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Simple liquid chromatographic method for the analysis of the blood brain barrier permeability characteristics of ceftriaxone in an experimental rabbit meningitis model

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Abstract

A simple LC method was developed and validated for the analysis of ceftriaxone in aqueous and biological samples. Chromatographic separation was achieved on a reversed-phase C_{18} microbore column (Hypersil 5 μm, 200×2.1 mm) with UV detection at 270 nm. This isocratic system was operated at ambient temperature and required less than 10 min of chromatographic time. The flow-rate was maintained at 0.5 ml min^{-1}. Cetyltrimethylammonium bromide (0.01 *M*) was utilized as the ion-pairing agent. For the analysis of the drug in the aqueous system, the mobile phase consisted of methanol–acetonitrile–phosphate buffer, pH 7.4 (20:20:60, v/v/v). The plasma and CSF systems used the same mobile phase constituents in a slightly different ratio (30:40:30, v/v/v). Lidocaine was used as an internal standard and the peak height ratios of the drug to that of the internal standard were linear over the concentration range of 0.0 to 16 μg ml^{-1} only in the case of aqueous systems. Within-day and day-to-day relative standard deviations ranged from 0.3 to 2.2% and 1.1 to 5.9%, respectively. This method was used to: (1) quantify ceftriaxone in an aqueous system, in rabbit plasma using a simple protein precipitation procedure, and in the CSF; (2) evaluate the permeability characteristics of ceftriaxone across the blood–brain barrier through quantification of ceftriaxone in the CSF using a microdialysis sampling technique; and (3) analyze the effects of dexamethasone (a synthetic fluorinated corticosteroid used for the relief of cerebral edema) on the permeability of ceftriaxone across the blood brain barrier through quantification of ceftriaxone in the dexamethasone-treated animals with meningitis. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

In the United States, approximately 5000 cases of *Streptococcus pneumoniae* (STP) meningitis occur annually [1]. Despite the introduction of newly modified antibiotics and improved diagnostic procedures, the morbidity and mortality associated with meningitis have not changed significantly over the past 30 years. In fact, the development of resistance to penicillin has caused worldwide concern regarding the treatment of pneumococcal infections. Indeed, morbidity from penicillin-resistant pneumococci has been reported as 50% higher than with penicillin-sensitive strains [2].

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Due to the development of penicillin-resistant pneumococcal infections it is important to achieve optimal therapeutic activity of a drug in bacterial meningitis. Thus, one should utilize an anti-microbial agent with bactericidal activity within the cerebrospinal fluid (CSF). Studies have indicated that rapid bactericidal action in the CSF is obtained when the concentration of an anti-microbial agent exceeds the minimal bactericidal concentration (MBC) by 10-fold [3–5]. Such bactericidal effects of an anti-microbial agent in the CSF depend on several factors including: the pH of the CSF, the elevated protein concentration and antibiotic binding, growth rate of bacteria in the CSF, the metabolism and excretion of the antibiotic from the CSF, and the large inoculum effect in the CSF found due to the presence of a high bacterial count [6]. Therefore, it is quite essential to determine the free drug concentration in the CSF in order to assess the therapeutic efficacy of an anti-microbial agent in the treatment of meningitis.

The restricted distribution of an anti-microbial agent from the blood to the brain depends upon the blood–brain barrier (BBB) permeability characteristics of the agent. The BBB consists of brain capillary endothelial cells which restrict the transport of drug molecules between the blood and the brain. Therefore, the BBB permeability affects the CSF concentration of a drug and has been shown to depend upon the drug's physical and chemical characteristics [7]. Ceftriaxone (Fig. 1) is a semi-synthetic third-generation cephalosporin antibiotic which is generally less active in vitro against susceptible strains of staphylococci than first-generation cephalosporins [8]. However, it has an expanded range of activity against gram-negative bacteria when compared with first- and second-generation antibiotics. Its action is usually bactericidal resulting from the inhibition of mucopeptide synthesis in the bacterial cell wall. Therefore, it is often used in the treatment of various infections including meningitis caused by STP. Ceftriaxone is only commercially available in the parenteral (Rocephin, Roche) dosage form [9] and has been used as a bactericidal agent in this rabbit meningitis model for investigative purposes.

Several high-performance liquid chromatography (HPLC) methods have been reported for the analysis of ceftriaxone in aqueous and biological samples at high concentrations [10–13]. In this investigation, an experimental rabbit meningitis model, in conjunction with microdialysis as the sampling tool, was utilized to analyze the pharmacokinetics of ceftriaxone. As such, this study demanded a highly sensitive method for the analysis of the drug in both the dialysate and plasma [14]. Since no net loss of CSF occurs when using microdialysis, the CSF concentration of the antibiotic was used as an indicator of the antibiotic's permeability characteristics across the BBB. Previous reports have indicated that meningitis may lead to elevated levels of protein in the CSF. Such elevated protein levels in the CSF can adversely affect the activity of highly protein-bound anti-microbial agents. Since only the free drug exhibits antibacterial action, it is essential to determine the free fraction of the drug in the CSF. Previously reported methods used either microbial or HPLC method to determine the total (free+bound) ceftriaxone concentration in the CSF. In contrast, a microdialysis sampling technique can be used to determine the free fraction and, thus, the active portion of the drug, in the CSF. Therefore, the objectives of this study included: (1) the development of a simple, sensitive, and specific LC method for determination of the ceftriaxone concentration in an aqueous system without the use of a complex extraction

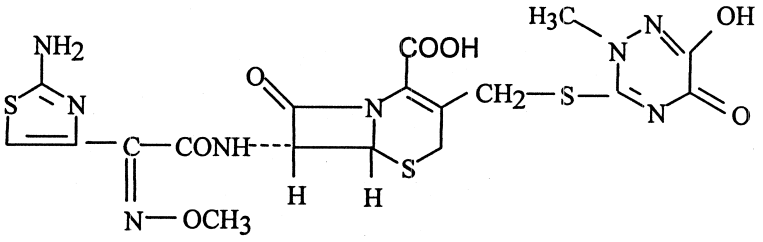

Fig. 1. Structure of ceftriaxone.

procedure and also in rabbit plasma using a simple protein precipitation procedure; (2) the evaluation of the permeability characteristics of ceftriaxone across the blood brain barrier through the analysis of the ceftriaxone concentration in both the CSF and microdialysis dialysates; and (3) evaluation of the effects of dexamethasone (a synthetic fluorinated corticosteroid used for the relief of cerebral edema) on the permeability of ceftriaxone across the blood brain barrier through analysis of the change in CSF ceftriaxone concentration due to the presence of dexamethasone.

2. Experimental

2.1. Materials

Ceftriaxone (Hoffmann-La Roche, Nutley, NJ, USA); lidocaine (Sigma, St. Louis, MO, USA); cetyltrimethylammonium bromide (CTAB), water (HPLC grade), acetonitrile, methanol, monobasic potassium phosphate, dibasic sodium phosphate (Fisher, Fairlawn, NJ, USA); and Lactated Ringer's solution, USP (Abbott, North Chicago, IL, USA) were used as received.

2.2. Chromatography

The HPLC system consisted of a pump (Model LC-600) programmed by a system controller (Model SCL-6B), a UV–Visible spectrophotometric detector (Model SPD-6AV) and a recorder (Model CR501), obtained from Shimadzu (Tokyo, Japan). The separation was carried out using a 200×2.1 mm C_{18} column (Hypersil, Hewlett-Packard). The mobile phase used in the aqueous system consisted of methanol–acetonitrile–phosphate buffer, pH 7.4 (20:20:60, v/v/v). In contrast, the plasma and CSF systems used the same mobile phase constituents in a slightly different ratio: (30:40:30, v/v/v). The exact composition of the phosphate buffer used in the preparation of the mobile phase is outlined below. CTAB (0.01 *M*) was used as an ion-pairing agent in the mobile phase. The flow-rate was maintained at 0.5 ml min^{-1} and the column effluent was monitored at 270 nm.

2.3. Solutions

2.3.1. Phosphate buffer (pH 7.4)

Monobasic potassium phosphate (9.07 g l^{-1}) (solution A) and disodium phosphate (9.48 g l^{-1}) (solution B) were prepared in water (HPLC grade). Solution A (643 ml) was mixed with solution B (357 ml) to prepare the phosphate buffer.

2.3.2. Mobile phase for the aqueous system

CTAB (3.65 g l^{-1}) was dissolved in methanol (100 ml). This solution was mixed with acetonitrile (100 ml) and phosphate buffer (300 ml). The resulting solution was then filtered through a prefilter and a 0.45-μm MAGNA Nylon, 47 mm filter (MSI, Westborough, MA, USA).

2.3.3. Mobile phase for both the plasma and CSF system

CTAB (3.65 g l^{-1}) was dissolved in methanol (150 ml). This solution was mixed with acetonitrile (200 ml) and phosphate buffer (150 ml). The resulting solution was then filtered through a prefilter and a 0.45-μm MAGNA Nylon, 47 mm filter (MSI).

2.3.4. Aqueous standard solutions

The stock standard solutions were prepared by dissolving 16.5 mg of ceftriaxone in 50 ml mobile phase (330 μg ml^{-1}). Various standard solutions (0–16 μg ml^{-1}) were then prepared from the resulting stock solution after adequate dilution with the mobile phase.

2.3.5. Plasma standard solutions

The standard stock solution was prepared by dissolving 2.87 mg ceftriaxone in HPLC grade methanol (100 ml) using a volumetric flask. Appropriate volumes (20 to 100 μl) of the stock solution (28.7 μg ml^{-1}) were pipetted into disposable test tubes, evaporated to dryness at 40°C using a drying oven (Stabil-Therma Model, Blue M Electric Company, Blue Island, IL, USA) and reconstituted with an appropriate volume of blank plasma defined as plasma without ceftriaxone.

2.3.6. CSF and microdialysis dialysate

Standard solutions (1 to 5 μg ml^{-1}) were prepared in Lactated Ringer's solution. The stock solution was prepared by dissolving 100 mg of ceftriaxone in 100 ml Lactated Ringer's solution utilizing a volumetric flask. Various standard solutions were then reconstituted utilizing the resulting stock solution.

2.3.7. Internal standard solution

Lidocaine (57.5 mg) was dissolved in methanol and the volume was then adjusted to 50 ml to prepare the internal standard solution (1.15 mg ml^{-1}).

2.4. Sample preparation for LC

2.4.1. Ceftriaxone in aqueous solutions

The internal standard solution (60 μl) was spiked to borosilicate glass test tubes and evaporated to dryness at 40°C. Standard solution (200 μl) was then added to each test tube, vortexed for 10 s, and injected directly to the HPLC. The peak height ratio of the drug to the internal standard vs. concentration was plotted.

2.4.2. Ceftriaxone in plasma solutions

Each plasma sample (20 μl) was spiked to borosilicate glass test tubes followed by mobile phase without the ion-pairing agent (380 μl). This solution was then vortexed for 10 s and centrifuged at 5000 rpm (4425 *g*) for 5 min. The supernatant was collected and analyzed using HPLC. The peak height vs. concentration was plotted and used as the calibration curve.

2.4.3. Ceftriaxone in CSF and microdialysis dialysates

Standard solutions (1 to 5 μg ml^{-1}) were prepared in Lactated Ringer's solution and analyzed using HPLC. The peak height vs. concentration was plotted and used as the calibration curve.

2.5. Calculation

The ratios of the peak heights of ceftriaxone to those of the internal standard were calculated for the ceftriaxone in aqueous solutions. The unknown ceftriaxone concentrations were then determined using the regression equation relating the peak-height ratio (PHR) of the standards to their nominal concentrations. In the case of ceftriaxone in plasma and microdialysis diasylate systems, the unknown ceftriaxone concentration was determined utilizing the regression equation relating to the peak height of the standards with respect to their nominal concentrations.

3. Results and discussion

3.1. Optimization of the analytical procedure

In this investigation, as discussed earlier, an experimental rabbit meningitis model, in conjunction with microdialysis as the sampling tool, was utilized to analyze the BBB permeability characteristics of ceftriaxone. Such a study demanded a highly sensitive method for the analysis of the drug in three different matrices, which included aqueous, plasma and dialysate systems.

In the analysis of ceftriaxone in the aqueous samples, no sample pretreatment prior to HPLC injection was required. Previously reported studies involving the determination of ceftriaxone in the plasma utilized complex extraction procedures prior to HPLC analysis [10–13]. In contrast, this method used a simple protein precipitation method, with the mobile phase. During the development of this method, it was noted that when mobile phase was used alone, a clean chromatogram was not achieved. This finding was possibly due to the interference of the ion-pairing agent in the mobile phase during the protein precipitation procedure. However, this problem was eliminated when mobile phase without ion-pairing agent was used as a protein precipitant. In addition, to obtain a cleaner sample, centrifugation was essential. From the preliminary studies, it was also evident that a cleaner sample could be obtained when the ratio of plasma to mobile phase was 1:19 (v/v) during protein precipitation. Representative chromatograms are shown in Fig. 2.

In the microdialysis study, simulated CSF (1.1 m*M* magnesium, 1.35 m*M* calcium, 3.0 m*M* potassium, 144.48 m*M* HPO_4, 131.9 m*M* chlorine, pH 7.6) in Lactated Ringer's solution was perfused through the microdialysis probe at a rate of 1 μl

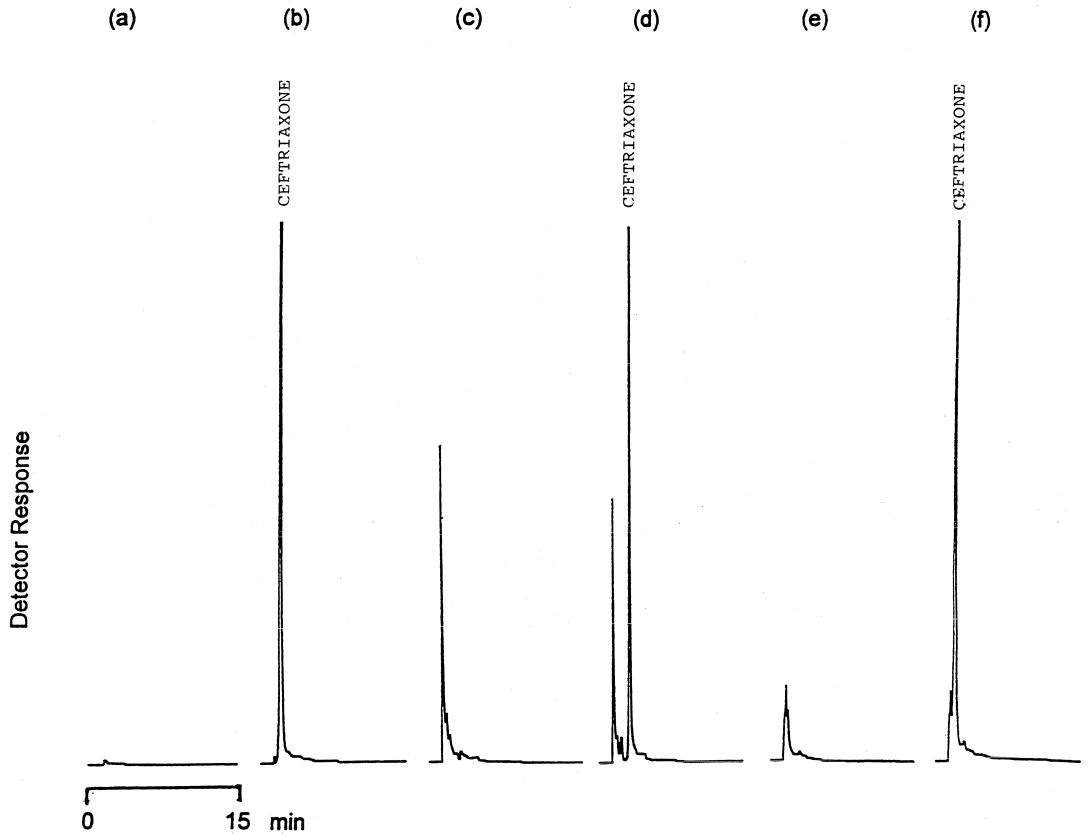

Fig. 2. Representative chromatograms obtained following injection of: (a) freshly prepared mobile phase, (b) freshly prepared ceftriaxone (5.7 μg ml^{-1}) in the mobile phase, (c) Lactated Ringer's solution, (d) ceftriaxone (3.0 μg ml^{-1}) in Lactated Ringer's solution, (e) blank rabbit plasma, and (f) ceftriaxone (5.7 μg ml^{-1}) in rabbit plasma.

min^{-1}. The dialysates were collected at predetermined time intervals and the drug content was measured using HPLC. Since protein cannot pass through the membrane of the microdialysis probe from the CSF to the dialysate samples during the microdialysis experiment, prior sample cleanup procedure was not required before injection onto the HPLC column.

3.2. Validation of the ceftriaxone assay

3.2.1. Linearity

The standard curves of ceftriaxone in the aqueous system were linear over the concentration range of 0 to 16 μg ml^{-1}. The equation of the standard curve relating the PHR to the ceftriaxone concentration (C in μg ml^{-1}) in this range was: $PHR = -0.0091 + 0.1685C$, $R^2 > 0.999$. In contrast, for the ceftriaxone in the plasma system, the standard curves were linear over the concentration range of 0 to 7 μg ml^{-1}. The equation of the standard curve relating the peak-height (PH) to the ceftriaxone concentration (C in μg ml^{-1}) in this range was: $PH = 100.8 + 1171.1C$, $R^2 > 0.99$. Standard curves of ceftriaxone in the CSF and microdialysis diasylate system were linear over the concentration range 0 to 5 μg ml^{-1}. The equation of the standard curve relating the PH to the ceftriaxone concentration (C in μg ml^{-1}) in this range was: $PH = 100.4 + 1171.1C$, $R^2 > 0.99$.

3.2.2. Precision

The within-day precision of the ceftriaxone assay in the plasma samples was determined by analysis of

Table 1
Within-day and day-to-day analytical precision

Concentration ($\mu g\ ml^{-1}$)	RSD (%)	
	Within-day[a]	Day-to-day[b]
0.00	–	–
1.4	6.7	6.2
2.9	2.5	5.6
4.3	3.8	3.3
5.7	1.9	2.4
7.2	0.9	1.8

[a] Analyzed on the same day.
[b] Analyzed on seven different days within a period of 30 days.

four different standard curves on the same day. Day-to-day precision was determined by analysis of the same solutions on seven different days over a period of 30 days. During this period, the stock solutions were stored under refrigeration (4°C) and solutions for the standard curves were prepared fresh daily from the stock solution. The plasma was stored in the freezer at 0°C. Variability in the peak height at each concentration was used to determine the precision of the assay procedure and the results are presented in Table 1. Within-day and day-to-day relative standard deviation (RSD) values ranged from 0.9 to 6.7% and 1.8 to 6.2%, respectively.

3.2.3. Accuracy

Two quality control samples and the standard solutions were refrigerated at (4°C) over a period of 30 days. These samples were analyzed seven times during this period and the accuracy of the plasma assay was determined by comparing the measured concentration to its nominal value (Table 2). The RSDs ranged from 1.3 to 4.7%.

3.2.4. Sensitivity for ceftriaxone assay

The sensitivity criteria were determined from five different standard curves using the lowest limit of reliable assay measurement criteria as described by Oppenheimer et al. [15]. The critical level is the assay response above which an observed response is reliably recognized as detectable. The critical level is also considered a threshold value, thus, defining detection. Therefore, if the measured value exceeds the value observed, then the presence of an analyte is detected, otherwise, it is not reliably recognized as detectable. In this study, the critical value was determined as $0.0001 \pm 0.0007\ \mu g\ ml^{-1}$ (mean±SD; $n=5$). The detection level is known as the actual net response, which may, a priori, be expected to lead to detection. This value is defined as the least value of the true concentration that is ''nearly sure'' to produce a measured value that results in detection [16]. In this study, the detection level was found to be $0.003 \pm 0.001\ \mu g\ ml^{-1}$ (mean±SD; $n=5$). The determination level is defined as the concentration at which the measurement precision will be satisfactory for quantitative determination. This value was found to be $0.008 \pm 0.004\ \mu g\ ml^{-1}$ (mean±SD; $n=5$) for a level of precision of 10% RSD

3.3. Applications of the LC method

This LC method was used to measure the concentration of ceftriaxone in rabbit plasma, CSF and microdialysis dialysate samples.

3.3.1. Ceftriaxone in plasma solutions

A simple protein precipitation procedure with mobile phase containing acetonitrile as a protein precipitant was utilized. The accuracy of this method in determining the ceftriaxone concentration in plasma was determined by comparing its nominal con-

Table 2
Accuracy in the analysis of ceftriaxone in quality control samples

Actual concentration ($\mu g\ ml^{-1}$)	Measured concentration[a] ($\mu g\ ml^{-1}$)	Accuracy[b] (%)	RSD (%)
3.56	3.61±0.17	101.8±4.8	4.7
6.46	6.28±0.09	97.40±1.3	1.3

[a] Mean±SD; $n=7$.
[b] Accuracy=(measured concentration/actual concentration)·100.

centration to that of the determined concentration. The determined concentration of three separate plasma samples ranged from 97.4 to 101.8% of the nominal value. Furthermore, no appreciable degradation product in the chromatogram was noticed. A representative plasma concentration versus time profile of ceftriaxone is shown in Fig. 3. The study also indicated that co-treatment of dexamethasone with ceftriaxone did not interfere with the assay of ceftriaxone and, as such, this method could be used to evaluate the pharmacokinetics parameters of ceftriaxone in this particular animal meningitis model.

3.3.2. Microdialysate samples

The microdialysate samples contained ceftriaxone in artificial CSF in Lactated Ringer's solution. The details of the microdialysis experiments performed in an experimental rabbit meningitis model are discussed elsewhere [14]. This LC method was used to determine the concentration of ceftriaxone in the CSF. Lactated Ringer's solution did not interfere with this assay and no protein precipitation was required for these samples prior to HPLC analysis. Furthermore, since no net loss of CSF occurred when utilizing the microdialysis sampling technique, the

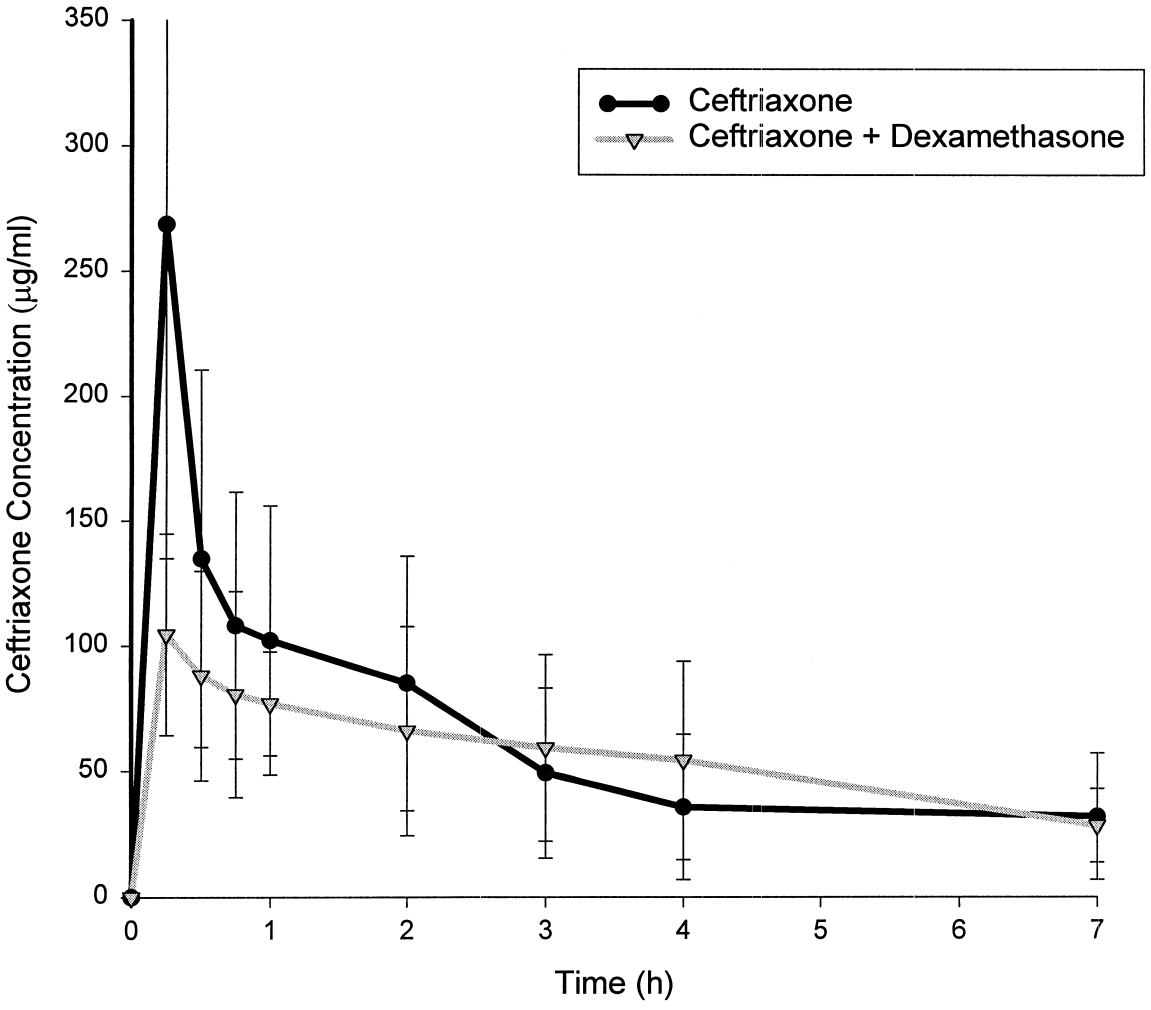

Fig. 3. Ceftriaxone plasma concentrations versus time profiles in an experimental animal model (rabbit) of *S. pneumoniae* meningitis.

concentration of ceftriaxone in the dialysate was used to determine the in vivo concentration of the drug in the brain and also the permeability of the drug across the BBB. The in vitro recovery of ceftriaxone in the probes used in the microdialysis sampling method was 15.7%. Both in vitro recovery and the drug concentration in the dialysate determined by the HPLC method were used to calculate the free ceftriaxone concentration in the CSF. A representative concentration versus time profile of ceftriaxone in the CSF is shown in Fig. 4. The ceftriaxone dose used in this study was 100 mg/kg of rabbit. This dose was administered over a period of 10 min by the use of a syringe, not an infusion pump. The peak CSF concentration of ceftriaxone (8.9 ± 1.6 μg ml^{-1}; $n=3$) was achieved within 0.5 h. However, the peak CSF concentration of ceftriaxone (6.3 ± 1.5 μg ml^{-1}; $n=3$) in conjunction with dexamethasone-treated animals, occurred 1 h after the initial administration of the drugs. The mean ceftriaxone concentrations for the ceftriaxone-treated groups at 60 and 120 min were: 6.9 ± 1.9 and 6.3 ± 1.0 μg ml^{-1}, respectively and 6.3 ± 1.1 and 3.6 ± 0.9 μg ml^{-1}, respectively for the ceftriaxone–dexamethasone treated groups. The results of this investigation were consistent with previously reported values [17,18]. There was no significant difference in CSF penetration of ceftriaxone between the ceftriaxone-treated and ceftriaxone–dexamethasone-treated groups. In addition, no significant differ-

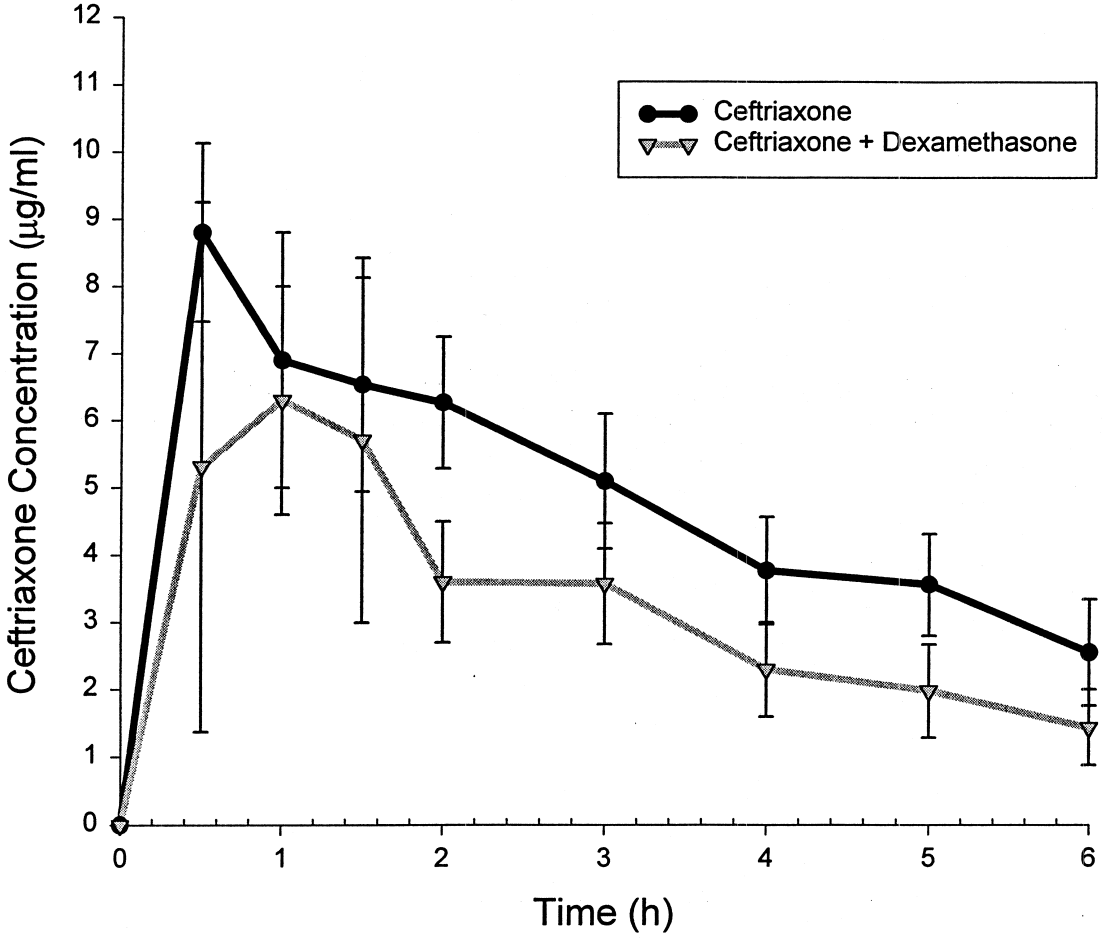

Fig. 4. Ceftriaxone cerebrospinal fluid concentrations versus time profiles in an experimental animal model (rabbit) of *S. pneumoniae* meningitis.

ences in the area under the curve (AUC_{CSF}) values were observed in the two groups.

4. Conclusions

A simple, sensitive method was developed for the analysis of ceftriaxone in aqueous solution, rabbit plasma, and CSF. In addition, no extraction or protein precipitation was necessary for the aqueous and CSF samples. For quantitation of ceftriaxone in the plasma, a simple protein precipitation method was used which avoided complex extraction procedures. Co-treatment with dexamethasone and the presence of Lactated Ringer's solution did not interfere with the assay of ceftriaxone. Although the onset of the peak CSF concentration of ceftriaxone varied between the control (0.5 h) and dexamethasone treated animals (1 h), the penetration of ceftriaxone was not affected by the addition of dexamethasone therapy. No significant differences in AUC_{CSF} were seen between the two groups. Future studies will focus on the development of a simple LC method for the determination of levofloxacin in animal plasma and CSF. It is hoped that such studies will further our current understanding of the BBB permeability characteristics of various antibiotics and the penetration into the CSF to enhance the therapeutic effectiveness of antibiotics in the treatment of meningitis caused by peniciiin-resistant pneumococci such as STP.

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