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Short communication

High performance liquid chromatographic determination of plasma free and total tazobactam and piperacillin

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ABSTRACT

A high-pressure liquid chromatography (HPLC) method with ultraviolet detection was developed for the measurement of plasma free and total tazobactam and piperacillin. This method is simple and fast, requiring only 11 min for the HPLC run and a sample preparation of about 11 min for total drugs and 10 min for free drugs. The procedure for the assay involves the treatment of plasma with acetonitrile for total drugs determination, and the use of a centrifugal filter device to deproteinize plasma for free drugs determination. The HPLC column, a Hypersil-ODS, was equilibrated with an eluent mixture composed of acetonitrile–potassium phosphate (pH 2.6). CVs for repeatability of tazobactam and piperacillin measurements ranged from 4.30 to 6.60; CVs for reproducibility ranged from 5.60 to 9.40. Mean analytical recoveries ranged from 100.4 to 103%. A linear relationship was obtained between peak area and drugs concentration in the range studied (0–62.5 mg/L for tazobactam and 0–500 mg/L for piperacillin). The equation for regression line were $y = 19x - 1.4$ for tazobactam and $y = 1.7x - 0.9$ for piperacillin; correlation coefficients were >0.999. The lower limit of quantitation (LLQ) for standard samples was about 0.12 mg/L for tazobactam and 0.49 mg/L for piperacillin, respectively. The lower limit of detection (LLD) was 0.06 mg/L for tazobactam and 0.24 mg/L for piperacillin. This HPLC assay for tazobactam and piperacillin is sensitive and accurate, and provides a reliable determination of both free and total tazobactam and piperacillin in human plasma, thus allowing the determination of these analytes in patients receiving tazocillin therapy.

1. Introduction

Antimicrobial resistance in bacteria is of great concern to healthcare practitioners [1] and is particularly important in critically ill patients admitted in intensive care units (ICU), who often experienced devastating infections. To minimize resistance development it is important to perform an early and appropriate antibacterial therapy. To optimize antibacterial therapy, the clinician should have knowledge of the pharmacokinetic and pharmacodynamic properties of commonly used antibacterial products. In addition, the important parameters may be affected by a constellation of pathophysiological changes that occur during infections, particularly sepsis. Sepsis increases renal preload and, via capillary permeability, leads to 'third-spacing', both resulting in higher antibacterial clearances. Alternatively, sepsis can induce multiple organ dysfunction, including renal and/or hepatic dysfunction, causing a decrease in antibacterial clearance [2]. In ICU patients many other factors such as burns, ipoalbuminemia, hemorrhages, continuous renal replacement therapies (CRRT), and massive transfusions, can affect blood concentration of hydrophilic antibacterial compounds such as β-lactams.

Piperacillin, a β-lactam antibiotic, exhibits a time-dependent bacterial killing activity. Maximizing the time above the minimum inhibitory concentration (MIC) for a pathogen is the best pharmacodynamic predictor of efficacy [3] Thus, conventional drug-dosing schemes may need continuous adjustments due to the pharmacokinetic and pharmacodynamic influences of infections in ICU patients, particularly in CRRT recipients [4]. To adjust drug dosage, a reliable method for monitoring drugs levels is required. Since the serum free and protein-bound fractions of drugs are found in dynamic equilibrium, and only the free fraction is available for therapeutic effects [5], as well as for metabolic and clearance by

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dialysis, distinguishing the free fraction from the total amount of drugs becomes critically important.

Tazobactam is a potent β-lactamase inhibitor belonging to a class of penicillanic acid sulfones. It is under development as an intravenous preparation in combination with piperacillin. Piperacillin–tazobactam (tazocillin) combined preparation has been shown by both *in vitro* and *in vivo* studies to be a potent inhibitor of β-lactam–β-lactamase [6].

Several methods for the measurement of tazobactam and piperacillin in biological fluids have been developed [6–11], but they are time-consuming or use laborious extraction protocols. Moreover, none reports the determination of both plasma free and total tazocillin in spite of, especially in critically ill patients, the correct dosage of antimicrobial drugs requires knowledge of several factors including the protein binding, a pharmacokinetic parameter with high interpatient variability. In addition, several papers report that piperacillin and tazobactam have nonlinear pharmacokinetics properties, further justifying the need for the measurement of both free and total drugs [12,13].

We present here a method for measuring plasma free and total tazobactam and piperacillin, which is based on high-pressure liquid chromatography (HPLC) followed by ultraviolet detection. The procedure for the assay involves the treatment of plasma with acetonitrile for total drugs determination, and the use of a centrifugal filter device (ULTRAFREE®–MC) to separate the unbound drug fraction for free drugs determination. The method is reproducible and sensitive, and it can be used in routine analysis of tazocillin.

2. Experimental

Blood was collected into a Vacutainer Tube (Becton Dickinson, Rutherford, NJ) containing EDTA. Plasma was obtained without delay by centrifugation of blood at 3,500 rpm for 5 min and stored at −20 °C until analysis. The procedure for total drugs determination has been performed, with little changes, as reported by Ocampo et al. [7]. 100 μL of plasma was spiked with 400 μL of acetonitrile. The solutions were vortex mixed for 30 s and the upper phase was then added to 2 mL of dichloromethane, vortex mixed for 30 s and centrifuged at 1000 × *g* for 10 min. 30 μL of the upper phase was then subjected to high-pressure liquid chromatography. For free drugs determination, 100 μL of plasma was centrifuged at 1000 × *g* for 10 min using an ULTRAFREE®–MC centrifugal filter device (Millipore Corporation, Bedford, MA, USA); 30 μL of ultra filtrate was then injected into a 150 mm × 4.6 mm Hypersil-ODS column equilibrated with a buffer prepared as follows: potassium phosphate monobasic (27 g) was added to 100 mL of acetonitrile and 900 mL of water and the pH was adjusted to 2.6 with 37% hydrochloric acid (buffer A).

Tazobactam and piperacillin were eluted from the column in 2.2 and 6.4 min, respectively, with a gradient of acetonitrile (buffer B) (0 min, 0% B; 1–7 min, linear gradient to 50% B), at a flow rate of 1.5 mL/min. Total HPLC run time was 9 min; re-equilibration time was 2 min. Room temperature was used, and the retention times for each analyte were calculated using external standards at five different concentrations.

The HPLC-system was an Agilent Technologies 1100 Series equipped with a diode-array detector operating at a wavelength of 220 nm. All operations, such as the injection cycle, were controlled by the ChemStation® program; the data obtained were analyzed with the ChemStation® program (Agilent Technologies Deutschland GmbH, Waldbronn, Germany).

A typical HPLC/DAD chromatogram of a standard sample (Fig. 1A) shows retention time of about 2.2 min for tazobactam and 6.4 min for piperacillin. Fig. 1B shows the chromatogram of a plasma sample obtained from a patient not on tazocillin therapy; Fig. 1C shows the chromatogram of free drugs determination in a sample from a subject after intravenous administration of known dose of tazocillin.

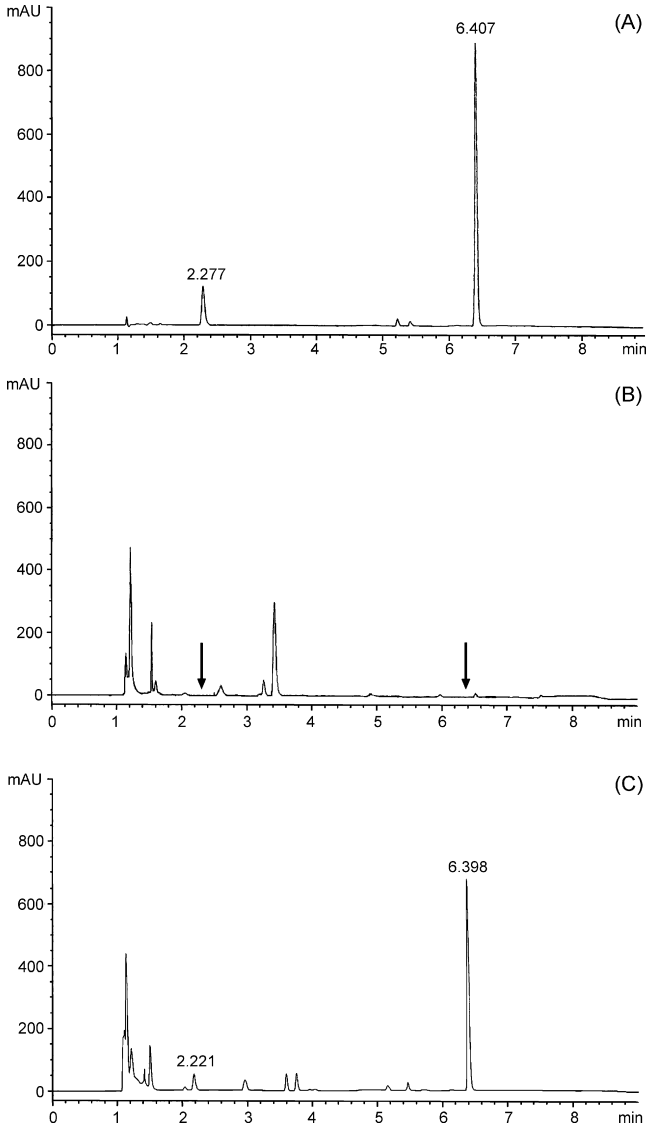

Fig. 1. Chromatograms of: (A) standard solution containing 31.2 mg/L tazobactam and 389 mg/L piperacillin. (B) Human plasma sample obtained from a patient not on tazocillin therapy. (C) Human plasma of a subject after administration of known dose of tazocillin. The peaks correspond to a free tazobactam concentration of 13 mg/L and a free piperacillin concentration of 160 mg/L. Retention times were 2.2 and 6.4 min for tazobactam and piperacillin, respectively.

We assessed the precision of the method by within and between run validation. Repeatability was studied by performing replicate analyses of pooled patient samples at high, middle and low concentrations against a calibration curve. Table 1A reports CVs for repeatability of tazobactam and piperacillin measurements; CVs for reproducibility, as determined by assaying on 10 different days over 1 month, are also reported.

The extraction efficiency (recovery) was determined assaying drug-free plasma spiked with known amounts of drugs (0.5, 5 and 50 mg/mL for tazobactam; 5, 50 and 500 mg/mL for piperacillin). Each sample was determined in quintuplicate. In Table 1B the mean analytical recoveries for tazobactam and piperacillin are shown. The accuracy of the method was expressed as percent deviation

Table 1A
Precision of the assay.

	Intra assay ($n = 10$)			Inter assay ($n = 10$)		
	Mean (mg/L)	SD	CV (%)	Mean (mg/L)	SD	CV (%)
Tazobactam (free)	5.08	0.22	4.30	5.10	0.30	5.90
	2.06	0.15	7.28	2.02	0.17	8.40
	1.26	0.10	7.90	1.28	0.11	8.60
Piperacillin (free)	50.50	3.90	7.70	49.94	4.69	9.30
	10.15	0.60	5.90	10.06	0.80	7.90
	1.03	0.05	4.80	1.00	0.09	9.00
Tazobactam (total)	12.88	0.63	4.89	13.00	0.80	6.10
	4.44	0.24	5.40	4.58	0.31	6.80
	2.06	0.09	4.37	2.15	0.12	5.60
Piperacillin (total)	173.20	10.64	6.10	174.00	12.00	6.90
	39.30	2.50	6.40	40.20	3.40	8.40
	5.26	0.35	6.60	5.30	0.50	9.40

Table 1B
Analytical recovery and accuracy of the assay.

	Added* (mg/L)	Measured[a] Total (mg/L)	Recovered Total (%)	Relative error (%)
Tazobactam	0.5	0.51 (0.03)	102.0	−2.00
	5	5.04 (0.31)	100.8	−0.80
	50	51.2 (3.20)	102.4	−2.40
Piperacillin	5	5.15 (0.34)	103.0	−3.00
	50	50.7 (3.9)	101.4	−1.40
	500	502 (13.4)	100.4	−0.40

SDs are indicated in parenthesis.
* Added to drug-free plasma.
[a] Mean of five replicate values.

of observed concentration from theoretical concentration with the relative error.

Calibration curve for tazobactam (0.12–62.5 mg/L) and piperacillin (0.49–500 mg/L) were prepared by diluting stock solutions with deionised water. A linear relationship was obtained between peak area and drugs concentration in the range studied; the equation for regression line was: $y = 19x - 1.4$ for tazobactam and $y = 1.7x - 0.9$ for piperacillin; correlation coefficients were >0.999 for both the analytes studied. The linearity of the assay was studied in the range: 0–62.5 mg/L for tazobactam and 0–500 mg/L for piperacillin. Linearity was confirmed by showing that the slopes of linear calibration curves were statistically different from zero and by comparison of intercept with zero and a correlation coefficient with 1.

The lower limit of detection (LLD) for standard samples, defined as the concentration resulting in a peak area of three times the signal-to-noise ratio, was about 0.06 mg/L for tazobactam and 0.24 mg/L for piperacillin, respectively.

To evaluate the specificity of the method, drug-free plasma was subjected to the assay procedure and the retention times of endogenous compounds were compared with those of tazobactam and piperacillin. As shown in Fig. 1B, no peak are observed at the retention times of tazobactam or piperacillin. Potential chromatographic interference by other commonly administered drugs was also studied. The following drugs were investigated: amikacin, ampicillin, amoxicillin, cefotaxime, cilastatin, cimetidine, ciprofloxacin, clavulanic acid, imipenem, meropenem, and sulbactam. No interference was found with any of the drugs tested (data not shown).

Conventional drug-dosing schemes may need continuous adjustments due to the pharmacokinetic and pharmacodynamic influences of infections in patients, particularly in CRRT recipients [4]. To adjust the drugs dosages, it is important to monitor free and protein-bound fractions of drugs, which are in dynamic equilibrium themselves. Since only the free, unbound fraction is available to interact with bacteria by diffusing in interstitial space of peripheral compartments [5], and it is the fraction that undergoes metabolic and dialytic clearances, it becomes of crucial importance to distinguish the free fraction from the total amount of drugs.

In conclusion, this HPLC assay for plasma is sensitive and accurate and provides a high sample throughput. In addition, the method is quick and simple, and requires low sample volumes, providing a reliable determination of both free and total tazobactam and piperacillin in human plasma, thus allowing the accurate determination of these analytes in all patients receiving tazocillin therapy, in particular in critically ill patients in which the correct dosing of antimicrobial drugs requires knowledge of several pharmacokinetic parameters, such as the exact amount of drugs bound to plasma protein. Determination of free and total drugs with this method allows the accurate quantification of the protein-bound fraction simply subtracting the free drugs concentration from the total amount.

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References

[1] M.J. Wood, R.C. Moellering Jr., Clin. Infect. Dis. 36 (2003) S2.
[2] J.A. Roberts, J. Lipman, Clin. Pharmacokinet. 45 (2006) 755.
[3] D.S. Burgess, T. Waldrep, Clin. Ther. 24 (2002) 1090.
[4] A. Arzuaga, A. Isla, A.R. Gascón, J. Maynar, A. Martín, M.A. Solinís, D. Toral, J.L. Pedraz, Biomed. Chromatogr. 19 (2005) 570.
[5] T. Dalla Costa, A. Nolting, A. Kovar, H. Derendorf, J. Antimicrob. Chemother. 42 (1998) 769.
[6] T. Marunaka, E. Maniwa, E. Matshushima, Y. Minami, J. Chromatogr. 431 (1988) 87.
[7] A.P. Ocampo, K.D. Hoyt, N. Wadgaonkar, A.H. Carver, C.V. Puglisi, J. Chromatogr. 496 (1989) 167.
[8] Y. Guillaume, E. Peyrin, C. Guinchard, J. Chromatogr. B Biomed. Appl. 665 (1995) 363.
[9] V. Augey, P.Y. Grosse, G. Albert, M. Audran, F. Bressolle, J. Chromatogr. B Biomed. Appl. 682 (1996) 125.
[10] C. Li, D. Xuan, M. Ye, C.H. Nightingale, D.P. Nicolau, Biomed. Chromatogr. 19 (2005) 99.
[11] C.H. Xia, Y.Q. Xiong, G.J. Wang, Biomed. Chromatogr. 21 (2007) 680.
[12] F. Sorgel, M. Kinzing, J. Antimicrob. Chemother. 31 (1993) 39.
[13] M.K. Kim, D. Xuan, R. Quintiliani, C.H. Nightingale, D.P. Nicolau, J. Antimicrob. Chemother. 48 (2001) 259.