +30 °C and the residue was reconstituted in 100 μL of phosphate buffer–acetonitrile (95:5, v/v). Samples were introduced into 200 μL HPLC microvials and a volume of 30 μL was used for HPLC analysis.

2.5. Validation of the method

The method has been validated following the general guidelines for validation of analytical methods with the Enoval program [16,17].

Calibration curves were obtained by plotting ratios of analyte peak area divided by internal standard peak area versus the

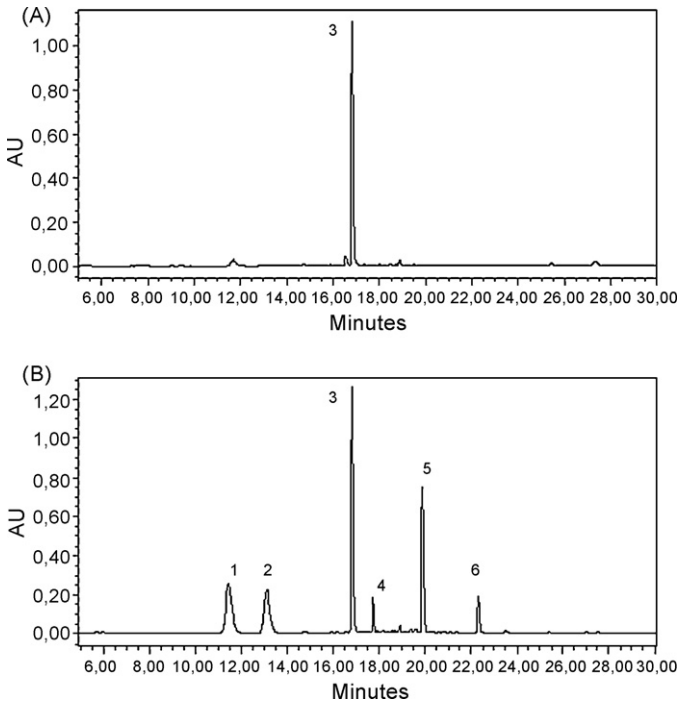

Fig. 1. HPLC chromatograms of (A) a blank patient plasma and (B) a plasma spiked with 15 μg/mL of each antibiotic and 20 μg/mL of the internal standard (1, ceftazidim; 2, cefepim; 3, ceforanide; 4, meropenem: 5, cefuroxim; 6, piperacillin).

analyte concentrations in spiked samples. The concentration of the unknown sample was calculated using linear regression analysis. The linearity of the method was demonstrated over the concentration range of 2.5–60 μg/mL for each compound by assaying six calibration standards in duplicate, one set at the beginning and the second at the end of the HPLC run, on three separate occasions. Control samples were analysed in triplicates at four concentration levels (5, 15, 30 and 50 μg/mL) of each compound, on three different days too. The control samples were used for determination of precision and accuracy of the method, calculated with the established calibration curves, and, of course, in each run. The limits of quantification (LOQ) were experimentally determined by analysing plasma samples spiked with antibiotics at 0.5, 1, 1.5, 2 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, in accordance with the FDA guidelines [18].

Different solutions were prepared to evaluate the absolute recovery: spiked plasma (5, 15, 30 μg/mL) subjected to SPE before being injected into the HPLC system and spiked aqueous solutions, not subjected to SPE, but injected directly into the system at equivalent concentrations of antibiotics. The absolute recovery (expressed in %) was evaluated as the ratio of the concentrations of antibiotics calculated for each solution.

The stability of antibiotics in plasma and in stock solutions was inspected during all the storage steps (i.e., at room temperature (22–25 °C), at +4 °C and at −80 °C). To determine the stability of β-lactams in the matrix, the plasma pool was spiked with the standard mixture to achieve a concentration of 20 μg/mL for each antimicrobial agent and then aliquoted in polypropylene Eppendorf tubes. Five aliquots from each storage condition were analysed at hour 0, 3, 6, 9, 12 and 24 for the room temperature stability study, at hour 3, 6, 9, 12, 24, 48 and 72 for the test at +4 °C, and at week 1, 2, 4 and 8 for samples stored at −80 °C. After being placed at the appropriated temperature, samples were stored if necessary at −80 °C until the day of analysis.

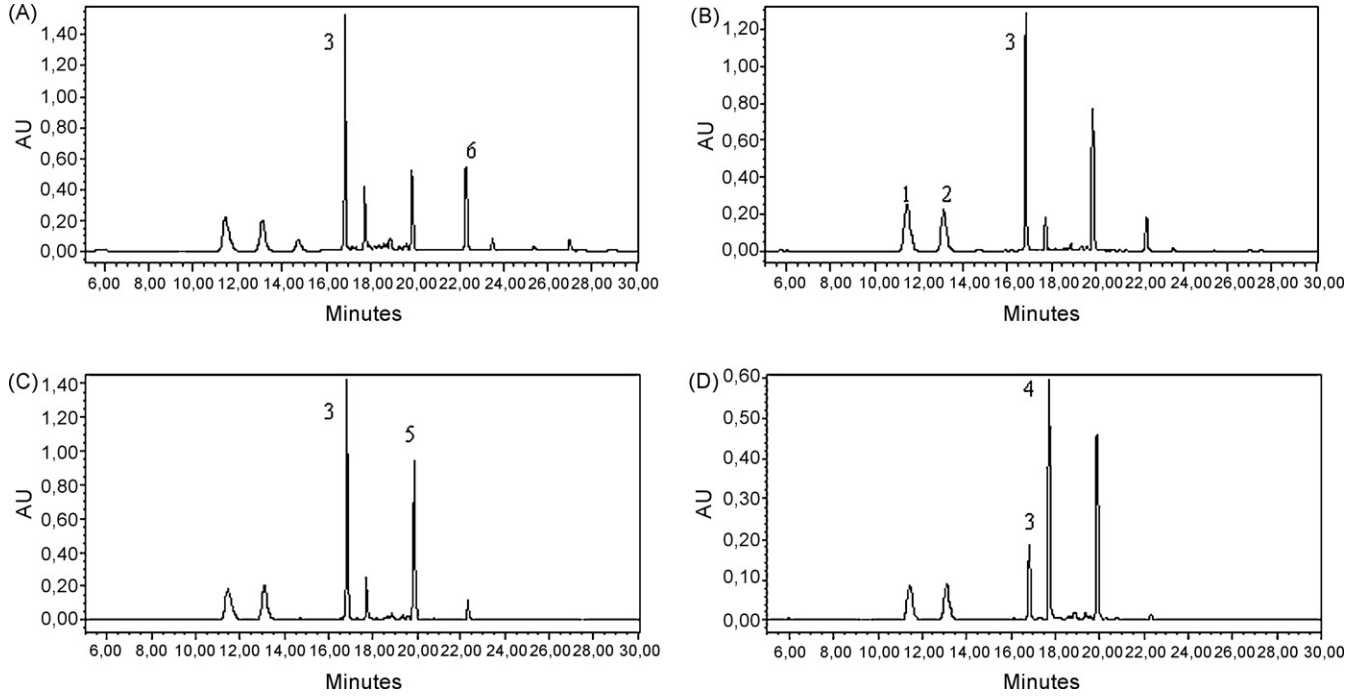

Fig. 2. The stacked chromatograms of a standard mixture of antibiotics at 15 μg/mL at 220 nm (A), at 256 nm (B), at 270 nm (C) and at 300 nm (D) (1, ceftazidim; 2, cefepim; 3, ceforanide; 4, meropenem: 5, cefuroxim; 6, piperacillin).

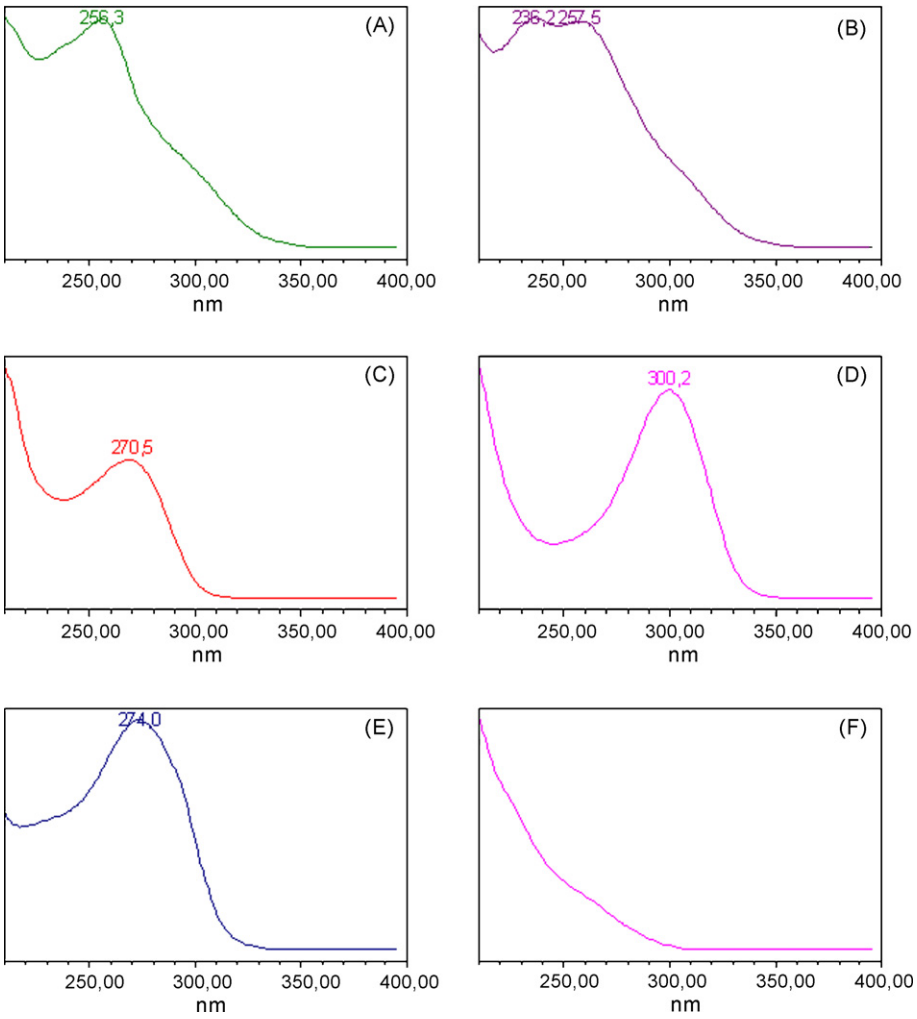

Fig. 3. The UV–vis spectra between 200 and 400 nm of each analyte (A, ceftazidim; B, cefepim; C, ceforanide; D, meropenem; E, cefuroxim; F, piperacillin).

Solutions of ceforanide at the concentration of 125 μg/mL in ultrapure water were tested too. Prior to their analysis, frozen samples were brought to room temperature and vortex-mixed well and concentrations were determined using calibration curves. The freezing-thawing stability of drugs at −80 °C was also confirmed. Samples were then analysed on a daily basis after repeated freezing-thawing cycles at −80 °C on three consecutive days. Drugs were considered stable in plasma if at least 90% of products were retained at the end of the study period and, stable if at least 95% were retained in the other solutions.

The influence of the matrix on the extraction by SPE was tested by varying the concentrations of proteins. Plasma, before spiked with antibiotics, was diluted with zero to ten volumes of physiological serum (NaCl 0.9%) to investigate the influence of proteins on the extraction procedure.

Each of the studied antibiotics were known to be instable, and specially at room temperature. The ruggedness of the procedure was estimated by checking its susceptibility to minor changes such as the time for the post-extracting of the samples. In normal conditions, the total time of extraction procedure was between 1 and 1.5 h. Eluates obtained from spiked plasma and ready to be injected in the HPLC system were stored during 0, 1, 2, 3, 4 and 5 h at room temperature before injection. Stability of β-lactam have been evaluated too, by storing eluates in an autosampler programmed at 4 °C during different periods of time (0, 6, 9, 12, 18, 24 h). Each determination was performed in triplicate.

Of course, control samples (15, 30 and 50 μg/mL) had been used in each run to validate the method.

2.6. Application

The method developed was used to investigate the plasma concentrations of β-lactam antibiotics in seriously infected patients. Blood samples were collected into heparinized test tubes and immediately centrifuged at 900 × *g* for 10 min. The plasma was immediately frozen at −80 °C in microtubes until analysis.

3. Results and discussion

3.1. Chromatograms

The proposed method enables the simultaneous quantification of five β-lactams antibiotics in plasma sample on the

Table 1
Assessment of the accuracy, precision and recovery

Analyte	Nominal concentration (μg/mL)	Accuracy (%)	Precision		Recovery (%)
			Intra-assay (%)	Inter-assay (%)	
Cefepim	5	96.2	3.7	6.8	70.0
	15	95.5	3.0	4.4	71.7
	30	93.2	3.8	4.8	57.4
	50	94.2	4.2	4.2	nt
Ceftazidim	5	98.2	6.6	8.4	66.3
	15	98.8	5.4	5.8	75.7
	30	94.8	3.3	4.3	65.6
	50	96.8	2.5	2.5	nt
Cefuroxim	5	97.8	3.5	4.0	82.7
	15	101.9	1.9	4.3	84.8
	30	104.0	9.6	9.7	83.4
	50	107.1	6.0	12.2	nt
Meroapenem	5	100.3	0.9	5.9	74.3
	15	96.7	3.1	3.2	76.9
	30	94.3	2.0	3.5	79.7
	50	96.5	1.3	1.3	nt
Piperacillin	5	100.0	3.6	3.8	81.1
	15	99.1	2.2	3.3	78.7
	30	98.3	5.2	5.2	78.3
	50	98.4	4.0	5.6	nt

nt: Not tested.

same HPLC run. The retention times of ceftazidim, cefepim, ceforanide, meropenem, cefuroxim, and pipéracillin were 11.50, 13.00, 16.80, 17.80, 19.95 and 22.36 min, respectively and the total run time of analysis was 30 min. As illustrated in Fig. 1, a blank patient sample and a plasma sample spiked with 15 μg/mL of each antibiotic are of high chromatographic quality. For specific quantifications, the wavelength were set at 256 nm for cefepim and ceftazidim, 270 nm for cefuroxim and ceforanide, 300 nm for meropenem and 220 nm for piperacilline, as shown in the chromatograms presented in Fig. 2. The identification of antibiotics was based on the retention time and UV spectrum (Fig. 3). Peak purity of the analytes was corroborated by comparing the UV spectra of antibiotics peaks in plasma samples with the spectra of the drugs in the working solution standards. As shown in the chromatogram of the blank plasma (Fig. 1), no interfering peaks due to the matrix components were observed at the retention time of antibiotics.

3.2. Calibration curves and linearity

The standard curves were satisfactorily described by unweighted linear regression analysis. The calibration parameters were stable with regression coefficients always ≥0.994. The curves were linear over the concentration range studied (2.5–60 μg/mL).

3.3. Limit of quantification

The limit of quantification in plasma samples was experimentally determined to be 0.5 μg/mL for ceftazidim, piperacillin ad meropenem, and 1.0 μg/mL for cefepim and cefuroxim. The mean precision and accuracy for this limit value was found to be less than the maximum tolerable CV of 20% and within 80–120%. These limits are better or comparable to those reported previously in published HPLC methods and are sufficient to determine plasma concentrations of each of the five antibiotics in the clinical pharmacokinetic studies [7–15]. Insofar as in all the spiked samples, each antibiotic was easily detected, the limits of detection were at least at 0.5 μg/mL. These limits were sufficient to establish correlations between the plasma concentrations and the MICs, generally comprised between 0.5 and 4 μg/ml for the most sensitive germs.

3.4. Precision, accuracy and recovery

The precision, the accuracy and the recovery of the HPLC developed method are given in Table 1. The accuracy and precisions were within the acceptable criteria: precision lower than 15% and mean accuracy within 85–115%. The intra-day and extra-day run precision were evaluated by replicating at three different moments the analyses of the plasma samples containing the antibiotics at four different concentrations. Recovery was determined at three different concentrations. Each value represents the mean of five measurements carried out.

3.5. Stability

Internal standard solutions, stock solutions and spiked plasmas did not reveal any appreciable degradation after 8 weeks of storage at −80 °C. At least three freeze-thaw cycles were able

Table 2
Result of analysis of several plasma samples obtained from patients undergoing chronic antibiotic treatments with continuous perfusion (A) or obtained from patients approximately 3 h after the end of a 30 min perfusion (B)

β-Lactams	Doses (g/day)	Concentrations (μg/mL)
(A)		
Meropenem	6	32.4
Meropenem	6	19.8
Meropenem	6	19.7
Piperacillin	20	86.0
Piperacillin	36	152.0
Piperacillin	24	59.3
Piperacillin	16	40.4
Cefepim	6	66.5
Ceftazidim	6	60.0
Ceftazidim	6	114.4
Ceftazidim	2	39.7
Ceftazidim	2	48.8
Ceftazidim	6	71.6
Ceftazidim	6	90.1
Ceftazidim	6	40.3
(B)		
Meropenem	4× 0.5	3.5
Meropenem	4× 1	48.0
Meropenem	3× 2	3.1
Cefepim	1	22.8
Cefepim	2	17.5
Cefepim	2	10.8
Cefepim	2	16.1
Cefepim	6	22.2

to be executed without losses higher than 5% for solutions and than 10% for plasmas.

Stock solutions could be stored at 4 °C for 24 h with no evidence of decomposition (<5%) and up to 48 h for ceftazidim. At 20 °C, all antibiotic stock solutions could be conserved for at least 12 h, excepting for the cefuroxim, which presented a degradation of 8% at this temperature. After 9 h, the stability remained acceptable (degradation <5%).

The antibiotics spiked in plasma presented more unstability. At room temperature, the degradation of cefuroxim was 11% after 9 h but less than 8% after 6 h. The other β-lactams presented acceptable stability after 9 h of storage at +20 °C. At 4 °C, all antibiotics were judged stable for at least 24 h, with degradation always lower than 10%.

Therefore, stock solutions and spiked plasmas could stay at least 2 months at −80 °C, at least 24 h at 4 °C and no more than 6 h at +20 °C. Treatments at room temperature had to be as short as possible. Plasma after collection had to be transferred to laboratories at +4 °C as quickly as possible and had to be frozen at −80 °C within the hour of reception.

3.6. Matrix effect

To evaluate the matrix effect, the concentration of proteins was changed by dilution of samples with physiological serum. No significant differences were observed. The antibiotic peak areas remained almost constant with decreased concentration of proteins for the entire series of measurements.

3.7. Ruggedness

The stability of β-lactams after processing had been evaluated on the bench-top at room temperature and in the autosampler at 4 °C. There was no statistical difference (<10%) between the results for each group according to the tested parameter. The procedure exhibited suitable ruggedness for some variations of analysis delays. Consequently, vials ready to be injected could stay at least 5 h at room temperature and at least 24 h in an autosampler programmed at 4 °C before injection with no significant difference in concentration determination.

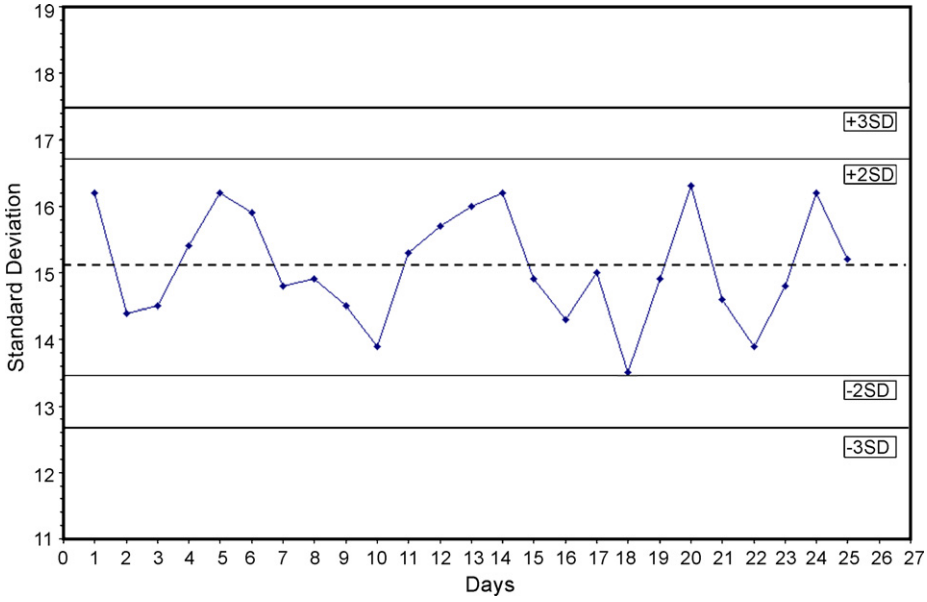

Fig. 4. Results of the quality controls obtained for ceftazidim (15 μg/mL) over a 6 months period (25 different runs).

3.8. Clinical application

The present method was successfully applied to perform the determination of concentrations of cefepim, ceftazidim, meropenem and piperacillin in several plasma samples obtained from patients undergoing chronic treatment, either as monotherapy or polytherapy with other antibiotic drugs. Results of some analysis are presented in Table 2. Plasma samples obtained from patients treated with continuous perfusions were systematically diluted with physiological serum (NaCl 0.9%) to be sure to obtain values included in the calibration curves. The results of the analyses of the ceftazidime control samples (spiked at 15 μg/mL) over a 6 months period are presented in Fig. 4.

4. Conclusion

Monitoring of the antibiotics concentration in plasma is an interesting tool in the treatment of patients seriously infected since the period during which the plasma concentration remains above the MIC is one of the most important parameters for the success of the treatment. The aim of this study was to develop a method for the determination of five β-lactam antibiotics in human plasma. The simultaneous determination of drugs is useful for routine application in the clinical laboratory. With this single sample preparation protocol, the analyst can monitor the most used antibiotic in intensive care units. This is the first report describing a simultaneous quantification of these β-lactam antibiotics. The method was simple, precise, accurate, selective and sufficiently sensitive. Analysis of plasma samples obtained from patients treated has shown the clinical suitability of the method for controlling the antimicrobial therapy of infected patients.

Further determinations will be done and comparison between the results of microbiological techniques and drug plasma monitoring will be performed to propose to the physicians a highly performing approach of the antibiotherapy in intensive care units.

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