Pharmacokinetic Studies of Methotrexate in Plasma and Synovial Fluid Following IV Bolus and Topical Routes of Administration in Dogs

G. W. Lu,[1] H. W. Jun,[1,3] M. T. Dzimianski,[2] H. C. Qiu,[1] and J. W. McCall[2]

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Purpose. The pharmacokinetic properties of methotrexate (MTX) in the plasma and synovial fluid (SF) after bolus IV and topical administration were studied in dogs to assess the feasibility of topical delivery of MTX for the treatment of rheumatoid arthritis.

Methods. A MTX gel in Poloxamer 407 containing an absorption enhancer was formulated and topically applied on the elbow and stifle joints of dogs. SF was collected by inserting a needle with syringe into the joint space. Drug concentrations in the plasma, SF and muscle tissues were determined using a HPLC method with fluorimetric detection.

Results. Peak MTX concentrations in SF occurrred at 38 ± 5 min following bolus IV dose, indicating the presence of a substantial diffusion barrier between the plasma and SF. The plasma/SF concentration ratios of 1.16 ± 0.25 were maintained after the attainment of distribution equilibrium between the two compartments. The t½ values in the plasma (11.2 ± 1.2 hr) and SF (12.7 ± 3.7 hr) were similar during the elimination phase, while the MRT in SF (3.24 ± 0.21 hr) was longer than that in plasma (2.56 ± 0.20 hr), probably due to the slow distribution of MTX to SF. After topical dose, MTX concentrations in plasma reached the steady state at ~4 hr, lasting for ~20 hr. The bioavailability of MTX from the gel was 11.8 ± 3.3% of the applied dose, but muscle tissues beneath the gel application site had significantly higher levels of MTX than untreated muscle tissues. There was no statistical difference in SF concentrations of MTX between drug treated and untreated joints 24 hr after topical dose.

Conclusions. Topical delivery of MTX in a hydrophilic gel achieved a sustained C/t profile in plasma and higher drug levels in muscle tissues underneath the dosing site, implicating the potential therapeutic value of the topical formulation.

KEY WORDS: methotrexate; pharmacokinetics; synovial fluid; poloxamer gel; muscle tissue.

INTRODUCTION

Methotrexate (4-amino-10-methylfolic acid, MTX) has been used as an anticarcinogenic agent for more than 40 years, but recently its clinical indication was extended to the treatment of rheumatoid arthritis and related diseases. The basis for its clinical efficacy as an anticarcinogenic agent and for the treatment of rheumatoid arthritis may be due to the inhibitory effect of MTX on DNA synthesis. It was reported that doses much less than those used for cancer treatments were found to be effective on the inhibition of the proliferation of endothelial cells in the synovium [1,2], and synovial membrane fibrosis in patients with rheumatoid arthritis was significantly improved after the weekly administration of MTX [3]. The results of pharmacokinetic studies using MTX for the treatment of rheumatoid arthritis were reviewed in the literature [4]. Since the initial signs of rheumatoid inflammation usually appear around the microvessels of the synovium, the concentration of MTX in synovial fluid (SF) might be a better indicator of the drug's pharmacological activity. It is therefore important to elucidate pharmacokinetic properties of MTX in the blood as well as in SF. The potential benefits of synovial pharmacokinetic data for improving the antirheumatic drug therapy were discussed by Wallis and Smikin [5]. The major adverse effects of oral doses of MTX are gastrointestinal irritation and hepatotoxicity [6-8]. In order to reduce the adverse effects caused by an oral route of administration, while sustaining therapeutic concentrations of MTX at the receptor site, a topical formulation of MTX is being developed in our laboratory. The present study was undertaken to investigate the bioavailability and pharmacokinetic behavior of MTX in the plasma and SF following IV bolus and topical doses in dogs.

MATERIALS AND METHODS

Materials

MTX (USP refined) was supplied by the American Cyanamid Co. (Pearl River, New York). Poloxamer 407 (PF-127, polyoxyethylene-polyoxypropylene block copolymer) was obtained from BASF (Parsippany, New Jersey). HPLC-grade acetonitrile and methanol, analytical-grade N,N-diethyl-m-toluamide (DET), and other chemicals were used as received from commercial sources. Bond-Elut cartridges (C_{18}, 100 mg) were supplied by Varian (Harbor city, California). The water used for the assay and gel preparation was treated with a Millipore purification system (Continental Water Systems Corporation, El Paso, Texas).

IV Administration

Three beagle dogs (12.0 ± 1.8 kg, two males and one female) were used in the study. Each dog received a bolus IV dose of 2.5 mg/kg of MTX. The MTX solution for injection was prepared by dissolving an appropriate amount of MTX powder in a phosphate buffer (pH 7.4). Three ml samples of blood collected from the jugular vein using vacutainer tubes containing EDTA at 0, 5, 15 and 30 min and 1, 2, 4, 8, 12, 24, 36, 48 and 72 hr post-dose were centrifuged at 2500 rpm for 15 min to separate the plasma. Using a 20-gauge needle with a 1-ml syringe, 50 - 100 μl of SF was removed from the elbow and stifle joints at 0.25, 1, 4, 8, 12, 24, 36, 48 and 72 hr following the IV administration. The multiple sampling of SF was achieved with relative ease after careful practice on other dogs. All the plasma and SF samples were frozen at −20°C until analysis which was performed within two weeks after collection.

[1] Department of Pharmaceutics, College of Pharmacy, The University of Georgia, Athens, Georgia 30602.
[2] Department of Parasitology, College of Veterinary Medicine, The University of Georgia, Athens, Georgia 30602.
[3] To whom correspondence should be addressed.

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Preparation of the MTX Gel

The topical gel consisting of 0.8% MTX, 4% DET, 16% ethanol and 20% PF-127 by weight was prepared by the cold method as described by Schmolka [9]. Twenty g of PF-127 was slowly dissolved in 44 ml of cold phosphate buffer (0.025 M, pH 6.5) in an ice-bath with mild stirring. Sixteen ml of 5% MTX in the buffer, 16 g of ethanol and 4 g of DET were mixed in the PF-127 solution with continuous stirring. The mixture was left in a refrigerator for 24 hr to form a clear solution. Due to the thermoreversibility of PF-127, the gel was formed quickly when the solution was left at room temperature.

Bioavailability of the Gel

Four different beagle dogs (two males and two females, weighing 13.8 ± 2.2 kg) were administered an IV bolus dose of 2.5 mg/kg MTX, and blood samples were collected from the jugular vein at 2, 10, and 30 min, and at 1, 2, 4, 8, 12, 24, 36, 48 and 72 hr post-dose. After a two-week wash-out period, the same four dogs participated in the topical dose study. After carefully shaving the skin overlying the elbow joints and stifle of the right foreleg and right hindleg a day prior to the study, a dose of approximately 3.0 g of the MTX gel (2.5 mg of MTX/kg BW) was evenly applied on the shaved skin areas of the right foreleg and right hindleg, covering an area of approximately 30 cm^2 and 70 cm^2, respectively. Blood samples were obtained from the jugular vein at 0.5, 1, 2, 4, 8, 12, 24, 36, 48 and 72 hr after the topical application. The dogs were closely observed for the first 12 hr, and none of the dogs ingested the gel which was applied to the skin covering the joints.

Preparation of SF and Muscle Samples

Another set of four dogs was used for this experiment. A dose of approximately 3 g of the gel (2.5 mg of MTX/kg BW) was applied to the shaved skin areas over the right elbow and right stifle joints of each dog. The left elbow and stifle joints served as non-treated controls. The dogs were deeply anesthetized 24 hr after the dose. A longitudinal skin incision was made on each of the elbow and stifle joints to expose bilateral tissues, and 100 μl of SF was obtained by carefully inserting a 20 gauge needle with the syringe into the joint space. After euthanizing the animals (procedure approved by the Animal Care and Use Committee of the University of Georgia), approximately 1 g of the triceps muscle (foreleg) and quadriceps muscle (hindleg) under the incised skin was obtained from both drug-treated and untreated joints of three dogs. The forceps and scissors used were thoroughly cleansed immediately after each step during the operation to avoid cross-contamination of MTX among the tissues. The samples were frozen at −20°C until analysis which was done within two weeks after dosing.

Quantitation of MTX in the Plasma, SF and Muscle Tissues

An improved HPLC assay for MTX in biological samples using the method of solid-phase extraction, reversed-phase HPLC and post-column photodegradation of MTX followed by fluorimetric detection was developed in our laboratory [10]. One ml of plasma was diluted with an equal volume of a phosphate buffer (0.05 M, pH 7.4), and 50 - 100 μl of SF was diluted 5-fold with the phosphate buffer. The muscle tissue (about 0.2 g) underneath the skin of drug application was removed and homogenized in one ml of phosphate buffer (0.05 M, pH 2.7) using a glass tissue grinder. After centrifuging at 3000 rpm for 10 min, the precipitate was again similarly homogenized, and the two supernatant layers were combined. Samples prepared from the plasma, SF or tissue extracts were applied on a pre-conditioned C_{18} cartridge column for the solid-phase extraction. The cartridge was sequentially washed with 2 ml of phosphate buffer (0.05 M, pH 2.7), 1 ml of sodium hydroxide (0.1 M) and 1 ml of phosphate buffer (0.05 M, pH 2.7). The adsorbed MTX was then eluted with 1.5 ml of methanol. The eluate was evaporated to dryness under nitrogen at 45°C. The residue was dissolved in 0.2 ml of the mobile phase by vortexing for 30 sec, and 0.1 ml of the aliquot was injected into HPLC for assay. The limit of quantitation and limit of detection for methotrexate in plasma using the present method were 100 pg/ml (CV of 17.3%) and 50 pg/ml (the signal/noise ratio of 3), respectively. Other analytical details were described previously [10].

Pharmacokinetic Analysis

The time courses of the plasma and synovial fluid concentrations of MTX were evaluated using a polyexponential equation in the form of $C = \Sigma C_i \exp(-\alpha_i t)$. The area under the C/t curve (AUC) in the plasma or SF was calculated by the trapezoidal rule. The elimination rate constant was estimated from the terminal phase of the plasma concentration/time profiles. The other pharmacokinetic parameters were calculated by the following equations:

Volume of distribution (Vd) = $Dose_{iv}/C_0$

Volume of distribution at steady stage (Vd_{ss}) = $Dose_{iv}AUMC/AUC^2$

Total body clearance (CI) = $Dose_{iv}/AUC$

Half-life ($t_{1/2}$) = $In2/\alpha_3$

MRT = AUMC/AUC.

Bioavailability (F) = $AUC_{topical}/AUC_{iv}$

The Rstrip (MicroMath Inc., Salt Lake City, Utah) and Medusa (Peter Varkonyi, University of Georgia, Athens, Georgia) programs were used for pharmacokinetic simulation and analysis. Data are expressed as the mean ± SD, and the students t test ($p<0.05$) was used for the test of significance.

RESULTS AND DISCUSSION

The time courses of MTX in the plasma and SF after a bolus IV administration in the dogs are shown in Figure 1. The plasma C/t profiles obtained from the three dogs were remarkably similar, and all exhibited three exponential phases. The coefficients of variation (n = 3) for half-life, AUC and MRT shown in Table I were 10.8%, 7.9% and 7.8% for the plasma data and 28.9%, 19.4% and 6.5% for the SF data, indicating relatively small interanimal variability. The calculated Vd, Vd_{ss} and CI were 1.57 ± 0.38 L, 4.52 ± 0.44 L and 1.79 ± 0.32 L/hr, respectively. Peak MTX concentrations in SF were reached at 38 ± 5 min after the IV doses. Thereafter, SF concentrations of MTX declined in parallel to

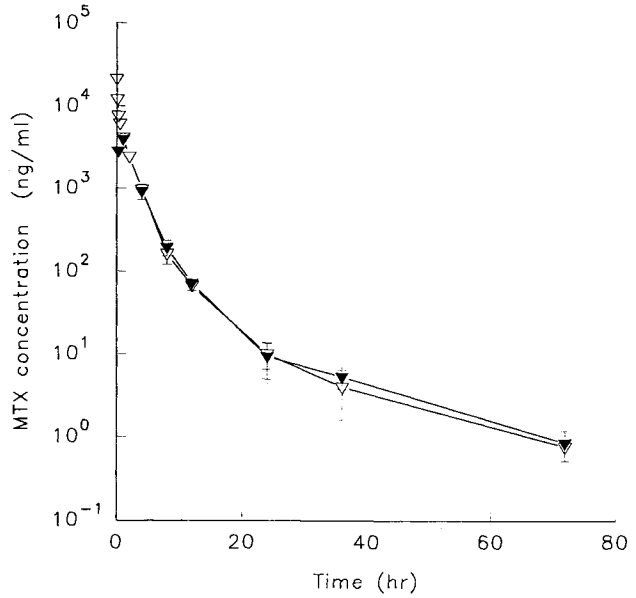

Fig. 1. The MTX concentration-time profiles in the plasma (▽) and synovial fluid (▼) of dogs after a bolus IV dose. (Data shown as mean ± SE, n = 3; Dose of 2.5 mg/kg).

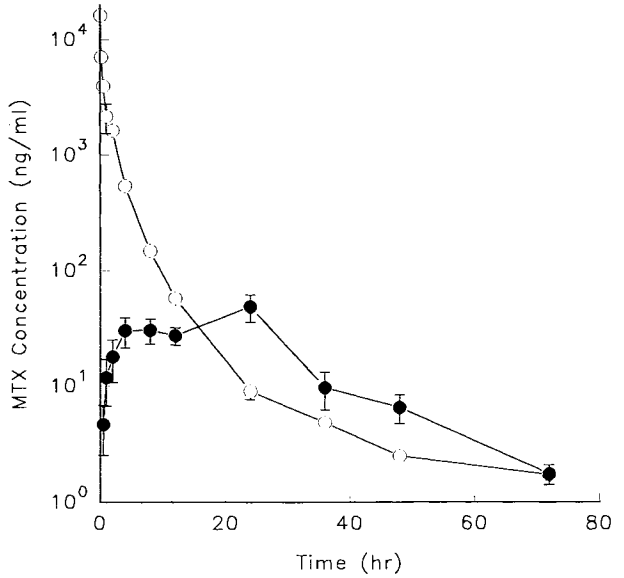

Fig. 2. The MTX concentration-time profiles in the plasma of dogs after administration of bolus IV (○) and topical gel (●). (Data shown as mean ± SE, n = 4; Dose of 2.5 mg/kg).

those in plasma, and no significant differences were observed between SF and plasma concentrations.

As shown in Table I, the half-lives of the elimination phases in the plasma (11.2 ± 1.2 hr) were similar to those in SF (12.7 ± 3.7 hr). However, the MRT for plasma (2.56 ± 0.20 hr) and SF (3.24 ± 0.21 hr) were statistically different ($p<0.05$). The longer MRT in SF than that in plasma could be due to the slow distribution of MTX into SF. The mean ratio of the MTX concentration in SF to that in the plasma found in the dogs was in close agreement with that observed in the patients suffering from rheumatoid arthritis [11]. In the present study, the mean plasma/SF concentration ratio of MTX following the IV dose was 1.16 ± 0.25, while the ratios reported for rheumatoid arthritic patients were 0.99 ± 0.40 between 4-7 hr and 1.06 ± 0.90 between 22-26 hr after an oral dose of MTX [11]. Despite the large individual variation found in the patients, the mean ratios of MTX in healthy dogs and human patients were both close to 1, indicating that the dog may be a good animal model for the study of synovial pharmacokinetics of MTX.

Figure 2 compares the MTX C/t profiles in the plasma following the bolus IV dose and the topical application of the gel on the dog's knee elbow and stifle joints. The topical dose of MTX resulted in a smooth and extended C/t profile without the dose dumping effect. The plasma concentrations reached the steady state in approximately 4 hr post-dose and were continuously sustained for about 20 hr. The mean bioavailability (n=4) of the MTX gel as compared to the IV dose was 11.8 ± 3.3% of the applied dose over the interval of 72 hr.

MTX is known to act as an immunomodulating agent with anti-inflammatory activity for the treatment of rheumatoid arthritis. According to O'Keefe *et al.* [12] and Wilke *et al.* [13], the efficacy and toxicity of MTX appeared to be better correlated with the duration of exposure to MTX than its peak concentrations. Therefore, the sustained blood levels of MTX achieved by the application of the topical gel may enhance the MTX activities on the inhibition of inflammation and cell proliferation in the synovium. As shown in Figure 2, the topical delivery of the MTX gel in dogs resulted in the steady state plasma concentrations in the range of 30-39 ng/ml.

Figure 3 shows that there was no statistical difference in the SF concentrations of MTX between drug treated and untreated joints at 24 hr after the dose. This observation suggests that the distribution of MTX in SF after topical application resulted primarily from the local microcapillaries rather than from direct permeation through the skin layers. However, as shown in Table II, the MTX concentrations in the muscle tissues taken from drug-treated forelegs of the three dogs at 24 hr post-dose were found to be approximately 63% higher than those of untreated forelegs, and the drug concentrations in the muscle tissues of drug-treated hindlegs were 185% higher than those of untreated hindlegs. These results are indicative of higher accumulation of MTX in the muscle tissue after the topical application. The lower concentration of MTX in drug-treated foreleg muscles as compared with drug-treated hindleg muscles was probably due to the greater clearance of MTX from these tissues than the muscle tissues of the hindlegs, which are known to have a

Table I. Pharmacokinetic Parameters of MTX in the Plasma and Synovial Fluid of Dogs Following a Bolus IV Dose of 2.5 mg/kg

	Plasma[a]	Synovial fluid[a]
$t_{1/2}$ (hr)	11.23 ± 1.21	12.71 ± 3.67
AUC (μg · hr/ml)	16.05 ± 1.27	13.60 ± 2.64
MRT (hr)	2.56 ± 0.20	3.24 ± 0.21[b]
T_{max} (hr)		0.64 ± 0.08

[a] Mean ± SD, n = 3.

[b] Significant difference between the plasma and synovial fluid ($p < 0.05$).

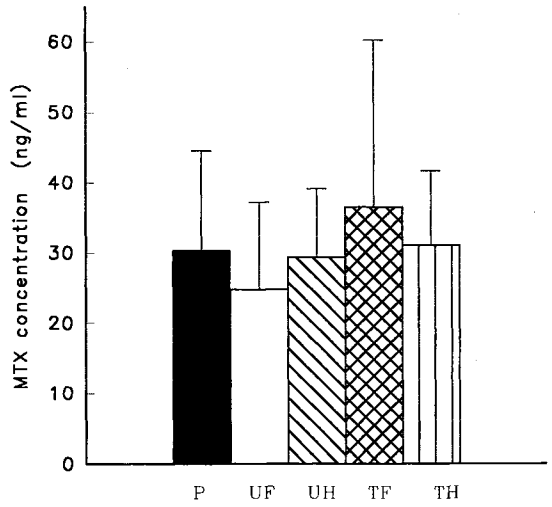

Fig. 3. Plasma and synovial fluid concentrations of MTX in the dogs 24 hr after topical application of the gel. (Data shown as mean ± SD, n = 4; Dose of 2.5 mg/kg; P-plasma, UF-untreated foreleg, UH-untreated hindleg, TF-treated foreleg, TH-treated hindleg).

smaller perfusion rate than the forelegs [14]. Similar results were reported for tissue uptake of topically applied non-steroidal anti-inflammatory drugs (NSAIDs). Singh and Roberts [15] recently demonstrated that in rats several topical NSAIDs, including salicylic acid, indomethacin and naproxen, quickly penetrated various layers of the skin, fascia and muscle tissues underneath the drug applied site. Rabinowitz *et al.* [16] compared the salicylate absorption among topical, articular, and oral routes in dogs and found that the topical dose resulted in the highest drug levels in the muscles, ligaments, tendons and cartilages associated with the drug-treated joint. Therefore, a topical drug delivery may be the route of choice for the treatment of arthritic conditions because higher drug concentrations could be achieved at the clinical sites. In this study, significantly higher levels of MTX were observed in the muscle tissue at joints beneath the drug-treated skin than in the muscle at untreated joints.

Since most studies measuring drug distribution in tissues use radio-labelled drugs, the total radioactivity measured in the tissues could represent both the parent drug and its metabolites. The HPLC method used in this study was specific for MTX and therefore, the results obtained should represent the intact MTX in the plasma, SF and muscle tissues.

In summary, the C/t profiles of MTX in the plasma and SF of dogs after IV bolus and topical routes of administration were presented. To our knowledge, this is the first time such data in dogs have been reported. MTX concentrations in plasma were quickly equilibrated with those in SF, and the plasma/SF concentration ratios remained close to 1 after equilibration. The dog was a good animal model for the study of synovial MTX disposition in man, including the correlation of the plasma and SF drug concentrations. The topical application of the MTX gel exhibited a considerably sustained C/t profile without the dose dumping effect. The distribution of MTX in SF after the topical application appeared to occur primarily through the systemic circulation. MTX accumulated in the muscle tissues underneath the skin where the drug was applied.

Table II. MTX Concentrations in Muscle Tissue (n = 3) Underneath Drug Treated and Untreated Sites at 24 hr after Topical Dose.

Sampling site	MTX concentration, ng/g mean[a] ± SD
Untreated foreleg	21.8[b] ± 8.8
Treated foreleg	35.5[c] ± 6.2
Untreated hindleg	26.2[b] ± 6.5
Treated hindleg	74.7[d] ± 4.0

[a] Significantly different ($p < 0.05$) between different letters.

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