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Simultaneous determination of ceftazidime and avibactam in patients by isocratic ion-pair liquid chromatography with photometric detection

Constantin Lier [a], Frieder Kees [b], Andrea Witowski [c], Tim Rahmel [c], Steffen Pockes [d], Christoph Dorn [a,*]

[a] Institute of Pharmacy, University of Regensburg, Universitätsstraße 31, 93053 Regensburg, Germany
[b] Department of Pharmacology and Toxicology, University of Regensburg, Regensburg, Germany
[c] Department of Anesthesiology, Intensive Care Medicine and Pain Medicine, University Hospital Knappschaftskrankenhaus Bochum, Bochum, Germany
[d] Department of Medicinal Chemistry I, University of Regensburg, Regensburg, Germany

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ABSTRACT

A simple and fast HPLC-UV method is described for the simultaneous determination of total or free ceftazidime and avibactam in serum, which is suitable for therapeutic drug monitoring (TDM) or pharmacokinetic studies in man. Sufficient retention of the very polar avibactam was obtained by addition of tetrabutylammonium hydrogen sulfate (TBA) as ion pairing agent, thus allowing the determination of both drugs in serum by isocratic elution. Total concentrations were determined after protein precipitation with acetonitrile, free concentrations after ultrafiltration. Separation was performed using an XBridge BEH C18 column with a mobile phase consisting of 20 mM sodium phosphate buffer/acetonitrile 90:10 (v/v), pH 6.5, containing 5 mM TBA. The lower limit of quantification was 1 mg/L for ceftazidime and 0.5 mg/L for avibactam, respectively. The imprecision of the determination of total drug was <3 %, the accuracy between 99.0 % and 104 %. Determination of free drug in quality control samples resulted in unbound fractions of 97.9 ± 2.0 % for ceftazidime and 99.4 ± 3.2 % for avibactam.

1. Introduction

Ceftazidime is a semisynthetic third-generation cephalosporin, which was introduced into clinical practice in the 1980s with broad spectrum activity against Gram-positive cocci and Gram-negative bacilli, including *Pseudomonas aeruginosa*. The utility of third-generation cephalosporins has become compromised by the increasing variety of beta-lactamases within Gram-negative bacilli. Avibactam is a potent inhibitor of many beta-lactamases, protecting the substrate drug from hydrolysis by Gram-negative organisms [1]. The ceftazidime-avibactam combination (Zavicefta®) has been approved in Europe in 2016 and is indicated for the treatment of complicated intra-abdominal infections, complicated urinary tract infections and hospital-acquired pneumonia [2].

Anti-infective drugs are commonly evaluated using pharmacokinetic/pharmacodynamic (PK/PD) indices, which should be expressed as a function of the pharmacologically active, i.e. free drug concentrations [3]. These can be calculated from total concentrations and the degree of protein binding (if available), or the free concentrations are measured directly.

To date, only gradient LC–MS/MS methods have been described for the simultaneous determination of ceftazidime and avibactam in humans [4–7]. This study aimed to develop an isocratic HPLC-UV method for the simultaneous determination of total or free ceftazidime and avibactam in serum, which is suitable for therapeutic drug monitoring (TDM) or pharmacokinetic studies in patients.

2. Materials and methods

2.1. Chemicals and materials

Ceftazidime (Ceftazidim EBERTH 2.0 g, powder for solution for infusion, Dr. Friedrich Eberth Arzneimittel GmbH, Ursensollen, Germany) and avibactam (Avibactam sodium, TargetMol, Boston, MA, USA) were used for calibration. Acetonitrile (HPLC gradient grade) and all other chemicals were obtained from Merck KGaA (Darmstadt, Germany). Vivafree 500 30 kD Hydrosart centrifugal ultrafiltration devices were purchased from Vivaproducts Inc. (Littleton, MA, USA). HPLC-

* Corresponding author.
E-mail address: christoph.dorn@ur.de (C. Dorn).

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grade water was produced using an Arium® basic water purification system (Sartorius, Göttingen, Germany). Blank serum was donated by healthy volunteers.

2.2. Stock solutions, calibration and quality control samples

Ceftazidime 5 g/L and avibactam 2 g/L stock solution were prepared in 10 mM phosphate buffer, pH 6.0. Calibration standards (STD) were prepared at concentrations of 300/150, 100/50, 30/15, 10/5, 3/1.5, and 1/0.5 mg/L ceftazidime/avibactam by spiking drug free human serum or 0.9 % saline (as surrogate for serum ultrafiltrate) with ceftazidime and avibactam (Supplementary Table S1 and S3). Quality control (QC) samples were prepared in serum at concentrations of 80/20, 20/5 or 4/1 mg/L for the determination of total, and in serum or 0.9 % saline at concentrations of 80/20 or 8/2 mg/L for the determination of free concentrations of ceftazidime/avibactam (Supplementary Tables S2, S4 and S5). Aliquots were stored at −70 °C until analysis.

2.3. Sample preparation

The sample treatment for determining total serum concentrations followed a repeatedly published protocol with minor modifications [8]. In brief, serum (100 µL) was mixed with 20 mM sodium phosphate buffer (pH 6.0, 200 µL) and acetonitrile (500 µL). After separation of the precipitated proteins and extraction of acetonitrile with dichloromethane (1.3 mL), an aliquot (1 µL) of the aqueous layer was injected into the HPLC system. The free serum concentrations of ceftazidime and avibactam were determined after ultrafiltration [9]. In brief, 2 M HEPES (pH 7.5 at room temperature, 10 µL) was pipetted into the ultrafiltration device and mixed with serum (300 µL). The sample was incubated for 10 min at 100 × g/37 °C (centrifuge 5417R with fixed-angle rotor 45-30-11, Eppendorf, Hamburg, Germany) and then centrifuged for 20 min at 1000 × g/37 °C. An aliquot (1 µL) of the ultrafiltrate was injected into the HPLC system.

2.4. HPLC system

The HPLC consisted of a Shimadzu Prominence modular system with a 3-channel degasser (DGU 20A3), quaternary solvent pump (LC 20AD, flow rate 0.4 mL/min), autosampler (SIL 20AC HT, set to 6 °C), column oven (CTO 20AC, set to 40 °C), photodiode array detector (SPD M30A) equipped with a high-sensitivity cell of 85 mm optical path length, system controller (CBM 20A) and LabSolution software (all from Shimadzu Europe, Duisburg, Germany). Separation was performed isocratically using an XBridge BEH C18 2.5 µm 100 × 3 mm column (Waters, Eschborn, Germany) preceded by a guard column (Nucleoshell RP18 2.7 µm 4 × 3 mm column protection system, MachereyNagel, Düren, Germany). The mobile phase consisted of 20 mM sodium phosphate buffer containing variable amounts of tetrabutylammonium hydrogen sulfate (TBA) and 8–10 % acetonitrile. Phosphate buffers were prepared by mixing ortho-phosphoric acid with sodium dihydrogen phosphate or disodium hydrogen phosphate solution. After addition of TBA and acetonitrile, the final pH was adjusted to 2.5 or 6.5 with 10 M sodium hydroxide or 37 % hydrochloric acid.

2.5. Method validation, calculations and statistical analysis

The HPLC method was validated following the ICH guideline M10 on bioanalytical method validation and study sample analysis [10]. The lower limit of detection (LOD) was defined as the amount of analyte injected into the column, which caused a signal with S/N = 3:1. The weighting factor $1/y^2$ was used for linear regression. Stability of ceftazidime and avibactam was analysed in serum of healthy volunteers, in patient serum, in the stock solutions (10 mM sodium phosphate buffer, pH 6), and in 0.9 % saline at different temperatures (−70 °C, −20 °C, 6 °C, and at 20–22 °C room temperature) over various time periods as indicated. Furthermore, autosampler stability of the processed samples (6 °C, 20–24 h) was assessed. Unbound fraction (f_u) was calculated according to the formula f_u (%) = $C_{free}/C_{total} \times 100$ %, with C_{free} being the free, and C_{total} the total concentration in the same sample, measured within the same assay. Prism 10 (GraphPad Software, La Jolla, CA, USA) was used for calculating statistics. Illustrations and structures were created with ChemDraw 20 (PerkinElmer Informatics Inc., Waltham, MA, USA).

3. Results and discussion

3.1. Chromatography

Cephalosporins including ceftazidime are hydrophilic drugs that are hardly retained at reversed-phase silica gel. Nevertheless, the typical separation system for cephalosporins includes a reversed-phase column and a mobile phase containing ion-pairing agents to ensure sufficient retention or more recently, acidic mobile phases with pH < 3, thus enhancing the lipophilicity of the drugs by suppressing the dissociation of the carboxylic acid moiety [11,12]. Avibactam exhibiting an O-sulfonate moiety, is an even more hydrophilic drug retaining its anionic properties even at pH 2.5. Therefore, ion-pair chromatography with TBA at pH 2.5 was applied to selectively increase the retention of avibactam. Indeed, the retention of avibactam increased steeply. However, the retention of ceftazidime decreased because of a repulsion reaction between the positively charged TBA, bound via the alkyl chains to the stationary phase, and the positively charged pyridinium moiety of ceftazidime referred to as “charge exclusion phenomenon” (Fig. 1) [13]. In contrast, at pH 6.5 addition of TBA increased the retention of both analytes, reaching a plateau for ceftazidime between 2 mM and 5 mM TBA (Fig. 2). The following moderate decrease at higher concentrations can be attributed to the charge exclusion effect as mentioned above.

Albeit the significant changes of the retention factor (k) at low TBA concentrations promised favorable selectivity of the chromatographic system, we failed to achieve chromatograms in patients, which were free of interferences. By increasing TBA concentration to 5 mM, an interference-free chromatogram was achieved (see Supplementary Figs. S2 and S3), unfortunately at the expense of sensitivity regarding the more demanding avibactam (poor UV absorption, lower dosed than ceftazidime) which now eluted after ceftazidime. However, in terms of robustness and reproducibility of preparing the mobile phase, this concentration of TBA should be favourable. Therefore, the final mobile phase consisted of 20 mM sodium phosphate buffer/acetonitrile 90:10 (v/v) containing 5 mM TBA with pH 6.5. It should be noted that, especially when using UV detection at short wavelengths, interferences from co-medication can never be completely ruled out, and therefore visual inspection of the chromatograms is strongly recommended.

3.2. Assay characteristic

Assay characteristics are summarized in Supplementary Table S6. The LOD for ceftazidime/avibactam was determined as 10/50 pg on column. Linearity in serum or 0.9 % saline has been shown from 300 to 1 mg/L for ceftazidime ($R > 0.999$), and from 150 to 0.5 mg/L for avibactam ($R > 0.999$), respectively. The lowest standard of the calibration curve (1/0.5 mg/L ceftazidime/avibactam) was conservatively chosen as the lower limit of quantification (LLOQ) [14]. Extrapolation using the 10σ method yielded lower values (see Supplementary Table S6) [15]. The recovery of ceftazidime/avibactam from spiked serum samples was 97.7 ± 1.8 %/98.7 ± 1.4 %. Based on in-process QC samples, the CV of intra-/inter-assay precision of the determination of total drug in serum was <2 %/<3 %, the accuracy was between 99.0 % and 104 %. Determination of free drug in QC samples (n = 12) resulted in unbound fractions of 97.9 ± 2.0 % for ceftazidime and 99.4 ± 3.2 % for avibactam, demonstrating good inter-assay precision. Of note, non-physiological experimental conditions during ultrafiltration

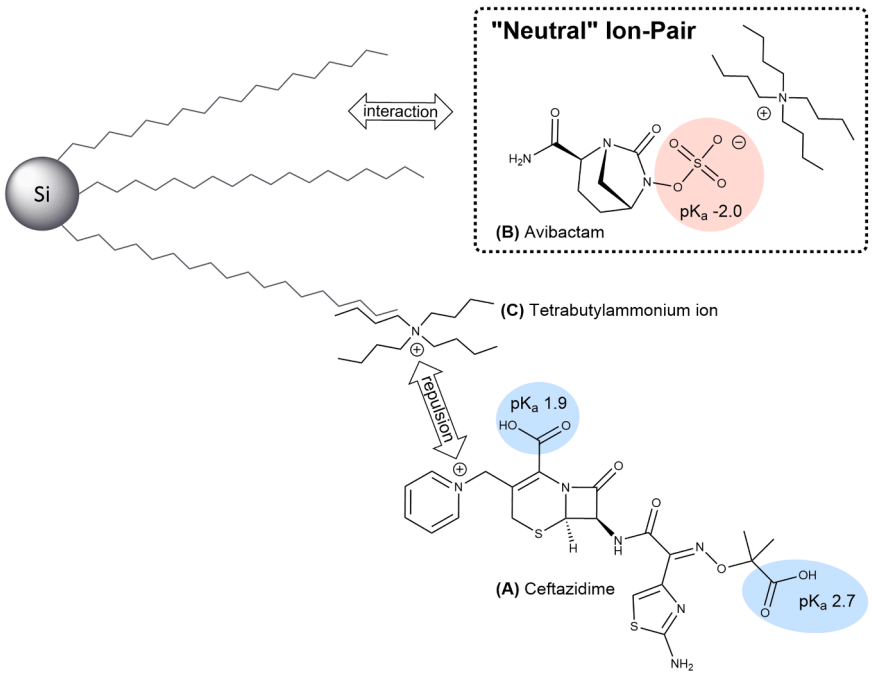

Fig. 1. Chemical structures of (A) ceftazidime and (B) avibactam demonstrating the interaction of the drugs with reversed-phase silica gel and (C) tetrabutylammonium ion at low pH.

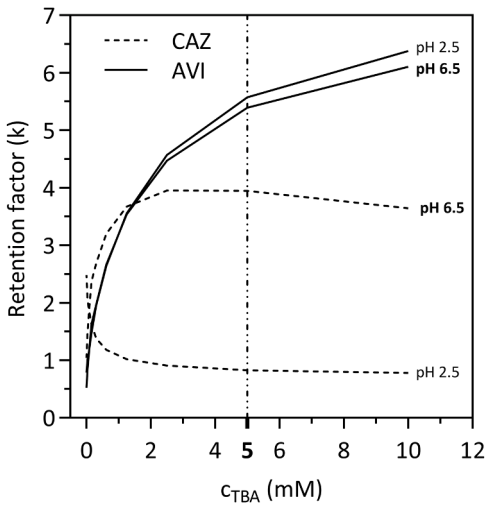

Fig. 2. Retention factor (k) of ceftazidime (dashed line) and avibactam (solid line) as a function of the tetrabutylammonium hydrogen sulfate (TBA) concentration at pH 2.5 or pH 6.5. Eluent: 20 mM sodium phosphate buffer/acetonitrile 92:8 (v/v) containing 0–10 mM TBA. Measured data points are shown on a semi-logarithmic scale in Supplementary Fig. S1.

(temperature <37 °C, pH > 8, high relative centrifugal force) may lead to lower values [9]. The intra-assay precision of the determination of free drug was not further examined, as in preliminary experiments the difference between spiked serum samples was as low as 1 %, i.e. in the range of the precision of the injection system. The accuracy cannot be specified as the extent of protein binding in a particular sample is not known [16].

3.3. Stability

The autosampler stability (20–24 h/6 °C) of ceftazidime/avibactam in processed serum samples was 102 ± 2.9 %/99.9 ± 5.5 % (total concentrations) and 99.0 ± 1.1 %/102 ± 3.0 % (free concentrations), respectively. The concentrations in plasma samples (n = 12) of patients reanalysed after storage at −70 °C for 11 months were 104 ± 2.5 % (total ceftazidime) and 101 ± 2.6 % (total avibactam) of the first analysis, respectively. At −70 °C stability of ceftazidime/avibactam in serum STD and QC samples has been shown for at least 4 months (101 ± 3.0 %/94.6 ± 4.8 %), stability in stock solutions (10 mM phosphate buffer, pH 6) for at least 5 months (101 ± 0.55 %/99.0 ± 1.4 %). Further results regarding stability of ceftazidime/avibactam at −20 °C (for 1 month), at 6 °C (up to 1 week) and at room temperature (up to 4 days) are shown in Supplementary Fig. S4. In 0.9 % saline and 10 mM phosphate buffer (pH 6, as used for stock solutions) both substances were stable under all tested conditions. In serum, ceftazidime was stable at −20 °C but showed instability at 6 °C (88 ± 0.44 %/80 ± 1.4 % after 4 days/7 days) and fast degradation at room temperature (71 ± 1.2 %/31 ± 0.32 % after 24 h/4 days), respectively. Avibactam showed good short-term stability in serum (up to 7 days at 6 °C, up to 4 days at room temperature), but revealed instability after mid-term storage at −20 °C (76 ± 0.52 % after 30 days). These stability data is in good agreement with previously published results in plasma [4,17].

3.4. Application to patient plasma

The method was applied to the analysis of plasma from patients undergoing elective abdominal surgery. Ceftazidime and avibactam eluted after 3.3 min and 4.8 min, respectively (Fig. 3). The injection sequence was set to 6.5 min, thus avoiding interference by a late eluting peak (LEP). The sensitivity of the assay was sufficient to describe the plasma pharmacokinetics of ceftazidime and avibactam in patients following intravenous infusion of ceftazidime/avibactam 2.0/0.5 g (Fig. 4). The mean unbound fractions in patient samples (n = 12) were 95,9 ± 1.5 % (ceftazidime) and 98,8 ± 4.0 % (avibactam), respectively.

4. Conclusion

The present HPLC-UV method allows the simultaneous determination of ceftazidime and avibactam in patients following therapeutic doses of ceftazidime/avibactam. Sufficient retention of the very polar avibactam was obtained by addition of tetrabutylammonium hydrogen

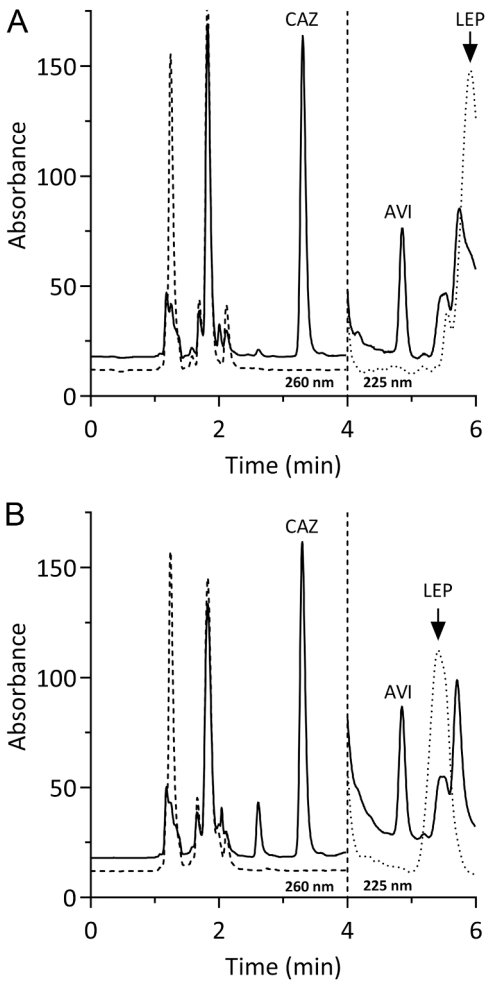

Fig. 3. Chromatogram of patient plasma predose (dotted line) or 8 h after intravenous infusion of ceftazidime/avibactam 2/0.5 g (solid line). (A) Total concentrations 20.3/3.6 mg/L, (B) free concentrations 19.5/3.6 mg/L ceftazidime/avibactam. The signal of avibactam is amplified by factor 20. Eluent: 20 mM sodium phosphate buffer/acetonitrile 90:10 (v/v), 5 mM TBA, pH 6.5. *Abbr.*: CAZ, ceftazidime; AVI, avibactam; LEP, late eluting peak.

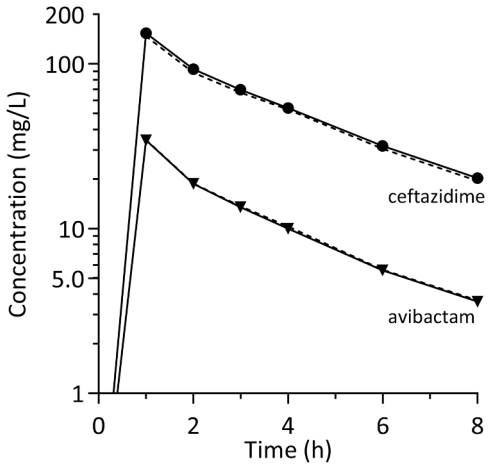

Fig. 4. Concentration-time course of ceftazidime (circles) and avibactam (triangles) in plasma of a patient receiving an intravenous infusion of ceftazidime/avibactam 2/0.5 g. Total concentrations are depicted as solid line, free concentrations as dashed line.

sulfate as ion pairing agent, thus allowing the determination of both drugs in plasma by isocratic elution.

CRediT authorship contribution statement

Constantin Lier: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Frieder Kees:** Writing – review & editing, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Andrea Witowski:** Writing – review & editing, Resources. **Tim Rahmel:** Writing – review & editing, Resources. **Steffen Pockes:** Writing – review & editing. **Christoph Dorn:** Writing – review & editing, Supervision, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jcoa.2025.100212.

Data availability

Data will be made available on request.

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