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Determination of diclofenac in rat bile and its interaction with cyclosporin A using on-line microdialysis coupled to liquid chromatography

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Abstract

Diclofenac is a potent inhibitor of prostaglandin synthesis, as well as an established antipyretic and analgesic agent. To determine diclofenac in rat bile and investigate its hepatobiliary excretion, a procedure using rapid and sensitive high-performance liquid chromatography coupled to microdialysis sampling system was developed. A shunt linear microdialysis probe was inserted into the common bile duct between the liver and the duodenum for continuous sampling of the drug from bile fluids following intravenous administration of diclofenac (1 mg/kg). Separation and quantitation of diclofenac in the bile dialysates were achieved using a microbore reversed-phase C_{18} column (150×1.0 mm I.D.; particle size 5 μm) maintained at ambient temperature. Samples were eluted with a mobile phase containing 100 m*M* sodium dihydrogenphosphate (pH 3.1)–acetonitrile (30:70, v/v), and the flow-rate of the mobile phase was 0.05 ml/min. The UV detector wavelength was set at 280 nm. The concentration–response relationship from the present method indicated linearity (r^2>0.995) over a concentration range of 5–5000 ng/ml for diclofenac. Intra-assay and inter-assay precision and accuracy of diclofenac fell well within the predefined limits of acceptability (≤15%). The diclofenac in rat bile appeared to have a slow elimination phase, with a peak concentration at 20 min following diclofenac administration. The results demonstrated that diclofenac might be secreted into bile in unconjugated form by a canalicular bile acid transporter, and then go through hepatobiliary excretion. These results may provide good clinical evidence showing the value of diclofenac for the treatment of biliary colic. The elimination half-life of diclofenac in the biliary elimination was prolonged by treatment with cyclosporin A, indicating that the drug–drug interaction might affect the hepatobiliary excretion of diclofenac.

Keywords: Diclofenac; Cyclosporin A

1. Introduction

Diclofenac, the first nonsteroidal anti-inflammatory agent to be approved being a phenylacetic acid derivative, competes with arachidonic acid for binding to cyclooxygenase, resulting in decreased forma-

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tion of prostaglandins. The drug has both analgesic and antipyretic effects. Diclofenac undergoes biotransformation to glucuronide and sulfate conjugations, which are excreted in urine. The liver is also the main organ that eliminates diclofenac from the body by metabolism and biliary excretion [1], and the hepatic extraction ratio in vivo of diclofenac was previously estimated to be 63% [2].

Analytical methods have been described in literature for the determination of diclofenac in biological fluids with high-performance liquid chromatography (HPLC) coupled to UV detection [3,4], as well as in plasma and cerebrospinal fluid with HPLC–electrochemical detection (ED) [5]. Other methods were developed for the determination of diclofenac in the plasma and urine by capillary gas chromatography–mass spectrometry (GC–MS) [6], and in the serum with HPLC–fluorimetric detection [7]. Moreover, a fully automated analytical system combining liquid–solid extraction with LC was developed for the measurement of plasma concentration [8]. Recently, a HPLC system linked to inductively coupled plasma mass spectrometry (ICP-MS) and orthogonal acceleration time-of-flight mass spectrometry (oa-TOF-MS) was also developed for the identification of urinary metabolites of diclofenac [9]. The above LC-based approaches reported to date all result in the measurement of total (protein bound and unbound) drug concentration, rather than the free fraction. Over the past several years, microdialysis has been used increasingly in various animal experiments for the in vivo sampling of unbound endogenous or exogenous compounds present in blood, brain, tissue, etc. [10]. Sampling by this technique involves continuous perfusion of fluid through microdialysis probes implanted in the appropriate tissue space. Because a high binding to plasma protein ratio (99%) of diclofenac has been reported [11], little of the protein-unbound diclofenac can be measured in plasma. Recently, microdialysis has been applied for the transdermal iontophoresis of diclofenac, which may be due to its high recovery [12]. More recently, P-glycoprotein medicated transport has been indicated to be responsible for the excretion of xenobiotics via the canalicular membrane of hepatocytes into the bile for hepatobiliary elimination of drugs [13]. To evaluate the biliary mechanism of diclofenac, the determination of the diclofenac concentration in bile is important for the research of hepatobiliary excretion of diclofenac.

The purpose of this study was to develop a microdialysis sampling method for measuring diclofenac concentrations in bile fluid, and to evaluate its interaction with cyclosporin A, a P-glycoprotein inhibitor. To explore the mechanism of interaction with P-glycoprotein on the hepatobiliary excretion of diclofenac, a single dose of cyclosporin A was co-administered with diclofenac.

2. Experimental

2.1. Chemicals and reagents

Diclofenac was purchased from Research Biochemicals International (South Natick, MA, USA). Cyclosporin A (Sandimmun) was obtained from Novartis (Basle, Switzerland). Liquid chromatographic grade solvents and reagents were obtained from E. Merck (Darmstadt, Germany). Triple deionized water (Millipore, Bedford, MA, USA) was used for all preparations.

2.2. Animals

Adult, male Sprague–Dawley rats (280–320 g) were obtained from the Laboratory Animal Center at 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 5 days before the experiments began. All experimental protocols involving animal were reviewed and approved by the institutional animal experimentation committee of National Research Institute of Chinese Medicine. The animals had free access to food (Laboratory Rodent Diet #5P14; PMI Feeds, Richmond, IN, USA) and water until 18 h prior to being used in experiments, at which time food only was withdrawn. At the start of experiments, the rats were initially anesthetized with urethane 0.8 g/ml and α-chloralose 0.08 g/ml (1 ml/kg, i.p.), and remained anesthetized throughout the experimental period. The femoral vein was exposed for further drug administration. The rat's

body temperature was maintained at 37 °C with a heating pad.

2.3. Chromatography

The 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 a UV detector (Soma S-3702, Tokyo, Japan). Diclofenac dialysate was separated using a microbore C_{18} column (150×1.0 mm I.D.; particle size 5 μm) maintained at ambient temperature. The mobile phase was composed of 100 m*M* sodium dihydrogenphosphate (pH 3.1)–acetonitrile (30:70, v/v), and the flow-rate of the mobile phase was 0.05 ml/min. The buffer was filtered through a Millipore 0.22 μm filter and degassed prior to use. Detecting UV wavelength was set at 280 nm. Output signal from the HPLC–UV system was recorded using an EZChrom chromatographic data system (Scientific Software, San Ramon, CA, USA).

2.4. Method validation

All calibration curves of diclofenac (external standards) were made prior to the experiments with correlation values of at least 0.995. The intra-day and inter-day variabilities for diclofenac were determined (six replicates) at concentrations of 5, 10, 50, 100, 500 and 5000 ng/ml on the same day, and on 6 consecutive days, respectively. The limit of detection (LOD) was determined at a signal-to-noise ratio of 3; whereas the limit of quantification (LOQ) was defined as lowest analyte concentration that could be measured with a stated level of confidence, in practice the lowest concentration in the calibration curve. The accuracy (% bias) was calculated from the nominal concentration (C_{nom}) and the mean value of observed concentration (C_{obs}) as follows: bias (%)=$[(C_{obs}-C_{nom})/(C_{nom})]\cdot 100$. The 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 [14].

2.5. Microdialysis experiment

The bile duct microdialysis probes were also constructed in our laboratory based on design originally described by Scott and Lunte [15]. In brief, a 7-cm piece of dialysis membrane (Spectrum Lab, composed of regenerated cellulose, 150 μm outer diameter with a cut-off at nominal molecular mass of 13 000, Laguna Hills, CA, USA) was inserted into a section of polyethylene tubing (PE-60; 0.76 mm I.D.×1.22 mm O.D.), with the ends of the dialysis membrane connected to a piece of silica tubing (40 μm I.D.×140 μm O.D.; SGE, Australia). A piece of PE-10 tubing (0.28 mm I.D.×0.61 mm O.D.) was then attached to both ends of the PE-60 tubing and all unions were cemented with epoxy synthetic resin at least 24 h was allowed for the epoxy to dry. The detailed construction of the flow-through microdialysis probe has been described in our previous reports [16–19].

After bile duct cannulation of the animal, the probe was perfused with Ringer's solution (147.0 m*M* Na^+, 4.0 m*M* K^+, 2.2 m*M* Ca^{2+}). Following stabilization (approximately 2 h), drug-free control samples were collected and then diclofenac (1 mg/kg) was intravenously (i.v.) administered via a femoral cannula. Dialysate sample (10 μl) from the bile microdialysis probe were connected to an on-line injector and automatically injected into the on-line HPLC system every 10 min.

2.6. Recovery of microdialysate

For in vivo recovery, Ringer's solution containing diclofenac (50, 500 and 1000 ng/ml) was pumped through the probes at a constant flow-rate (1.0 μl/min) using the infusion pump (CMA/100). After a stabilization period of 2 h, the inlet (C_{in}) and outlet (C_{out}) concentrations of diclofenac were determined by HPLC. The in vivo recovery ratios were then calculated by the following equation [20]:

$$\text{Recovery}_{\text{in vivo}} = 1 - (C_{out}/C_{in})$$

2.7. Pharmacokinetic study

Calibration curves were constructed from HPLC analyses of various concentrations of diclofenac. The

concentrations of diclofenac in rat bile dialysates were determined from the calibration curves. Absolute concentrations in extracellular fluid were calculated from the concentrations in dialysates by the following equation [20]:

Concentration = dialysate/recovery

Pharmacokinetic calculations were performed using the observed data. All data were subsequently processed by the computer pharmacokinetic program WinNonlin standard version 1.1 (Science Consulting, Apex, NC, USA) for the calculation of pharmacokinetic parameters according to the non-compartmental model. All data are presented as means±standard errors. The area under the concentration curves (AUC), the area under the first moment curve (AUMC), and the mean residence time (MRT) were calculated by using statistical moments. The mean residence time was calculated as follows: MRT = AUMC/AUC.

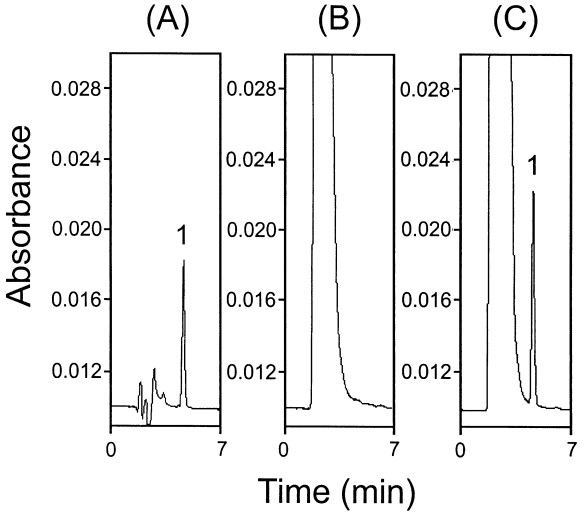

Fig. 1. Typical chromatogram for injection of (A) standard diclofenac (500 ng/ml), (B) blank bile dialysate, and (C) bile dialysate sample containing diclofenac (815 ng/ml) collected from bile fluid at 20 min after diclofenac administration (1 mg/kg, i.v.). 1: Diclofenac.

3. Results and discussion

The present liquid chromatographic method was applied to determine diclofenac from rat bile using microdialysis. Typical chromatograms of standards and dialysates containing diclofenac are shown in Fig. 1. Isocratic separation of diclofenac from certain endogenous chemicals in the bile dialysate was achieved in an appropriate mobile phase containing acetonitrile–100 m*M* sodium dihydrogenphosphate (pH 3.1) (70:30, v/v), with the flow-rate of the mobile phase at 0.05 ml/min. The retention time of diclofenac was 4.8 min. Each analysis was completed within 7 min. Peak areas of diclofenac were linear ($r^2 > 0.995$) over a concentration range of 5–5000 ng/ml. Compared with other liquid chromatographic methods with solid-phase extraction [21] and liquid-phase extraction [22] for diclofenac assay, the on-line microdialysis and microbore HPLC method requires less time for the complex sample extraction procedures and consumes less solvent.

Fig. 1A shows a typical chromatogram of a standard containing diclofenac (500 ng/ml). The blank sample (Fig. 1B) shows that the chromatographic conditions revealed no biological substances that would interfere with the determination of the drug. Fig. 1C depicts a chromatogram of actual diclofenac in rat bile which contains diclofenac (815 ng/ml) collected from the bile fluid at 20 min following diclofenac administration (1 mg/kg, i.v.). Owing to the high level of protein binding (99%) [10] and low loading dose (1 mg/kg), the protein unbound diclofenac in blood was not detectable in this experiment. However, in vivo microdialysis has been applied in transdermal iontophoresis of diclofenac with various polymer formulations [12]. The synergistic effect of polymer formulation and diclofenac significantly enhance drug delivery.

Intra-assay and inter-assay (Table 1) accuracy of diclofenac levels fell well within predefined limits of acceptability. All % bias and RSD values were within ±15%. The LOD and LOQ for diclofenac were 3 and 5 ng/ml, respectively. The in vivo recovery of diclofenac is shown in Table 2, which indicates that this method is sufficiently sensitive to measure diclofenac in rat bile for its pharmacokinetic study.

To minimize the bile fluid loss, we constructed an automatic on-line flow-through microdialysis probe

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

Nominal concentration (ng/ml)	Observed concentration (ng/ml)[a]	RSD (%)	Accuracy (% bias)
Intra-assay ($n=6$)			
5	5.7±0.3	6.0	14.0
10	9.6±0.6	5.9	−4.3
50	50.6±2.4	4.7	1.2
100	103.6±3.5	3.4	3.6
500	497.5±7.6	1.5	−0.5
5000	4979.1±132.6	2.7	−0.4
Inter-assay ($n=6$)			
5	5.6±0.3	5.5	11.7
10	10.3±0.7	6.5	2.7
50	49.8±1.3	2.5	−0.5
100	97.9±2.5	2.6	−2.1
500	501.7±4.0	0.8	0.3
5000	4999.9±0.4	0.1	−0.1

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

for bile duct sampling, coupled with a HPLC system, thus providing near real-time analysis of diclofenac in bile dialysate samples after drug administration.

Fig. 2 shows the relationship between measured diclofenac concentrations versus time in rat bile and co-administration with cyclosporin A (20 mg/kg, i.v.) after diclofenac administration (1 mg/kg, i.v.). These data have been corrected for in vivo recoveries. The average concentration of diclofenac in the bile increased during the first 20 min following drug administration. From these microdialysis sampling data, the pharmacokinetics of unbound diclofenac in the rat bile was calculated according to the non-compartmental model.

The pharmacokinetic parameters are shown in Table 3. The C_{max} and AUC of diclofenac were increased by adding cyclosporin A. The $t_{1/2}$ and

Table 2
In vivo microdialysis recoveries (%) of diclofenac in rat bile with a flow-rate of 1.0 μl/min

Concentration (ng/ml)	Recovery (%)
50	69.4±5.6
500	73.9±1.8
1000	73.0±3.3

Data are expressed as mean±SD ($n=6$).

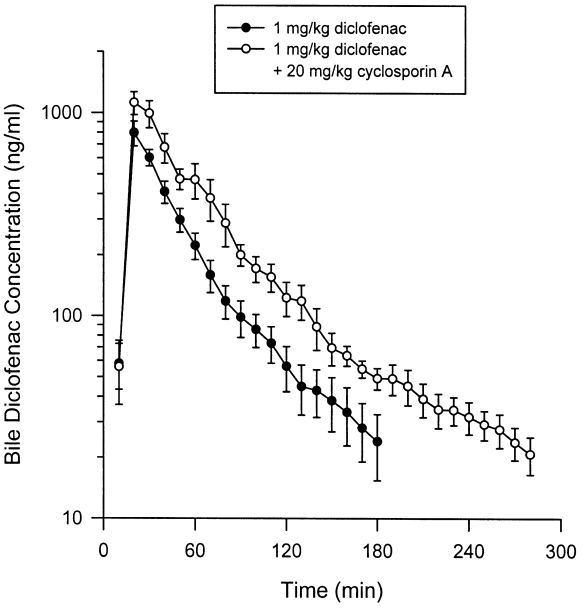

Fig. 2. Pharmacokinetic curve of diclofenac in rat bile concentration versus time following intravenous administration of diclofenac (1 mg/kg, i.v.), both alone and pretreated with cyclosporin A (20 mg/kg, i.v., $n=6$). Data are presented as mean±S.E.M.

MRT were not significantly altered between the groups with and without cyclosporin A. Previously, Seitz et al. indicated that the reactive diclofenac glucuronide is selectively transported into the bile via Mrp2 and that hepatobiliary transport is critical for diclofenac covalent binding to proteins in the biliary tree [23]. Biotransformation studies suggest that the metabolism of diclofenac is associated with hepatotoxicity [24,25]. Diclofenac was metabolized via phase I and phase II reactions. The biliary elimination of diclofenac increased as cyclosporin A was administered, which may be because the cyclosporin A was a CYP 3A inhibitor [26]. This observation was best explained by the decreased protein binding and increased biliary excretion of diclofenac in the presence of cyclosporin A.

In summary, we describe a rapid and sensitive chromatographic method for the determination of diclofenac in rat bile using in vivo microdialysis with HPLC–UV. The disposition of diclofenac in bile indicates that it may undergo biliary elimination and that the bile elimination of diclofenac was increased by administration of cyclosporin A.

Table 3
Pharmacokinetic data of diclofenac in rat bile by i.v. administration of 1 mg/kg diclofenac, both with and without received cyclosporin A (20 mg/kg)

Drug treatment	Diclofenac (1 mg/kg)	
	Without cyclosporin A	With cyclosporin A
Bile		
C_{max} (ng/ml)	798±110	1187±146*
$t_{1/2}$ (min)	31±4	35±4
AUC (min ng/ml)	32 700±2560	60 100±6880*
MRT (min)	55±7	65±4

Data are expressed as mean±S.E.M. ($n=6$). *$P<0.05$ Significantly different from the group without cyclosporin A.

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