Pharmacokinetic Study of Ketoprofen Isopropyl Ester-Loaded Lipid Microspheres in Rat Blood Using Microdialysis

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A blood microdialysis technique coupled with high-performance liquid chromatography was used to investigate the pharmacokinetics of unbound ketoprofen in rats after intravenous administration of a lipid-soluble ketoprofen derivate, ketoprofen isopropyl ester (KPI), loaded into lipid microspheres (LM) and ketoprofen solution. A microdialysis probe was inserted into the jugular vein of male Wistar rats. KPI-loaded LM or ketoprofen solution (24 mg/kg, i.v.) was then administered via a femoral vein. Dialysate samples were analyzed using HPLC.

The in vitro and in vivo recovery rate of the microdialysis probe was 30.42±0.74% (n=3) and 40.27±2.74% (n=3), respectively. The pharmacokinetic parameters for ketoprofen after intravenous administration of KPI-loaded LM and ketoprofen solution exhibited no statistically significant differences. The results of this pharmacokinetic study indicate that the microdialysis technique can be widely applicable to investigations of in vivo free-drug of microcarrier systems.

Key words ketoprofen isopropyl ester; ketoprofen; lipid microsphere; microdialysis; pharmacokinetics; HPLC

Lipid microspheres have been widely studied as a microcarrier system. They are made of lecithin and soybean oil in which a drug is dissolved and are widely used in clinical medicine for parenteral nutrition. Currently, pharmacokinetic studies of such microcarrier systems are usually performed on small laboratory animals, such as rats and mice. Blood samples are usually collected at a series of intervals that could lead to blood loss, which could adversely affect the plasma-concentration profile of the drug. However, compared with common formulations (e.g., injection), the biological samples (blood and tissues) of microcarrier systems include not only the drug released from the carriers, but also the drug that is retained in the carriers. Conventional extraction methods for biological samples also extract the drug that is retained by the carriers. Thus, in pharmacokinetic studies of microcarrier systems, the concentration of free drug in biological samples is difficult to determine using conventional extraction methods.

In recent years, microdialysis has been used as an in vivo sampling technique that allows determination of free-drug concentrations in blood and most tissues. Microdialysis is a sampling technique based on the passive diffusion of compounds down a concentration gradient across a semipermeable membrane.2 This technique has been used successfully in pharmacokinetic and pharmacodynamic studies in animals and humans.3–5 Microdialysis provides several advantages for pharmacokinetic and pharmacodynamic studies by allowing in vivo sampling of extracellular fluid in many kinds of tissues and fluids.6 In contrast to other methods of sampling biological fluids and tissues, microdialysis offers a relatively clean and protein-free dialysate because the membrane is only permeable to small molecules and, consequently, no further clean-up procedures are required. No fluid is removed, and therefore continuous sampling can be performed with small laboratory animals without affecting the pharmacokinetic profiles while allowing determination of concentration–time profiles involving a high number of samples over short time periods. The microdialysis technique has been used to determine the in vitro drug release from microcarrier systems7,8 and it has been found to be a powerful tool for determination of the in vivo pharmacokinetics of drug-loaded microcarrier systems.

Ketoprofen, a potent non-steroidal anti-inflammatory drug (NSAID), is a racemic propionic acid derivative with well-recognized analgesic, anti-inflammatory, and antipyretic properties and is widely used clinically. Pre-emptive analgesia with intravenous ketoprofen (100 mg) produces better postoperative pain-relief in patients undergoing breast surgery.9 A combination of intravenous ketoprofen 200 mg and bupivacaine 0.25% intraperitoneal spray 40 ml achieved the best pain management following elective laparoscopic cholecystectomy.10

In this study, we incorporated a lipid-soluble derivate of ketoprofen (Fig. 1a), ketoprofen isopropyl ester (KPI) (Fig. 1b), into lipid microspheres (LM). Then, an in vivo microdialysis sampling method coupled with high-performance liquid chromatography (HPLC) was used to determine the free, unbound ketoprofen in rat blood. The pharmacokinetics of unbound ketoprofen in rat blood following intravenous administration of 24 mg/kg KPI-loaded LM and ketoprofen solution were also investigated using this sampling and assay system.

MATERIALS AND METHODS

Materials Ketoprofen was purchased from Wuxue XunDa Pharmaceutical Co. (Wuxue, China). KPI, (purity 99.5%), a colorless, transparent, oily ester of ketoprofen, was kindly synthesized and supplied by the Laboratory of Organic Chemistry of Shenyang Pharmaceutical University.

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Fig. 1. Chemical Structure of Ketoprofen (a) and Its Lipophilic Prodrug, Ketoprofen Isopropyl Ester (KPI) (b)
was used for all preparations. were of analytical reagent grade, and double-distilled water was used for all preparations.

**Liquid Chromatography** The HPLC system was equipped with a mobile phase delivery pump (PU-1580, Jasco Corporation; Japan) and a UV–VIS detector (UV-1575, Jasco Corporation, Japan). A C18 reverse-phase column (HiQ Sil, 250×4.6 mm, i.d. 5 μm, KYA TECH Corporation; Japan) was used, and the chromatography was performed at ambient temperature. The mobile phase was acetonitrile–0.01% aqueous H₃PO₄ (pH 3.0, 55:45, v/v) and the flow rate was 1.0 ml/min. The eluate was monitored at 200 nm and the AUFS of the detector was set to 0.001. The dialsates from the blood were injected directly into the HPLC system. The samples for total blood levels of ketoprofen were treated as follows: A 0.2 ml plasma sample was placed into a 15-ml centrifuge tube, and flurbiprofen 50 μl (300 μg/ml, internal standard) and 100 μl of 1 N HCl were added. Then they were extracted with 5 ml of ether. After vortexing for 10 min, the mixture was centrifuged at 4000 rpm for 15 min. The organic phase were transferred to other tubes and evaporated to dryness under a gentle stream of nitrogen in a 40°C water bath. The residues were dissolved with 200 μl of acetonitrile and aliquots of 20 μl were injected into the HPLC system for analysis.

**Method Validation** A stock solution of ketoprofen was prepared by dissolving the appropriate amount of drug in methanol. Seven microdialysate standards containing ketoprofen 50, 100, 200, 500, 1000, 1500, and 2000 ng/ml ketoprofen were prepared by the diluting stock solutions of ketoprofen with perfusate.

The linearity, precision, and reproducibility of the chromatographic method were examined. The limits of quantification and detection were also determined. Microdialysate calibration curves in the range of 50—2000 ng/ml for ketoprofen were constructed by plotting the ketoprofen peak area against the ketoprofen standard concentrations using the least-squares method. The intraday and interday precisions of ketoprofen were determined (n=3) at concentrations of 100, 200, 500, 750, and 1000 ng/ml on the same day and on 3 sequential days, respectively. The accuracy (%Bias) was calculated from the theoretical concentrations (C_{the}) and the mean value of the observed concentrations (C_{obs}) as follows: %Bias=[(C_{obs}−C_{the})/C_{the}]×100. The precision coefficient of variation (RSD) was calculated from the observed concentrations as follows: %RSD=[standard deviation (S.D.)/C_{obs}]×100.

**Animals** Male Wistar rats, weighing 250±20 g, were purchased from the China Medical University Animal Center. All animal experiments complied with the requirements of the National Act on the Use of Experimental Animals (People’s Republic of China). All the rats were fasted for 12 h before the experiments but had free access to water. The rats for microdialysis experiments were anesthetized with urethane (1.2 g/kg, i.p.), and remained anesthetized throughout the experimental period. The body temperature of the rats was maintained at 37°C with an infrared lamp.

**Microdialysis Experiment** The jugular probe was a U-shaped microdialysis probe made of hollow cellulose fiber (DM-22, 200 μm in inner diameter and 220 μm in outer diameter with a cut-off at nominal molecular weight of 5000 Da, EICOM CORP, Japan), and the active region of the microdialysis probe was 10 mm in length.

The blood microdialysis probe was positioned within the jugular vein and then perfused with anticoagulant citrate dextrose (ACD) solution 13) (citric acid 3.5 mM; sodium citrate 7.5 mM; dextrose 13.6 mM), at a flow rate of 4 μl/min using an S200 microdialysis syringe pump (KD Scientific Company, U.S.A.) for both delivery of the perfusion solution and sample collection.

The in vitro recovery by gain was determined by immersing the microdialysis probes in stirred ACD solution containing ketoprofen (100, 500, or 1000 ng/ml) as a dialysis medium (C_{med}). The probes were perfused with drug-free ACD solution at a flow rate of 4 μl/min. The concentration of ketoprofen in the microdialysate samples was determined using HPLC (C_{dial}). The in vitro relative recovery rate (R) of ketoprofen was calculated using the following equation: R=C_{dial}/C_{med}.

To determine the unbound ketoprofen concentration in the blood from the microdialysis data, the concentration of ketoprofen in the microdialysis samples was adjusted with the in vivo recovery rate of the probe. A retrodialysis technique was used for the study of in vivo recovery. 14) The blood microdialysis probe was inserted into the rat jugular vein under anesthesia. The probe was perfused with ACD solution containing ketoprofen (100, 500, or 1000 ng/ml) at a flow rate of 4 μl/min. The in vivo recovery rate of ketoprofen was calculated with the following equation: R_{dial}=1−C_{dial}/C_{perf}, where R_{dial} is the ketoprofen in vivo recovery rate, C_{perf} is the concentration of ketoprofen in the perfusate, and C_{dial} is the concentration of ketoprofen in the dialysate.

**Drug Administration** After a 90-min stabilization period following implantation of the probes, KPI-loaded LM or ketoprofen solution (24 mg/kg ketoprofen equivalent, i.v.) was administered via the femoral vein. The volume of each injection was 2.4 ml/kg. The dialsates from the blood were collected at 20-min intervals and analyzed using HPLC as described above.

The rats for determining the total blood level of ketoprofen were anaesthetized lightly with ether and administered the same drugs and doses as above. Venous blood (0.5 ml) was collected via the orbital vein at 0, 0.0833, 0.15, 0.5, 1, 1.5, 2, 4, 6, and 8 h after administration.

**Pharmacokinetic Analysis** The pharmacokinetic parameters, elimination rate constant (Ke), total body clearance (CL), and half-life (T_{1/2}), were calculated using a noncompartamental model and Excel software. The area under the concentration–time curve (AUC) was calculated using the trapezoidal method. The half-life (T_{1/2}) values were calculated using the equation: T_{1/2}=0.693/Ke, where Ke is obtained from the terminal slope of the individual ketoprofen concentration–time curves after logarithmic transformation of the ketoprofen concentration values and application of linear regression. The clearance (CL) was calculated as: CL=dose/AUC.

The data obtained from the pharmacokinetic parameters of KPI-loaded LM and ketoprofen solution were analyzed sta-
tistically by one-way analysis of variance and Student’s t-test using SPSS version 11.0 software. Statistically significant differences were assumed when \( p < 0.05 \). All data are presented as mean \( \pm S.D. \)

RESULTS AND DISCUSSION

Validation of Chromatographic Methods Chromatograms for standard ketoprofen, blank blood dialysis, and a typical chromatogram of ketoprofen obtained from a rat sample after intravenous administration of KPI-loaded LM are shown in Fig. 2. The retention time of ketoprofen was approximately 6.6 min. These chromatographic conditions revealed no biological substances that would significantly interfere with the accurate determination of the drug. The peak area of ketoprofen versus the ketoprofen concentration curve was linear over the range of 50—2000 ng/ml, and the detection limit was 10 ng/ml. The regression equations obtained were 

\[
A = 15.0821C + 65.4587
\]

\( n = 7, r = 0.9988 \), where \( A \) is the peak area of ketoprofen, and \( C \) is the ketoprofen concentration in ng/ml. The intraday and interday accuracy and precision were determined at five concentrations of ketoprofen in ACD solution and the results are presented in Table 1.

Microdialysis Various conditions can affect the recovery of the probe, such as the perfusion flow rate, membrane length, and temperature. The perfusion flow rate is an important factor that defines the performance of a microdialysis probe and directly influences on the recovery by the probe. Recovery increases with decreasing perfusion flow rate. In this study, a perfusion rate of 4 \( \mu l/min \) was selected. At this perfusion rate, the \textit{in vitro} and \textit{in vivo} recoveries of ketoprofen at concentrations of 100, 500, and 1000 ng/ml were 30.42±0.74\% and 40.27±2.74\%, respectively (Table 2). Each value was determined using three independent microdialysis probes. Since the surrounding environment of the probes implanted in blood vessels was completely different from aqueous solution, the \textit{in vitro} recovery would differ significantly from the \textit{in vivo} recovery, and therefore we calibrated microdialysis probes \textit{in vivo}.

Pharmacokinetics The clinical dose of ketoprofen for the treatment of postoperative pain is about 100—200 mg.\(^9,10\) It is assumed that the intravenous dose of ketoprofen was 2 mg/kg for humans. The intravenous dose of ketoprofen for rats was 12 mg/kg as calculated using the body surface area conversion equation: \( \text{Dose}_{\text{rat}} \times \text{Conversion factor}_{\text{human}} / \text{Conversion factor}_{\text{rat}} \), where the conversion factors were 36 and 6 for humans and rats, respectively. In this study, twice of the calculated dose was used, i.e., 24 mg/kg, to enhance the accuracy of determination because ketoprofen is highly bound to plasma protein (more than 99\%).

The concentration-versus-time curves of unbound ketoprofen in rat blood is shown in Fig. 3. The concentration of ketoprofen in rat blood dialsates was determined from the calibration curve. The unbound concentration of ketoprofen in blood (\( C_{\text{blood}} \)) was calculated from its concentration in the dialysate (\( C_{\text{dial}} \)) using the following equation: 

\[
C_{\text{blood}} = C_{\text{dial}} / R_{\text{dial}}
\]

\( R_{\text{dial}} \) is the \textit{in vivo} recovery of ketoprofen.

![Fig. 2. Typical Chromatograms of (a) Standard Ketoprofen, (b) Blank Blood Dialysate from the Microdialysis Probe before Drug Administration, and (c) Blood Dialysate Sample Containing Ketoprofen Collected from Rat Blood after Administration of KPI-Loaded LM (24 mg/kg, Ketoprofen Equivalent, i.v.); 1, Ketoprofen](image)

![Table 1. Intra- and Inter-Day Accuracy and Precision of the Determination of Ketoprofen in Microdialysis](image)

<table>
<thead>
<tr>
<th>Theoretical concentration (ng/ml)</th>
<th>Observed concentration ( ^a ) (ng/ml)</th>
<th>%RSD</th>
<th>Accuracy ( % \text{Bias} )</th>
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<td><strong>Intra-day</strong></td>
<td></td>
<td></td>
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<tr>
<td>100</td>
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<tr>
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<tr>
<td>1000</td>
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<tr>
<td><strong>Inter-day</strong></td>
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</table>

\( a \) Data expressed as mean \( \pm S.D. \) (\( n = 3 \)).

![Fig. 3. Mean Protein-Unbound Blood Concentration–Time Profile of Ketoprofen after i.v. Administration of KPI-Loaded LM and Ketoprofen Solution in Rats](image)

Dose, 24 mg/kg. Data are expressed as mean \( \pm S.D. \) \( n = 6 \).
intravenous administration of KPI-loaded LM and ketoprofen solution were calculated and the results are shown in Table 3. There was no statistical difference in any of the pharmacokinetic parameters between these two preparations ($p > 0.05$). The total ketoprofen plasma pharmacokinetic of KPI-LM and ketoprofen solution were investigated in previous study. The total plasma profiles of ketoprofen in these two sampling methods were similar (Fig. 4). The pharmacokinetic parameters (Table 3) calculated based on total blood levels of ketoprofen also did not differ statistically between these two preparations ($p > 0.05$). The half-life of ketoprofen was somewhat longer when considering the total plasma concentrations of 241.11 ± 45.94 min and 255.42 ± 49.72 min for KPI-LM and ketoprofen solution, respectively, as compared with the unbound concentrations of ketoprofen 166.36 ± 15.72 min and 180.83 ± 16.26 min. This phenomenon may be explained by the concentration-dependent plasma binding of ketoprofen, and a similar effect of concentration-dependent plasma protein binding on the half-life was demonstrated for flurbiprofen. As shown in Fig. 3, during the early period after drug administration, the concentration of unbound ketoprofen following intravenous administration of the LM was lower than that after intravenous administration of ketoprofen solution, implying the removal of LM from the blood circulation, probably by the reticuloendothelial system-rich organs. Since ketoprofen is highly bound to plasma protein, after intravenous administration of ketoprofen solution, ketoprofen will bind to plasma protein immediately and remain in the circulation. At approximately 2 h, the concentration-versus-time curves of the two preparations crossed, and the ketoprofen concentration of LM became higher than that of the solution. This may be accounted for by the uptake of the KPI-loaded LM by some tissues in which KPI was degraded to ketoprofen. After that, ketoprofen was slowly removed from these tissues, passing into the blood circulation. As far as the in vitro fate of KPI-loaded LM is concerned, it appears that after intravenous administration of KPI-loaded LM, KPI is released rapidly from the LM and then degraded immediately to ketoprofen in blood or tissues by the action of enzymes. The same result was obtained in the in vitro degradation testing of KPI-loaded LM in rat plasma, where it was found that KPI incorporated in LM was released rapidly from the microcarrier and then degraded to ketoprofen with a degradation $T_{1/2}$ of less than 1 min.

CONCLUSIONS
A simple and sensitive liquid chromatographic system coupled to a microdialysis technique has been developed for the determination of unbound ketoprofen in rat blood. This method was used to monitor the pharmacokinetics of ketoprofen following i.v. administration of KPI-loaded LM 24 mg/kg i.v. and compared with the pharmacokinetics of ketoprofen solution. The possible fate of KPI-loaded LM in vivo was also discussed. 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. In addition, this sampling method facilitates pharmacokinetic studies by eliminating the effects of blood volume changes, compared with conventional blood sampling techniques. The results of this study show that the microdialysis technique may have wide applications prospective in the investigation of in vivo free drug concentrations of microcarrier systems, such as LM, liposomes, and solid lipid nanoparticles.

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REFERENCES