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Simultaneous blood and brain sampling of cephalexin in the rat by microdialysis and microbore liquid chromatography: application to pharmacokinetics studies

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Abstract

To circumvent the need for laborious sample clean-up and multiple blood sampling, a system was developed consisting of on-line microdialysis coupled to microbore liquid chromatography and ultraviolet detection. The system was designed for the simultaneous and continuous monitoring of unbound blood and brain cephalexin in the rat following single bolus intravenous administrations (10 mg/kg, $n=6$). Microdialysis probes were inserted into the jugular vein and brain striatum, respectively, for blood and brain sampling. Chromatographic conditions consisted of a mobile phase of methanol–100 m*M* monosodium phosphoric acid (20:80, v/v, pH 5.0) pumped through a microbore reversed-phase column at a flow-rate of 0.05 ml/min. Detection wavelength was set at 260 nm. The method was validated for response linearity as well as intra- and inter-day variabilities. Rapid appearance of cephalexin in the striatal dialysate suggested good blood–brain barrier penetration. This study provided pharmacokinetics information for cephalexin as well as demonstrated the applicability of this continuous sampling method for pharmacokinetics studies.

Keywords: Microdialysis; Microbore liquid chromatography; Pharmacokinetics; Rat; Blood; Brain; Cephalexin

1. Introduction

Cephalexin belongs to the β-lactam class of cephalosporin antibiotics [1] that are bactericidal against streptococci, pneumococci and sensitive gram-negative bacilli (*Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*) and bacteriostatic against *Haemophilus influenzae* [2]. As it has been prescribed for the treatment of serious bacterial infections in the central nervous system such as meningitis in humans there is a need to examine its pharmacokinetics both in the periphery and the central nervous system. Various methods have been developed for the determination of cephalexin in biological fluids, all of which involve various clean-up procedures prior to reversed-phase liquid chromatography [3–8]. These include liquid-phase [6] or solid-phase [3–5] extraction, or deproteination by

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organic solvent [8]. These clean-up procedures can be time consuming and difficult particularly if sample volumes are already small. Another difficulty with conventional pharmacokinetics studies is the need for multiple sampling to complete a reasonable profile. This could be traumatic and the cumulative consumption of blood, as necessitated in these processes, may alter the hemodynamics and possibly introduce factors that may affect drug distribution.

Microdialysis sampling, being relatively less invasive and requiring no loss of biological fluids, offers an attractive alternative through which some of these undesirable factors can be circumvented. In the present study microdialysis probes were inserted into the striatum and jugular vein, respectively, for blood and brain sampling. Microbore high-performance liquid chromatography (HPLC) enhanced resolution and reduced the sample size required, permitting the setting up of an on-line microdialysis system for convenient and continuous monitoring. The system conceivably can be applied for pharmacokinetics studies of other drugs possessing central nervous system actions.

2. Experimental

2.1. Reagents

Cephalexin (Fig. 1) and chemical reagents were purchased from Sigma (St. Louis, MO, USA). HPLC-grade solvents were obtained from BDH (Poole, UK). Triple deionized water from Millipore (Bedford, MA, USA) was used for all preparations.

2.2. Liquid chromatography

The microbore liquid chromatographic system consisted of a chromatographic pump (BAS PM-80, West Lafayette, IN, USA), an on-line injector (CMA 160, Stockholm, Sweden) equipped with a 10-μl sample loop and a Dynamax UV–Vis absorbance detector (Walnut Creek, CA, USA). Dialysates were separated using a reversed-phase C_{18} microbore column (150×1 mm I.D.; particle size 5 μm, BAS) maintained at ambient temperature. The mobile phase, consisting of methanol–100 m*M* monosodium phosphoric acid (20:80, v/v, pH 5.0), was filtered through a 0.22-μm Millipore membrane and then degassed prior to use. Flow-rate was maintained at 0.05 ml/min. Detection wavelength was set at the reported optimal of 260 nm [9]. Output data from the detector were integrated using an EZChrom chromatographic data system (Scientific Software, San Ramon, CA, USA).

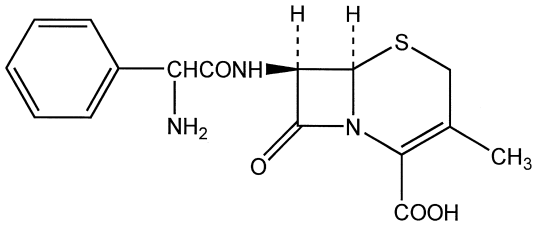

Fig. 1. Chemical structure of cephalexin.

2.3. Animals

Adult male Sprague–Dawley rats (280–320 g) were obtained from the Laboratory Animals Center at The National Yang-Ming University (Taipei, Taiwan). These animals were specifically pathogen-free and were allowed to acclimate in environmentally controlled quarters (24±1°C and 12:12 h light–dark cycle) for at least 5 days before being used for experiments. The rats were initially anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and remained anesthetized throughout the experimental period. The rat's body temperature was maintained at 37°C with a heating pad. The femoral vein was cannulated for drug administration.

2.4. Method validation

All calibration curves of cephalexin (external standards) were constructed prior to the experiments with correlation values of at least 0.995. The intra- and inter-day variabilities of cephalexin were assayed (six replicates) at concentrations of 0.05, 0.1, 0.5, 1, 5 and 10 μg/ml on the same day and on six sequential days, respectively. The accuracy (% bias) was calculated from the nominal concentration (C_{nom}) and the mean value of observed concentration (C_{obs}) as follows: bias (%)=[($C_{obs}-C_{nom}$)/(C_{nom})]·100. The precision relative standard deviation (RSD) was calculated from the observed concentrations as follows: % RSD=[standard deviation (SD)/C_{obs}]·100. Accuracy (% bias) and precision (% RSD)

values of within ±15% covering the range of actual experimental concentrations were considered acceptable [10].

2.5. Blood and brain microdialysis

Blood and brain microdialysis systems consisted of a CMA/100 microinjection pump (CMA) and microdialysis probes. Blood (10 mm in length) and brain (3 mm in length) dialysis probes were made of silica capillary in a concentric design and covered at the tips by dialysis membranes (Spectrum, 150 μm outer diameter with a cut-off at nominal molecular mass of 13 000, Laguna Hills, CA, USA). The blood microdialysis probe was positioned within the jugular vein/right atrium (toward the heart). Following implantation of the blood microdialysis probe, the rat was turned around and mounted on a Kopf stereotaxic frame for the implantation of the brain microdialysis probe in the striatum (coordinates: AP 0.2 mm; ML 3.2 mm; DV 7.0 mm) according to the Paxinos and Watson Atlas [11]. The blood and brain dialysis probes were, respectively, perfused with the anticoagulant ACD solution (3.5 m*M* citric acid; 7.5 m*M* sodium citrate; 13.6 m*M* dextrose) [12] and Ringer's solution (147 m*M* Na^+; 2.2 m*M* Ca^{2+}; 4 m*M* K^+; pH 7.0) at a flow-rate of 1 μl/min. Following a 2 h baseline collection of dialysates, cephalexin (10 mg/kg) was intravenously administered via the femoral vein cannula. Blood dialysates were automatically injected into the microbore chromatographic system by an on-line injector (CMA/160) at 10-min intervals while brain dialysates were collected by a fraction collector (CMA/140) at 10-min intervals. Aliquots of 10 μl were then injected into the chromatographic system for analysis. Body temperature of the animals was maintained at 37°C throughout the experiment with a heating pad. The position of each brain microdialysis probe was verified histologically at the end of the experiments [13,14].

2.6. Recovery of microdialysis probe

For in vivo recovery determinations, the blood and brain microdialysis probes were, respectively, inserted into the rat jugular vein and brain striatum while the animal was under anesthesia with sodium pentobarbital. ACD solution (for blood microdialysis) containing cephalexin (500 or 1000 ng/ml) or Ringer's solution (for brain microdialysis) containing cephalexin (100 or 500 ng/ml) was perfused through the probe at a constant flow-rate (1 μl/min) using the infusion pump. The perfusate (C_{perf}) and dialysate (C_{dial}) concentrations of cephalexin during a 2-h post probe implantation stabilization period were determined by HPLC. The relative loss of cephalexin during retrodialysis L_{retro} or relative recovery (R_{dial}) by dialysis, was then calculated as follows: $[L_{retro}=R_{dial}=(C_{perf}-C_{dial})/C_{perf}]$ [15].

2.7. Pharmacokinetics

Cephalexin concentrations were corrected by in vivo recoveries of the respective microdialysis probes. Pharmacokinetic calculations were performed on each individual set of data using the pharmacokinetic software WinNonlin Standard Edition Version 1.1 (Scientific Consulting, Apex, NC, USA). The areas under the concentration–time curves (AUC_{inf}) were calculated by the linear trapezoid rule up to the last detectable concentration and extrapolated to infinite time using the terminal elimination rate. The area under the first moment curve ($AUMC_{inf}$) was similarly calculated using the concentration vs. time data. Mean residence time (MRT) was calculated as $AUMC_{inf}/AUC_{inf}$.

3. Results and discussion

In order to lower the limit of detection, we investigated the possible effects of using the relatively large volume of injection of 10 μl and found that in both blood and brain dialysates cephalexin was adequately resolved with good peak symmetry using microbore LC (Fig. 2). The retention time of 7.1 min was relatively short. Fig. 2A and B show typical chromatograms, respectively, of standard cephalexin (5 μg/ml) and a blank blood dialysate. Although a number of peaks also appeared in the chromatogram, none of them interfered with the analysis of cephalexin. Fig. 2C shows a chromatogram of a blood dialysate sample containing cephalexin (3.69 μg/ml) collected from rat blood 20 min after cephalexin administration (10 mg/kg, i.v.).

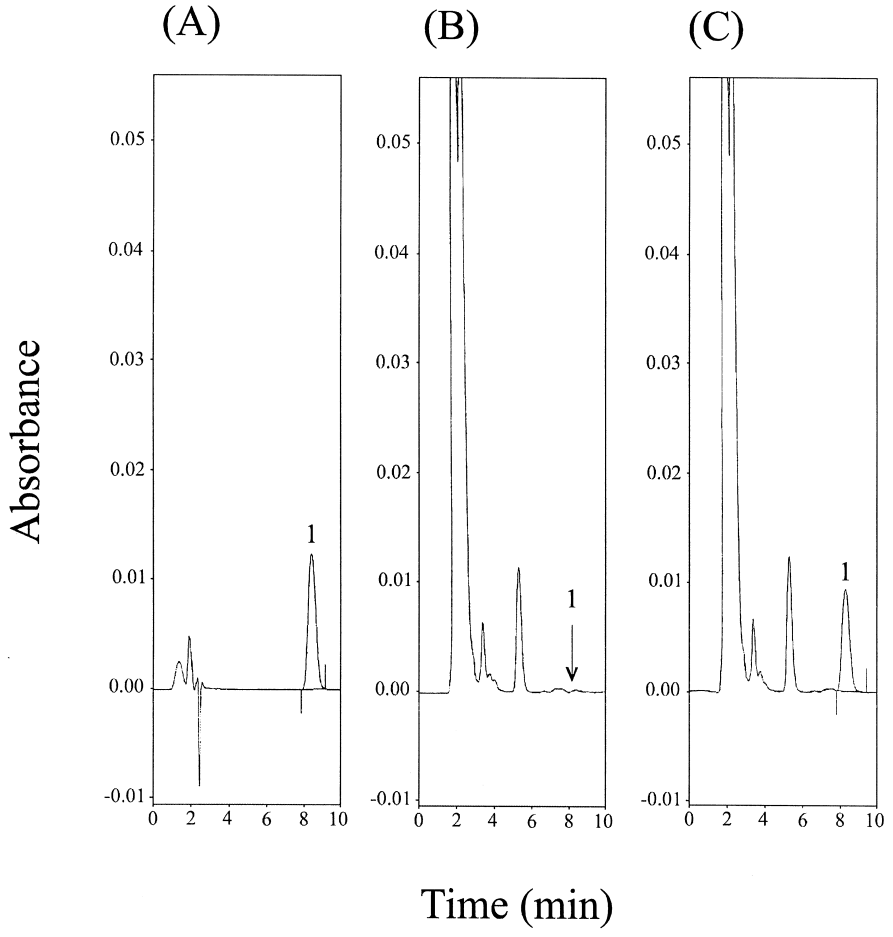

Fig. 2. Typical chromatograms of (A) standard cephalexin (5 μg/ml), (B) a blank blood dialysate from the microdialysis probe prior to drug administration, and (C) a blood dialysate sample containing cephalexin (3.69 μg/ml) collected from a rat blood microdialysate 20 min post cephalexin administration (10 mg/kg, i.v.). 1: Cephalexin.

Fig. 3A and B show typical chromatograms of standard cephalexin (0.5 μg/ml) and a blank brain dialysate. Again none of the observed peaks interfered with the analysis of cephalexin. Fig. 3C shows a chromatogram of a brain dialysate sample containing cephalexin (0.07 μg/ml) collected from the rat brain (striatum) 20 min following cephalexin administration (10 mg/kg, i.v.).

Concentration–response relationship in the present method indicated linearity (r^2>0.995) over a concentration range of 0.01–10 μg/ml for cephalexin. Intra- and inter-day precision and accuracy of cephalexin fell well within the predefined limits of acceptability (<10%). All % bias and % RSD values were within ±10% (Table 1).

Although microdialysis offers certain advantages including relative less invasiveness and non-consumption of biological fluids, it is nonetheless not free of limitations including the exclusion of larger molecules such as proteins and therefore the inability to monitor protein-bound drugs. However, it is generally believed that the unbound fraction is mostly responsible for a drug's actions. Protein binding for cephalexin in human has been reported to be a moderate 12.4% at 37°C and physiological pH of 7.4 [16].

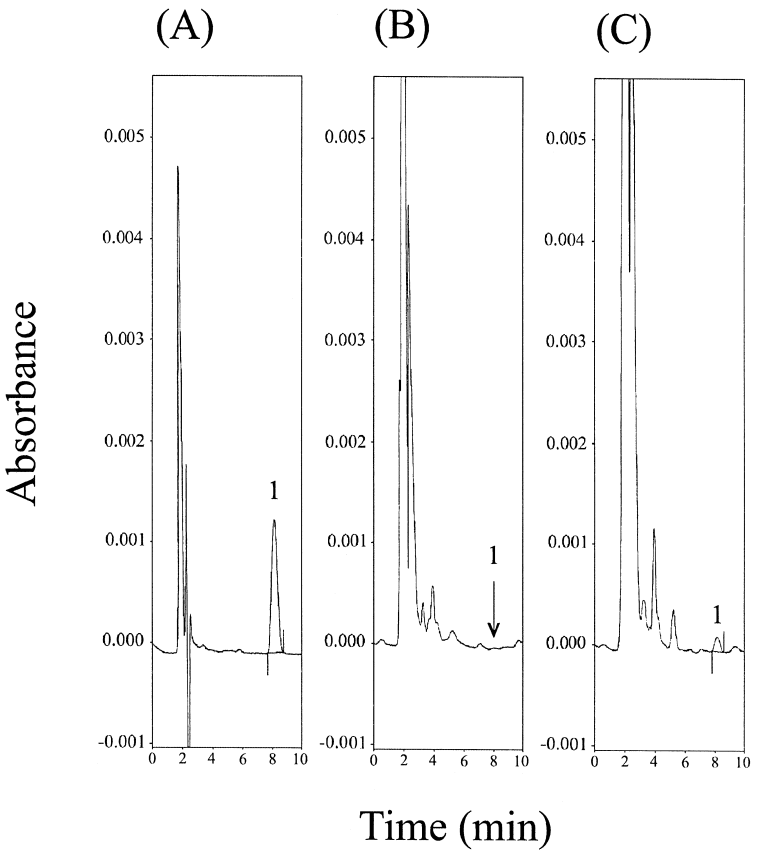

Fig. 3. Typical chromatograms of (A) standard cephalexin (0.5 μg/ml), (B) a blank brain dialysate from the microdialysis probe prior to drug administration, and (C) a brain dialysate sample containing cephalexin (0.07 μg/ml) collected from a rat brain microdialysate 20 min post cephalexin administration (10 mg/kg, i.v.). 1: Cephalexin.

A major concern regarding the application of dialysis and the reliability of data obtained surrounds the dialytic efficiency and reproducibility. Recovery tends to be relatively low, casting doubt on its ability to reflect in vivo changes. In vitro recoveries are usually affected by physical factors such as materials and dimensions of the probe, the perfusion rate, medium viscosity and temperature. Thus the probes need to be calibrated and the physical constants in a study kept constant as much as possible. In in vivo situations additional factors such as body temperature, tortuosity volume fraction may affect the recovery in highly variable manners [17]. In vitro calibration is used for the optimization of the probe production technique and screening for usability and uniformity of the probes while in vivo recoveries are actually used for conversion calculations. However, after having screened several hundred probes, we were satisfied with the production technique and in the present study only concentrated on the more relevant in vivo recovery. The difficulty in accurately calibrating in vivo recovery prompted many workers to simply report treatment-induced changes relative to the basal levels, assuming that the factors that may affect recovery remain essentially constant throughout the course of the experiments. However, for studies that require absolute values such as the present one, dialytic data need to be corrected for in vivo recovery (Table 2). As it is impossible to assess recovery in the actual outside-in direction, in vivo calibration is often done in retrograde fashion. Our results indicated that in vivo recoveries in blood

Table 1
Intra- and inter-day accuracy and precision of the HPLC method for the determination of cephalexin

Nominal concentration (μg/ml)	Observed concentration[a] (μg/ml)	RSD (%)	Accuracy (% bias)
Intra-assay (n=6)			
0.05	055±0.005	8.9	10
0.1	001±0.0036	3.6	1
0.5	0.495±0.008	1.7	−1
1	1.01±0.0063	0.6	1
5	4.97±0.02	0.4	−0.6
10	10.02±0.01	0.1	0.2
Inter-assay (n=6)			
0.05	0.048±0.0039	8.2	−5
0.1	0.098±0.007	7.1	−2
0.5	0.499±0.01	2.0	−0.1
1	1.01±0.0017	1.7	1
5	5.01±0.023	0.5	−0.1
10	10.01±0.011	0.11	1

[a] Observed concentration data are expressed as rounded means±SD.

were higher than in vivo recoveries in brain. On-line microdialysis coupled with microbore LC also proved to be sufficiently sensitive to allow measurement of small amounts of unbound cephalexin in rat blood vessel and brain.

Pharmacokinetics parameters of cephalexin in blood and brain, calculated on a non-compartmental basis, are presented in Table 3. Although the relative proportions of cephalexin in the brain was relatively small, as indicated by the brain–blood AUC ratio of 1.5%, it still suggested blood–brain barrier penetration, which was consistent with some previous findings [18–20]. Fig. 4 shows the measured cephalexin concentrations over time in the blood and brain following cephalexin administration (10 mg/kg, i.v.), the data having been corrected for in vivo recoveries. The mean concentration of cephalexin in the brain increased during the first 10 min and reached the peak concentration at 20 min.

In conclusion, our results indicated that cephalexin rapidly (within 10 min) entered the extracellular fluid of brain striatum following i.v. administration. On-line microdialysis sampling coupled with using microbore LC and ultraviolet detection permitted automated and continuous monitoring requiring no clean-up preparations or consumption of body fluids. Simultaneous sampling provided information regarding blood and brain pharmacokinetics as well as blood–brain barrier penetration. With optimized conditions, this simple and convenient method can be

Table 2
In vivo microdialysis recoveries (%) of cephalexin in rat blood and brain

Concentration (μg/ml)	Recovery[a] (%)
In rat blood	
0.5	28.14±7.82
1	31.96±5.14
In rat brain	
0.1	11.99±3.78
0.5	9.25±1.22

[a] Data are expressed as mean±standard error of the mean (S.E.M.) (n=6).

Table 3
Pharmacokinetic parameters of cephalexin in rat blood and brain following cephalexin administration (10 mg/kg, i.v., n=6)

Parameters	Estimate[a]
Blood	
AUC_{inf} (min μg/l)	1.42±0.21
$AUMC_{inf}$ (min μg/l)	193±66
MRT (min)	117±27
Brain	
AUC_{inf} (min μg/l)	0.021±0.006
$AUMC_{inf}$ (min μg/l)	2.29±1.39
MRT (min)	84±31

[a] Data are expressed as mean±S.E.M. (n=6).

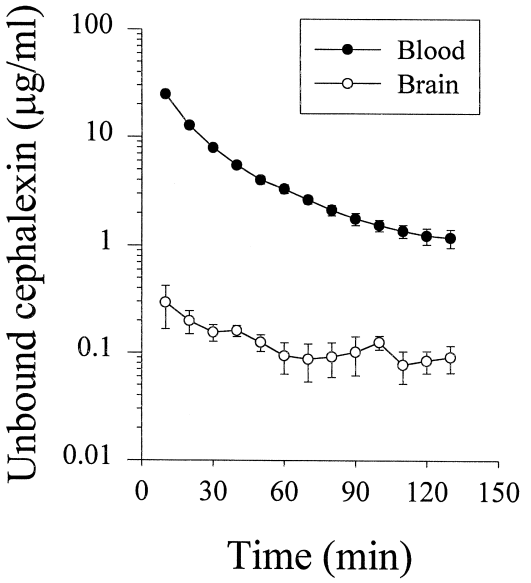

Fig. 4. Unbound cephalexin concentrations in rat blood and brain following 10 mg/kg intravenous administration. Data are presented as the mean±S.E.M. (n=6).

applied to studies of drugs possessing central nervous system penetrability.

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