ELSEVIER

Journal of Chromatography B, 742 (2000) 125–130

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Determination of unbound cephalothin in rat blood by on-line microdialysis and microbore liquid chromatography

Y.L. Chang[a], Y.F. Chen[b], C.F. Chen[b], T.H. Tsai[b,c,*]

[a]Department of Pharmacy, Veterans General Hospital-Taipei, Taipei 112, Taiwan
[b]Department of Pharmacology, National Research Institute of Chinese Medicine, Section 2, 155-1, Li-Nong Street, Shih-Pai, Taipei 112, Taiwan
[c]Institute of Traditional Medicine, National Yang-Ming University, Taipei 112, Taiwan

Received 6 December 1999; received in revised form 15 February 2000; accepted 16 February 2000

Abstract

A method of analysis for the determination of unbound cephalothin in rat blood has been developed. The method was fully automated using an on-line microdialysis procedure. A microdialysis probe was inserted into the jugular vein/right atrium of male Sprague–Dawley rats to examine the unbound cephalothin level in the rat blood after cephalothin administration (50 mg/kg, i.v.). Dialysates were directly input to a liquid chromatographic system using an on-line injector. Samples were eluted with a mobile phase containing methanol–acetonitrile–100 m*M* monosodium phosphate (pH 5.0) (20:20:60, v/v). The UV wavelength was set at 254 nm for monitoring the analyte. Using the retrograde method, at infusion concentrations of 1 and 5 μg/ml of cephalothin, the in vivo microdialysis recoveries were 48.4±4.5% and 52.9±4.7% for the rat blood (n=6). Intra- and inter-assay accuracy and precision of the analyses were≤10% in the range of 0.01 through 10 μg/ml. Pharmacokinetic parameters were calculated from the recovery corrected dialysate concentrations of cephalothin versus time data. The results suggest that the pharmacokinetics of unbound cephalothin in blood fitted best to the two-compartmental model following cephalothin administration (50 mg/kg, i.v.). © 2000 Elsevier Science B.V. All rights reserved.

Keywords: On-line microdialysis; Cephalothin

1. Introduction

Cephalothin, classified as the first-generation cephalosporin, is very effective in severe staphylococcal infections, such as endocarditis. It is not well absorbed orally and is available for parenteral administration [1]. Several liquid chromatographic methods [2–7] and micellar electrokinetic capillary chromatographic method [8] are available for the determination of cephalothin levels in biological fluids with different extractive techniques. For example, protein precipitation with acetonitrile followed by vortex and centrifugation and then injection of the upper aqueous phase directly into the column [7], or involving a plasma protein precipitation with trichloroacetic acid [4] have been developed. These techniques, however, require a more complex procedure for sample clean up. Therefore, in this paper, we use a microdialysis method to exclude the protein-bound compounds out of the microdialysis membrane in order to sample only the protein-un-

*Corresponding author. Fax: +886-2-2826-4276.
E-mail address: thtsai@cma23.nricm.edu.tw (T.H. Tsai)

0378-4347/00/$ – see front matter © 2000 Elsevier Science B.V. All rights reserved.
PII: S0378-4347(00)00148-1

bound fraction of the drug [9,10]. We constructed a blood microdialysis probe and inserted it into the rat's jugular vein for the sampling of cephalothin in biological fluids after cephalothin administration intravenously.

2. Experimental

2.1. Chemicals and reagents

Cephalothin (Fig. 1) was purchased from Sigma (St. Louis, MO, USA). Liquid chromatographic grade solvents and reagents were obtained from E. Merck (Darmstadt, Germany). Triply deionized water (Millipore, Bedford, MA, USA) was used for all preparations.

2.2. Animals

Adult, male Sprague–Dawley rats (280–350 g) were obtained from the Laboratory Animal Center of the National Yang-Ming University (Taipei, Taiwan). These animals were specifically pathogen-free and were allowed to acclimate to their environmentally controlled quarters (24±1°C and 12:12 h light–dark cycle) for at least five days before the experiments began. In the beginning of experiments, the rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). Supplements of sodium pentobarbital were given as needed throughout the experimental period.

2.3. Chromatography

The microbore HPLC system consisted of a chromatographic pump (BAS PM-80, Bioanalytical System, West Lafayette, IN, USA), an on-line injector (CMA/160, Stockholm, Sweden) equipped with a 10 μl sample loop and Dynamax UV/Vis absorbance detector (Walnut Creek, CA, USA). Cephalothin and dialysate were separated using a microbore column (BAS, reversed-phase C_{18}, 150×1 mm I.D.; particle size 5 μm) maintained at ambient temperature. The mobile phase was comprised of methanol–acetonitrile–100 m*M* monosodium phosphate (pH 5.0) (20:20:60, v/v), and the flow-rate of the mobile phase was 0.05 ml/min. The concentration of eluent buffer was referring to the previous reports [3,6]. Following preparation, the buffer was then filtered through a Millipore 0.22 μm filter and degassed prior to use. The UV wavelength was set at 254 nm for detection. The UV detector cell volume is 1.2 μl. Output signal from the HPLC–UV was recorded using an EZChrom chromatographic data system (Scientific Software, San Ramon, CA, USA).

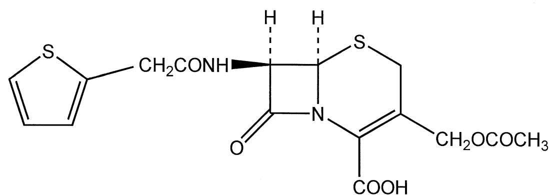

Fig. 1. Chemical structure of cephalothin.

2.4. Method validation

The intra-day and inter-day variabilities for cephalothin were assayed (six replicates) at concentrations of 0.05, 0.1, 0.5, 1, 2, 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 the observed concentration (C_{obs}) as follows: Bias (%) = $[(C_{nom} - C_{obs})/(C_{nom})] \times 100$. The precision (coefficient of variation; C.V.) was calculated from the observed concentrations as follows: % C.V. = [standard deviation (SD)/C_{obs}]×100. Accuracy (% Bias) and precision (% C.V.) values within ±15% covering the actual range of experimental concentrations were considered acceptable [11].

2.5. Microdialysis experiment

The on-line microdialysis system consisted of a microinjection pump (CMA/100) and an on-line injector (CMA/160) [12,13]. Microdialysis probes were made of silica capillary and concentrically designed dialysis membrane (Spectrum, 10 mm length, 150 μm outer diameter with a cut-off at nominal molecular weight of 13000, Laguna Hills, CA, USA). Prior to the experiment, perfusate anticoagulant (Acid Citrate Dextrose; ACD) solution (citric acid 3.5 m*M*; sodium citrate 7.5 m*M*; dextrose 13.6 m*M*) was degassed. Each microdialysis probe was perfused with the degassed ACD solution for at

least 60 min prior to use. A microdialysis probe was inserted into the jugular vein/right atrium (toward the heart) of the anesthetized rat and perfused with ACD solution at a flow-rate of 1 μl/min. The body temperature of the rat was maintained at 37°C with a heating pad. Dialysates were collected at 10 min intervals (each with 10 μl of dialysate) into the on-line injector (CMA/160) and assayed with the microbore LC system [14].

2.6. Recovery of microdialysate

A retrograde calibration technique was used for the assessment of in vivo recovery. The microdialysis probes were inserted into the rat jugular vein under sodium pentobarbital anesthesia. The ACD solution containing cephalothin (1 or 5 μg/ml) was passed through the blood microdialysis probe at a constant flow-rate (1 μl/min) using an infusion pump (CMA/100) into the rat jugular vein. After a 2-h stabilization period subsequent to the probe implantation, the perfusate (C_{perf}) and dialysate (C_{dial}) concentrations of cephalothin were determined by HPLC. The relative loss of cephalothin during retrodialysis (L_{retro}) or relative recovery (R_{dial}) by dialysis, was then calculated as follows [15]: $L_{retro} = R_{dial} = (C_{perf} - C_{dial})/C_{perf}$.

2.7. Pharmacokinetic study

All calibration curves of cephalothin (external standards) were made prior to the experiments with concentration range from 0.05 to 50 μg/ml and with correlation values of at least 0.995. The concentrations of cephalothin in rat blood dialysates were determined from the calibration curves. Following a 2-h period of stabilization, cephalothin (50 mg/kg, i.v.) was administered. Dialysates were injected every 10 min by an on-line injector (CMA/160) for an additional 100 min following cephalothin administration. Microdialysate concentrations of cephalothin (C_m) were converted to unbound concentration (C_u) as follows [15]: $C_u = C_m/R_{dial}$.

Pharmacokinetic analyses of the rat blood concentration–time data were performed on each individual animal set of data by compartmental method. Blood data were fitted to a biexponential decay given by the following formula: $C = A\ e^{-\alpha t} + B\ e^{-\beta t}$. Here A and B are the concentrations for fast and slow disposition phases, respectively. The distribution and elimination rate constants, α and β were calculated using the equation: α or $\beta = (\ln C_2 - \ln C_1)/(t_2 - t_1)$; where C_1 is the value of C at time t_1 and C_2 is the value of C at time t_2. Formation rate constants were calculated by extrapolation of the formation slope determined by the method of residuals. The areas under the concentration curves (AUC) were calculated by the trapezoid method. Half-life ($t_{1/2}$) values were calculated using the equations: $t_{1/2,\alpha} = 0.693/\alpha$ and $t_{1/2,\beta} = 0.693/\beta$ for distribution and elimination half-life, respectively. All data were subsequently processed by the computer program WinNonlin (version 1.1, SCI Software Inc., Apex, NC, USA) for the calculation of pharmacokinetic parameters.

3. Results and discussion

The chromatograms obtained with microbore liquid chromatographic method are shown in Fig. 2. Each analysis was completed within 10 min. Separation of cephalothin from endogenous chemicals in blood dialysate was achieved in an optimal mobile phase containing 60% of 100 m*M* monosodium phosphate (pH 5.0) 20% acetonitrile, and 20% of methanol. Cephalothin retention time was 6.2 min (Fig. 2). Peak areas of cephalothin were linear ($r^2 >$ 0.995) over a concentration range of 0.05–50 μg/ml. Comparison with other liquid chromatographic methods [2–7], the automatic hyphenation of microdialysis and microbore HPLC provides several time-saving advantages in the complex sample extraction procedures and also minimize solvent consumption. To extend the life of the microbore column, flushing it with water and methanol daily at the end of experiment was necessary.

Fig. 2A shows a typical chromatogram of a standard mixture containing cephalothin (5 μg/ml). The chromatogram of the blank sample (Fig. 2B) shows that the chromatographic conditions revealed no biological substances that would significantly interfere with the accurate determination of the drug in rat blood. Fig. 2C depicts a chromatogram of actual unbound cephalothin in rat blood. The dialysate sample contains cephalothin (2.49 μg/ml)

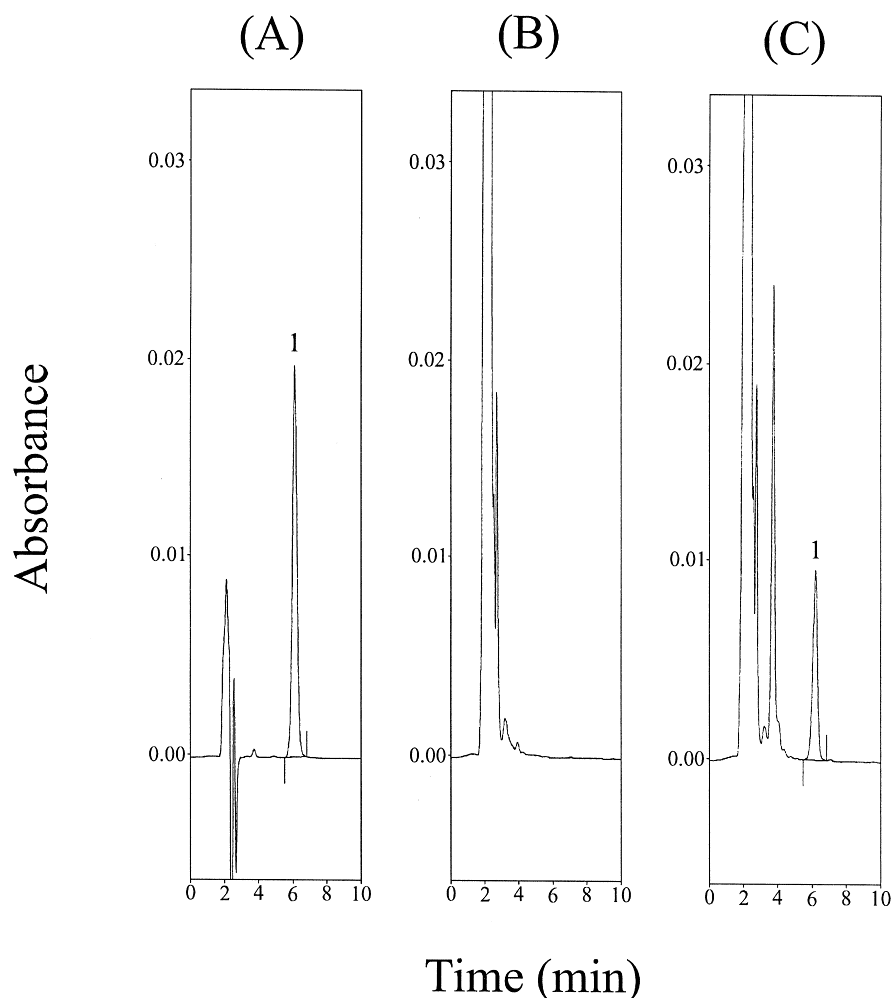

Fig. 2. Typical chromatogram for injection of (A) standard cephalothin (5 μg/ml), (B) blank blood dialysate, and (C) blood dialysate sample containing cephalothin (2.49 μg/ml) collected from jugular vein 20 min after cephalothin administration (50 mg/kg, i.v.). 1: cephalothin.

collected from the jugular vein after 20 min following cephalothin administration (50 mg/kg, i.v.).

Intra-assay and inter-assay (Table 1) accuracy of cephalothin levels are well within the predefined limits of acceptability. All% bias and% C.V. values were within ±10%. This method has quantitative limits of 10 ng/ml for cephalothin. The in vivo recoveries of cephalothin based on 1 and 5 μg/ml are shown in Table 2. It can be seen that this method is sufficiently sensitive to allow measurement of unbound cephalothin in rat blood for pharmacokinetic study.

In order to simplify the analytical steps, we have set-up a method that combines automatic sampling and rapid assay procedure with the microdialysis technical device. To minimize the degradation of cephalothin in the physiological environment, an automatic sampling system and a stable analytical condition are required. Therefore, in the present study we have used an in vivo on-line microdialysis sampling method coupled with microbore HPLC analytical system for the measurement of cephalothin in the rat blood.

The curve of protein unbound cephalothin concentration in blood versus time after the intravenous administration of cephalothin (50 mg/kg) to rats is

Table 1
Intra-assay and inter-assay accuracy of cephalothin

Nominal concentration (μg/ml)	Observed concentration (μg/ml)[a]	C.V. (%)	Accuracy (% Bias)
Intra-assay ($n=6$)			
0.01	0.009±0.0005	5.6	−10
0.05	0.051±0.0014	2.7	2.0
0.1	0.102±0.005	4.9	2.0
0.5	0.497±0.006	1.2	−0.6
1	0.98±0.05	5.1	−2.0
5	5.06±0.13	2.6	1.2
10	9.97±0.06	0.6	−0.3
Inter-assay ($n=6$)			
0.01	0.011±0.001	9.1	10
0.05	0.051±0.002	3.9	2.0
0.1	0.102±0.003	2.8	2.0
0.5	0.512±0.016	3.1	2.5
1	1.01±0.011	1.1	1.0
5	5.04±0.082	1.6	0.8
10	10.05±0.063	0.6	0.5

[a] Observed concentration data are expressed as rounded means±SD ($n=6$).

shown in Fig. 3. These data have been corrected for in vivo recoveries. Based on these microdialysis sampling data, the pharmacokinetics of unbound cephalothin in rat blood was fitted best to a two-compartment model by the computer program WinNonlin resulting in the following equation: $C = 478e^{-0.28t} + 15e^{-0.074t}$. The pharmacokinetic parameters are shown in Table 3. The elimination half-life of unbound cephalothin in rat blood was 16.1±7.2 min.

In summary, we introduced a rapid and sensitive chromatographic method for the determination of cephalothin in rat blood vessel using in vivo microdialysis with microbore HPLC–UV. This method provides an on-line microdialysis system and exhibits no endogenous interference with sufficient sensitivity in blood dialysates. Based on the compartmental pharmacokinetics model, unbound cephalothin in rat blood was fitted best to a two-compartment model.

Table 2
In vivo microdialysis recovery (%) of cephalothin in rat blood

Concentration (μg/ml)	Recovery[a] (%)
1	48.4±4.5
5	52.9±4.7

[a] Data are expressed as means±SD ($n=6$).

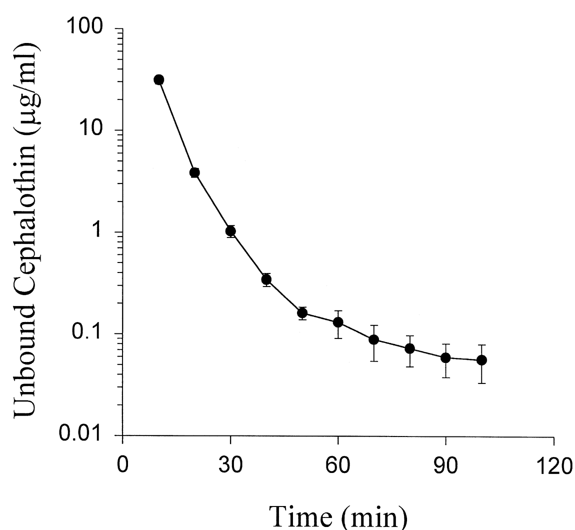

Fig. 3. Mean unbound levels of cephalothin in rat blood after cephalothin administration (50 mg/kg, i.v., $n=6$).

Acknowledgements

This study was supported in part by research grants (NSC89-2113-M-077-004; NSC89-2314-B-

Table 3
Estimated pharmacokinetic parameters following cephalothin administration (50 mg/kg, i.v.)

Parameters	Estimated[a]
A (μg/ml)	478.2±113.7
B (μg/ml)	15.4±5.9
α (1/min)	0.3±0.02
β (1/min)	0.07±0.02
$t_{1/2,\alpha}$ (min)	2.5±0.2
$t_{1/2,\beta}$ (min)	16.1±7.2
AUC (μg min/ml)	1975±298
Vd[b] (ml)	130.8±28.8
Cl[b] (ml/min/kg)	32.2±5.4
AUMC[b] (μg min^2/ml)	7744±757
MRT[b] (min)	4.6±0.5

[a] Data are expressed as means±S.E.M. ($n=6$).
[b] Vd: volume of distribution; Cl: clearance; AUMC: area under the moment versus time curve; MRT: mean residence time.

077-006) from the National Science Council, Taiwan.

References

[1] G. Renzini, G. Ravagnan, B. Oliva, Chemotherapy 21 (1975) 289.
[2] I. Nilsson-Ehle, P. Nilsson-Ehle, J. Infect. Dis. 139 (1979) 712.
[3] V.D. Gupta, K.R. Stewart, J. Pharm. Sci. 69 (1980) 1264.
[4] J.S. Wold, S.A. Turnipseed, Clin. Chim. Acta 78 (1977) 203.
[5] C.G. Pinto, J.L.P. Pavon, B.M. Cordero, Analyst 120 (1995) 53.
[6] R.P. Buhs, T.E. Maxim, N. Allen, T.A. Jacob, F.J. Wolf, J. Chromatogr. 99 (1974) 609.
[7] J.G. Gambertoglio, S.L. Barriere, E.T. Lin, J.E. Conte, Am. J. Med. 75 (1983) 54.
[8] C.J. Sciacchitano, B. Mopper, J.J. Specchio, J. Chromatogr. B 657 (1994) 395.
[9] M.J. Johansen, R.A. Newman, T. Madden, Pharmacotherapy 17 (1997) 464.
[10] M.I. Davies, Anal. Chim. Acta 379 (1999) 227.
[11] R. Causon, J. Chromatogr. B 689 (1997) 175.
[12] T.H. Tsai, F.C. Cheng, L.C. Hung, C.F. Chen, J. Chromatogr. B 720 (1998) 165.
[13] T.H. Tsai, Y.F. Chen, I.F. Chen, C.F. Chen, J. Chromatogr. B 729 (1999) 119.
[14] T.H. Tsai, C.F. Chen, J. Chromatogr. A 762 (1997) 269.
[15] P.A. Evrard, G. Deridder, R.K. Verbeeck, Pharm. Res. 13 (1996) 12.