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High-performance liquid chromatographic determination of tazobactam and piperacillin in human plasma and urine

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Abstract

A high-performance liquid chromatographic (HPLC) method with ultraviolet (UV) absorbance was developed for the analysis of piperacillin–tazobactam (tazocillin), in plasma and urine. The detection was performed at 218 nm for tazobactam and 222 nm for piperacillin. The procedure for assay of these two compounds in plasma and of piperacillin in urine involves the addition of an internal standard (ceftazidime for tazobactam and benzylpenicillin for piperacillin) followed by a treatment of the samples with acetonitrile and chloroform. To quantify tazobactam in urine, diluted samples were analysed using a column-switching technique without internal standard. The HPLC column, LiChrosorb RP-select B, was equilibrated with an eluent mixture composed of acetonitrile–ammonium acetate (pH 5). The proposed technique is reproducible, selective, and reliable. The method has been validated, and stability tests under various conditions have been performed. Linear detector responses were observed for the calibration curve standards in the ranges 5–60 μg/ml for tazobactam, and 1–100 μg/ml for piperacillin and spans what is currently though to be the clinically relevant range for tazocillin concentrations in body fluids. The limit of quantification was 3 μg/ml for tazobactam and 0.5 μg/ml for piperacillin in plasma and urine. Extraction recoveries from plasma proved to be more than 85%. Precision, expressed as C.V., was in the range 0.4–18%.

Keywords: Tazobactam; Piperacillin; Tazocillin

1. Introduction

Piperacillin is an ureidopenicillin (Fig. 1), susceptible to hydrolysis by a range of β-lactamases, including the plasmid-mediated enzymes. Tazobactam, [2*S*-(2α,3β,5α)]-3-methyl-7-oxo-3-(1*H*-1,2,3-triazol-1-ylmethyl)-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid 4,4-dioxide, is an irreversible inhibitor of many bacterial β-lactamases belonging to a class of penicillanic acid sulfones. When combined with piperacillin, tazobactam has demonstrated notable synergy against β-lactamase-producing strains including *Staphylococcus aureus*, *Haemophilus influenzae*, *Bacteroides spp.* and many of the Enterobacteriaceae [1,2]. Piperacillin–tazobactam (tazocillin) is currently undergoing clinical investigation as empirical therapy for moderate to severe polymicrobial infections. This drug is administered as a fixed ratio (eight parts piperacillin to one part tazobactam) [3].

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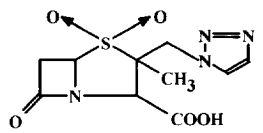
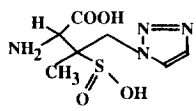
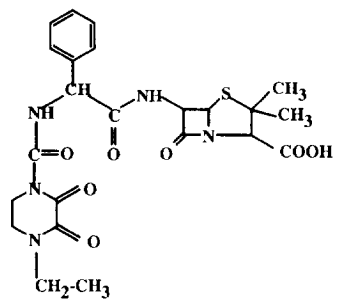


Tazobactam, I



Metabolite M1



Piperacillin, II

Fig. 1. Structural formulae of tazobactam, its main metabolite and piperacillin.

Clinical trials have shown the efficacy of tazocillin in the treatment of intra-abdominal infections, skin and soft-tissue infections, respiratory tract infections and febrile episodes in patients with neutropenia [3].

Several methods for detection and quantification of tazobactam and piperacillin in biological fluids have been developed [4–11]. In most of them, liquid–liquid extraction was followed by evaporation of the organic phase under nitrogen stream. These methods included microbiological assay [4], classical reversed-phase HPLC [5,7–11] and ion-pair HPLC [6]. Ocampo et al. [5] reported an HPLC gradient system which allowed simultaneous measurement of tazobactam and piperacillin with a limit of quantitation (LOQ) for both compounds of 1 μg/ml in plasma and 50 μg/ml in urine. After deproteinisation of plasma with acetonitrile and extraction of the samples with dichloromethane, a column-switching technique was used by Muth et al. [7] to quantify tazobactam in plasma; concentrations as low as 0.1 μg/ml were detected. Several methods have been described to quantify piperacillin in serum or plasma and urine [5,6,9–11]. All these methods have a LOQ higher or equal to 1 μg/ml in plasma and urine.

The purpose of this study was to develop reproducible, reliable, rapid and selective methods for the determination of tazobactam and piperacillin in plasma and urine, for therapeutic drug monitoring on patients with peritonitis. Two different methods were developed, a liquid–liquid extraction to quantify tazobactam in plasma and piperacillin in plasma and urine, and a solid-phase extraction (SPE) method based on column switching for urine assay of tazobactam. These methods have enhanced precision due to the use of internal standards with retention times very close to that of the drug under analysis. These methods were validated according to Good Laboratory Practice Guidelines [12,13].

2. Experimental

2.1. Materials and reagents

Tazobactam and piperacillin were obtained from Wyeth Lederle (Paris La Défense, France). The internal standards, ceftazidime (III) and benzylpenicillin sodium (IV), were obtained from Glaxo (Paris, France) and Sarbach (Suresnes, France), respectively. The structural formulae of these compounds are shown in Fig. 1. Acetonitrile and chloroform were Chromasol grade (SDS, Peypin, France) and used without further purification. Ammonium acetate, sodium dihydrogenphosphate, sodium hydroxide and acetic acid were all analytical grade (Merck, Nogent sur Marne, France). Sodium dihydrogenphosphate (0.1 *M*, pH 6) was prepared in purified water (Laboratoires Fandre, Ludres, France) and adjusted with sodium hydroxide (10%). The buffer consisted of 5.4 g ammonium acetate in 1 l of purified water adjusted to pH 5.0 with 10% acetic acid.

Stock solutions of tazobactam and piperacillin (1 mg/ml) were prepared in purified water and absolute ethanol, respectively. Further dilutions of the working solutions were made with purified water (0.5 μg/ml). The internal standard stock solutions (1 mg/ml) were prepared in purified water.

For validation of the method, human plasma was obtained from pooled blood samples collected from healthy volunteers. Coagulation was prevented by adding EDTA-sodium salt. The blood was then centrifuged at 2000 *g* for 10 min. The obtained

drug-free plasma and drug-free human urine were stored at −30°C before use.

2.2. Instrumentation

The system consisted of the following components: a Model P4000 quaternary gradient pump from Thermo Separation Products (Orsay, France) with a Rheodyne loading valve (Model 7010) fitted with a 100-μl sample loop, an automatic sample injection system (Model 231, Gilson Medical Electronic, Villiers le Bel, France), a guard column (20× 4.6 mm I.D.; SFCC, Neuilly Plaisance, France) packed with Hypersil ODS, and a stainless-steel column (250×4.6 mm I.D., Merck, Nogent sur Marne, France) packed with LiChrosorb RP-select B (particle size, 5 μm). The column effluent was monitored with a Spectra Focus spectrophotometric detector (Thermo Separation Products). The HPLC system was interfaced with an IBM compatible-DX computer–data station and controlled through Thermo Product PC 1000 software which allowed post-data analysis whilst allowing further on-line acquisition of data.

For the on-line SPE clean-up and pre-concentration of tazobactam in urine, analysis by HPLC was performed using a Gilson instrument (Model 305) with a Rheodyne loading valve (Model 7010) fitted with a 50-μl sample loop, an automatic sample injection system (Gilson 232) and the previously described stainless-steel column (LiChrosorb, 5μm). The basic chromatographic apparatus was supplemented with a pre-column (30×4.6 mm I.D.), dry filled with Spheri-5 amino (5 μm, Brownlee, Touzart Matignon, Paris, France), a constant-flow pump (Gilson, Model 305) for pumping the necessary solvent for the clean-up and pre-concentration on the pre-column, a six-way high-pressure valve and a Gilson sample controller for the complete automation of the switching operations. All the chromatographic conditions were controlled using the GME 712 Gilson software. The scheme of chromatographic apparatus used for on-line clean-up of samples with column switching is shown in Fig. 2. The column effluent was monitored with a variable wavelength UV detector (Model SPD-6AV; Shimadzu Instruments, Touzart Matignon, France).

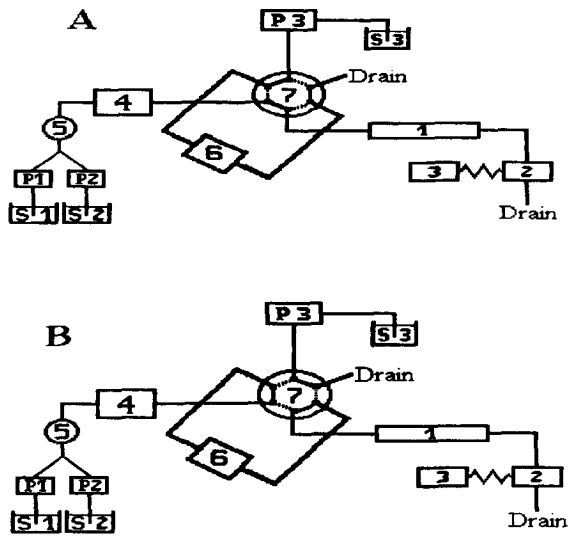


Fig. 2. Scheme of the HPLC system for quantitation of tazobactam in urine. P1, P2 and P3=pumps; S1 and S2=solvents of the mobile phase; S3=washing solvent; 1=analytical column; 2=UV detector; 3=data processor; 4=automatic injection system with a Rheodyne loading valve fitted with a 50-μl sample loop; 5=mixer; 6=pre-column for the on line SPE; 7=Rheodyne loading valve. (A) Rheodyne valve is positioned for the on line SPE clean-up and preconcentration of the urine. (B) Rheodyne valve is positioned to transport sample to the analytical column.

2.3. Chromatographic conditions

The mobile phases containing acetonitrile and ammonium acetate buffer were used for the determination of tazobactam in plasma (3.5:96.5, v/v), tazobactam in urine (1.5:98.5, v/v), and piperacillin in plasma and urine (18:82, v/v).

The flow-rates were 1.0 ml/min, which corresponds to a pressure of about 155 bar for tazobactam and 167 bar for piperacillin. Acetonitrile and the aqueous phases were filtered through a membrane filter (0.45 μm, Millipore, Molsheim, France). The oven temperature was 30°C.

Mobile phases were desaerated ultrasonically prior to use and vacuum desaerated during use.

The detector was set at 218 nm for the first 10.5 min and at 254 nm from 10.5 to 16 min to quantify tazobactam in plasma. Two different wavelengths were used in order to avoid an interfering compound with the same retention time as that of the internal standard which absorbs at 218 nm. The detection was performed at 218 nm to quantify tazobactam in

urine, and at 222 nm to quantify piperacillin as well as the internal standard in plasma and urine.

2.4. Sample processing

To quantify tazobactam in plasma and piperacillin in plasma and diluted urine (1:10 in purified water), samples (0.5 ml) were spiked with internal standard (25 μl of ceftazidime (III) for tazobactam and 40 μl of benzylpenicillin (IV) for piperacillin) and homogenised. Acetonitrile (1 ml) was added to all samples and the mixture vortex-mixed for 10 s, then all vials were centrifuged at 2000 *g* for 10 min. The supernatant was pipetted into a 10-ml glass tube and chloroform (3 ml) was then added. The samples were vortex-mixed for 10 s. After centrifugation, the aqueous solution was separated; 100 μl of this solution was injected onto the column.

To quantify tazobactam in urine, samples were diluted (1/10) with purified water. A 50-μl volume of this solution was injected. The sample was loaded on the pre-column, where the clean-up and pre-concentration took place; the pre-column, after the sample injection, was flushed for 5 min with 0.1 *M* NaH_2PO_4 (pH 6.0) at a flow-rate of 0.8 ml/min in order to eliminate endogenous compounds. Then, after valve switching, the pre-column was connected to the analytical column where analytes were transferred by the HPLC mobile phase, the pre-column was disconnected after 7 min and then, while chromatography took place on the analytical column, it was re-equilibrated with 0.1 *M* NaH_2PO_4 for 5 min. Afterwards, the next sample was injected.

2.5. Data analysis

For assay of tazobactam in plasma, and piperacillin in plasma and urine, peak-area ratios of tazobactam or piperacillin to internal standard were used to construct the standard curves. Unweighted least squares linear regression of the peak-area ratios as a function of the theoretical concentrations was applied to each standard curve. For assay of tazobactam in urine, the peak area was used as the assay parameter. Peak areas were plotted against theoretical concentrations.

The linearity of the method was confirmed by showing that the slopes of linear calibration curves were statistically different from zero, and by comparison of intercepts with zero and of correlation coefficients with 1.

2.6. Specificity

To evaluate the specificity of the method, 0.5 ml of drug free plasma and urine samples were subjected to the assay procedure and the retention time of endogenous compounds were compared with those of tazobactam, piperacillin and internal standards.

The interference from the main inactive metabolite M1 of tazobactam (Fig. 1) was checked. The interference from other drugs that could be co-administered was also studied. The following drugs were checked: amikacin, tobramycin, cefotaxime, amoxicillin, clavulanic acid, famotidine, cimetidine, phenacetin, ofloxacin and ciprofloxacin.

2.7. Precision and accuracy

Inter-day and intra-day repeatabilities of the assay were assessed by performing replicate analyses of spiked samples at high, middle, and low concentrations in plasma and diluted urine (7.5, 25, and 40 μg/ml for tazobactam; 1.5, 30, and 70 μg/ml for piperacillin) against a calibration curve. The procedure was repeated on different days on the same spiked standards to determine inter-day repeatability. Intra-day repeatability was determined by treating spiked samples in replicate the same day. The accuracy, expressed as percent deviation of observed concentration from theoretical concentration, with the relative error, was evaluated.

2.8. Determination of LOQ and limits of detection (LOD)

The LOQ was determined from the peak and the standard deviation of the signal-to-noise ratio (*S/N*). The LOQ was defined as the sample concentration of tazobactam and piperacillin resulting in a peak area of ten times the *S/N*. The estimated *S/N* was determined by extrapolation to zero. Spiked samples were used to determine the analytical error in the LOQ.

The LOD was defined as the sample concentration resulting in a peak area of three times the *S/N*.

2.9. Recovery

The extraction efficiency (recovery) was determined by comparing peak areas from drug-free plasma and urine spiked with known amounts of drugs (7.5, 25, and 40 μg/ml for tazobactam; 1.5, 30 and 70 μg/ml for piperacillin) and assayed accordingly, versus peak areas of the same concentrations prepared in purified water injected directly onto the analytical column. Each sample was determined in triplicate. The extraction efficiencies were also determined for the internal standards.

In order to study the effect of co-extracted biological material, recoveries were also computed by comparison of extracts from spiked samples with blank extracts spiked after the extraction.

2.10. Stability study

For stability studies, control human plasma and urine samples were spiked with 7.5, 25, and 40 μg/ml of tazobactam and with 1.5, 30 and 70 μg/ml of piperacillin.

The stability of tazobactam and piperacillin was inspected during all the storage steps and during all steps of the analytical method, in plasma and urine (i.e., at room temperature, at +4°C, at −30°C and at −80°C). Spiked samples were analysed immediately after preparation (reference values) and after storage. Each determination was performed in triplicate.

The freeze–thaw stability was also determined. Spiked plasma and urine were analysed immediately after preparation and on a daily basis after repeated freezing/thawing cycles at both −20°C and −80°C on four consecutive days.

3. Results

3.1. Retention times

The retention times and corresponding capacity factors are reported in Table 1. Representative chromatograms are shown in Fig. 3 and Fig. 4.

3.2. Specificity

Representative chromatograms are shown in Fig. 4 and Fig. 5. No peak interfered at the retention times

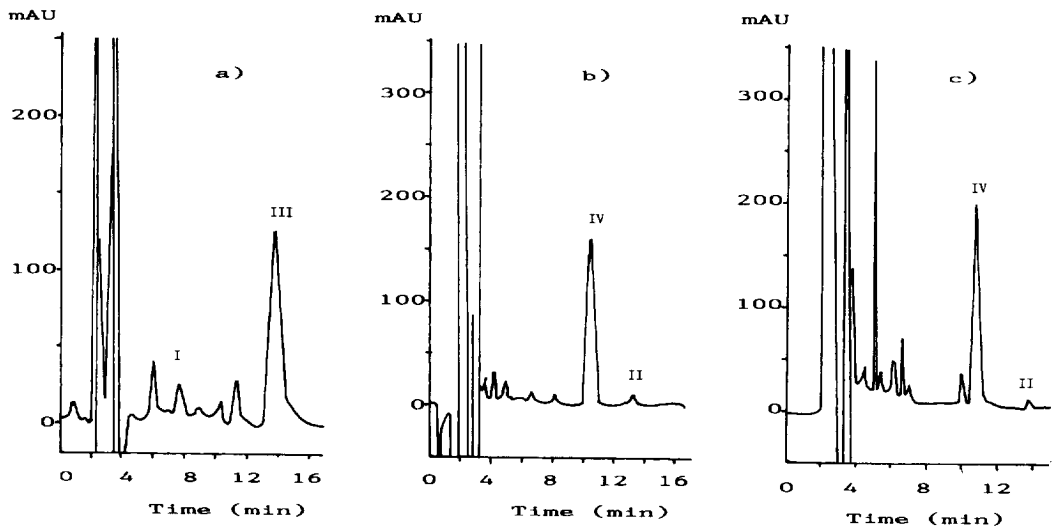


Fig. 3. Chromatograms of plasma spiked with 5 μg/ml of tazobactam (a), and of plasma (b) and urine (c) spiked with 2 μg/ml of piperacillin. Peak I is tazobactam, peak II is piperacillin, and peaks III and IV are the internal standards. For chromatographic conditions see Section 2.3.

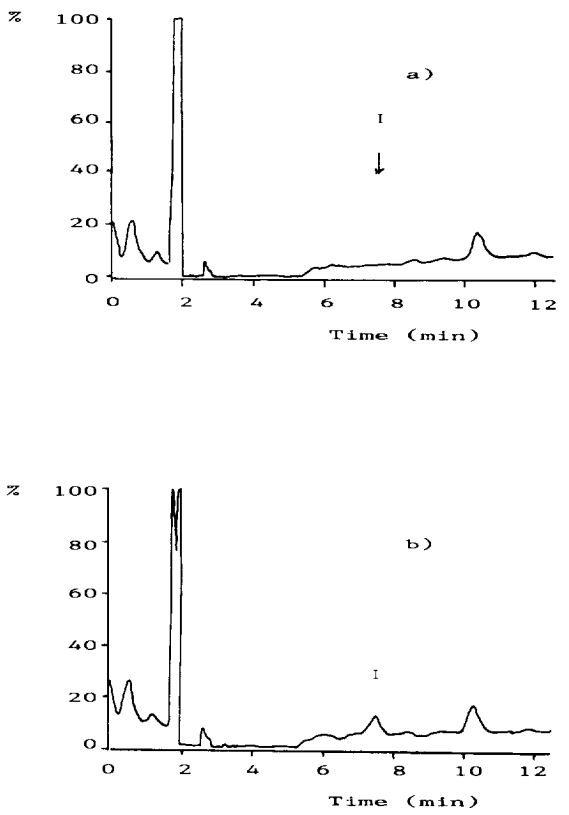


Fig. 4. Chromatograms of blank urine (a) and of urine (b) spiked with 5 μg/ml of tazobactam. Peak I is tazobactam. Analysis: 50 mV FS. For chromatographic conditions see Section 2.3.

Table 1
Observed retention times (min) of tazobactam, piperacillin and internal standards

Compound	Retention time (min)	Capacity factor	Resolution
I in plasma[a]			
I	7.4	2.7	
III	12.8	5.4	10.8
I in urine[b]			
I	6.7	2.91	–
II in plasma and urine[c]			
II	13.7	5.3	
IV	10.6	3.95	6.2

I=tazobactam, II=piperacillin, III=ceftazidime, IV=benzylpenicillin.

[a] Liquid–liquid extraction, mobile phase: acetonitrile–ammonium acetate (3.5:96.5, v/v).

[b] SPE method based on column switching, mobile phase: acetonitrile–ammonium acetate (1.5:98.5, v/v).

[c] Liquid–liquid extraction, mobile phase: acetonitrile–ammonium acetate (18:82, v/v).

of tazobactam, piperacillin or internal standards.

No interference was found with the metabolite M1 nor with any of the drugs tested that could be co-administered with tazobactam and piperacillin.

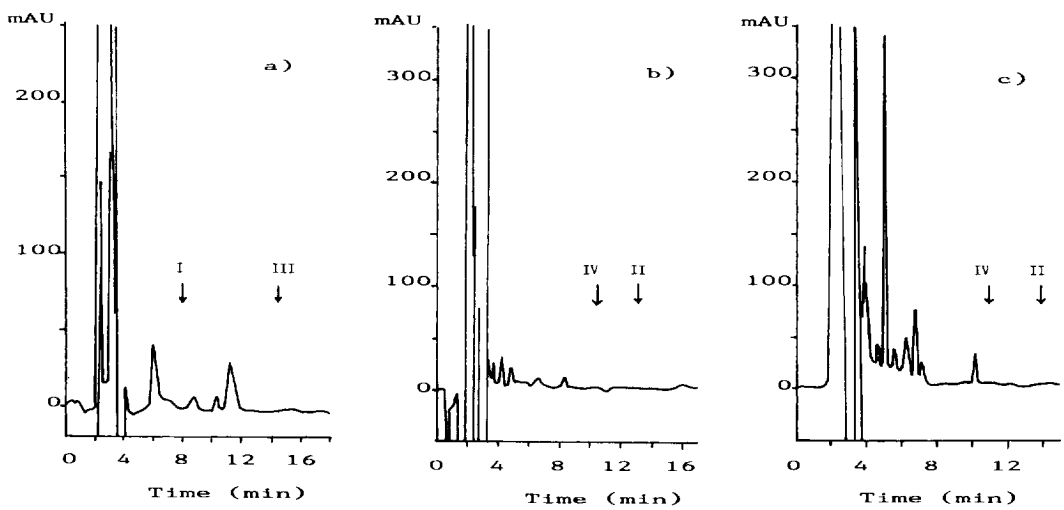


Fig. 5. Chromatograms of blank plasma (a, b) and urine (c). For chromatographic conditions see Section 2.3.

3.3. Linearity

Peak-area ratio of tazobactam in plasma, and of piperacillin in plasma and urine over the internal standard, and peak area of tazobactam in urine varied linearly with concentration over the range used. The correlation coefficients (r) for calibration curves were equal or better than 0.993. Intra-assay reproducibility was determined for calibration curves prepared the same day in replicate ($n=6$) using the same stock solutions. Inter-assay reproducibility was determined for calibration curves prepared on different days ($n=11$). Results are given Table 2. For each point of the calibration standards, the concentrations were recalculated from the equation of the linear regression curves (experimental concentrations) and the coefficients of variation (C.V.) were computed. Inter-day and intra-day variabilities at various concentrations of calibration standards are presented in Table 3 for tazobactam and in Table 4 for piperacillin.

The linearity of this method was statistically confirmed. For each calibration curve, the slope was statistically different from 0, and the intercept was not statistically different from zero.

3.4. Precision and accuracy

For concentrations of calibration standards, the precision around the mean value did not exceed 15% (Table 3 and Table 4). The results for accuracy, intra-day, and inter-day precision are presented in Table 5.

3.5. Recovery

The mean recoveries of tazobactam in plasma, and of piperacillin in plasma and urine, computed by comparison of extracts from spiked samples with the same concentrations prepared in water, averaged 95.4±4.0, 90.0±8.8 and 83.5±8.2%, respectively ($n=9$). Recovery averaged 99.5±3% ($n=9$) for tazobactam in urine. The extraction efficiency was not statistically different over the range of concentrations studied. The mean recovery of the internal standards (III and IV) were 85±2.2 and 90.4±7.1% ($n=6$), respectively. No effect of the co-extracted biological material was detected.

3.6. LOQ and LOD

The LOQ was 3 μg/ml for tazobactam and 0.5 μg/ml for piperacillin, in plasma and urine. At these levels the analytical error averaged 20%. The LOD was 1 μg/ml for tazobactam and 0.1 μg/ml for piperacillin.

3.7. Stability

In aqueous solutions tazobactam and piperacillin were stable for 24 and 12 h at 4°C, respectively. Losses of 8 and 12.5% were observed after 3 days, respectively. Losses were 23% for tazobactam and 50% for piperacillin after 14 days

After bench-top storage at room temperature, tazobactam was stable in plasma for 8 h; the percent recovery ranged from 95 to 112%. At 4°C, tazobactam was stable for 24 h, a significant decrease ranging from 10 to 26% was observed after 48 h. Frozen at −30°C tazobactam was stable for 4 days; a significant degradation averaging 15% was observed after 8 days.

Tazobactam was stable in urine for 8 h, 48 h and 8 days at ambient temperature, 4°C and −30°C, respectively; at each time study, no statistical difference appeared by comparison with the reference values. Degradation of tazobactam is slower in urine than in plasma.

The stability of piperacillin in plasma indicated that no statistically significant degradation occurred over a span of 1 h at ambient temperature for the concentration of 1.5 μg/ml (percent recovery ranging from 98 to 107%). For the highest concentrations tested, after 1 h, a significant decrease was observed, the percent recovery averaged 91.4% for 30 μg/ml and 84.1% for 70 μg/ml. At 4°C, piperacillin was stable for 3 h; after 4 h, a significant decrease was observed for the highest concentration tested and the percent recovery averaged 84.6%, less than 10% degradation occurred at the other concentrations (1.5 and 30 μg/ml). After 6 h, a ≤20% decrease in all concentrations tested was observed. At −30°C, piperacillin was stable for 3 days, the percent recovery averaged 101%; a mean of 15% decrease in concentrations was observed after 6 days.

Piperacillin was stable in urine for 6 h, 48 h and 8 days at ambient temperature, 4°C and −30°C, re-

Table 2
Assay linearity for tazobactam and piperacillin

Sample[a]	Correlation coefficient (r) of the linear[b] regression analysis (mean±S.D.)		Slope (b) (mean±S.D.)		Intercept (a) (mean±S.D.)	
	Intra-day reproducibility	Inter-day reproducibility	Intra-day reproducibility	Inter-day reproducibility	Intra-day reproducibility	Inter-day reproducibility
I in plasma	$0.997\pm1.97\cdot10^{-3}$ C.V.=0.197%	$0.997\pm2.15\cdot10^{-3}$ C.V.=0.216%	$0.0108\pm2.1\cdot10^{-4}$ C.V.=1.94%	$0.0109\pm5.56\cdot10^{-4}$ C.V.=5.1%	$1.64\ 10^{-3}\pm3.66\ 10^{-3}$	$0.0156\pm7.7\cdot10^{-3}$
I in urine	$0.998\pm5.47\cdot10^{-4}$ C.V.=0.055%	$0.998\pm1.98\cdot10^{-3}$ C.V.=0.20%	74.5±9.6 C.V.=12.9%	70.4±5.5 C.V.=7.82%	16.1±37.1	−51.7±43.7
II in plasma	$0.9997\pm3.00\cdot10^{-4}$ C.V.=0.0302%	$0.999\pm2.21\cdot10^{-4}$ C.V. =0.0221%	$0.0227\pm2.08\cdot10^{-3}$ C.V.=9.15%	$0.0208\pm1.05\cdot10^{-3}$ C.V.=5.05%	$8.53\ 10^{-4}\pm5.70\ 10^{-4}$	$7.82\cdot10^{-3}\pm0.0116$
II in urine	$0.999\pm4.02\cdot10^{-4}$ C.V.=0.0402%	$0.9998\pm1.57\cdot10^{-4}$ C.V.=0.0157%	$0.0269\pm3.24\cdot10^{-4}$ C.V.=1.20%	$0.0240\pm1.40\cdot10^{-3}$ C.V.=5.82%	$6.57\ 10^{-3}\pm2.66\ 10^{-3}$	0.0054±0.0103

I=tazobactam, II=piperacillin; r=correlation coefficient.

[a] $n=6$ for intra-day reproducibility; $n=11$ for inter-day reproducibility.

[b] Linear unweighted regression, formula: $y=a+bx$.

Table 3
Intra- and inter-assay reproducibilities of the HPLC analysis of tazobactam

Theoretical concentration (μg/ml)	Intra-assay reproducibility ($n=6$)		Inter-assay reproducibility ($n=11$)	
	Experimental concentration (mean±S.D.) (μg/ml)	C.V. (%)	Experimental concentration (mean±S.D.) (μg/ml)	C.V. (%)
Tazobactam in plasma				
5	5.03±0.28	5.57	5.20±0.710	13.6
10	10.2±1.28	12.5	10.5±0.775	7.38
20	20.2±0.823	4.07	20.4±0.943	4.62
30	29.2±0.754	2.58	30.8±0.984	3.19
40	40.7±1.33	3.27	39.9±1.36	3.41
50	50.1±1.61	3.21	49.9±1.32	2.64
60	59.7±1.40	2.34	59.4±0.641	1.08
Tazobactam in urine				
5	5.04±0.30	5.95	5.16±0.336	6.51
10	10.2±0.23	2.26	9.61±0.747	7.77
20	20.1±0.86	4.27	19.2±1.38	7.17
30	29.9±0.68	2.27	30.2±0.69	2.29
40	40.2±0.87	2.16	40.0±1.11	2.77
50	49.4±0.98	1.99	49.9±0.73	1.46
60	60.7±0.22	0.36	60.4±0.78	1.29

Table 4
Intra- and inter-assay reproducibilities of the HPLC analysis of piperacillin

Theoretical concentration (μg/ml)	Intra-assay reproducibility ($n=6$)		Inter-assay reproducibility ($n=11$)	
	Experimental concentration (mean±S.D.) (μg/ml)	C.V. (%)	Experimental concentration (mean±S.D.) (μg/ml)	C.V. (%)
Piperacillin in plasma				
1	0.906±0.0646	7.13	0.985±0.135	13.7
2	1.95±0.217	11.13	1.91±0.266	13.9
5	5.27±0.412	7.82	5.34±0.339	6.35
10	9.83±0.419	4.26	9.84±0.420	4.27
20	20.5±0.385	1.88	20.3±0.625	3.08
50	49.9±1.25	2.51	50.2±0.961	1.91
100	99.9±0.688	0.688	99.8±0.541	0.542
Piperacillin in urine				
1	0.906±0.106	11.7	1.02±0.15	14.7
2	1.96±0.115	5.87	2.05±0.188	9.19
5	5.22±0.237	4.54	5.06±0.275	5.43
10	9.80±0.204	2.08	10.1±0.425	4.21
20	20.1±0.256	1.27	19.9±0.598	3.00
50	50.6±1.18	2.33	50.2±0.657	1.31
100	99.7±0.527	0.528	99.9±0.393	0.40

Table 5
Intra-day and inter-day precision and accuracy of the HPLC method

Theoretical concentration (μg/ml)	n	Experimental concentration mean ± S.D. (μg/ml)	C.V. (%)	Mean recovery (%)	Relative error (%)
Tazobactam in plasma					
Intra-day precision					
7.5	6	7.22±0.774	10.7	96.3	3.73
25	6	24.8±1.09	4.39	99.2	0.8
40	6	39.9±0.766	1.92	99.7	0.25
Inter-day precision					
7.5	6	7.76±0.43	5.53	103.5	3.50
25	8	26.8±1.22	4.55	107.2	7.2
40	8	40.6±2.84	7.00	101.5	1.5
Tazobactam in urine					
Intra-day precision					
7.5	6	7.41±0.42	5.67	98.8	1.2
25	6	23.5±2.11	8.97	94.0	5.96
40	6	40.4±2.32	5.75	100.9	0.87
Inter-day precision					
7.5	6	6.67±0.50	7.49	88.9	11.1
25	6	24.1±1.01	4.19	96.5	3.52
40	6	39.9±0.766	5.14	103.0	3.02
Piperacillin in plasma					
Intra-day precision					
1.5	5	1.73±0.27	15.6	115.3	15.3
30	5	28.9±0.701	2.43	96.3	3.67
70	6	68.9±0.372	0.54	98.4	1.60
Inter-day precision					
1.5	6	1.75±0.188	10.7	116.7	16.7
30	7	30.4±2.31	7.60	101.3	1.33
70	7	71.3±5.48	7.70	101.9	1.86
Piperacillin in urine					
Intra-day precision					
1.5	6	1.67±0.109	6.53	111.3	11.3
30	6	29.9±0.159	0.532	99.7	0.33
70	5	66.0±4.71	7.14	94.3	5.71
Inter-day precision					
1.5	7	1.55±0.27 4	17.7	103.3	3.33
30	7	29.3±2.25	7.69	97.7	2.33
70	7	69.5±2.46	3.54	99.3	0.71

spectively; compared to the reference values, no statistical difference appeared. Degradation of piperacillin is slower in urine than in plasma.

The long term freezer stability indicated that tazobactam and piperacillin were stable at −80°C for 3 months.

Run-time stability at room temperature for 24 h of processed samples after extraction of tazobactam and

piperacillin was determined for each point of calibration standard. After 5 h for tazobactam and 6 h for piperacillin, recoveries were lower than 90%. Losses were decelerated at 4°C.

At least three freeze–thaw cycles can be tolerated without losses higher than 10% for both tazobactam and piperacillin in plasma and urine. Indeed, after the third cycle, losses ≤7% for tazobactam and ≤8.5% for piperacillin were observed.

4. Discussion and conclusion

The present HPLC method enables a rapid assay of tazobactam and piperacillin in plasma and urine with a run time lower than 17 min. The liquid–liquid extraction used to quantify tazobactam and piperacillin in plasma and piperacillin in urine involves the addition of an internal standard followed by a treatment of the samples with acetonitrile and chloroform without an evaporation step to avoid the risk of degradation of these two drugs. The quantitation of tazobactam in urine was performed without internal standard. The use of ceftazidime as internal standard for tazobactam was possible. However, its use increased two-fold the time of each analysis. As the recovery of tazobactam was ~100% without influence from the urine components, the use of an internal standard was not essential.

In the present method, tazobactam and piperacillin were quantified in two separative runs. Indeed, the use of the gradient elution HPLC assay described by Ocampo et al. [5] allowing simultaneous determination of these two compounds has not been possible due to the risk of interference with some drugs which could be co-administered to patients admitted into intensive care units.

Assay performance was assessed both on the basis of the statistical characteristics of individual calibration lines and from the results of quality control samples. This method has been validated for concentrations ranging from 5 to 60 μg/ml for tazobactam and from 1 to 100 μg/ml for piperacillin spanning what is currently though to be the clinically relevant range for tazocillin concentrations in body fluids. This assay is selective, reliable and has good efficiency in terms of run time and sample throughput. It also has sufficient sensitivity for pharmacokinetic study.

For the determination of tazobactam in plasma, LOD similar to that published by Ocampo et al [5] was found; however, it was higher than that reported by Marunaka et al. [6] and Muth et al. [7]. These latter workers, using deproteinisation with acetonitrile followed by evaporation [6] or by a HPLC column switching technique [7], improved widely the sensitivity of the assay (0.2 and 0.1 μg/ml, respectively). Compared to the published methods [5,6,9–11], an improvement of the LOQ was reported in the present study for the assay of tazobactam in urine and piperacillin in plasma and urine. The separation between the analytes and endogenous substances brought by plasma and urinary matrices was satisfactory. Moreover, the specificity from drugs that could be co-administered is good. Stability studies carried out directly in plasma and urine showed that samples should both be processed as promptly as possible and stored at −80°C.

The method described was found to be suitable for the analysis of all samples collected during pharmacokinetic investigations in human. The column switching method to quantify tazobactam in urine with a LOQ of 3 μg/ml, which generally satisfies pharmacokinetic needs, is very quick as automatic clean-up and pre-concentration of the urine samples is performed during the chromatographic run.

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