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Pharmacokinetics of pefloxacin and its interaction with cyclosporin A, a P-glycoprotein modulator, in rat blood, brain and bile, using simultaneous microdialysis

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1 *In vivo* microdialysis with HPLC was used to investigate the pharmacokinetics of pefloxacin and its interaction with cyclosporin A. Microdialysis probes were inserted into the jugular vein/right atrium, the striatum and the bile duct of male Sprague-Dawley rats. Biological fluid sampling thereby allowed the simultaneous determination of pefloxacin levels in blood, brain and bile.

2 Following pefloxacin administration, the brain-to-blood coefficient of distribution was 0.036. This was calculated by dividing the area under the concentration curve (AUC) of pefloxacin in brain by its AUC in blood ($k = AUC_{brain}/AUC_{blood}$).

3 When the P-glycoprotein cyclosporin A ($10\ mg\ kg^{-1}$) was co-administered with pefloxacin ($10\ mg\ kg^{-1}$), the AUC and the mean residence time in rat blood did not differ significantly ($P > 0.05$). Similarly, the pharmacokinetics of pefloxacin in rat brain was not affected by the presence of cyclosporin A.

4 The AUC of unbound pefloxacin in bile was significantly greater than that in blood. The disposition of pefloxacin in rat bile shows a slow elimination phase following a peak concentration 30 min after pefloxacin administration ($10\ mg\ kg^{-1}$, i.v.). The bile-to-blood coefficient of distribution ($k = AUC_{bile}/AUC_{blood}$) was 1.53.

5 The results indicated that pefloxacin was able to penetrate the blood-brain barrier and that the concentration in bile was greater than that in the blood, suggesting active biliary excretion of pefloxacin. Current data obtained from rats show no significant impact of cyclosporin A on the pharmacokinetics of pefloxacin in rat blood and brain when administered by concomitant i.v. bolus.
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Abbreviations: AUC, area under the concentration curve; MDR, multidrug resistance; MIC, minimum ihibitory concentration

Introduction

Pefloxacin, a fluoroquinolone, (classified as 4-quinolone), has a broad spectrum of activity against Gram-negative and Gram-positive bacteria. Pefloxacin also demonstrates favourable cellular penetration characteristics, yielding high tissue/serum ratios. This has obvious implications for the treatment of infections caused by intracellular pathogens (Hooper & Wolfson, 1991). Most *in vitro* and *in vivo* studies have examined the relationship between minimum inhibitory concentration (MIC) and *in vivo* efficacy when testing the effect of antimicrobial agents (Frimodt-Moller *et al*., 1986; Frimodt-Moller & Thomsen, 1987). These studies have generated contradictory results as the *in vivo* concentration of antimicrobial agents relates both to protein-bound and protein-unbound forms. The protein-bound form of an antimicrobial agent cannot exert its antimicrobial effect.

With respect to the pharmacokinetic profile of quinolones, the serum protein binding capacity of pefloxacin is in the range of 20–30% (Montay *et al*., 1984; Lode *et al*., 1990). Binding of quinolones to bovine serum albumin has been studied using equilibrium dialysis with moderate binding, (40–60%) reported (Mrhar *et al*., 1991). To determine the pharmacokinetics of the protein-unbound form of an antimicrobial agent in the body, we employed *in vivo* microdialysis techniques to obtain the protein-free pefloxacin from simultaneously derived rat blood, brain and bile samples (Davies, 1999; de Lange *et al*., 1997).

Microdialysis sampling techniques were originally developed to allow *in vivo* sampling of neurotransmitters released in the brain (Tossman & Ungerstedt, 1986; Zetterstrom *et al*., 1983) Techniques have subsequently been extended to encompass pharmacological and pharmacokinetic studies (Stenken *et al*., 1997; Johansen *et al*., 1997). Over the past several years, microdialysis has been increasingly used, in various animal experiments, for the *in vivo* sampling of unbound endogenous or exogenous compounds present in blood, brain or tissue etc. (Scott *et al*., 1991; Stahle, 1991). Sampling by this technique involves continuous perfusion of fluid through microdialysis probes implanted in the appropriate tissue space.

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It has been reported that pefloxacin concentrations in brain are significantly lower than those observed in plasma (Ooie *et al*., 1997; Okezaki *et al*., 1988). Thus, the brain distribution of pefloxacin seems to be restricted. P-glycoprotein is encoded by the MDR gene (multidrug resistance) and is located in the luminal membrane of brain capillary endothelial cells (Jette *et al*., 1995). It contributes to the function of the blood-brain barrier (BBB) (Schinkel *et al*., 1996). The P-glycoprotein modulator could reverse the drug resistance in chemotherapy, altering either the pharmacokinetics or the BBB penetration of simultaneously administered drugs. Therefore, it is of particular interest to study the effects of P-glycoprotein inhibitors on pefloxacin distribution. Although several P-glycoprotein inhibitors have been described (e.g., cyclosporin A, verapamil, quinine, nifedipine, etc.), in consideration of the minimal acute peripheral cardiovascular side effects attributable to cyclosporin A, it was decided to use cyclosporin A in this study. In clinical application, it is possible to combine pefloxacin and cyclosporin A in the treatment of organ transplant patients and therefore the interaction of these two drugs merits investigation. Recent research indicates that P-glycoprotein may act as a door-keeper at the BBB (Jette *et al*., 1995; Schinkel *et al*., 1996). Very few studies have been published concerning the effects of P-glycoprotein inhibitor drugs on the pharmacokinetics of pefloxacin. Therefore, the pharmacokinetics of pefloxacin, both alone and in combination with cyclosporin A in rats, as determined by a microdialysis on-line system, was evaluated in this study.

In elimination, pefloxacin is mainly excreted *via* the bile duct and it undergoes enterohepatic circulation (Neuman, 1988). Biliary excretion of pefloxacin, mainly as a glucuronide conjugate of the drug, occurs and is extensive in rats and dogs. In rat and human bile, the main active compound is unchanged pefloxacin (Montay *et al*., 1984). To investigate the biliary excretion of pefloxacin, we designed a flow-through microdialysis probe (Scott & Lunte 1993; Hadwiger *et al*., 1994; Tsai *et al*., 1999c) and inserted the probe into the rat bile duct for the sampling of pefloxacin from biliary fluid following intravenous pefloxacin administration.

Methods

Chemicals and reagents

Pefloxacin mesylate and Cyclosporin A (Sandimmun) were purchased from the Phone-Poulenc Rorer Group (Neuilly-Sur-Seine, France) and Bristol-Myers Squibb (Sermoneta Latina, Italy), respectively. Liquid chromatographic grade solvents and reagents were obtained from E. Merck (Darmstadt, Germany). Triple deionized water (Millipore, Bedford, MA, U.S.A.) was used for all preparations.

Animals

All experimental protocols involving animals were reviewed and approved by the institutional animal experimentation committee of the National Research Institute of Chinese Medicine. Male specific pathogen-free Sprague-Dawley rats were obtained from the Laboratory Animal Center of the National Yang-Ming University, Taipei. Animals had free access to food (Laboratory rodent diet #5P14, PMI Feeds Inc., Richmond, IN, U.S.A.) and water until 18 h prior to experimentation, at which time only food was removed. The rats were initially anaesthetized with sodium pentobarbitone (50 mg kg^{-1}, i.p.), and remained anaesthetized throughout the experimental period. The femoral vein was exposed for further drug administration. During the experiment, rat body temperature was maintained at 37°C using a heating pad.

Chromatography

The chromatographic system consisted of a chromatographic pump (BAS PM-80, West Lafayette, IN, U.S.A.), an on-line injector (CMA/160, Stockholm, Sweden) equipped with a 20 μl sample loop and a fluorescence detector (Waters 474 scanning fluorescence detector, Milford, MA, U.S.A.). Samples obtained were separated using a reverse-phase C18 column (150×4.6 mm; 5 μm; Cosmosil, Kyoto, Japan). Chromatography was performed at ambient temperature.

The mobile phase consisted of citrate buffer (pH 5.0), acetonitrile and triethylamine (83 : 17 : 0.1, v/v/v). One litre of citrate buffer, (25 mM), was prepared using 25 mM of sodium acetate and 25 mM of citric acid dissolved in triple distilled HPLC grade water and buffered to pH 5.0 using orthophosphoric acid. This mobile phase was filtered with a 0.45 μm Millipore membrane prior to being used for elution. Chromatographic pump flow rate was set at 1 ml min^{-1}. The excitation and emission wavelengths for optimal fluorescence response for pefloxacin were determined to be 330 and 440 nm, respectively. The output signal from the HPLC-fluorescence detector was recorded using an EZChrom chromatographic data system (Scientific Software, San Ramon, CA, U.S.A.).

Microdialysis experiment

Blood, brain and bile microdialysis systems consisted of a CMA/100 microinjection pump (CMA, Stockholm, Sweden) and the appropriate microdialysis probes. The dialysis probes for blood (10 mm in length), brain (3 mm in length) and bile (7 cm in length; Figure 1) were made of silica glass capillary tubing arranged in a concentric design (Tsai *et al*., 1999c; 2000a,b). Their tips were covered by dialysis membrane (150 μm outer diameter with a nominal molecular weight cut-off of 13,000, Spectrum Co, Laguna Hills, CA, U.S.A.) and all unions were cemented with epoxy. To allow adequate time for the epoxy to dry, the probes were made at least 24 h prior to use. The blood microdialysis probe was positioned within the jugular vein/right atrium and then perfused with ACD solution (citric acid 3.5 mM; sodium citrate 7.5 mM; dextrose 13.6 mM) at a flow rate of 2 μl min^{-1} using the CMA microinjection pump (Tsai *et al*., 1999a). The bile duct microdialysis probes were constructed in house, based on the design originally described by Scott & Lunte (1993) and Hadwiger *et al*. (1994). This bile microdialysis method has been reported in our previous studies (Tsai *et al*., 1999b,c). For brain microdialysis, the rat was mounted on a stereotaxic frame and perfused with Ringer's solution (147 mM Na^{+}; 2.2 mM Ca^{2+}; 4 mM K^{+}; pH 7.0). After being washed with Ringer's solution at a flow-rate of 2 μl min^{-1}, the microdialysis probe was implanted in the right striatum (coordi-

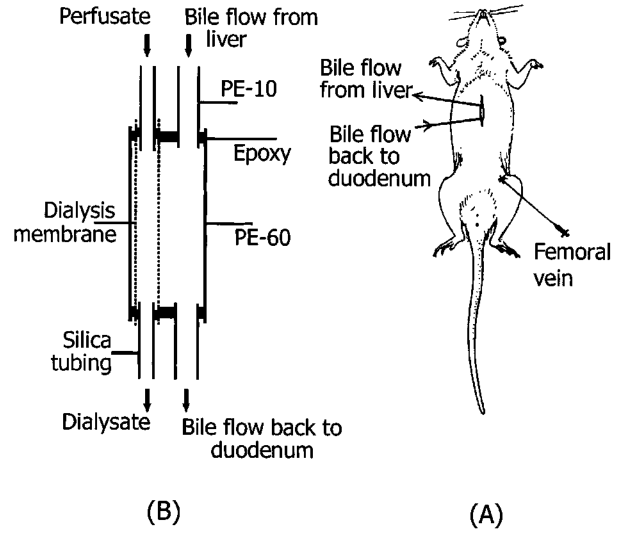

Figure 1 (A) Animal model for bile sampling, flow-through microdialysis probe used for rat bile sampling. (B) Detailed description of a homemade bile microdialysis probe.

nates: AP 0.4 mm, ML −3.0 mm, DV −7.0 mm) according to the Paxinos & Watson (1986) atlas. The positions of the probes were verified by standard histological procedure at the end of experiments. This method has been previously reported (Tsai *et al.*, 1999a,d).

Drug administration

After a 2 h post-implantation period, an intravenous dose of drug was administered *via* the femoral vein. Cyclosporin A, 10 mg ml^{-1}, was produced by diluting cyclosporin A injectable solution with a 5% dextrose/water solution. Pefloxacin, 10 mg kg^{-1} ($n=6$) was administered intravenously to control group rats. For the cyclosporin A treated group ($n=6$), cyclosporin A, 10 mg kg^{-1}, was injected *via* femoral vein 10 min prior to pefloxacin; also 10 mg kg^{-1} injection. The total volume of each injection was 1 ml kg^{-1}. The blood, brain and bile dialysates were connected to an on-line injector (CMA 160) and a fraction collector (CMA/140). The sampling interval was 10 min for each probe. Blood, brain and bile dialysates were measured by HPLC on the same day as the experiment.

Recovery of microdialysate

For *in vivo* recovery, the blood, brain and bile microdialysis probes were inserted into the jugular vein, striatum and bile duct under anaesthesia with sodium pentobarbitone. Ringer's solution containing pefloxacin (1 μg ml^{-1}) was passed through the microdialysis probe at a constant flow rate (2 μl min^{-1}) using an infusion pump (CMA/100). Two hours after probe implantation, the perfusate (C_{perf}) and dialysate (C_{dial}) concentrations of pefloxacin were determined by HPLC. The relative recovery (R_{dial}), *in vivo*, of pefloxacin across the microdialysis probe inserted in the bile duct was calculated according to the following equation, $R_{\mathrm{dial}}=(C_{\mathrm{perf}}-C_{\mathrm{dial}})/C_{\mathrm{perf}}$, (Evrard *et al.*, 1996).

Pharmacokinetic application

Pefloxacin microdialysate concentrations (C_m) were converted to unbound concentration (C_u) as follows: $C_u=C_m/R_{\mathrm{dial}}$, (Evrard *et al.*, 1996). Pharmacokinetic calculations were performed on each individual set of data using the pharmacokinetic calculation software WinNonlin Standard Edition Version 1.1 (Scientific Consulting Inc., Apex, NC, U.S.A.) by noncompartmental method. The area under the concentration-time curve (AUC), the area under the first moment curve (AUMC) and the mean residence time (MRT) were calculated using statistical moments (Gabrielsson & Weiner, 1994). Formation rate constants were calculated from the extrapolated formation slope determined by the method of residuals. The AUCs from time zero to time infinity were calculated by the trapezoidal rule and extrapolated to time infinity by the addition of $\mathrm{AUC}_{t-\mathrm{inf}}$. The AUC values were thus given by the sum of the products of the measured concentrations and the collection time interval, plus the residual area, that is: $\mathrm{AUC}=\mathrm{AUC}_{0-t}+\mathrm{AUC}_{t-\mathrm{inf}}$. An analogous method was used for the calculation of the AUMC by using the concentration *vs* time data. MRT was calculated as AUMC/AUC.

The differences in pharmacokinetic data between the control and treated groups was determined by Students *t*-test with significance set at $P<0.05$.

Results

Typical chromatograms of standard containing pefloxacin are shown in Figure 2. Separation of pefloxacin from endogenous chemicals in blood dialysate was achieved in an optimal mobile phase containing 83% of 25 mM citrate buffer (pH 5.0), 0.1% triethylamine and 17% of acetonitrile. Retention time of pefloxacin was 5.8 min. The calibration curve of pefloxacin was obtained prior to LC analysis of dialysates over concentration ranges of 0.05–10 μg ml^{-1}. This chromatographic system has been validated for both inter- and intra-day accuracy and the determined limits of this precision assay deemed acceptable. The chromatograms of a blank blood, brain and bile dialysate indicate that none of the observed peaks interfered with the analysate.

Figure 2A shows a standard injection of pefloxacin (1 μg ml^{-1}), whereas Figure 2B shows the chromatogram of a rat blood dialysate sample containing pefloxacin (0.74 μg ml^{-1}) collected 60 min after pefloxacin administration (10 mg kg^{-1}, i.v.). Figure 2C shows the chromatogram of a striatal dialysate sample containing pefloxacin (0.036 μg ml^{-1}). This sample was collected 20 min after pefloxacin administration (10 mg kg^{-1}, i.v.). Figure 2D shows a chromatogram of bile dialysate sample obtained 60 min after pefloxacin administration (10 mg kg^{-1}, i.v.). To optimize the pefloxacin separation from the dialysate of bile fluid, the acetonitrile concentration and the pH value of the buffer solution were modified from the conditions described in a previous report (Abanmi *et al.*, 1996).

In vivo recovery of pefloxacin in blood (1 μg ml^{-1}) was 41.6±2.6% ($n=6$), in brain (0.1 μg ml^{-1}) was 14.7±3.1% ($n=6$) and in bile (1 μg ml^{-1}) was 86.4±1.6% ($n=6$). The *in vivo* recovery (or dialysis efficiency) can be affected by

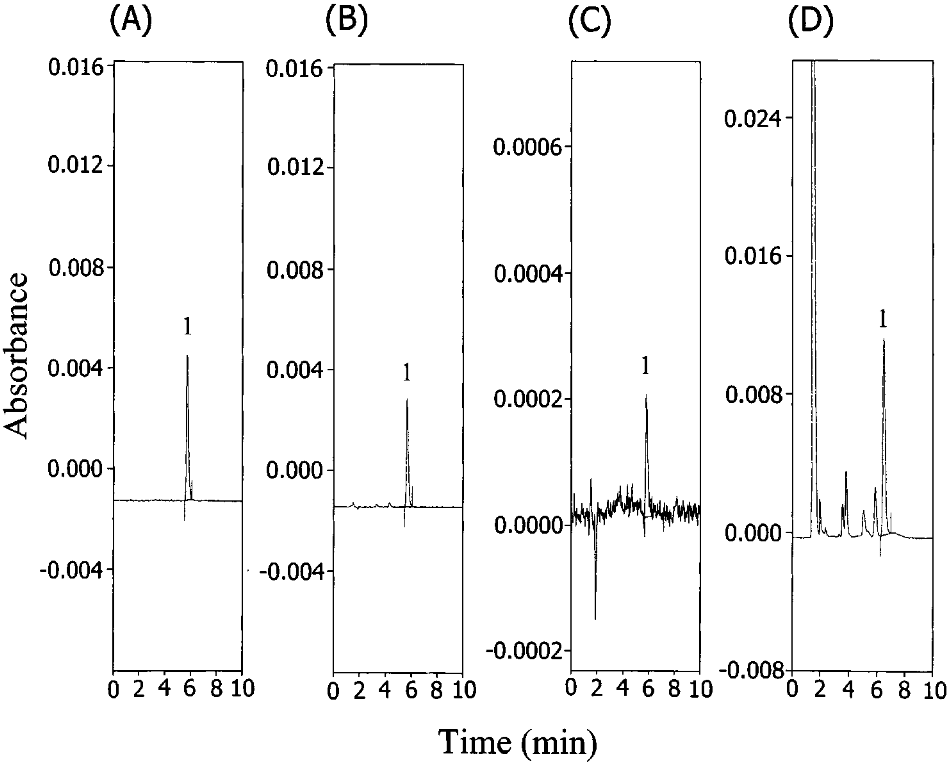

Figure 2 Typical chromatograms of (A) standard pefloxacin (1 μg ml^{-1}). (B) Blood dialysate sample containing pefloxacin (0.74 μg ml^{-1}) collected from the rat blood microdialysate 60 min after pefloxacin administration (10 mg kg^{-1}, i.v.). (C) Brain dialysate sample containing pefloxacin (0.036 μg ml^{-1}) collected from the rat brain microdialysate 20 min after pefloxacin administration (10 mg kg^{-1}, i.v.). (D) Bile dialysate sample containing pefloxacin (2.03 μg ml^{-1}) collected from a rat bile microdialysate 60 min after pefloxacin administration (10 mg kg^{-1}, i.v.). 1: pefloxacin.

certain factors, mostly physical in nature, such as temperature and perfusion rate. Also the materials used in the construction of the probe and the final dimensions of the probe can affect dialysis efficiency. Thus, each probe must be calibrated prior to use and all physical components must be kept constant.

The concentration versus time curve of pefloxacin in blood and brain are shown in Figures 3 and 4, respectively. The pharmacokinetic profiles indicate that cyclosporin A treated animals did not show significant changes in the pharmacokinetics of pefloxacin in blood and brain (Table 1). The result indicates that a lower concentration of pefloxacin penetrates BBB.

The pharmacokinetic profiles of unbound pefloxacin in rat blood, brain and bile in both control and cyclosporin A treated groups are presented in Table 1. The AUC of pefloxacin in bile, brain and blood were 544.1±23.4 min μg ml^{-1}, 12.7±2.7 min μg ml^{-1} and 356.1±32.8 min μg ml^{-1}, respectively. The mean residence time of pefloxacin in bile is significantly greater than that in blood and brain. The average concentration of pefloxacin in the bile increased during the first 30 min following drug administration. The amount of pefloxacin, as estimated from the AUC, in bile set against the concentration gradient was significantly greater than that in blood, suggesting that pefloxacin might be actively excreted into the bile.

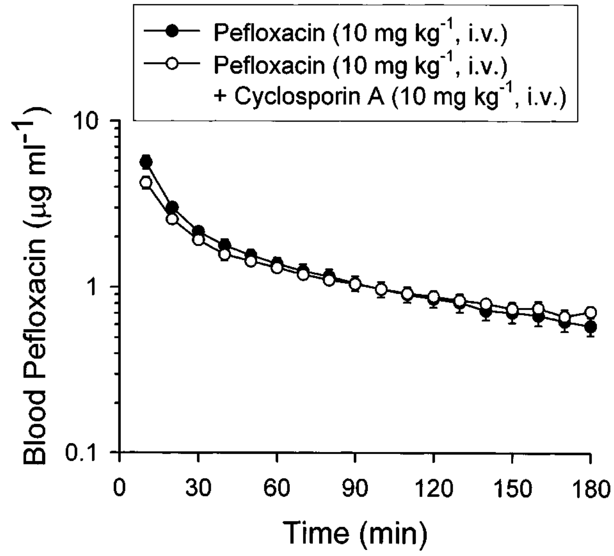

Figure 3 Mean unbound levels of pefloxacin in rat blood after pefloxacin (10 mg kg^{-1}, i.v.) administration and co-administration of pefloxacin (10 mg kg^{-1}, i.v.) and cyclosporin A (10 mg kg^{-1}, i.v.) ($n=6$). Data are presented as mean±s.e.mean.

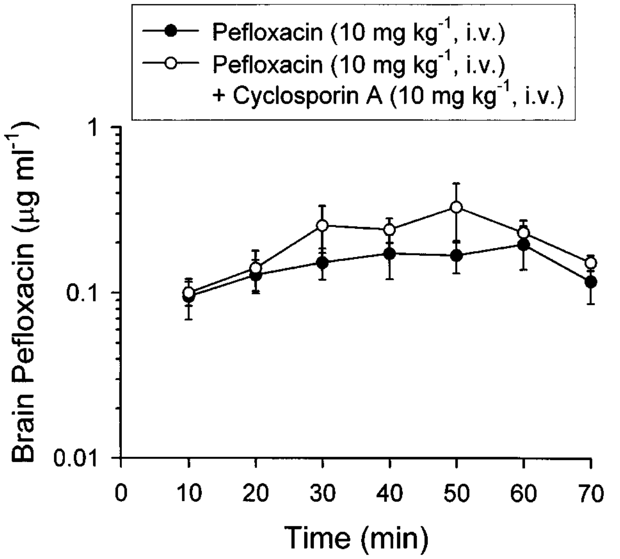

Figure 4 Mean unbound levels of pefloxacin in rat brain after pefloxacin (10 mg kg^{-1}, i.v.) administration and co-administration of pefloxacin (10 mg kg^{-1}, i.v.) and cyclosporin A (10 mg kg^{-1}, i.v.) ($n=6$). Data are presented as mean ± s.e.mean.

Table 1 Pharmacokinetic parameters of the control group, pefloxacin administration (10 mg kg^{-1}, i.v.) and the treated group, cyclosporin A 10 mg kg^{-1} was injected *via* femoral vein 10 min prior to pefloxacin 10 mg kg^{-1} injection

Parameters	*Control group*	*Treated group*
Blood		
AUC (min μg ml^{-1})	356.1 ± 32.8	394 ± 44.5
MRT (min)	92.4 ± 6.9	128.3 ± 30.4
Brain		
AUC (min μg mlμg ml^{-1})	12.7 ± 2.7*	14.0 ± 2.6
MRT (min)	55.4 ± 6.4*	51.7 ± 9.3
Bile		
AUC (min μg ml^{-1})	544.1 ± 23.4*	
MRT (min)	136.8 ± 13.2*	
AUC_{brain}/AUC_{blood}	0.036	0.036
AUC_{bile}/AUC_{blood}	1.53	

Data are expressed as mean ± s.e.mean ($n=6$). Significant difference from the group of blood values, $^{*}P<0.05$.

Discussion

The brain penetration of pefloxacin, defined as the brain-to-blood coefficient of distribution (k value), was calculated by dividing the pefloxacin AUC in brain by the pefloxacin AUC in blood ($k = AUC_{brain}/AUC_{blood}$) (de Lange *et al.*, 1997). The k value (Table 1) of unbound pefloxacin (10 mg kg^{-1}, i.v.) alone is 0.036 and that of the cyclosporin A (10 mg kg^{-1}, i.v.) treated group is 0.036. Ooie *et al.* (1996) reported that the concentration ratio of steady-state cerebrospinal fluid (CSF) to unbound serum was about 1.0%. Steady-state brain interstitial fluid concentrations of the quinolones were 7–30 times lower than the unbound serum concentrations due to restricted distribution in the brain. The steady state brain-to-unbound serum concentration ratio showed an 11 fold difference after intravenous infusion (Ooie *et al.*, 1997). The results of the present experiments suggest that pefloxacin may penetrate the BBB, in agreement with the earlier observations of Ooie and colleagues (Ooie *et al.*, 1996; 1997). In addition, these pharmacokinetic profiles indicate that the pharmacokinetics of pefloxacin in rat blood and brain were not altered by cyclosporin A treatment. This result is in agreement with that of Janknegt (1990), who found a pharmacodynamic interaction between pefloxacin and cyclosporin A, but observed no pharmacokinetic interaction.

Several techniques have been employed for the study of drug transport to the brain (Pardridge, 1998). The brain tissue homogenate technique is one of the traditional methods for determining drug distribution in the brain. However, only single concentration-time points can be provided in such studies, as the experimental animals need to be sacrificed to obtain samples. Thus to obtain a complete brain drug concentration-time profile, it is necessary to use many animals and inter-animal variation often compromises the accuracy of the results. Although possessing the advantage of not being invasive, modern methods such as positron emission topography (PET) and nuclear magnetic resonance (NMR) require expensive materials as well as highly sophisticated and expensive equipment not commonly available in a general laboratory. On the other hand, microdialysis is relatively inexpensive and reproducible. Probes can be constructed in the laboratory reducing expense and allowing the necessary customization for specialized needs sampling in biological fluids. By taking certain precautions, such as allowing time for the subject to stabilize after the surgical implantation of the probe and for partial recovery from other surgical traumas, any disadvantages of this procedure can be minimized. The dialysates can be analysed using any appropriate analytical methods. However, the sensitivity of the chosen method dictates the amount of dialysate required and therefore the sampling time. High sensitivity is required in the analytical apparatus to keep sampling time to a minimum. As the values expressed for each collection period actually represent mean values over the dialysate sample collection time, it is disadvantageous to have a long interval between samples. In this case, HPLC was relatively accessible and the analytical method was optimized so that only 10 min of collection time was required.

The AUC of pefloxacin in bile was 544.1 ± 23.4 min μg ml^{-1} (mean ± s.e.mean, $n=6$). The bile-to-blood coefficient of distribution ($K = AUC_{bile}/AUC_{blood}$) was 1.53. The pharmacokinetic results show that the disposition of pefloxacin in bile appeared to be in a slow elimination phase and then reached a peak concentration 30 min after pefloxacin administration (10 mg kg^{-1}, i.v.). The AUC profiles indicated significantly higher levels of pefloxacin in the bile than in the blood for the same time periods. A bile-to-blood concentration ratio greater than unity indicates that the compound is concentrated in the bile by an active transport mechanism or by the formation of micelles in the bile (Scott & Lunte, 1993). Active biliary secretion and recirculation in the enterohepatic loop would be compatible with the significantly longer elimination half-life in the bile compared with that in blood.

Pharmacokinetic studies indicate that quinolone antibiotics diffuse rapidly in extravascular fluid, saliva, urine, kidney, prostate, bile and peritoneal fluid (Bergan, 1989; Montay *et al.*, 1984). Montay *et al.* (1984) reported that protein binding of pefloxacin was weak, about 20% in plasma and the concentrations of pefloxacin in bile were higher than those in plasma. The current protein unbound pharmacokinetic data are also in good agreement with those of Neuman (1987), who found high peak biliary concentrations of pefloxacin, markedly excreted from bile fluid for the treatment of acute cholecystitis patient (Neuman, 1987).

The microdialysis technique provides protein-free samples that can be directly injected into a liquid chromatographic system for continuous *in vivo* monitoring of unbound drugs in blood, other biological fluids and tissues (Davies, 1999; de Lange *et al*., 1997). Compared with other assay methods, where drugs are obtained from biological samples by extraction or protein precipitation (Abanmi *et al*., 1996), microdialysis offers many advantages. Microdialysis allows the continuous monitoring of analyte concentrations in the extracellular compartment of the same animal over a period of time, there is less biological fluid loss and therefore, minimal stress on haemodynamics (de Lange *et al*., 1997).

Other methods for measuring drug concentration in the bile fluid have been described using liquid–liquid extraction of bile fluid collection (Vielnascher *et al*., 1997). However, such samples may have to go through a relatively complicated process of sample clean up before they can be analysed. By applying the microdialysis technique to a biliary excretion study, the number of animals needed can be substantially reduced as the technique involves a sampling procedure which does not incur large body fluid losses and therefore does not disturb blood homeostasis in the subject.

In conclusion, we have developed a specific, rapid and cost-saving HPLC fluorescence method for the determination of protein-unbound pefloxacin in rat blood, brain and bile. This method exhibits no endogenous interference and its sensitivity is sufficient for the determination of biological samples. Current data obtained from rats show no significant impact of cyclosporin A on the pharmacokinetics of pefloxacin in rat blood and brain when they are concomitantly injected. Results using such techniques confirm that pefloxacin is excreted in the bile.

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