Simultaneous Determination of 12 β-Lactam Antibiotics in Human Plasma by High-Performance Liquid Chromatography with UV Detection: Application to Therapeutic Drug Monitoring

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A rapid and specific high-performance liquid chromatography method with UV detection (HPLC-UV) for the simultaneous determination of 12 β-lactam antibiotics (amoxicillin, cefepime, cefotaxime, ceftazidime, ceftriaxone, cloxacillin sodium salt, imipenem, meropenem, oxacillin sodium salt, penicillin G, piperacillin, and ticarcillin) in small samples of human plasma is described. Extraction consisted of protein precipitation by acetonitrile. An Atlantis T3 analytical column with a linear gradient of acetonitrile and a pH 2 phosphoric acid solution was used for separation. Wavelength photodiode array detection was set either at 210 nm, 230 nm, or 298 nm according to the compound. This method is accurate and reproducible (coefficient of variation [CV] < 8%), allowing quantification of β-lactam plasma levels from 5 to 250 µg/ml without interference with other common drugs. This technique is easy to use in routine therapeutic drug monitoring of β-lactam antibiotics.

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FIG. 1. HPLC chromatograms of an internal quality control with 10 μg/ml of each antibiotic at 298 nm (a), 230 nm (b), and 210 nm (c). Values after the antibiotic names are retention times.
Instrumentation and chromatographic conditions. The HPLC system included a P1000XR pump equipped with a degasser, an AS3000 refrigerated autosampler, and a UV microarray spectrophotometer detector (UV6000LP, ThermoFisher Scientific, Villebon sur Yvette, France). The compounds were separated on an Atlantis T3 analytical column (150 by 4.6 mm, 5 μm; Waters, Saint Quentin, France), coupled with an Atlantis T3 guard column (20 by 4.6 mm, 5 μm; Waters, Saint Quentin, France). The mobile phase, filtered and degassed, consisted of a 10 mM phosphoric acid solution, adjusted to pH 2 with hydrochloric acid, and acetonitrile. A linear gradient from 7% to 19% acetonitrile in 6 min and 19% to 49% from 6 to 16 min was used with a flow rate of 2 ml/min. Run time was prolonged to 22 min to return to initial conditions. The effluent was monitored at 210 nm for cloxacillin, oxacillin, penicillin G, piperacillin, and ticarcillin, at 230 nm for amoxicillin, celepime, ceftazidime, cefotaxime, and ceftriaxone, and at 298 nm for imipenem and meropenem. Data were recorded with ChromQuest software (version 2.5.1). Quantification was based on the peak height of each compound; baselines were inspected visually and adjusted manually when necessary.

Standard solutions and QC materials. Stock solutions of each beta-lactam were prepared at 50 or 100 mg/ml in water and stored at −80°C. Under these conditions they are stable for a few months (9, 18, 25). They were further mixed in water to obtain a 1-mg/ml solution, which was used to produce standard solutions at 250, 125, 40, and 10 μg/ml. Three working solutions of beta-lactams at 50, 1,000, and 2,000 μg/ml were prepared from stock solutions and diluted 10-fold in blank plasma to obtain quality control (QC) materials at 5 μg/ml, 100 μg/ml, and 200 μg/ml. The recovery of each compound was calculated at three concentrations (9, 18, 25).

Sample preparation. Venous blood samples were collected in heparinized tubes, immediately transported on ice to the laboratory, and centrifuged for 10 min at 3,000 × g at 4°C. If necessary, the plasma samples were stored at −20°C until analysis. For the extraction procedure, 200 μl of water and 700 μl of acetonitrile were added to 100 μl of control or patient plasma samples. For the calibration curve, 100 μl of corresponding working solution, 100 μl of water, and 700 μl of acetonitrile were mixed with 100 μl of blank plasma. Each sample was vortex mixed and centrifuged at 3,000 × g for 10 min at 4°C. One hundred microliters of the supernatant were diluted with 900 μl of water and 700 μl of acetonitrile amount increased. The Atlantis T3 column en-

RESULTS AND DISCUSSION

Chromatography. Separation of the 12 compounds was satisfactory, as shown in Fig. 1. No interference with endogenous compounds was observed, allowing use of specific wavelengths of 210, 230, and 298 nm, as shown in Fig. 2 (blank plasma sample) and Fig. 3 (patient plasma samples). Deviation from the baseline is related to the gradient of acetonitrile, which absorbs UV. So the baseline absorbance was higher when the acetonitrile amount increased. The Atlantis T3 column en-
abled a simplified HPLC method allowing good retention of polar drugs. A gradient of acetonitrile enabled elution of less-polar compounds. This was the only column we know allowing good separation of all compounds and supporting this extreme pH. With other C18 columns, retention times ranged from 1 min (passage through the column without retention) to above 1 h (cloxacillin) and separation was not optimal. The pH of the mobile phase was another parameter that modified retention time. Changes in retention times of the different compounds according to pH of the mobile phase are shown in Fig. 4. The curve shows that extreme pH (2) provided the best separation while maintaining retention times under 17 min. For most of the compounds, retention time decreased as the pH increased, with the risk of elution with the solvent front or coelution of several compounds (penicillin G plus piperacillin at pH 5 or amoxicillin plus ceftazidime plus ceftriaxone at pH 7). We found the best compromise between stationary phase and pH. In this way, the 12 antibiotics were assayed within 22 min. We recommend the use of a guard column to extend the lifetime of the column subjected to acid pH. At this pH, imipenem pre-

FIG. 3. Chromatograms of extracted plasma samples from patients treated with amoxicillin (residual concentration, 33.5 mg/liter) (a) and cloxacillin (residual concentration, 15.3 mg/liter) (b).

FIG. 4. Retention times of beta-lactam antibiotics by pH of the mobile phase.
sented 2 peaks because of its tautomeric form (31). We attempted to stabilize our samples and the stock solutions by diluting with MES (morpholineethanesulfonic acid) buffer at pH 7.4 or phosphate buffer at pH 10 but were unable to avoid this double peak. However, we noticed that this tautomeric form was stable, and the accuracy and precision assays confirmed that we could determine the plasma concentration of imipenem under these conditions by considering the first of the two peaks.

**Sample preparation.** Different types of sample preparation were tested, solid phase extraction (SPE) with three different stationary phases (SCX, NH2, and OASIS HLB; Waters, St Quentin, France) and protein precipitation. SPE was not effective for the 12 compounds. Protein precipitation was the most reproducible and allowed the best recovery. As performed by McWhinney et al. (25), we removed lipid-soluble components by a chloroform or hexane wash after protein precipitation. The procedure with chloroform increased the repeatability and reproducibility tests give a variation of less than 8%.

**Method validation.** Values for retention time, detection wavelength, linear regression, correlation coefficient, and extraction recovery for the different compounds are given in Table 1.

The calibration curves, fitted using linear regression between the beta-lactam peak heights and the concentrations of the respective compounds, appeared linear from 2 to 250 μg/ml for amoxicillin, cefepime, cefotaxime, ceftazidime, imipenem, and meropenem and from 5 to 250 μg/ml for ceftaxone, cloxacillin, oxacillin, penicillin G, piperacillin, and ticarcillin. All calibration curves were well described using a weighted least-squares regression (weighting factor: 1/concentration) to achieve homogeneity of variances. The coefficients of determination \( R^2 \) were all above 0.99.

**Specificity and selectivity.** There was no evidence of interference with the different compounds tested. Not all the beta-lactam metabolites were available and could be evaluated. Therefore, few metabolites were verified, and one of them, desacetyl-cefotaxime, was determined in another run because of a risk of interference with amoxicillin or ceftazidime (retention time = 3.8 min) (34). This interference was a problem for standard solutions and QC but not in patients treated with ceftaxone, amoxicillin, or ceftazidime. Beta-lactams are antibiotics which are very rarely prescribed simultaneously. Patients treated with ceftaxone present detectable concentrations of desacetyl-cefotaxime but are never simultaneously treated with amoxicillin or ceftazidime. Conversely, patients treated with amoxicillin or ceftazidime are never treated with ceftaxone at the same time. Currently, determination of desacetyl-cefotaxime is not routinely prescribed in our hospital.

**Accuracy and precision.** The LODs were <1 μg/ml for amoxicillin, cefepime, cefotaxime, ceftazidime, imipenem, and meropenem and 2 μg/ml for the other compounds. The LOQ was 2 or 5 μg/ml for the different compounds. Data on the accuracy and precision analysis are summarized in Table 2. All the repeatability and reproducibility tests give a variation of less than 8%.

Target residual concentrations were within the 10 to 50 μg/ml range and were 30 to 100 μg/ml for piperacillin and ticarcillin (13, 17, 20, 28, 32). The efficacy of beta-lactams was proved for plasma concentrations over 4 to 5 times greater than the MIC more than 50% of the time between two injections. Clinicians asked for the determination in severe infections, for which they request concentrations above 10 times the MIC 100% of the time. MICs for strains sensitive to beta-lactams are between <0.5 and <8 μg/ml. When the residual concentration is lower than 4 times this value, the antibiotic is no longer sufficiently effective. We concluded that it was not necessary to determine concentrations lower than 2 or 5 μg/ml for this application.

**Recovery.** Mean recovery values were greater than 90% for the different compounds.

**Stability.** The stability study, performed on extracts preserved in a refrigerated automatic injector for 15 h at 9°C, demonstrated a reduction in spike height from 3 to 18% depending on the antibiotic. Ceftazidime exhibited the most pronounced degradation pre- and postextraction: in plasma preserved at 4°C or room temperature, concentrations declined 3.7 and 7%, respectively, after 12 h.

**Therapeutic drug monitoring.** Every day we receive 1 or more requests for TDM of several beta-lactams, hence the

### Table 1. Chromatographic data and extraction recovery for the different compounds

| Compound        | Wavelength (nm) | Retention time (min) | Linear regression 
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<td>( y = ax + b ) value for:</td>
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<td></td>
<td></td>
<td></td>
<td>( a )</td>
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<td>Imipenem</td>
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<td>0.01085</td>
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<td>Amoxicillin</td>
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<td>Ceftazidime</td>
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<td>Meropenem</td>
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<td>Ceftriaxone</td>
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<td>Cefotaxime</td>
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<td>6.8</td>
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<td>Ticarcillin</td>
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<td>11.3</td>
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<td>Piperacillin</td>
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<td>12.8</td>
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<td>Penicillin G</td>
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<td>Oxacillin</td>
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<td>Cloxacillin</td>
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<td>16.5</td>
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importance of having a unique technique to be able to meet these requests whatever the compound. During the past 18 months, 2,196 measurements (30 per week) have been performed in our laboratory on these 12 drugs for 761 patients. Amoxicillin and cloxacillin were the two beta-lactam agents most determined. Samples were drawn at steady state to determine trough concentration for intermittent administrations or plateau concentration for continuous infusions. Analyses performed in response to the first requests for plasma concentrations \( (n = 746) \) showed that 15.3% of patients had inadequate concentrations, either <10 \( \mu g/ml \) or >100 \( \mu g/ml \), for all compounds included. The limit of 10 \( \mu g/ml \) was established based on MIC breakpoints of most strains involved (35), and the limit of 100 \( \mu g/ml \) was set as the threshold for toxicity. Based on our clinical experience, effective and nontoxic concentrations are mostly between 10 and 50 \( \mu g/ml \) and up to 100 \( \mu g/ml \) for piperacillin and ticarcillin, whose MIC breakpoints are higher. Considering this range, 35.0% of patients showed inadequate plasma concentrations, whatever the administration schedule and despite doses adapted according to weight and renal function. Target concentrations have to be precisely determined for each patient, depending on the strain and MIC, consistent with the recommendations of the French Society for Microbiology (35). Because plasma concentrations of beta-lactams remain largely unpredictable, TDM of these antibiotics is now an everyday tool, in addition to antimicrobial susceptibility testing of each strain, for the proper management of patients receiving high intravenous doses.

**Conclusion.** A rapid, robust, and specific HPLC method with UV detection for the simultaneous determination of 12 beta-lactams in small samples of human plasma is described. Daily, this method can be applied to meet all requests in a single sequence run. Compared with other analytical methods published previously, the method presented here offers the advantage of a rapid simultaneous assay of commonly prescribed antibiotics using one rapid extraction protocol and satisfying all validation criteria. It is complementary to the method published by McWhinney et al. (25) because it does not concern the same compounds.

In clinical practice, this technique is already in routine use for TDM (37). Around 30 assays of beta-lactam concentrations have been performed each week on patient plasma since the validation. In addition, this method will be applied to more-recent beta-lactams such as ertapenem and doripenem.

**ACKNOWLEDGMENT**

We thank all students who participated in the daily implementation of these assays for method validation.

**REFERENCES**


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