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Pharmacokinetic study of levofloxacin in rat blood and bile by microdialysis and high-performance liquid chromatography

F.C. Cheng[a], T.R. Tsai[b], Y.F. Chen[c], L.C. Hung[c], T.H. Tsai[c,d,*]

[a]*Department of Medical Research, Taichung Veterans General Hospital, Taichung 407, Taiwan*
[b]*School of Pharmacy, Kaohsiung Medical University, Kaohsiung 807, Taiwan*
[c]*National Research Institute of Chinese Medicine, 155-1, Li-Nong Street, Section 2, Shih-Pai, Taipei 112, Taiwan*
[d]*Institute of Traditional Medicine, National Yang-Ming University, Taipei 112, Taiwan*

Abstract

The aim of this study was to develop a rapid and sensitive method for the simultaneous determination of unbound levofloxacin in rat blood and bile using high-performance liquid chromatography coupled with microdialysis for further pharmacokinetic study. Microdialysis probes were simultaneously inserted into the jugular vein toward the right atrium and the bile duct of male Sprague–Dawley rats for biological fluid sampling after administration of levofloxacin 3 mg/kg through the femoral vein. Levofloxacin and dialysates were separated using a Merck LiChrospher reversed-phase C_{18} column maintained at ambient temperature. The mobile phase was comprised of acetonitrile–1 m*M* 1-octanesulfonic acid (40:60, v/v, pH 3.0 adjusted with orthophosphoric acid). The fluorescence response for levofloxacin was observed at excitation and emission wavelengths of 292 and 494 nm, respectively. The detection limit of levofloxacin was 50 ng/ml. Intra-day and inter-day precision and accuracy of levofloxacin measurements fell well within the predefined limits of acceptability. The disposition of levofloxacin in the blood and bile fluid suggests that there was rapid exchange and equilibration between the blood and hepatobiliary systems, and the plasma level of levofloxacin was greater than that of the bile. Thus, levofloxacin undergoes hepatobiliary excretion but might not be related to the P-glycoprotein transport system.

Keywords: Microdialysis; Levofloxacin; Fluoroquinones; Ofloxacin

1. Introduction

Levofloxacin, an effective new respiratory fluoroquinone, is separated from the racemic compound ofloxacin, and is the pure *S*-(−) isomer. Levofloxacin possesses a wide spectrum of bactericidal activity against both Gram-positive and Gram-negative bacteria, as well as atypical pathogens such as *Mycoplasma*, *Chlamydia* and *Legionella* [1,2]. The bactericidal effect of levofloxacin is achieved through reversible binding to DNA gyrase and subsequent inhibition of bacterial DNA replication and transcription [3]. Levofloxacin is significantly more active against bacterial pathogens than *R*-(+)-ofloxacin. Therefore, investigation of the pharmacokinetic characteristics of levofloxacin is important for the elucidation of dosage regimens. Recently, a capillary electrokinetic chromatographic technique was used for the enantioseparation of ofloxacin, enabling detection of *R*-(+)-ofloxacin at 2 μg/ml in urine without sample clean-up [4]. Several high-performance liquid chromatography (HPLC) assays have

*Corresponding author. Tel.: +886-2-2820-1999; fax: +886-2-2826-4276.
E-mail address: thtsai@cma23.nricm.edu.tw (T.H. Tsai).

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been reported for the determination of levofloxacin in biological fluids [5–8]. These assays involved the use of one-step protein precipitation extraction [5] and single-step liquid–liquid extraction [6] followed by UV detection. Solid-phase extraction followed by fluorescence detection has also been reported for the determination of levofloxacin in biological fluids [9,10].

The general pharmacokinetics of levofloxacin has previously been discussed [11]. Levofloxacin is widely distributed throughout the body, and penetrates well into most body tissues and fluids. Drug concentrations in tissues and fluids are generally greater than those observed in plasma, but penetration into the cerebrospinal fluid is relatively poor. Levofloxacin is approximately 24–38% bound to serum plasma proteins (primarily albumin); serum protein binding is independent of serum drug concentrations. Approximately 80% of levofloxacin is eliminated as unchanged drug in the urine through glomerular filtration and tubular secretion [2].

Despite numerous studies describing the pharmacokinetics of levofloxacin, no researchers have described the simultaneous determination of the pharmacokinetics of levofloxacin in rat blood and bile employing microdialysis. The LC-based approaches reported to date all result in the measurement of total drug concentration rather than the free fraction. In the present study, we measured protein-unbound levofloxacin in rat blood and bile by inserting two microdialysis probes into the blood and bile of rats for simultaneous biological fluid sampling and analysis using HPLC. This method disturbs the hemodynamics and physiological processes of the experimental animal minimally and provides the basis for the construction of pharmacokinetic profiles and analyses.

2. Experimental

2.1. Chemicals and reagents

Levofloxacin was purchased from Ortho-McNeil Pharmaceutical (Raritan, NJ, USA). Cyclosporin A (Sandimmun) was obtained from Novartis Pharma (Basle, Switzerland). 1-Octanesulfonic acid and orthophosphoric acid were purchased from Sigma (St Louis, MO, USA), and E. Merck (Darmstadt, Germany), respectively. Chromatographic 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 liquid chromatographic system was comprised of a chromatographic pump (Waters 510, Bedford, MA, USA), an injector (Rheodyne 7125, Cotati, CA, USA) equipped with a 20-μl sample loop, and a fluorescence detector (Jasco FP-920, Hachioju City, Japan). Dialysates were separated with a C_{18} column (Merck LiChrospher, 250×4.6 mm I.D.; particle size 5 μm) maintained at ambient temperature. The mobile phase was composed of acetonitrile–1 m*M* 1-octanesulfonic acid (40:60, v/v, pH 3.0 adjusted with orthophosphoric acid), with a flow-rate of 1.0 ml/min. The mobile phase mixture was filtered through a 0.45-μm Millipore membrane, and then degassed before use. The fluorescence response for levofloxacin was observed at excitation and emission wavelengths of 292 and 494 nm, respectively. Chromatographic peak recording and integration were performed with an EZChrom chromatographic data system (Scientific Software, San Ramon, CA, USA).

2.3. Assay validation

The concentration of levofloxacin in dialysate samples was determined using the linear regression line (unweighted) of the concentration standard versus peak area. The precision of the method was expressed as the intra-day and inter-day coefficients of variation (%), which were assayed (six replicates) at concentrations of 0.1, 0.5, 1, and 5 μg/ml of levofloxacin on the same day and on six sequential days, respectively. The accuracy (% bias) was calculated from the nominal concentrations (C_{nom}) and the mean value of the observed concentrations (C_{obs}) as follows: bias (%)=$[(C_{obs}-C_{nom})/(C_{nom})]\times 100$. The relative standard deviation (RSD) was calculated from the observed concentrations as follows: precision (% RSD)=[standard deviation (SD)/C_{obs}]×100. Accuracy and precision values within ±15% covering the actual range of experimental concentrations were considered acceptable [12].

2.4. Animals

Specific pathogen-free male Sprague–Dawley rats were obtained from the Laboratory Animal Center of the National Yang-Ming University, Taipei. The animals had free access to food (Laboratory rodent diet No. 5P14, PMI Feeds, Richmond, IN, USA) and water until 18 h prior to being supplied for experiments, at which time only food was removed. The rats were initially anaesthetized with urethane 1.0 g/ml and α-chloralose 0.1 g/ml (1 ml/kg, i.p.) for probe insertion, and remained anaesthetized throughout the experimental period. The femoral vein was exposed for further drug administration. The rats' body temperature was maintained at 37 °C with a heating pad during the experiment.

2.5. Blood and bile microdialysis

Blood and bile microdialysis systems were comprised of a CMA/100 microinjection pump (CMA, Stockholm, Sweden) and microdialysis probes. The dialysis probes for blood (10 mm in length) and bile (7 cm in length) were made of silica capillary using a concentric design [13–16]. Their tips were covered with a dialysis membrane (Spectrum Laboratories, 200 μm I.D. with a nominal molecular mass cut-off of 13 000, Laguna Hills, CA, USA) and all unions were cemented with epoxy. At least 24 h was allowed for the epoxy to cure. The blood microdialysis probe was located within the jugular vein/right atrium and then perfused with anticoagulant dextrose (ACD) solution (citric acid 3.5 m*M*; sodium citrate 7.5 m*M*; dextrose 13.6 m*M*) at a flow-rate of 2 μl/min employing the microinjection pump [17]. The bile duct microdialysis probes were fabricated in our laboratory based on the design originally described by Scott and Lunte [18] and Hadwiger et al. [19] as reported in our previous studies [13–16].

A retrograde calibration technique was used during in vivo recovery. Following a stabilization period of 2 h post probe implantation, the perfusate (C_{perf}) and dialysate (C_{dial}) concentrations of levofloxacin were determined by HPLC. ACD solution (for blood microdialysis) containing levofloxacin or Ringer's solution (for bile microdialysis) containing levofloxacin was perfused through the probe at a constant flow-rate (2 μl/min) employing the infusion pump (CMA/100). The in vivo relative recovery (R_{dial}) of levofloxacin across the microdialysis probe was calculated by the following equation: $R_{dial}=(C_{perf}-C_{dial})/C_{perf}$. The microdialysate recovery and concentration calculations were carried out according to our previous report [16]. Levofloxacin microdialysate concentrations (C_m) were converted to unbound concentration (C_u) as follows: $C_u=C_m/R_{dial}$.

2.6. Drug administration

Six animals were used in each group. The control group received 3 mg/kg of levofloxacin by i.v. injection. For the cyclosporin A-treated group, cyclosporin A 20 mg/kg was injected via the femoral vein 10 min before levofloxacin injection of 3 mg/kg. Outflow dialysates from blood, and bile were collected in a fraction collector (CMA/140) every 10 min. These dialysate samples were measured by HPLC on the day of sampling.

2.7. Pharmacokinetics

Pharmacokinetic calculations were performed on each individual animal's data using the pharmacokinetic calculation software WinNonlin Standard Edition Version 1.1 (Scientific Consulting, Apex, NC, USA) by a 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 by statistical moments [20]. Formation rate constants were calculated from the extrapolated formation slope determined by the residual method. The AUCs from time zero to time infinity (AUC_{0-inf}) were calculated by the trapezoidal rule and extrapolated to time infinity by the addition of AUC_{t-inf}. An analogous method was employed to calculate the AUMC with the concentration versus time data, as follows:

$$AUC = AUC_{0-t} + AUC_{t-inf} = AUC_{0-t} + C_{last}/\lambda_z$$

$$AUMC = AUMC_{last} + (t_{last} \times C_{last}/\lambda_z) + C_{last}/(\lambda_z)^2$$

where C_{last} and t_{last} are the last observed concentration and time, respectively; and λ_z is the terminal slope which is estimated by linear regression of the

logarithmic value of the last observed data. MRT was calculated as follows: MRT = AUMC/AUC. Blood to tissue distribution was calculated as follows: AUC_{tissue}/AUC_{blood}.

2.8. Statistical analysis

Statistical analysis was performed with SPSS version 10.0 (SPSS, Chicago, IL, USA). One-way analysis of variance (ANOVA) was followed by a Dunnett's post-hoc test comparison between the control (levofloxacin treated alone) and P-glycoprotein modulators-treated groups. All statistical tests were carried out at the two-sided 5% level of significance.

3. Results and discussion

Typical chromatograms of levofloxacin are shown in Figs. 1 and 2. The retention time of levofloxacin was 4.0 min. The calibration curve of levofloxacin was obtained prior to HPLC analysis of the dialysates over the concentration range of 0.1–5 μg/ml. The concentrations of levofloxacin were linearly related to peak areas in the chromatogram ($r^2 > 0.995$). This chromatographic system was validated for both intra- and inter-day accuracy (0.1–6%) and precision (0.1–5.9%) as shown in Table 1. In vivo recovery of levofloxacin (0.5 μg/ml) in blood, and bile were 13.5±3.6, and 62.7±1.0%, respectively ($n = 6$). Fig. 1A shows the injection of standard levofloxacin (0.1 μg/ml), and Fig. 1B shows the chromatogram of a blank blood dialysate. None of the observed peaks interfered with the analyte. Fig. 1C shows the chromatogram of a blood dialysate sample containing levofloxacin (0.21 μg/ml) collected from a rat blood microdialysis probe 30 min after levofloxacin administration (3 mg/kg, i.v.).

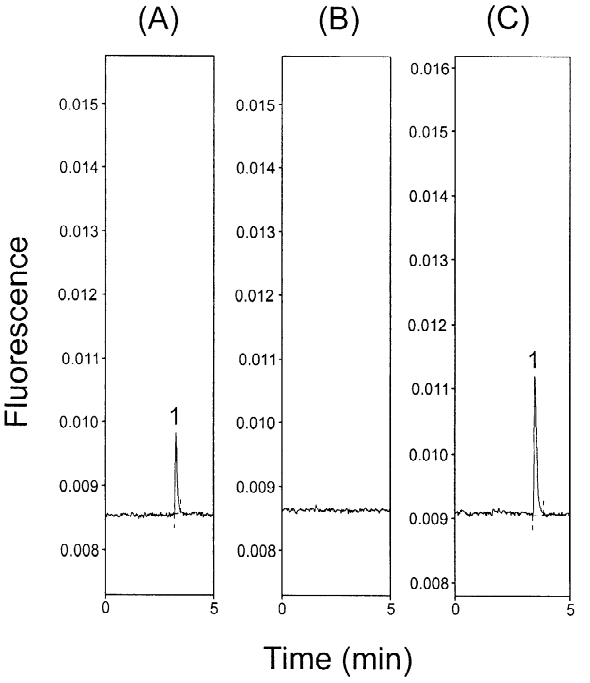

Fig. 1. Typical chromatograms of (A) standard levofloxacin (0.1 μg/ml), (B) blank blood dialysate from the microdialysis probe before drug administration, and (C) blood dialysate sample containing levofloxacin (0.21 μg/ml) collected from the rat blood microdialysate 30 min after levofloxacin administration (3 mg/kg, i.v.); 1, levofloxacin.

Fig. 2A shows a standard injection of levofloxacin (0.5 μg/ml). Fig. 2B shows a chromatogram of a blank bile dialysate sample obtained from the bile duct microdialysis probe before drug administration. Again, none of the observed peaks interfered with the analyte in the chromatogram of the bile sample. Fig. 2C shows the chromatogram of the bile dialysate sample containing levofloxacin (0.67 μg/ml) collected from the bile duct microdialysis probe 30 min after levofloxacin administration (3 mg/kg, i.v.).

The concentration versus time curves of levofloxacin in rat blood and bile are shown in Figs. 3 and 4, respectively. Disposition of levofloxacin in rat bile exhibited a peak concentration 20 min after levofloxacin administration (3 mg/kg) and was followed by a slow elimination phase. The AUCs of levofloxacin in blood and bile were 237.9±24.1 and 88.7±14.1 min μg/ml, respectively (Table 2) suggesting that levofloxacin may be excreted from blood to bile. The distribution ratio of levofloxacin from blood to bile (AUC_{bile}/AUC_{blood}) in the control group was 0.37±0.06 revealing that hepatobiliary excretion is not the dominant elimination route for levofloxacin. Actually, a previous report demonstrates that levofloxacin is primarily excreted through the kidney [21]. Renal distribution of levofloxacin may be mediated

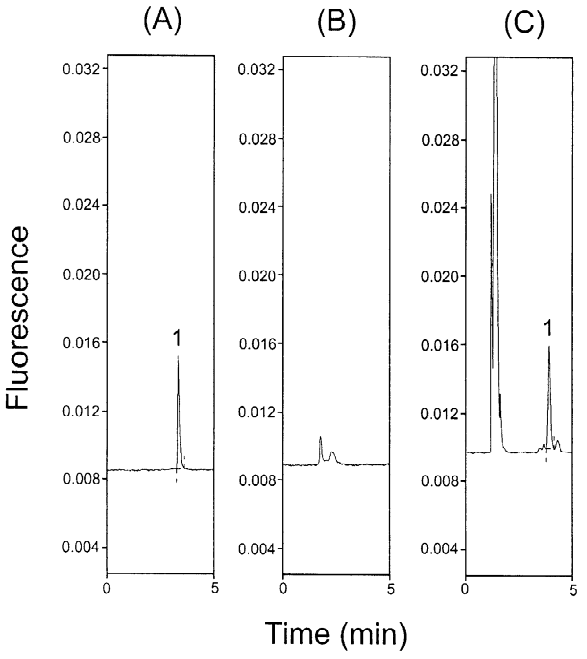

Fig. 2. Typical chromatograms of (A) standard levofloxacin (0.5 μg/ml), (B) blank bile dialysate from the flow-through microdialysis probe before drug administration, and (C) bile dialysate sample containing levofloxacin (0.67 μg/ml) collected from rat bile microdialysate 30 min after levofloxacin administration (3 mg/kg, i.v.); 1, levofloxacin.

by a specific transport system for quinolones, which is distinct from the organic cation and organic anion transport systems in the kidney [21].

Table 1
Intra-day and inter-day precision (RSD) and accuracy (bias) of the HPLC method for the determination of levofloxacin

Nominal concentration (μg/ml)	Observed concentration[a] (μg/ml)	RSD (%)	Bias (%)
Intra-day			
0.10	0.106±0.001	0.9	6.0
0.50	0.487±0.005	1.0	-2.6
1.00	0.989±0.008	0.8	-1.1
5.00	5.003±0.001	0.2	0.1
Inter-day			
0.10	0.102±0.006	5.9	2.0
0.50	0.472±0.026	5.5	-5.6
1.00	1.016±0.029	2.8	1.6
5.00	4.997±0.006	0.1	-0.1

[a] Data expressed as means±S.D. ($n=6$).

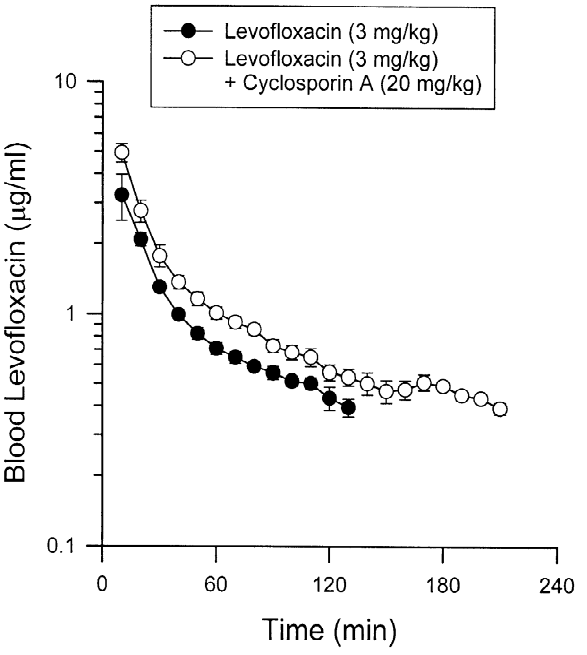

Fig. 3. Mean unbound levels of levofloxacin in rat blood after levofloxacin (3 mg/kg, i.v.) administration, and co-administration of levofloxacin (3 mg/kg, i.v.) and cyclosporin A (20 mg/kg, i.v.) ($n=5$). Data presented as mean±S.E.M.

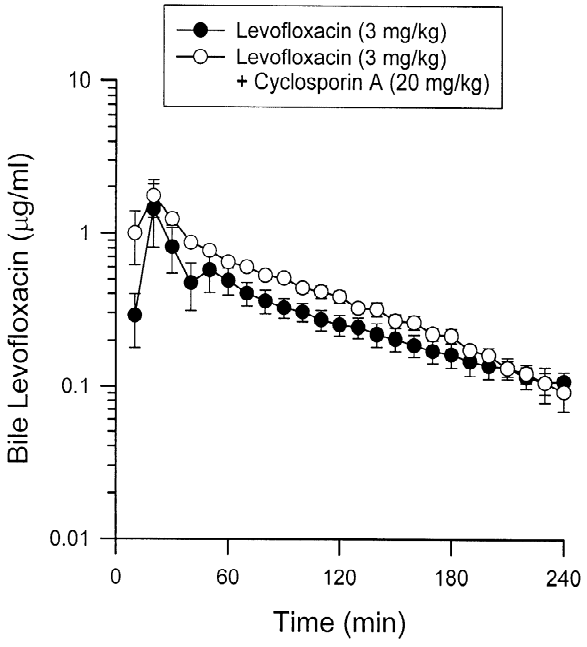

Fig. 4. Mean levofloxacin in rat bile after levofloxacin (3 mg/kg, i.v.) administration, and coadministration of levofloxacin (3 mg/kg, i.v.) and cyclosporin A (20 mg/kg, i.v.) ($n=5$). Data presented as mean±S.E.M.

Table 2
Pharmacokinetic parameters of the control group, levofloxacin administration (3 mg/kg, i.v.) and the treated group, cyclosporin A 20 mg/kg was injected via the femoral vein 10 min prior to 3 mg/kg levofloxacin injection

Parameters	Control group	Treated group
Blood		
AUC (min μg/ml)	237.9±24.1	306.3±30.9
MRT (min)	99.8±34.6	80.0±11.4
Bile		
AUC (min μg/ml)	88.7±14.1*	127.2±10.4*
MRT (min)	117.1±12.6	95.4±7.9
AUC_{bile}/AUC_{blood}	0.37±0.06	0.41±0.03

Data expressed as mean±S.E.M. ($n=5$). Significantly different from the group of blood values, *$P<0.05$.

The distribution ratio of levofloxacin from blood to bile (AUC_{bile}/AUC_{blood}) in the cyclosporin A-treated group was 0.41±0.03, not significantly different from the control group (0.37±0.06) (Table 2). Cyclosporin A (20 mg/kg) did not markedly affect the biliary distribution ratio of levofloxacin implying that hepatobiliary excretion of levofloxacin might not be regulated by P-glycoprotein. However, levofloxacin is transported by P-glycoprotein, and this may contribute at least in part to the renal tubular secretion of levofloxacin, as reported [22]. Recently, levofloxacin has been demonstrated for penetration into the CSF using microdialysis in an experimental rabbit meningitis model [23] which may relate to the action of multiple efflux transporters, including P-glycoprotein, MRP1, and an unknown anion-exchange transporter [24].

In summary, a rapid and sensitive HPLC system free of endogenous interference was developed for the determination of levofloxacin not bound to protein in rat blood and bile. The results reveal that levofloxacin undergoes hepatobiliary excretion.

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