Development of a Dog Microdialysis Model for Determining Synovial Fluid Pharmacokinetics of Anti-Arthritis Compounds Exemplified by Methotrexate

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Purpose. The purpose of this study was to develop and validate an animal model of drug disposition in synovial fluid (SF) by comparing microdialysis with arthrocentesis using the anti-arthritic drug methotrexate (MTX).

Methods. Microdialysis probes were calibrated *in vitro* with the no net flux method using dog synovial fluid. The probes were implanted surgically into the stifle joint space of four dogs and were dialyzed overnight using a portable microinfusion pump. The membrane integrity of the probes was monitored by retrodialysis using an internal standard. After an intravenous bolus of 2.5 mg/kg of MTX, unbound concentrations in synovial fluid, as well as total plasma concentrations, were measured by liquid chromatography tandam mass spectrometer (LC/MS/MS) in samples collected from 0 to 48 h postdose.

Results. The probe membrane remained intact at least 48 h after implantation. The mean probe recovery and unbound fraction of MTX in SF were 46.8% and 44.8%, respectively. The unbound fraction of MTX was 44% in synovial fluid. MTX penetrated into the joint space rapidly, with maximal concentrations of 6.6 μM reached at approximately 1 h postdose. The unbound MTX area under the curve in SF was approximately 40% of the total area under the curve in plasma. These data agree well with the previous data obtained for MTX using arthrocentesis.

Conclusion. In contrast with arthrocentesis, microdialysis enables the collection of multiple serial SF samples from individual animals with minimal trauma and potential blood contamination. This animal model should prove valuable for studying the disposition of new anti-arthritis compounds or biomarkers in SF.

KEY WORDS: microdialysis; synovial fluid pharmacokinetics; methotrexate; rheumatoid arthritis.

INTRODUCTION

Synovial fluid (SF) is the most relevant biologic fluid surrogate for the major site of action of anti-arthritic therapeutics. Measurement of the free drug in SF is important because the unbound fraction of drugs in SF most likely accounts for the therapeutic effect in the joint space, the site of anti-inflammatory action for many anti-arthritic drugs, including methotrexate (MTX), nonsteroidal anti-inflammatory drugs, and anti-TNF therapeutics. Techniques that would allow sampling of this important biophase with minimal local trauma, contamination from other fluids like blood, and general anesthesia would be useful in evaluating the disposition of new drugs and validation of possible biomarkers of disease progression or modulation.

Historically, studies designed to characterize the disposition and fate of drugs in SF have been difficult to pursue, especially in preclinical animal models of arthritis. This was largely because of the difficulty of sequential sampling by arthrocentesis of small-volume compartments from individual animals. Microdialysis is a valuable *in vivo* sampling technique that has been extensively applied in central nervous system research to monitor changes in endogenous and exogenous analyte concentrations (1). It has more recently been applied to sampling of extracellular fluids in organs like the liver (2), tumors (3), and specialized compartments in the eye (4). Microdialysis has gained increasing recognition in the area of pharmacokinetics and drug metabolism; however, the application of this technique for SF pharmacokinetics has been rarely explored. St. Claire and Brouwer successfully demonstrated the potential utility of microdialysis to determine the SF concentration–time profile of an anti-arthritic compound in a rat (5). In their study, only one rat was used, and the rat was anesthetized during the entire study period, which is not ideal for a pharmacokinetic study.

Lu *et al.* (6) have characterized the pharmacokinetics of methotrexate (4 amino-10-methylfolic acid), a cytotoxic immunosuppressive agent used in low oral doses for second-line therapy in rheumatoid arthritis, in blood and SF from the same dogs using arthrocentesis to collect serially 50–100 μL of SF from joints, and found similar concentration-time profiles of MTX in plasma and SF. The MTX concentrations determined represent total concentrations in SF, and additional *ex vivo* experiments would be needed to calculate free or unbound MTX. In addition, arthrocentesis is an invasive method, and repetitive collection of SF using this method from the same joints would certainly increase the chance of contaminating the SF samples with blood, as well as disturbing the homeostasis of the joint space from the resulting trauma.

To support the discovery and development of novel anti-arthritic compounds, we sought to develop and validate an animal model to evaluate SF pharmacokinetics directly, using MTX as the model compound. Serial microdialysis sampling and LC/MS/MS analysis were used to collect and measure the free concentrations of MTX in SF, and the results were compared with those reported previously using arthrocentesis.

MATERIALS AND METHODS

Chemicals and Reagents

MTX and the internal standard aminopterin were purchased from Sigma (St. Louis, MO, USA). Ethanol was bought from AAper Alcohol and Chemical Co., (Shelbyville, KY, USA) and Dulbecco's phosphate-buffered saline (PBS) was bought from Gibco (BRL Life Technologies, Grand Island, NY, USA). High-performance liquid chromatography-

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grade acetonitrile was purchased from Fisher Scientific (Fair Lawn, NJ, USA) and all other reagents were of analytical grade and purchased from Fisher Scientific as well.

Microdialysis System

The microdialysis system used a syringe infusion pump (CMA-107, CMA/Microdialysis, Acton, MD, USA), and a 2-mL microliter syringe to deliver the perfusate solution (PBS). The syringe was connected to a customized flexible CMA-20 probe (tip length 4 mm, molecular weight cut off 20,000 Da) with a fused silica connecting tube. The lag time (because of the dead volume between the sampling site and the point of dialysate collection) was calculated to be 27 s at a flow rat of 1 μL/min, and was considered negligible; therefore, no corrections were made in the time points.

Animals

Four male beagle dogs (10.6 ± 1.9 kg), obtained from Marshall Farms (North Rose, NY, USA) were used in this study. The animals were individually housed in an AAALAC-accredited facility under controlled environmental conditions in accordance with the *Guide for the Care and Use of Laboratory Animals* and the Animal Welfare Act. They were fed 350 g of a certified canine diet (#5007 PMI, St. Louis, MO, USA) daily and provided water *ad libitum.* All procedures performed on the dogs were approved by the Institutional Animal Care and Use Committee, and were adherent to the "Principles of Laboratory Animal Care" (NIH publication #85-23, revised 1985).

Implantation of the Microdialysis Probe

Each probe was implanted using aseptic techniques while the dog was under general anesthesia (Isoflurane, minimal alveolar concentration 0.75–1.5%; Abbott Laboratories, North Chicago, IL, USA). A 1-cm incision was made along the lateral aspect of the stifle joint, followed by a stab incision along the medial aspect of the joint. A sterile 18-gauge needle was then inserted through the joint space and exteriorized at the lateral aspect. The probe was inserted into the hub of the needle and advanced into the joint space as the needle was removed. The unit was secured using 4-0 PDS (Ethicon, Somerville, NJ, USA). To facilitate the collection of SF, a trocar was advanced subcutaneously from the stifle area and exteriorized on the lateral dorsum near the seventh to eighth rib space, and the catheters attached to the probe were fed through as the trocar was removed. The inlet catheter was connected to the pump unit, which was stored in a torso jacket. The outlet catheter was placed in a collection vial. The incision on the medial aspect of the knee was closed with one 3-0 nylon skin suture (Ethicon, Somerville, NJ, USA), and the lateral incision was closed using 4-0 PDS for subcutaneous tissue and 3-0 nylon for skin. The pump was primed with 2.5 mL of sterile saline and the infusion rate was set at 1 μL/min. Each animal received a subcutaneous injection of Buprenex® (Reckitt and Colman Pharmaceuticals, Richmond, VA, USA) at a 0.01 mg/kg dosage for postoperative analgesia. The microdialysis probe was allowed to equilibrate inside the joint space overnight before drug administration.

Experimental Design

Approximately 20 h after the surgical implantation of the microdialysis probes, each animal was given an intravenous bolus of 2.5 mg/kg of MTX in PBS (pH 7.4). Whole blood was collected by repeated venipuncture of the jugular vein, and serial SF samples were collected at a flow rate of 1 μL/min. Blood samples (1 mL) were collected into tubes containing EDTA immediately prior to dosing and at 0.08, 0.25, 0.5, 1, 2, 4, 6, 8 h, 10, 12, 24, 32, and 48 h after the start of the infusion. Plasma was collected after centrifugation and stored at –20°C until analysis. SF was collected into a glass microvial at 10-min intervals surrounding the time points described above. To monitor the integrity of the probe membrane, an *in vivo* retrodialysis was done simultaneously by perfusing the structurally distinct molecule TA789 (7) at a concentration of 0.5 μM and monitoring the loss of the compound over time.

Determination of SF Binding, MTX Recovery, and Probe Integrity

The relative recovery in % for the microdialysis probe is defined as the ratio of dialysate concentration to outside concentration × 100 and was determined *in vitro* by no net flux (NNF; Refs. 8,9). The microdialysis probe was inserted into a small hole drilled through the screw cap of a 1.5-mL tube containing PBS (pH 7.4) or pooled donor dog SF. The tube was then placed in 37°C water bath and stirred continuously while the microdialysis probe was perfused with PBS at a flow rate of 1 μL/min. After about 40 min stabilization, a 20-min fraction was collected. Additionally, the binding of MTX in SF was determined by microdialysis, and the probe integrity and recovery *in vitro* were determined by retrodialysis.

Point of NNF Method

This method was used to determine the *in vitro* recovery. A solution of known concentration of MTX (0.5 μM) in PBS or dog SF was used as surrounding medium. The microdialysis probe was perfused at a flow rate of 1 μL/min using a PBS solution containing different concentrations of MTX (0, 0.1, 0.5, 1, and 2 μM) and the net change in MTX concentration in the dialysate was determined. Forty minutes of stabilization separated each new concentration of MTX before dialysate samples were collected. A total of four experiments were performed under these conditions. The net change in the MTX concentration in the dialysate was plotted against the initial perfusate concentration. The intercept of the plot with the x-axis (at net flux = 0) is the point of no net flux and is equal to the free concentration of MTX in the dog SF. The slope of the line can be used to determine the *in vitro* recovery (*in vitro* recovery = –slope × 100). Recovery is reported here as the slope of the regressed line for dog SF. The unbound (free) concentrations (Cu) of MTX *in vivo* were obtained from MTX microdialysate concentrations (Cmioro) during the *in vivo* study as follows:

$$C_u = C_{micro}/Recovery_{in\ vitro}$$

SF Binding by Microdialysis

MTX (0.5 μM) was added to the medium surrounding the probe and the probe was perfused with PBS at a flow rate

of 1 μL/min. The concentration of MTX collected was determined by LC/MS/MS (10).

Retrodialysis

The relative loss of MTX and the internal standard TA789 to the surrounding medium was determined with the same probes used *in vivo* (n = 4). A solution of the two compounds (0.5 μM) was perfused at a rate of 1 μl/min through probes immersed in dog SF. The relative loss of solute during retrodialysis under ideal conditions is identical to the relative recovery by microdialysis, and can be calculated by;

$$L_{retro} = R_{dial} = C_{in} - C_{out}/C_{in}$$

Where C_{in} and C_{out} represent the concentrations of solutes in the perfusate and the microdialysate, respectively. A total of four experiments were performed under these conditions.

Sample Analysis

Microdialysis samples were analyzed by microbore high-performance liquid chromatography/MS/MS. Separation was performed using a Shimadzu 10 AV binary pump (Shimadzu, Tokyo) with a CTC HTS PAL autosampler (LEAP Technologies, Carrboro, NC, USA). A microbore C18 column (Luna, 50 × 1.0 mm, 3 μm) from Phenomenex (Torrance, CA, USA) was used for the separation. Compounds were eluted with a gradient of 0.1% formic acid in water as solvent A and 0.1% formic acid in acetonitrile as solvent B. A flow rate of 0.1 mL/min and an injection volume of 5 μL were used. The Shimadzu pump was equipped with a micro-volume mixing T, which allowed the delivery of a fast gradient under low flow rates, and the CTC autosampler was capable of accurately injecting low microliter volumes of samples without any waste. A Quattro Ultima triple-quadrupole mass spectrometer (Micromass, Manchester, UK) with a Z-spray ionization source operated in the positive ion electrospray mode was used as the detector. Analytes were quantitated by multiple reaction monitoring with a unit mass resolution on both mass analyzers. Transitions from m/z 455 → 308 and 441 → 294 were monitored for MTX and aminopterin (internal standard), respectively with a dwell time of 500 ms per channel. LC/MS/MS data were processed using Masslynx software (Micromass) using linear regression of standard curves and a weighting of $1/x^2$. The linear range was 1 to 1000 nM, and all QC samples were within 15% of the nominal concentration.

Dog plasma was analyzed using LC/MS/MS with on-line turbulent flow solid phase extraction similar to that reported by Wu *et al.* (11). Plasma was injected directly onto a microbore extraction column (Oasis HLB column, 1 × 50 mm, 30 μm, Waters Corporation, Milford, MA, USA) operated under extremely high flow rate (4 mL/min). The separation pump, autosampler, mass spectrometer were the same as those used in the microdialysis sample assay. A linear assay range from 1 to 2500 nM was achieved, and all QC samples were routinely within 15% of the nominal concentrations.

Data Analysis

The pharmacokinetic parameters of MTX were determined for each dog using standard non-compartmental methods. The terminal elimination rate constant (β) was obtained from the terminal slope of a log linear plot of individual plasma or SF concentrations vs. time. The area under the concentration-time curve (AUC), the area under the first moment curve, the mean residence time, the half-life (t1/2), the systemic clearance (Cls), and the volume of distribution at steady state were calculated using standard pharmacokinetic equations.

Determination of the partition coefficient of total MTX between SF and plasma (penetrance), *Ks*, was obtained using the following relationship: *Ks* = area under the curve $(AUC)_{synovial\ fluid}/AUC_{plasma}$ from time zero to infinity following a single dose (12). A z-test was performed for a mean of each parameter against the corresponding mean and standard deviation from either the plasma or SF MTX data reported previously (6). A significant difference existed if $p < 0.05$.

RESULTS

All dogs tolerated the surgery well, with no apparent distress. These dogs could be acclimated to slings or portable infusion pump jackets, thus enabling systemic infusion of test compounds and the serial collection of blood and SF samples. After practice the probe placement was robust, with minimal breakage of the probes because of physical trauma.

Determination of *in Vitro* Recovery and Membrane Integrity

Figure 1 shows the effect of varying MTX concentrations in the perfusate on the net gain or loss of MTX in dialysate using the NNF technique. Linear regression of the data from the four probes and dog SF demonstrated the average periprobe concentration was 0.448 ± 0.028 μM, and the average recovery was 48.4 ± 2.8%. In a separate *in vivo* study, the retrodialysis recovery from SF was 43.5 ± 19.7% from 10 fractions over a 12-h period. This result is in reasonable agreement with that found using the *in vitro* NNF method and dog

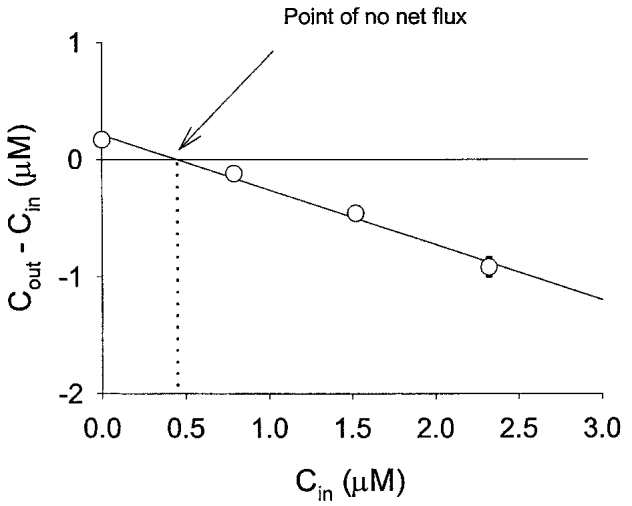

Fig. 1. *In vitro* no net flux determination of recovery of methotrexate from dog synovial fluid. Linear regression of the combined data (n = 4) provides the synovial concentration and the recovery. Synovial concentration (0.448 ± 0.028 μM) is indicated by the dotted vertical line while the slope of the regression line is an estimate of recovery; 48.4 ± 2.8%. Error bars indicate standard deviations.

SF, indicating that microdialysis can be used to monitor quantitatively changing MTX concentrations in SF and that an *in vitro* calibration can provide valuable *in vivo* recovery values if surrounding medium properties, perfusion flow-rate, temperature and stirred conditions are well controlled. Figure 2 illustrates that the membrane integrity was intact for at least 48–50 h after surgical implantation, as the averaged probe recovery (loss in retrodialysis) of the reference compound was constant (83.5 ± 4.8%).

Determination of MTX Pharmacokinetics in Plasma and SF

Figure 3 shows mean ± SD (n = 4) concentration-time profiles of MTX in plasma (total) and SF (free) after intravenous administration of 2.5 mg/kg MTX. Table I summarizes the pharmacokinetic parameters of total MTX in plasma and SF determined by microdialysis and compared to those determined by Lu *et al.* (6) using arthrocentesis. No statistically significant differences were noted among any of the parameters. The unbound fraction of MTX in dog SF determined by *in vitro* microdialysis or *in vitro* NNF was similar, 43.2 ± 2.1 and 49.1 ± 2.8%, respectively (n = 4).

DISCUSSION

Determining the unbound concentrations of drugs at the site of action is a goal of all pharmacokinetic-pharmacodynamic studies, but is often extremely difficult because of the practical limitations of sampling deep tissues. Microdialysis, when combined with sensitive LC/MS/MS analytical methods, offers the potential to collect samples and measure unbound concentrations in real time with minimal trauma. The present study extends the elegant work of St. Clair and Brouwer (5), and demonstrates that this technique can be a powerful approach in defining the systemic and intra-articular pharmacokinetics/pharmacodynamics (PK-PD) of antiarthritic drugs. Methotrexate was chosen as a model drug because it is an important adjunctive therapy for rheumatoid arthritis whose disposition in SF of dogs dosed with MTX has been studied previously by a conventional arthrocentesis method (6).

Microdialysis has been used successfully to determine the drug distribution in many specialized tissues. This technique was chosen here because the synovium is the site of joint destruction in arthritis and the anatomy of the synovial space in larger animals, including dogs and humans, enables the robust placement of the probes and serial collection of interstitial SF. Historically, SF has been collected by arthrocentesis, an invasive and traumatic technique in humans and animals. This method, especially when used for multiple time points, may damage capillaries and cause bleeding in and around the synovium, potentially contaminating the SF with blood. SF concentrations might, therefore, be overestimated, especially after intravenous doses of a drug wherein blood concentrations are very high during the early distribution phase. SF concentrations might also be underestimated by the dilution of SF by edema during the inflammatory response to repeated tissue trauma. Normal homeostasis and drug distribution in the joint space might be altered because of the reduced volume of SF (ca. 50% in stifle joints with 100 μL withdrawn) resulting from serial sampling. Because of these physiologic restrictions as well as the technical difficulty involving drug level measurements in SF clinically, only sparse SF data are available from the same individuals. This may lead to a greater interpatient variability for data analysis when characterizing transsynovial distribution of drugs, as noted by Elmquist *et al.* (12).

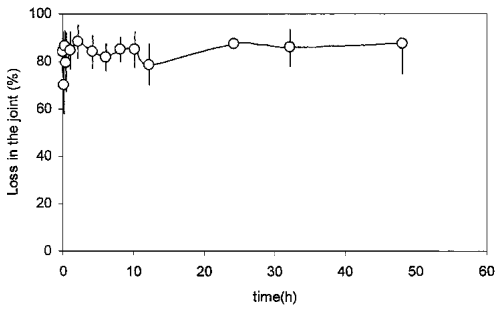

Fig. 2. Membrane integrity vs. time for the microdialysis probes *in vivo* (n = 4). Data are mean ± SD of the loss of the reference compound TA789 measured by retrodialysis.

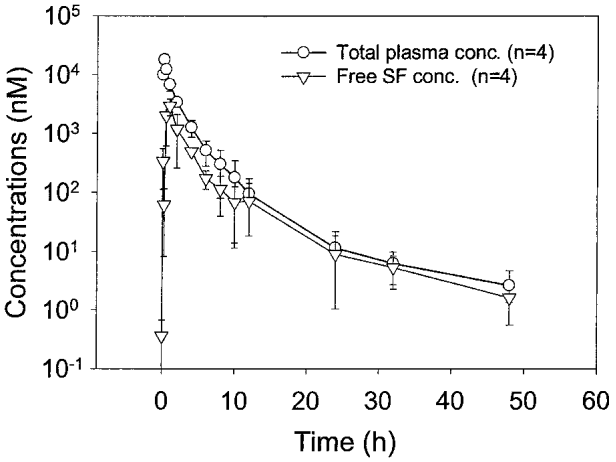

Fig. 3. The concentration-time profiles of unbound MTX in the synovial fluid collected by microdialysis and the total MTX in plasma after an IV bolus of 2.5 mg/kg (mean ± SD, N = 4)

Verification of the performance of the probes both *in vitro* and *in vivo* is critical to ensure the accuracy of the concentrations measured and the calculated pharmacokinetics. The integrity of the probe remained constantly high for approximately 48 h after dosing, long enough to study the disposition of MTX. This result suggests that if any inflammatory cells surrounded the probe, as described by Davies and Lunte (2), they did not significantly diminish the probe's performance. Among commonly used *in vitro* methods for microdialysis calibration, the NNF method is suggested to give robust estimates of recoveries and tissue concentrations (13). DeSousa Maia *et al.* (14) made the similar observations by comparing NNF method with other methods for MTX protein binding and also found that the protein content can directly affect recovery of the microdialysis probe. In the present study, the mean recovery of MTX, 48.4% determined using the no net flux method *in vitro* with SF, was good, and compares reasonably well to the previous results of 45.5% using human plasma (14).

The profile of unbound MTX in synovium shows a rapid penetration into the joint, reaching peak concentrations within 1 h after dosing, then distributing and clearing in parallel with the plasma concentrations of MTX. This profile,

Table I. Pharmacokinetic Parameters of Total Methotrexate in the Plasma and Synovial Fluid of Dog Given an Intravenous Dose of 2.5 mg/kg

	Plasma				Synovial fluid			
	Present		Lu *et al.,* 1995		Present		Lu *et al.,* 1995	
PK parameters	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Dose (mg/kg)	2.5		2.5					
Sampling method	Jugular venipuncture				Microdialysis		Arthrocentesis	
CI (L/h/kg)	0.22	0.04	0.15	0.02				
Vss (L/kg)	0.48	0.13	0.38	0.04				
MRT (h)	2.0	0.5	2.6	0.2	3.1	0.3	3.2	0.2
t1/2 (h)	10.1	4.3	11.2	1.2	14.0	4.1	12.7	3.7
AUC (uM.h)	25.339	4.619	35.317	2.794	17.844	6.174	29.926	5.809
T_{max} (h)					0.9	0.3	0.6	0.1
C_{max} (uM)					6.601	2.115	12.102[a]	
Ks					0.7	0.1	0.8[a]	

[a] Estimated from Lu et al., 1995.

when corrected for total SF concentrations based on an unbound fraction of 44.6%, looks almost identical to the plasma and SF profile of total MTX from three dogs obtained with arthrocentesis (6). The advantage of the microdialysis method, in addition to minimizing trauma, is that unbound concentrations are determined directly, without the need for additional binding studies with SF. For either plasma or SF, the calculated pharmacokinetic parameters (Cl, Vss, mean residence time, t1/2, and T_{max}) were not statistically different between the two methods. It is reasonable to speculate, however, that the 68% greater AUC in SF determined using arthrocentesis may represent an overestimate resulting from blood contamination. This difference is also suggested by the approximately 2-fold greater C_{max} in SF when compared with the published data. The partition coefficient or penetrance of total MTX, *Ks,* reflects the ratio of clearances into and out of the synovial space, and was similar in both studies, at 0.7 and 0.8, respectively. For most nonsteroidal antiinflammatory drugs, the partition coefficient between SF and plasma ranges from 0.4 to 1.3 (15).

Microdialysis has also been used for the determination of the *in vitro* and *ex vivo* plasma protein binding of small molecules, including MTX (14). Human serum and SF differ in the concentration of protein and the presence of other components like hyaluronic acid (16) and, therefore, the unbound and total concentrations may differ between the two matrices. In the present study, the unbound fraction of MTX in dog SF was similar when determined by *in vitro* microdialysis or by the NNF method, 43 or 49%. These results demonstrate that microdialysis provides a useful tool to determine directly the unbound fraction of drugs in SF.

CONCLUSIONS

Overall, microdialysis enables the collection of multiple SF samples from alert individual animals with minimal contamination from blood, and is much less invasive than arthrocentesis. The method used in dogs is mechanically robust and reliable. The consistency of the results from the present study and the previous study using arthrocentesis demonstrates microdialysis provides an accurate estimate of unbound drug concentrations in SF directly. We believe this animal model should prove valuable for the study of the disposition of new anti-arthritis compounds in SF.

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