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Original Article

METHOD DEVELOPMENT AND VALIDATION OF CEFOPERAZONE AND SULBACTAM IN DRIED BLOOD SPOTS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY PHOTODIODE ARRAY DETECTOR

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ABSTRACT

Objective: The primary purpose of this research was to develop a simple, precise, fast, and accurate method for measuring cefoperazone and sulbactam simultaneously in dried blood spots (DBS) using HPLC PDA.

Methods: A simplified analytical method for quantifying cefoperazone and sulbactam in DBS samples using a High-Performance Liquid Chromatography photodiode array detector with isocratic elution was developed and validated. The best chromatographic conditions were obtained by using a reversed-phase column (250 x 4.6 mm; 5 µm); phosphate buffer 10 mmol pH 3.2–acetonitrile (83:17, v/v) as a mobile phase; a flow rate of 1.0 ml/min; a column temperature of 35 °C; a photodiode array detector at 210 nm, and cefuroxime as internal standard. Samples were prepared by liquid-liquid extraction with 100 µl hydrochloric acid 0.5 mol/l and 1000 µl ethyl acetate, evaporated with nitrogen and reconstituted with 100 µL phosphate buffer–acetonitrile (4:1).

Results: The total chromatography run time was 15 min, and the elution times for sulbactam, cefoperazone, and IS (cefuroxime) were 3.46, 10.221, and 6.987 min, respectively. A linear response function was established at 0.5-30 µg/ml with (r) 0.995 for sulbactam and 2.5-250 µg/ml with (r) 0.999 for cefoperazone in dried blood spots. The lower limit quantification (LLOQ) concentration of sulbactam 1 µg/ml and cefoperazone were 5 µg/ml.

Conclusion: This method has successfully fulfilled the validation requirement referring to the 2011 EMA and 2018 FDA guidelines.

Keywords: Cefoperazone, Sulbactam, Dried blood spots (DBS), HPLC

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INTRODUCTION

Acinetobacter baumanii is a gram-negative bacterial disease that causes primary nosocomial infection. Infectious diseases caused by these bacteria include pneumonia, urinary tract infections, meningitis, bacteremia, gastrointestinal infections, and skin or wound infections [1, 2]. The World Health Organization (WHO) has published a list of antibiotic resistance priorities for Acinetobacter baumannii, a critical category as a severe threat to global public health. This bacterial infection is linked to community-acquired pneumonia (CAP), the leading cause of death in the Asia-Pacific area, and causes antibiotic resistance [3]. Cefoperazone-sulbactam is a susceptible antibiotic for *Acinetobacter baumannii* [4]. According to studies conducted at Dr Soetomo Hospital in Surabaya, up to 27% resistance to cefoperazone-sulbactam antibiotics will continue to rise [3]. Antibiotic concentrations in the blood must be determined to measure treatment effectiveness and avoid harmful effects and resistance [5].

Quantitative analytical methods for the determination of cefoperazone and sulbactam in plasma using LCMS MS [6] and rat plasma with HPLC gradient elution [7] have already been reported. However, there has never been a quantitative analysis of cefoperazone and sulbactam in dried blood spots utilizing an HPLC Photodiode array (PDA) detector and isocratic elution. Dried blood spots (DBS) have been widely applied in drug development and discovery, drug level monitoring, drug toxicology, blood heavy metals, forensic purposes, and animal experiments [8]. This method has several advantages: it is non-invasive because samples are collected through the fingertip or heel; the number of samples required is limited; it is simple to store and transport, and it reduces the risk of transmission of infectious diseases [9].

The isocratic elution method is preferred over the gradient approach because it has various advantages, including easier transfer to different apparatus, laboratories, and column brands. Other advantages of this approach are its simplicity, quick re-equilibration time, and low frequency of baseline disturbances [10]. This investigation employed cefuroxime as an internal standard, with an HPLC PDA detector and a short-time analysis.

MATERIALS AND METHODS

Material

Cefoperazone, sulbactam, and cefuroxime were purchased from the Indonesia pharmacopeia reference standards (Indonesia), acetonitrile (HPLC grade), phosphoric acid, and ethyl acetate, and potassium dihydrogen phosphate was purchased from Merck (Darmstadt, Germany), ultrapure water. Perkin Elmer 226 paper was obtained from PerkinElmer (USA). Whole blood was acquired from the Indonesian Red Cross.

Instrument and chromatographic conditions

High-Performance Liquid Chromatography (Waters 2965) consisted of an autosampler, photodiode array detector (Waters 2996) (Waters, Amerika Serikat), C18 column Xbridge Waters (250 x 4.6 mm; 5 µm). Optimization of chromatography methods adopted by research on simultaneous analysis of amoxicillin and sulbactam in human plasma by HPLC-DAD performed by Pei *et al.*, modifications were made to the use of mobile phase, elution method, and pH [11]. The optimal chromatography system utilized a mobile phase of 10 mmol phosphate buffer, pH 3.2–acetonitrile (83:17, v/v), a flow rate of 1.0 ml/min, a column temperature of 35 °C, and a wavelength analysis at 210 nm.

Preparation of stock solution, calibration samples, and quality control samples

Cefoperazone, sulbactam and cefuroxime were prepared in 80% acetonitrile. The calibration ranges of 2.5–250 µg/ml for

cefoperazone and 0.5–30 µg/ml for sulbactam, at eight levels of concentration for each, were obtained by diluting working solutions in the blood at a concentration of 1:10. Quality control (QC) solutions were prepared by diluting working solutions in the blood with 1:10 concentration of blood at 15.0 µg/ml (QCL); 50.0 µg/ml (QCM) and 187.5 µg/ml (QCH) for cefoperazone and 3.0 µg/ml (QCL); 5.0 µg/ml (QCM); and 22.5 µg/ml (QCH) for sulbactam. Standard solutions were stored in the refrigerator at 4 °C.

Preparation of DBS samples

In developing this method, DBS sample preparation was evaluated using various extraction methods, spotting volume, blood spots drying time, the volume of extraction solvent, sonication, and centrifugation time. The optimum sample preparation was using liquid-liquid extraction, an adopted experiment from Zhou Yingjie *et al.*, with modification [6]. Initially, the DBS paper was spotted in 30 µl samples and left to dry for 2 h at room temperature. Blood spots were cut off entirely and placed in a 1.5 ml microtube, then added 50 µl internal standards 100 µg/ml and 0.5 mol/l hydrochloric acid, vortex for 1 min, 1000 µl ethyl acetate was added, vortex for 1 min, sonicated for 10 min and centrifuged at 8000 rpm for 10 min. Aliquots of 900 µl supernatant were evaporated for 15 min under N_2 gas flow. The residue was reconstituted by 100 µl phosphate buffer–acetonitrile (4:1), vortex for 1 min, and then centrifuged for 5 min at 8000 rpm. Afterwards, 20 µl of aliquot was injected into the chromatography system.

Validation of cefoperazone and sulbactam

The complete validation of the cefoperazone and sulbactam analysis method in dried blood spots was performed with parameters such as selectivity, LLOQ, the linearity of calibration, accuracy, precision, recovery, carryover, dilution integrity, and stability [12–14].

RESULTS AND DISCUSSION

Method development

The initial experiments were performed to decide the basic analytical requirements of the method, such as the type of buffer, organic modifier in the mobile phase, the concentration of buffer, pH range, flow rate and column temperature.

Optimization of buffer and mobile phase combination

The organic phase ratio to buffer is appropriate for achieving a better separation. Selectivity can be achieved by selecting an appropriate mobile phase and organic-buffer phase composition [13]. In the research conducted by Korake *et al.*, simultaneous analysis of cefoperazone and sulbactam using the gradient elution method with a run time of more than 20 min [7]. The analysis in this study was carried out using a simpler isocratic elution method with a shorter analysis time than in previous studies, which was only 15 min. The mobile phase was tested using an isocratic method with phosphate/acetate buffer–methanol and phosphate buffer–acetonitrile. Phosphate buffer with acetonitrile as an organic modifier produced better resolution, tailing factor, and peak shapes. Four types of mobile phase combinations were phosphate buffer–acetonitrile (78.5:21.5, v/v); (79:21, v/v); (80:20, v/v); and (83:17, v/v). Based on the result, a phosphate buffer-acetonitrile (83:17, v/v) was selected because it produced the best chromatogram and good resolution.

Optimization of buffer concentration

The buffer concentration was tested at 10, 20, and 30 mmol. Good separation and retention time depend on buffer concentration [4]. The optimum buffer concentration at 10 mmol gave the best chromatogram with a lower tailing factor than other concentrations.

Optimization of buffer pH

pH plays an important role in achieving chromatographic separation as it controls the elution properties based on the ionization characteristics. The pH of the buffer is selected based on the analyte's pKa and the analyte's molecular structure. Analyte retention varies with pKa, and acidic analytes can increase retention when the pH is low, whereas alkaline compounds decrease retention at low pH [15]. In this study, buffer pH variation optimization at 2.8, 3.2, and 3.6. The optimum pH of 3.2 gave the best chromatogram with a lower tailing factor and short-time analysis.

Optimization of flow rate

The flow rate of the mobile phase influences the separation of the analyte mixture. Flow rate is essential for producing a tailing factor of analyte peaks. The flow rate can be optimized depending on retention time, column backpressure, and separation of peaks adjacent to impurities [15]. The recommended maximum flow rate is 2.0 ml/min. Flow rate of mobile phase was optimized with variation 0.5; 0.6; 0.7 and 1.0 ml/min. A flow rate of 1.0 ml/min was chosen to produce the best chromatogram for separation and fast analysis time.

Optimization of column temperature

Control of the column temperature is crucial for the method's reproducibility, where the temperature impacts selectivity. The temperatures commonly used are between 30 °C and 40 °C. In reverse-phase chromatography, an increase in column temperature is predicted to decrease retention time by 1% to 2% for each 1 °C increase in column temperature [16]. The column temperature has been optimized with variations of 30, 35 and 40 °C. The 35 °C was selected for column temperature because this temperature gave the best chromatogram with a lower tailing factor and short-time analysis.

System suitability test

The System Suitability Test ensures that the system works well during analysis to produce accurate data. Based on this study, the resulting % CV, the peak area ratio, time retention, theoretical plate and tailing factor made by cefoperazone and sulbactam were not more than 2 % and met the requirements that the system was running well. The result of the system suitability test was summarized in table 1.

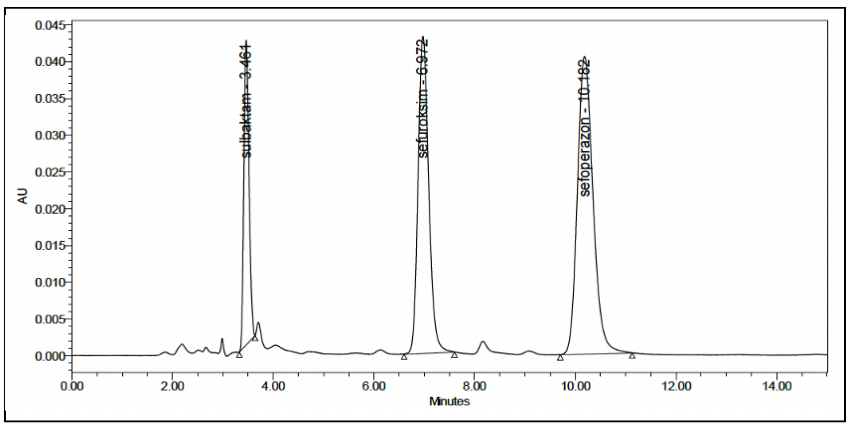

Fig. 1: Chromatogram in the system suitability test

Table 1: System suitability test of cefoperazone and sulbactam

Parameter	Mean % CV			
	Cefoperazone	Sulbactam	Cefoperazone	Sulbactam
Retention time (min)	10.196	3. 466	0.35	0.05
Area	811450	265295	1.90	0.39
USP Plate Count	6786	7330	1.72	1.06
USP Tailing	1.18	1.20	1.31	0.23

(n=6)

Optimization of sample preparation

Several methods were investigated to establish an efficient method for extracting cefoperazone and sulbactam from DBS. The sampling method using DBS provides the advantage of using a minimum sample of only 30 µl of whole blood, compared to the previous method [6] using 200 µl of plasma. Protein precipitation was initially used using acetonitrile, methanol, and mixing acetonitrile-methanol for sample preparation. Extraction with the protein precipitation method yields poor results; very low sulbactam recovery. Therefore, solvents such as hexane, dichloromethane, and ethyl acetate are utilized for liquid-liquid extraction. DBS was prepared optimally by the liquid-liquid extraction method using ethyl acetate because it could produce a good recovery and linear calibration curve [6]. Ethyl acetate was chosen as the best extractor solvent with 1000 µl optimum volume. The optimum sample preparation was performed by taking 1 min vortex time, 10 min sonication time, and 10 min centrifugation at 8000 rpm. The optimization results were selected based on the area of analytes and the internal standard also chromatogram form of each compound.

Method validation

LLOQ measurement

The concentration of LLOQ of cefoperazone was 5 µg/ml, with CV of 10.72% and with % diff range-4.07–17.56%, sulbactam was 1 µg/ml with CV of 12.96% and with % diff-3.00–16.47%. The measurement of LLOQ meets the criteria that are <±20%. The concentration measurement was half to 2.5 µg/ml for cefoperazone and 0.5 µg/ml for sulbactam. The measurement result, the % diff, and % CV values do not meet the requirements. Final LLOQ values for cefoperazone is 5 µg/ml and 1 µg/ml for sulbactam. The analyte Cmax is 155.1 ± 46.66 µg/ml for cefoperazone and 34.9 ± 12.55 µg/ml for sulbactam. LLOQ values meet the requirements because they do not exceed 5% of maximal concentration (Cmax). The previous study by Korake *et al.* [7] had 213.60 and 1.08 µg/ml as the LLOQ concentration of cefoperazone and sulbactam, respectively, with a range of calibration curves of cefoperazone and sulbactam, was 600–1000 µg/ml and 6–10 µg/ml respectively. Compared with the previous study, our study has better sensitivity for cefoperazone with LLOQ of 5 µg/ml and a range of calibration curves of 2.5–250 µg/ml and 0.5–30 µg/ml for cefoperazone and sulbactam respectively.

Calibration curve and LLOQ

The calibration curve was linear and had a correlation coefficient (r) of 0.999 in the concentration range of 2.5–250 µg/ml for cefoperazone and (r) of 0.995 in the concentration range of 0.5–30 µg/ml for sulbactam. The calibration curve consisted of blank samples (DBS without analyte and internal standard), zero samples (DBS with internal standard), and DBS with the analyte and internal standard. The analysis was performed by observed the linearity and % diff ≤ 20% for LLOQ and ≤15% for other concentrations.

Accuracy, precision, and recovery

Accuracy precision was performed in 5 replicates from every four concentrations (LLOQ, QCL, QCM, and QCH) 3 times at a minimum of 2 d. Accuracy and precision data from within and between days are shown in tables 2 and 3.

Table 2: Data of within-run accuracy and precision

Compounds	Actual concentration (µg/ml)	Measured concentration (Mean±SD; µg/ml)	Mean % CV	% diff
Cefoperazone	5	5.62±0.50	8.92	-5.50–18.35
	15	16.20±0.90	5.57	-1.05–14.34
	50	54.72±1.72	3.14	4.50–12.37
	187.5	205.31±6.82	3.32	4.18–14.36
Sulbactam	1	1.03±0.12	11.27	-8.10–16.22
	3	3.15±0.13	4.06	-1.11–10.06
	5	5.20±0,20	3.90	-1.94–7.45
	22.5	19.72±0.46	2.35	-9.54–14.51

(n=5)

Table 3: Data of between-run accuracy and precision

Compounds	Actual concentration (µg/ml)	Measured concentration (Mean±SD; µg/ml)	Mean % CV	% diff
Cefoperazone	5	5.54±0.12	2.19	-5.50–18.91
	15	16.44±0.27	1.61	-4.50–17.72
	50	55.30±0,50	0.90	4.50–13.92
	187.5	204.18±4.47	2.19	-5.24–14.36
Sulbactam	1	1.04±0.01	1.26	-8.10–19.24
	3	3.20±0.05	1.54	-4.79–14.38
	5	5.19±0.08	1.61	-8.85–11.25
	22.5	19.88±0.45	2.25	-9.54–18.42

(n=15)

This study conducted absolute recovery tests and relative recovery tests. The extracted analyte area is compared to the analyte area in the absolute recovery test without extraction. A comparison is made between the measured concentration and the actual concentration during relative recovery. Meanwhile, a comparison between the measured concentration and the actual concentration in relative

recovery is made. The mean relative recovery results for cefoperazone at concentrations of QCL, QCM, and QCH were 110.73%, 109.17%, and 104.86%, and sulbactam were 104.75%, 106.22%, and 88.58%, respectively. The recovery absolute of cefoperazone at three-level concentrations of QCL, QCM, and QCH was 48.72-50.54%; 46.08-51.66%, and 46.33-54.55%, respectively. Recovery of sulbactam at three-level concentrations of QCL, QCM, and QCH was 48.71-55.80%, 43.69-53.10%, and 48.09-52.71%, respectively.

Selectivity

The method's selectivity was assessed by analyzing six whole blood samples for potential interferences in the chromatography region for analytes and IS. The experiment's acceptance criterion must have an area response within 20% of the LLOQ level response in the same matrix. In all whole blood responses, the retention of cefoperazone and sulbactam was less than 20% of the LLOQ response, whereas the retention of IS was less than 5% of the mean LLOQ response for IS. The chromatogram of the blank sample can be seen in fig. 2. The chromatogram LLOQ of cefoperazone and sulbactam can be seen in fig. 3. The chromatogram ULOQ of cefoperazone and sulbactam can be seen in fig. 4.

Carryover

The result showed no carryover in DBS blank after injection of the highest concentration (ULOQ) cefoperazone and sulbactam. The carryover percentage still meets requirements for analytes<20 % and<5 % for the internal standard. The result can be seen in table 5.

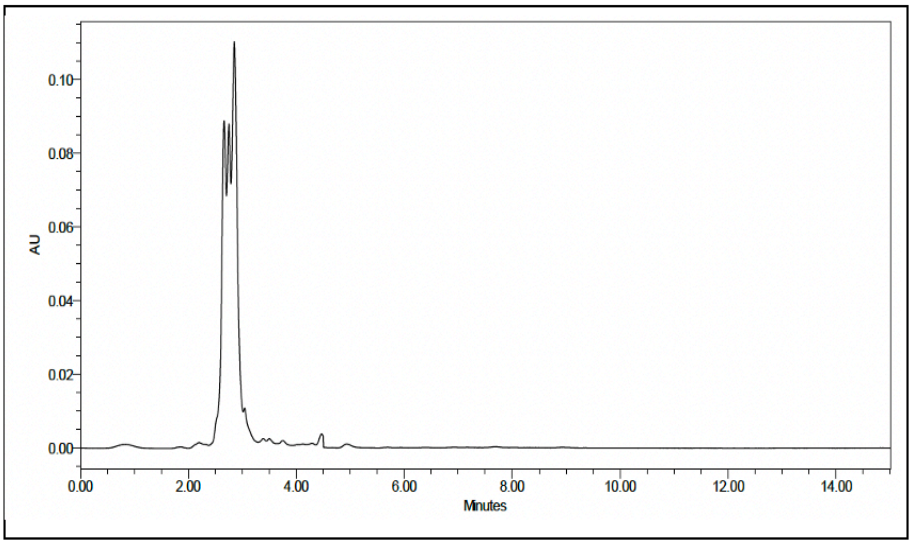

Fig. 2: Chromatogram of blank

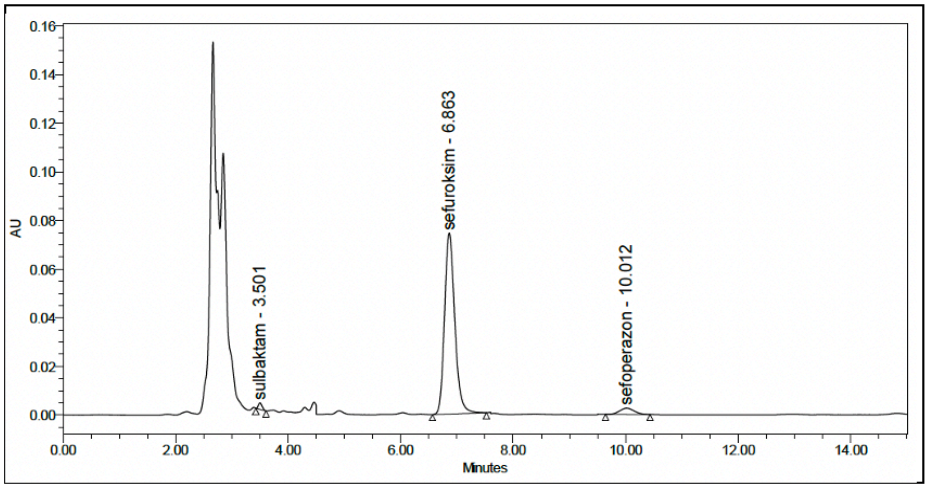

Fig. 3: Chromatogram of cefoperazone (5 µg/ml) and sulbactam (1 µg/ml) at the lower limit of quantitation

Table 5: Data of carryover

Blank sample	Cefoperazone carryover (%)*	Sulbactam carryover (%)*	Internal standard carryover (%)*
Blank 1	0.16	9.81	0.01
Blank 2	0.14	12.26	0.01
Blank 3	0.00	9.95	0.01
Blank 4	0.00	10.63	0.01
Blank 5	0.00	9.03	0.01

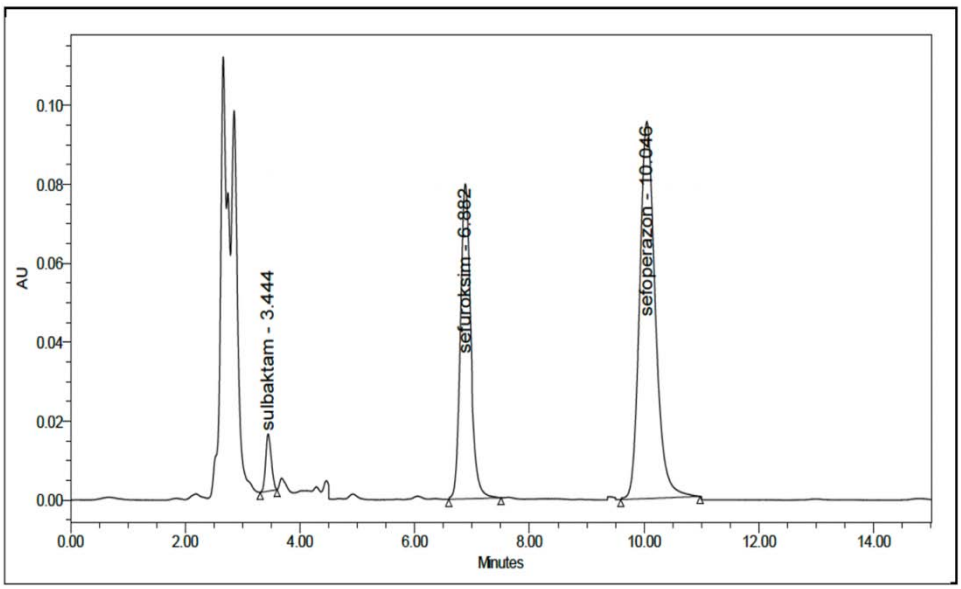

Fig. 4: Chromatogram of cefoperazone (250 µg/ml) and sulbactam (30 µg/ml) at the upper limit quantitation

Dilution integrity

Dilution integrity was performed to ensure that dilutions were accurate and reliable during the analysis. The test was performed with a concentration above ULOQ of 300 µg/ml for cefoperazone and 40 µg/ml for sulbactam diluted to half, a one quarter, and one per eight using a whole blood blank. The analysis was conducted in five replicates on each dilution. The result showed that until one per eight dilutions, the CV and bias (% diff) were not more than 15%.

Stability

The stability of cefoperazone, sulbactam, and IS was tested to determine whether degradation occurred during storage and preparation. Stability tests were performed using QCL and QCH samples with three replicates for each sample. In short-term stability tests, samples were stored at room temperature and were stable at 24 h. The result was that cefoperazone and sulbactam were stable in DBS for at least 24 h at room temperature. The study also performed long-term stability, and samples were stored at 4 °C for 0, 3, 5, 7, 14, and 21 d. These test cefoperazone and sulbactam samples are stable until 21 d.

To determine the stability of cefoperazone and sulbactam in the autosampler after the preparation of DBS samples, post-preparation stability tests were necessary for 24 h at the autosampler temperature. Cefoperazone and sulbactam preparations were stable for at least 24 h in the autosampler. Short-term stability tests of standard cefoperazone, sulbactam, and internal standard solution were conducted at room temperature for 24 h, and long-term stability tests of standard solution were performed after storage at 4 °C for 21 d. These data suggest that stock solutions of cefoperazone and sulbactam are stable at room temperature for at least 24 h and at 4 °C for 21 d.

CONCLUSION

The developed method for cefoperazone and sulbactam in the DBS sample was valid in concentrations of 2.5–250 µg/ml, 0.5–30 µg/ml, respectively. Moreover, cefoperazone and sulbactam samples are stable during storage at room temperature for at least 24 h, and stock solutions of cefoperazone and sulbactam are stable at room temperature for at least 24 h and at 4 °C for 21 d.

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AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICTS OF INTERESTS

Declare none

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