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Simultaneous determination of vancomycin and ceftazidime in cerebrospinal fluid in craniotomy patients by high-performance liquid chromatography

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ABSTRACT

A simple, accurate and rapid method for simultaneous analysis of vancomycin and ceftazidime in cerebrospinal fluid (CSF), utilizing high-performance liquid chromatography (HPLC), has been developed and thoroughly validated to satisfy strict FDA guidelines for bioanalytical methods. Protein precipitation was used as the sample pretreatment method. In order to increase the accuracy, tinidazole was chosen as the internal standard. Separation was achieved on a Diamonsil C18 column (200 mm × 4.6 mm I.D., 5 μm) using a mobile phase composed of acetonitrile and acetate buffer (pH 3.5) (8:92, v/v) at room temperature (25 °C), and the detection wavelength was 240 nm. All the validation data, such as accuracy, precision, and inter-day repeatability, were within the required limits. The method was applied to determine vancomycin and ceftazidime concentrations in CSF in five craniotomy patients.

1. Introduction

Vancomycin is a glycopeptide antibiotic that is primarily active as an inhibitor of cell-wall synthesis of susceptible organisms. It is widely used in the prophylaxis and therapy against infections by many Grampositive bacteria, including methicillin-resistant staphylococci [1]. Ceftazidime is a semisynthetic, broad-spectrum cephalosporin antibiotic that has been described as a β-lactamase-resistant third-generation cephalosporin antibiotic. Ceftazidime has a zwitterionic structure, which may enable it easily cross the lipid layer and achieve excellent penetration to cerebrospinal fluid (CSF) [2]. The chemical structures of vancomycin and ceftazidime are shown in Fig. 1.

The leading pathogens of surgical site infection (SSI) in craniotomy patients are Grampositive bacteria, especially *Staphylococcus aureus* and CoNS [3]. Antimicrobial therapy is often administered prior to the availability of microbiology and antibiotic susceptibility data, so empirical treatment for SSI in craniotomy patients should offer bactericidal activity against the most likely pathogens, including those with only moderate susceptibility [4–6]. Vancomycin in combination with ceftazidime is frequently used to prevent and treat such infections. Vancomycin penetrates the blood–brain barrier (BBB) poorly compared to ceftazidime, so vancomycin concentrations in CSF are very low and even no vancomycin is found in CSF in the absence of inflammation. However, after craniotomy or with meningeal inflammation, antibiotic concentrations in CSF are greatly increased [2,7]. Although intrathecal injection is an effective method to enhance antibiotic concentrations in CSF, it is traumatogenic and has several side effects, such as point of puncture bleeding, injury of spinal nerve root, local infection or central nervous system infection, and side effects in the central or peripheral nervous system irritated by the drugs. Rodríguez Guardado et al. [8] studied the clinical features and the outcome of a group of patients with nosocomial neurosurgical meningitis. These patients were treated with different therapeutic options, including intravenous monotherapy, intravenous combination therapy, and intravenous in combination with intrathecal therapy. In their study, no statistical significant differences were observed in the mortality among the groups with different treatments. The findings indicated that intrathecal injection was not necessarily superior to intravenous injection. After weighing the advantages and disadvantages of each method, intravenous administration is preferred in our hospital.

As for craniotomy patients, concentrations of antibiotics in CSF are more meaningful than that in plasma. Antibiotics can only be effective when they achieve therapeutic concentrations at the site

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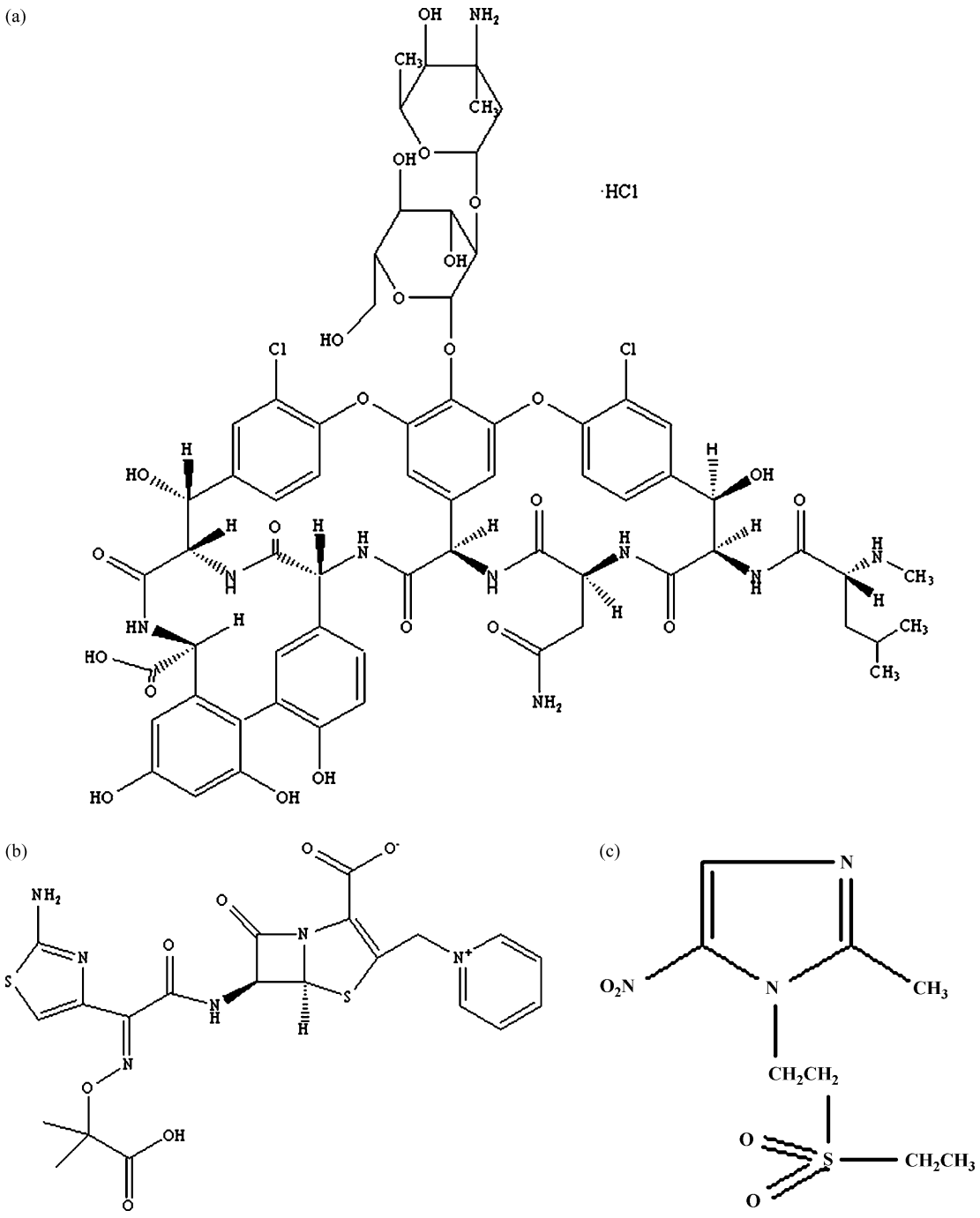

Fig. 1. Chemical structures of: (a) vancomycin; (b) ceftazidime; (c) tinidazole (IS).

of infection and knowledge of the penetration into the CSF is important in the treatment of craniotomy patients. Due to the differences of individual craniotomy patients, CSF penetration of vancomycin and ceftazidime varies greatly. As a result, the exact concentrations of the two drugs in CSF cannot be interpreted from their concentrations in plasma. In addition, vancomycin and ceftazidime are primarily excreted by the kidney functions. Consequently, it takes longer to clear the drugs for those with impaired renal function. The elimination half-life increases significantly in correlation with the severity of the renal failure [9,10]. Moreover, excessively high concentrations of these two drugs may lead to serious side effects [11,12]. To build personalized treatment plan and to minimize side effects, it is extremely important to monitor the CSF concentrations of vancomycin and ceftazidime. It is especially true to infants and those renally impaired.

A variety of methods have been developed for determination of vancomycin or ceftazidime in biological samples including immunoassays [13–15], high-performance liquid chromatography (HPLC) [16–18], liquid chromatography–tandem mass spectrometry (LC–MS–MS) [19,20] and capillary electrophoresis (CE) [21,22]. In general, immunoassays lack specificity and cannot distinguish multiple antibiotics or active metabolites or degradation products from the parent compound. Although LC–MS–MS method can provide excellent sensitivity, the apparatus is expensive and the matrix effects are difficult to control. CE is a powerful separation technique for the determination of ionic and neutral components. Compar-

ing to HPLC, few CE methods have been developed to determine vancomycin or ceftazidime concentrations in biological matrices. Among all these reported methods, HPLC is the most popular because of appropriate sensitivity and specificity and has the ability to detect low levels with high precision and accuracy, although not the simplest or least time consuming.

Compared to the reports of vancomycin or ceftazidime concentrations in plasma, only few data are available on those in CSF [13–22]. In this paper, we demonstrate a simple, rapid and accurate HPLC method for simultaneous determination of vancomycin and ceftazidime in CSF. Application of the proposed method to analyze the two drugs in five craniotomy patients was evaluated and it proved to be satisfactory.

2. Experimental

2.1. Materials

Vancomycin, ceftazidime and tinidazole (IS: internal standard) were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, PR China). The purities of the three compounds were >99.5%. Acetonitrile (chromatographic grade) was purchased from Merck (Darmstadt, Germany). Acetic acid, zinc sulfate and sodium acetate were all analytical reagent grade and were obtained from Shanghai Chemical Reagent Company (Shanghai, PR China). Deionized (18 MΩ/cm) water was generated in-house using a Millipore (Bedford, MA, USA) Milli-Q Plus system.

2.2. Instrumentation and chromatographic conditions

Experiments were carried out using an Agilent 1100 pump and an UV-VWD detector set at 240 nm. Separation was achieved on a Diamonsil C18 column (200 mm × 4.6 mm I.D., 5 μm) using a mobile phase composed of acetonitrile and acetate buffer (pH 3.5) (8:92, v/v) at room temperature (25 °C). The mobile phase was degassed by an ultrasonic bath before use. The flow rate was 1.0 ml/min and the injection volume was 20 μl. Data processing was handled by means of Agilent Chemstation A.10.02 software.

2.3. Preparation of standards and quality control samples

Stock solutions of vancomycin, ceftazidime and tinidazole were prepared at 1.0 mg/ml in water. Working standards were the mixture of the two compounds at the concentration levels of 10–1000 μg/ml for vancomycin and 100–10000 μg/ml for ceftazidime in water. They were prepared by serial dilutions from the stock solutions. A 50 μg/ml IS working solution was obtained by diluting the stock solution of tinidazole with water. All the solutions were stored at 4 °C and were brought to room temperature before use.

Calibration standards were prepared daily by spiking appropriate amounts of the mixed standard solutions to blank human CSF. The final concentrations in CSF standards were 0.1, 0.2, 0.5, 1, 2, 5 and 10 μg/ml for vancomycin and 1, 2, 5, 10, 20, 50 and 100 μg/ml for ceftazidime. QC samples were prepared by specific dilution of the stock solution in drug-free CSF to obtain the following high, medium and low concentrations, respectively, 5, 1 and 0.2 μg/ml of vancomycin and 50, 10 and 2 μg/ml of ceftazidime. All QC samples were to be prepared in bulk, and stored at −20 °C until analysis.

2.4. Sample preparation

CSF samples were removed from −20 °C storage and immersed in a heated (37 °C) water bath to thaw. A 0.5 ml volume of CSF was transferred to a 1.5 ml capped microcentrifuge plastic tube together with 0.25 ml of IS working solution. To the mixed samples, 0.25 ml of 10% zinc sulfate aqueous solution was added and vortexed for 20 s. After centrifugation at 12,000 rpm at room temperature for 10 min, 0.4 ml of supernatant was centrifuged again at 12,000 rpm for 5 min and 20 μl of supernatant was injected onto the analytical column.

2.5. Drug administration and sample collection

With the approval of the Scientific Research Committee in 101st Hospital of PLA, and with written informed consent obtained from patients or their appropriate relatives, five patients who underwent neurosurgical operation received intravenously 0.5 g of vancomycin and 2 g of ceftazidime within 60 min. CSF samples were collected instantly by ventricle drainage at 0, 0.25, 0.5, 1, 2, 4, 6, 8 and 12 h after drug administration and were centrifuged immediately at 3500 rpm for 5 min, then the upper liquid was collected and stored at −20 °C prior to analysis.

3. Results and discussion

3.1. Method development

The chromatographic conditions were optimized through several trials to achieve good resolution and symmetric peak shapes for the analytes and the IS, as well as a short run time. The influence of pH of the mobile phase was studied by using 0.075 M solutions of sodium acetate at different pH adjusted to 2.9–5.5 with acetic acid. The vancomycin retention time is much more affected by the pH than ceftazidime. Finally, the pH of the acetate buffer in mobile phase was adjusted to 3.5. The organic modifier content was also optimized and the increasing proportion of acetonitrile caused a decrease in retention time. It was found that a mixture of acetonitrile-acetate buffer (pH 3.5) (8:92, v/v) was appropriate and was finally adopted as the mobile phase. Protein precipitation was used for the sample preparation in this work. The method was rapid and robust, as only two simple steps are needed. The chromatogram for a blank CSF sample is clean and the recovery for the analytes from CSF is high.

3.2. Method validation

The method was validated according to the guidelines of the main regulatory agencies, such as those issued by the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) [23], by the United States Pharmacopeia (USP) [24] and by the US Food and Drug Administration (FDA) [25]. The validation experiments and results obtained are described below.

3.2.1. Robustness

Some experimental conditions were varied with respect to those reported in Section 2 to test their influence on method performance. The chosen parameters were: mobile-phase buffer pH (±0.3 pH units), mobile-phase composition (±2% to the acetonitrile percentage) and flow rate (±0.1 ml/min).

When the buffer pH was modified (by ±0.3 pH units), only small changes of retention times were noticed. Slightly longer run times were observed when increasing the pH, and slightly shorter times when decreasing it, without significant effect on resolution or method applicability. Compared to the buffer pH, mobile-phase composition had a stronger influence on the separation. A small change of acetonitrile percentage (by ±2%) caused general change

in run times, but had no great influence on separation performance. Changes to the flow rate (by ±0.1 ml/min) caused inverse changes in run times, without generating problems with respect to resolution, efficiency or peak shape.

3.2.2. Selectivity

Assay selectivity was evaluated by analyzing six separate lots of drug-free control CSF. All CSF lots were found to be free of interferences with the compounds of interest. The retention time for vancomycin, ceftazidime and IS was about 7, 11 and 20 min, respectively. Typical chromatograms are shown in Fig. 2.

3.2.3. Sensitivity and linearity

The lower limit of quantification (LLOQ) of the assay, defined as the lowest concentration on the standard curve that can be quantitated with accuracy within 20% of nominal and precision not exceeding 20%, was 0.1 and 1 μg/ml for vancomycin and ceftazidime, respectively. The reproducibility of LLOQ was determined by examining five LLOQ samples independent from the standard curve.

Calibration curves were constructed by plotting the peak area ratios (analytes/IS) of plasma standards versus nominal concentration. Weighted ($1/x^2$, where x = nominal standard concentration) linear least squares regression was employed. Calibration curves showed a linear range of 0.1–10 μg/ml for vancomycin and 1–100 μg/ml for ceftazidime. Unknown sample concentrations exceeding the range were diluted appropriately with control blank CSF and re-assayed. The difference between the nominal standard concentration and the back-calculated concentration from the weighted linear regression line was within 15% for each point on the standard curve indicating that the linear regression analysis applied provided an adequate fit of the data.

3.2.4. Accuracy and precision

The intra-day accuracy and precision of the assay were determined by analyzing replicates ($n = 5$) containing vancomycin and ceftazidime at three different concentration levels. The inter-day accuracy and precision were determined by analyzing three concentrations of QC samples, five times at each concentration. Table 1 presents the intra- and inter-day accuracy and precision for each of the QC samples. Our intra- and inter-day accuracy and precision (%CV) acceptance criteria for each QC was ≤15%. The assay successfully met the criteria.

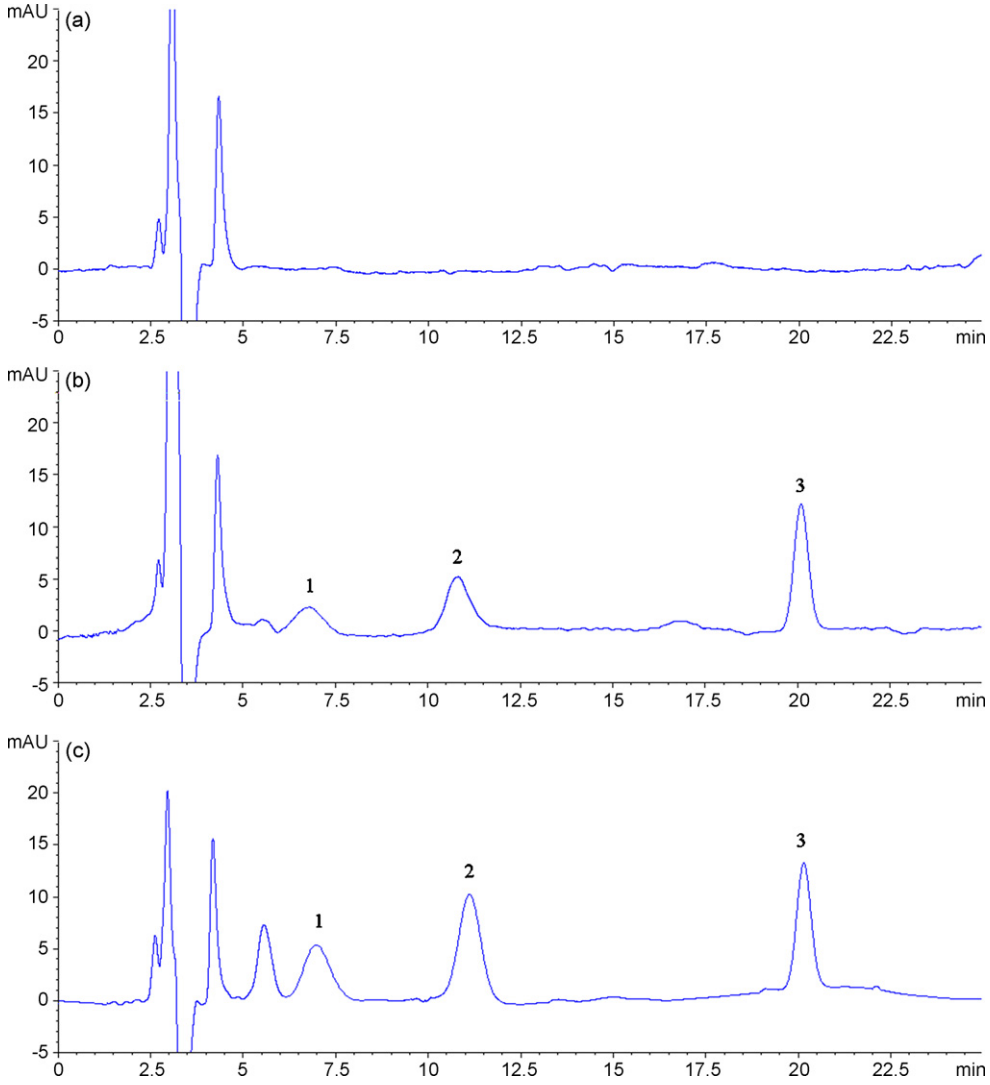

Fig. 2. Representative chromatograms of: (a) blank CSF; (b) CSF spiked with vancomycin (0.5 μg/ml), ceftazidime (5 μg/ml) and IS (12.5 μg/ml); (c) CSF sample from a patient 12 h after receiving 0.5 g of vancomycin and 2.0 g of ceftazidime intravenously. (1) Vancomycin, (2) ceftazidime and (3) tinidazole (IS).

Table 1
Intra- and inter-day accuracy and precision of QC samples of vancomycin and ceftazidime in CSF

Nominal concentration (μg/ml)	Intra-day (n = 5)			Inter-day (n = 5)		
	Mean determined concentration (μg/ml)	Accuracy (%)	CV (%)	Mean determined concentration (μg/ml)	Accuracy (%)	CV (%)
Vancomycin						
0.2	0.22	110.0	7.3	0.19	95.0	6.8
1.0	0.97	97.0	3.8	1.04	104.0	4.5
5.0	4.93	98.6	2.1	4.94	98.8	1.8
Ceftazidime						
2.0	2.13	106.5	6.9	1.92	96.0	7.9
10.0	10.28	102.8	4.2	10.13	101.3	3.2
50.0	49.53	99.1	1.9	50.27	100.5	2.0

3.2.5. Recovery

To investigate extraction recovery, a set of samples (n = 5 at each concentration in unique lots of CSF) was prepared by spiking vancomycin/ceftazidime into CSF at 0.2/2, 1/10, and 5/50 μg/ml. Each of the samples was also spiked with tinidazole at the working concentration of 50 μg/ml. The samples were subsequently processed using the procedure described previously. A second set of CSF samples was processed and spiked post-extraction with the same concentrations of two analytes and tinidazole that actually existed in the pre-extraction spiked samples. Extraction recovery for the analyte was determined by calculating the ratios of the raw peak areas of the pre-extraction spiked samples to the raw peak areas of the samples spiked after extraction. Mean extraction recoveries of vancomycin/ceftazidime at concentrations of 0.2/2, 1/10, and 5/50 μg/ml were 97.17/102.04%, 105.18/103.02% and 100.77/100.98%, respectively, and the mean extraction recovery of the IS was 96.0%. The recoveries were comparable to the reported ones of vancomycin and ceftazidime extracted from plasma [17,26].

3.2.6. Stability

Table 2 lists all the data for stability study.

3.2.6.1. Freeze and thaw stability. Freeze–thaw stability was evaluated for vancomycin and ceftazidime using QC samples. The QCs were exposed to three freeze–thaw cycles, and each cycle consisted of removing the QCs from the freezer, thawing them unassisted to room temperature, kept at room temperature for 4 h and re-freezing at −20 °C. The samples were processed along with a standard curve and concentrations were determined. The results indicated that vancomycin and ceftazidime had acceptable stabilities after three freeze–thaw cycles in human CSF.

3.2.6.2. Long-term stability. The long-term stability at −20 °C was tested using QC samples. The stability was monitored during validation and sample analysis periods, and no degradation of the compounds was observed. The 1-month stability data listed in Table 2 indicated that vancomycin and ceftazidime were stable in CSF for at least 1 month.

3.2.6.3. Short-term stability. Short-term stability was investigated to ensure that analytes were not degraded in CSF samples at room temperature for a time period to cover the sample preparation. Three sets of QC samples were left at room temperature for 12 h. The samples were then processed and analyzed. The results indicated that vancomycin and ceftazidime were stable during the exposure period.

3.2.6.4. Post-preparative stability. Due to the need for occasional delayed injection or reinjection of extracted samples, stability of compounds in the final extraction fluid was evaluated in the autosampler at 25 °C. A group of QC samples were extracted, loaded onto the autosampler and kept in the autosampler for 24 h before injection. The quantitative results indicated that vancomycin and ceftazidime were stable in the autosampler up to at least 24 h.

3.2.7. Sample dilution

To demonstrate the ability to dilute and analyze samples containing vancomycin and ceftazidime at concentration above the assay upper limits of quantitation, a set of CSF samples were prepared containing vancomycin and ceftazidime at a concentration of 25 and 250 μg/ml, respectively, and placed in a −20 °C freezer overnight prior to analysis. After thawing, a 0.2 ml aliquot was withdrawn for analysis (n = 5), diluted with 0.8 ml of control human CSF,

Table 2
Stability data of vancomycin and ceftazidime

Nominal concentration (μg/ml)	Freeze–thaw stability[a] (n = 3)		Long-term stability[b] (n = 3)		Short-term stability[c] (n = 3)		Post-preparative stability[d] (n = 3)	
	Found concentration (μg/ml)	CV (%)	Found concentration (μg/ml)	CV (%)	Found concentration (μg/ml)	CV (%)	Found concentration (μg/ml)	CV (%)
Vancomycin								
0.2	0.19	5.3	0.21	9.5	0.19	5.3	0.20	5.0
1.0	0.99	4.0	0.98	3.1	1.04	5.8	0.98	2.0
5.0	4.88	2.5	5.13	2.9	4.97	2.2	4.95	1.0
Ceftazidime								
2.0	2.12	7.1	2.04	5.4	1.92	7.3	2.03	3.0
10.0	9.89	3.2	10.07	3.6	10.10	2.4	9.95	1.7
50.0	50.18	2.1	49.89	2.2	50.21	2.0	49.94	1.4

[a] after three freeze–thaw cycles.
[b] stored at −20 °C for 1 month.
[c] exposed at room temperature (25 °C) for 12 h.
[d] kept at 20 °C for 24 h.

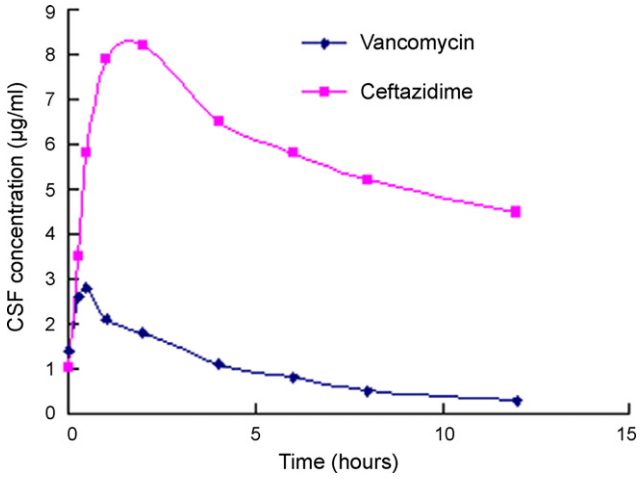

Fig. 3. Mean CSF concentration–time curve for vancomycin (♦) and ceftazidime (■) in craniotomy patients after intravenous administration of 0.5 g of vancomycin and 2.0 g of ceftazidime.

then prepared and analyzed. The accuracy of the test was above 90% and the precision was good.

3.3. Application of the assay

This method was successfully applied to determine the concentrations of vancomycin and ceftazidime in craniotomy patients after intravenous administration. The mean CSF concentrations–time profiles of vancomycin and ceftazidime are shown in Fig. 3. For most susceptible strains of *S. aureus*, including MRSA, MICs for vancomycin are below 2 μg/ml and for Gramnegative bacilli susceptible strains have MICs for ceftazidime below 1 μg/ml. The concentrations of vancomycin exceeded 2 μg/ml for atmost 1 h after administration. However, ceftazidime concentrations in CSF reached or exceeded 1 μg/ml for at least 12 h. The results showed that neurosurgical operation might enhance the BBB penetrability of vancomycin and ceftazidime, and the administration dose of vancomycin could be increased. Wang et al. [7] studied CSF concentration of vancomycin in craniotomy patients after intravenously administration of 1 g vancomycin. Compared with their results, our results indicated that the CSF concentration of vancomycin is generally lower in consideration of the different dosages. It might attribute to the existence of ceftazidime, which could competitively inhibit BBB penetrability of vancomycin. In consistent with earlier reports [27], the CSF concentration of ceftazidime at 12 h is still high, suggesting that the clearance from CSF is slower than that from plasma.

4. Conclusion

The method reported in this paper describes a selective and accurate HPLC method for simultaneous determination of vancomycin and ceftazidime in CSF with rapid and simple sample pretreatment. The results of the study indicated that neurosurgical operation disrupted BBB and thus enhanced vancomycin and ceftazidime penetrability. However, in this study vancomycin reached therapeutic concentration in CSF for quite a short time after IV dosing. This suggested that the administration dose could be increased or some other administration methods should be adopted.

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