Species Differences in Oral Bioavailability of Methotrexate between Rats and Monkeys

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The contributions of incomplete absorption and a first-pass effect to the low bioavailability (BA) of methotrexate (MTX) were evaluated pharmacokinetically in rats and monkeys which respectively have a lower and higher aldehyde oxidase (AO) activity than humans. Plasma concentration profiles of MTX in rats showed linear and nonlinear pharmacokinetics respectively after intravenous (i.v.) and oral dosing of 0.1, 0.5 or 2.5 mg/kg MTX. In rats, most of the dose was excreted as the parent compound into bile and urine after i.v. dosing of 0.5 mg/kg MTX, while the radioactivity was largely eliminated in expired air after oral dosing of 0.5 mg/kg 14C-MTX. Elimination in expired air fell markedly following antibiotics treatment. 7-Hydroxymethotrexate (7-OH-MTX), formed from MTX by AO, was detected in monkey plasma after i.v. and oral dosing of 0.5 mg/kg MTX, but not in rat plasma. The ratio of the cumulative urinary excretion of 7-OH-MTX to MTX in monkeys was higher after oral dosing than after i.v. dosing. The low BA in rats (10% at 0.5 mg/kg) was shown to be mainly due to incomplete absorption, including limited absorption and degradation to 2,4-diamino-N10-methylpteroyl acid (DAMPA) and glutamic acid (Glut) by the carboxypeptidase of intestinal bacteria. The low BA in monkeys (5% at 0.5 mg/kg) was shown to be mainly due to the extensive first-pass effect, including metabolism to 7-OH-MTX.

Key words methotrexate; 7-hydroxymethotrexate; bioavailability; pharmacokinetics; rat; monkey

Methotrexate (MTX, 2,4-diamino-N10-methylpteroyl-L-glutamic acid), a folic acid antagonist, is a potent inhibitor of dihydrofolate reductase.1) Since it has been widely used as an anti-cancer agent for over 40 years,2) many clinical and non-clinical studies of the pharmacokinetics of MTX have been published. Recently, MTX has been also used in the treatment of rheumatoid arthritis (RA), as a low oral dose intermittent regimen (2.5–25 mg/1 or 3 doses/week).3–5) It has been reported that the plasma concentrations of MTX in RA patients exhibit wide variability,6,7) and that this variability cannot be explained by food intake8) or glomerular filtration rate (GFR).9) It is important to identify the cause of this variable bioavailability (BA) since MTX is severely cytotoxic.

MTX is metabolized to 7-hydroxymethotrexate (7-OH-MTX) by aldehyde oxidase (AO),10) and is degraded to 2,4-diamino-N10-methylpteroyl acid (DAMPA) and glutamic acid (Glut) by the carboxypeptidase of intestinal bacteria.11) The AO activity exhibits species12,13) and strain14) differences in rats. The AO activities in Sprague-Dawley (S.D.) rats from Charles River Japan and Cynomolgus monkeys were lower than that in humans, respectively.12,15,16) In this study, therefore, we compared the pharmacokinetics of MTX at the clinical dose used in RA, 0.5 mg/kg, corresponding to 25 mg per 50 kg body weight, in rats and monkeys. Excretion of 14C-MTX into urine, feces and expired air was studied in control and antibiotic-treated rats in order to evaluate degradation by intestinal bacteria. We also evaluated the reduction in BA not only in rats and monkeys but also in humans.

MATERIALS AND METHODS

Chemicals MTX was supplied by American Cyanamid Co. (Pearl River, New York).14C-MTX (radiochemical purity: 98.2%, specific activity: 1.67 GBq/mmol), radiolabeled at the C1 position of glutamic acid, was obtained from Amer sham Life Science (Little Chalfont, Buckinghamshire). 7-OH-MTX was synthesized at Junsei Chemical Co., Ltd. (Tokyo). All other reagents and solvents were obtained from commercial sources, and were used without additional purification.

Animal Experiments Male SPF S.D. rats (7–10 weeks old, 293 ± 5 g, Charles River, Tokyo) and male Cynomolgus monkeys (6–7 years old, 4.98 ± 0.06 kg, C. V. Primaco Indonesia) were used. Each animal was fasted overnight prior to each study, except for the rat study involving excretion into feces, urine and expired air. Each test compound (MTX, 14C-MTX and 7-OH-MTX) was dissolved in sterile saline containing 2 mol sodium hydroxide per 1 mol test compound to obtain a dosing volume of 2 and 0.5 ml/kg body weight of rats and monkeys, respectively. The solution of 14C-MTX was prepared by mixing equal volumes of MTX and 14C-MTX solutions. Each animal received a single intravenous (i.v.) or oral dose. The i.v. dose was injected into the caudal vein of the rat and the cephalic vein of the monkey. About 250 μl of rat blood and about 1.5 ml of monkey blood were collected using heparinized syringes to obtain plasma at 5, 15 min, 0.5, 1, 2, 4, 6 and 8 h after dosing via the jugular and femoral vein, respectively. To sample bile and urine after i.v. dosing, rats underwent cannulation of the bile-duct and urinary-bladder under ether anesthesia, and were then kept in Bolimian cages and injected after recovery from the anesthesia. Bile and urine were collected up to 8 h after dosing via the bile-duct and urinary-bladder catheters, respectively. After oral dosing, feces, urine and expired air were collected from rats without surgery every 24 h up to 168 h after dosing using glass metabolism cages (Metabolica MC-CO2, Sugiya-
magen). Urine was collected from monkeys without surgery up to 8 h after i.v. or oral dosing.

Rats were orally dosed with 200 mg/kg bacitracin, 200 mg/kg neomycin sulfate and 200 mg/kg streptomycin sulfate, dissolved in sterile saline, twice daily (every 12 h) for 5 d in order to kill the aerobic and anaerobic bacteria in the upper and lower intestinal tract without damaging host tissue cells. Four hours after the final treatment, the animals were given 0.5 mg/kg 14C-MTX orally, and feces, urine and expired air were collected as described above.

Assay MTX and 7-OH-MTX concentrations were simultaneously determined by a slight modification of the method involving solid-phase extraction and reversed-phase HPLC with post-column photo-degradation and fluorometric detection as reported by Beck10 One hundred microliter of plasma, bile or urine was diluted with 50 μl of saline and 1 ml of 50 mM phosphoric acid. One milliliter of each dilution was applied to a solid-phase extraction cartridge (Bond Elut Certify II, Varian), sequentially pre-conditioned with 2.5 ml of methanol and 2.5 ml of 50 mM phosphoric acid. The cartridge was sequentially washed with 2 ml of 50 mM phosphoric acid containing 5% methanol, 2 ml of 50 mM sodium phosphate buffer (pH 8.0) and 2.5 ml of 25% methanol. It was then dried by pumping air through it, followed by elution with 1 ml of methanol containing 2% formic acid. The eluate was collected and evaporated to dryness under nitrogen gas at 60°C. The residue was then dissolved in 0.2 ml of 50 mM phosphoric acid and 0.1 ml was injected onto an ODS column (3 μm, 75X4.6 mm i.d., Develosil® ODS-UG-3, Nomura Chemical Co., Ltd.). The HPLC system consisted of an HPLC unit (LC Module 1, Waters), a post-column photochemical reactor (PHRED, Supelco) equipped with a 254 nm lamp plus a reaction coil (5 m×0.25 mm i.d.) and a fluorometric detector (Exc 350 nm and Em 435 nm, Model 470, Waters). The mobile phase, 50 mM sodium phosphate buffer (pH 6.5)–N,N-dimethylformamide–30% hydrogen peroxide (940:60:2, v/v/v), was delivered at a flow rate of 1.1 ml/min, and MTX and 7-OH-MTX were eluted at retention times of 9 and 18 min, respectively. Fifty microliter of standard solution containing MTX and 7-OH-MTX was added instead of 50 μl of saline to obtain the calibration curve. The calibration curves of MTX and 7-OH-MTX ranged from 0.005 to 10 μg/ml plasma, bile and urine. Each sample for HPLC was kept at 4°C and was measured within a day of sample preparation.

Radioactivity was determined using a liquid scintillation counter (LSC-1000, Aloka) and an automatic quenching correction was applied using the external standard channel ratio method following the mixing with scintillation cocktail (ACS II, Amersham) or combustion using a sample oxidizer. After i.v. dosing, 100 μl of bile or urine was mixed with 10 ml of the scintillation cocktail. After oral dosing, urine was diluted to 100—150 ml with distilled water for cage washing and 500 μl of the dilution was mixed with 10 ml of the scintillation cocktail. Feces were homogenized in 100—200 ml of distilled water and 500 μl of the homogenate was used in the sample oxidizer. Expired air was passed through 250 ml of trapping solution, 2-aminoethanol—2-methoxyethanol (1:1, v/v), in order to collect 14C-CO₂ and 500 μl of the trapping solution was mixed with 3 ml of methanol and 10 ml of another scintillation cocktail (Permafluor® E⁻, Packard).

Pharmacokinetic Analysis Concentrations below the lower limit of quantitation were treated as zero, and the area under the plasma concentration–time curve (AUC0–t) and the mean residence time (MRT0–t) up to 8 h after dosing were calculated by the linear trapezoidal method. The half-life could not be estimated due to entero-hepatic recycling. Bioavailability (BA) was estimated as the ratio of the MTX AUC value or cumulative urinary excretion after oral and i.v. dosing.

When a compound is completely absorbed from the gastrointestinal tract, eliminated only by hepatic and renal routes and obey linear pharmacokinetics, its BA is estimated from the following equation based on the clearance concept:

\[ BA = \frac{E}{D} - \frac{Q_b}{Q_e} \times AUC_{0\rightarrow t} \]  

where \( D \) represents the dose, \( E \) represents the urinary excretion and \( Q_b \) represents the hepatic blood flow (14.7, 92 and 1190 ml/min for rats,20 monkeys31 and humans22 respectively).

Statistical Analysis The significance of any differences among groups was evaluated by the Student Newman-Keuls method (p<0.05) using SigmaStat (SPSS Inc., Chicago).

RESULTS AND DISCUSSION

Plasma Concentrations of MTX in Rats Plasma concentrations of MTX following i.v. and oral dosing of 0.1, 0.5 or 2.5 mg/kg MTX to rats are shown in Fig. 1 and the pharmacokinetic parameters are shown in Table 1. After i.v. dosing, \( C_{max} \) and \( AUC_{0\rightarrow t} \) increased almost proportionally with the dose and no significant difference was observed in the dose-normalized values or \( MRT_{0\rightarrow t} \). After oral dosing, \( C_{max} \) and \( AUC_{0\rightarrow t} \) increased less than proportional to the dose and significant differences were observed in the dose-normalized \( C_{max} \) and \( MRT_{0\rightarrow t} \) for the three doses. The BAs of MTX at 0.1, 0.5 and 2.5 mg/kg were low and dose-dependent, 21, 10 and 8.1%, respectively. 7-OH-MTX, the main metabolite of MTX, was not detected in rat plasma after any of the doses.

Excretion of MTX in Rats The biliary and urinary excretion of MTX and 7-OH-MTX following i.v. dosing of 0.5 mg/kg MTX to rats is shown in Fig. 2. MTX was rapidly excreted into bile and urine, and the cumulative biliary and urinary excretion up to 8 h after dosing was 56±3 and 36±5% of the dose, respectively. 7-OH-MTX was detected in bile.
Table 1. Pharmacokinetic Parameters of MTX in Rats

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>$T_{max}$ (h)</th>
<th>$C_{max}$ (µg/ml)</th>
<th>$AUC_{0-8}$ (µg·h/ml/kg)</th>
<th>MRT$_{0-8}$ (h)</th>
<th>BA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1$^{e}$</td>
<td>i.v.</td>
<td>—</td>
<td>0.208 ± 0.014</td>
<td>0.122 ± 0.014</td>
<td>0.889 ± 0.126</td>
<td>—</td>
</tr>
<tr>
<td>0.5$^{e}$</td>
<td>i.v.</td>
<td>—</td>
<td>(2.08 ± 0.14)</td>
<td>(1.22 ± 0.14)</td>
<td>0.999 ± 0.096</td>
<td>—</td>
</tr>
<tr>
<td>2.5$^{e}$</td>
<td>i.v.</td>
<td>—</td>
<td>1.18 ± 0.07</td>
<td>0.629 ± 0.062</td>
<td>0.646 ± 0.032</td>
<td>—</td>
</tr>
<tr>
<td>0.1 Oral</td>
<td>0.95 ± 0.28</td>
<td>—</td>
<td>0.012 ± 0.002</td>
<td>0.026 ± 0.006</td>
<td>1.42 ± 0.21</td>
<td>21</td>
</tr>
<tr>
<td>0.5 Oral</td>
<td>0.80 ± 0.12</td>
<td>—</td>
<td>0.024 ± 0.003</td>
<td>(0.12 ± 0.02)</td>
<td>0.065 ± 0.013</td>
<td>1.83 ± 0.28</td>
</tr>
<tr>
<td>2.5 Oral</td>
<td>1.6 ± 0.7</td>
<td>—</td>
<td>0.055 ± 0.009</td>
<td>(0.048 ± 0.007$^{h}$)</td>
<td>0.191 ± 0.049</td>
<td>2.74 ± 0.37$^{e}$</td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± S.E. of 4$^{e}$ or 5 animals. The values in parentheses represent the dose-normalized values. $C_{max}$ for the i.v. dose is the plasma concentration at 5 min after dosing. $C_{max}$ and $T_{max}$ for the oral dose are the observed values. BA was estimated as the ratio of the AUC of MTX after oral dosing to that after i.v. dosing. The significance of the difference was evaluated by the Student Newman-Keuls method ($p<0.05$). $^{a}$Significantly different from rats given 0.1 mg/kg MTX orally. $^{b}$ Significantly different from rats given 0.5 mg/kg MTX orally.

Fig. 2. Cumulative Biliary (A) and Urinary (B) Excretion of MTX (●) and 7-OH-MTX (▲) Following Intravenous Dosing of 0.5 mg/kg MTX to Fasted Rats

Bile and urine were collected via bile-duct and urinary-bladder catheters. Data are expressed as the mean ± S.E. of 5 animals.

and urine, but the cumulative biliary and urinary excretion was low, 0.63±0.05 and 0.039±0.013% of the dose, respectively.

Excretion of $^{14}$C-MTX in Rats The excretion of radioactivity following i.v. and oral dosing of 0.5 mg/kg $^{14}$C-MTX to rats is shown in Fig. 3. The cumulative biliary and urinary excretion up to 8 h after i.v. dosing was 63±2 and 30±2% of the dose, respectively, similar to that of MTX. After oral dosing to rats untreated with antibiotics, the excretion into expired air was marked, 46±3% of the dose, while the excretion into feces and urine was 9.9±1.4 and 33±3% of the dose, respectively. Antibiotic treatment did not affect the increase in body weight and the intestinal morphology, except for the enlarged cecum (data not shown). After oral dosing to the antibiotic-treated rats, the excretion into expired air fell to 0.7±0.1% of the dose, and the cumulative excretion into feces (55±2% of dose) and urine (34±2% of dose) was comparable with that into bile and urine, respectively, after i.v. dosing of $^{14}$C-MTX. These findings indicate that the elimination of radioactivity in expired air after oral dosing of $^{14}$C-MTX is due to the degradation of $^{14}$C-MTX to
DAMPA and $^{14}$C-Glu by the carboxypeptidase of intestinal bacteria.\(^{(1)}\)

**Plasma Concentrations and Excretion of MTX in Monkeys**

The plasma concentrations and urinary excretion of MTX and 7-OH-MTX following i.v. and oral dosing of 0.5 mg/kg MTX to monkeys are shown in Fig. 4 and the pharmacokinetic parameters are shown in Table 2. The BA of MTX in monkeys was estimated using the cumulative urinary excretion of MTX due to the very low plasma concentration profile after oral dosing. Although the assessment of BA on the basis of the amount excreted is not valid for drugs with highly variable elimination, the assessment for MTX in monkeys is considered to be acceptable due to the small variability in the urinary excretion. The BA of MTX at 0.5 mg/kg was low, 4.8%, and 7-OH-MTX was detected in monkey plasma and urine. The ratio of the cumulative urinary excretion of 7-OH-MTX to that of MTX was 0.49 and 0.83 for i.v. and oral dosing, respectively.

**Pharmacokinetic Analysis of the Low BA of MTX in Rats and Monkeys**

Plasma concentrations of MTX following i.v. dosing of 0.1, 0.5 or 2.5 mg/kg MTX to rats obeyed linear pharmacokinetics, i.e., no significant difference was observed in the dose-normalized $C_{\text{max}}$, AUC$_{0-\infty}$ or MRT$_{0-\infty}$ (Table 1). Most of the dose was excreted as unchanged compound into bile and urine after i.v. dosing of 0.5 mg/kg MTX to rats (Fig. 2), and 7-OH-MTX was not detected in plasma after i.v. or oral dosing of MTX. These results suggest less metabolism to 7-OH-MTX and less degradation to DAMPA and Glu in rat tissue. Accordingly, it is valid to estimate the BA of MTX in rats using Eq. 1, assuming linear pharmacokinetics and only hepatic and renal elimination. When MTX was completely absorbed from the gastrointestinal tract, the BA of MTX at 0.5 mg/kg in rats was estimated to be 86%. The actual BA at 0.5 mg/kg, however, was 10% (Table 1). These findings suggest that the reduction of 14% ($=100-86$) and 76% ($=86-10$) can be attributed to a first-pass effect and incomplete absorption, respectively, i.e., 24% ($=100-76$) is absorbed from intestine and 10% ($=24-14$) circulates systemically (Fig. 5). The incomplete absorption was considered to result from limited absorption and degradation in the intestinal tract, since non-linearity with saturated absorption was observed in the plasma concentrations after oral dosing (Table 1) while antibiotic treatment reduced the excretion of radioactivity into expired air (Fig. 3).

When Eq. 1 was applied to monkeys, the BA of MTX at

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### Table 2. Pharmacokinetic Parameters of MTX and 7-OH-MTX in Monkeys

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>Compound</th>
<th>$T_{\text{max}}$ (h)</th>
<th>$C_{\text{max}}$ ($\mu$g/ml)</th>
<th>AUC$_{0-\infty}$ (mg·h/ml)</th>
<th>MRT$_{0-\infty}$ (h)</th>
<th>BA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>i.v.</td>
<td>7-OH-MTX</td>
<td>0.88±0.13</td>
<td>1.92±0.09</td>
<td>0.615±0.087</td>
<td>0.583±0.047</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>MTX</td>
<td>1.5±0.3</td>
<td>0.008±0.000</td>
<td>n.e.</td>
<td>n.e.</td>
<td>4.8±1.1</td>
</tr>
<tr>
<td>0.5</td>
<td>Oral</td>
<td>7-OH-MTX</td>
<td>2.0±0.0</td>
<td>0.016±0.006</td>
<td>n.e.</td>
<td>n.e.</td>
<td></td>
</tr>
</tbody>
</table>

Data are expressed as the mean±S.E. of 4 animals. The values in parentheses represent the dose-normalized values. $C_{\text{max}}$ of MTX for the i.v. dose is the plasma concentration at 5 min after dosing. $C_{\text{max}}$ of 7-OH-MTX for the i.v. dose, $C_{\text{max}}$ and $T_{\text{max}}$ of MTX and 7-OH-MTX for the oral dose are the observed values. BA was estimated as the ratio of the cumulative urinary excretion of MTX after oral dosing (0.9±0.2% of dose) to that after i.v. dosing (21±4% of dose). n.e., not estimated.

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![Fig. 4. Plasma Concentrations (A) and Urinary Excretion (B) of MTX (●, ○) and 7-OH-MTX (▲, △) Following Intravenous (Closed Symbol) and Oral (Open Symbol) Dosing of 0.5 mg/kg MTX to Fasted Monkeys.](image)

Data are expressed as the mean±S.E. of 4 animals.

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![Fig. 5. Scheme of the Intestinal Absorption of MTX Following Oral Dosing of 0.5 mg/kg MTX in Rats (Open Arrow) and Monkeys (Closed Arrow). Each value is the mean % of the dose.](image)
0.5 mg/kg was estimated to be 43%. Since the actual BA at 0.5 mg/kg was approximately 5% (Table 2), this suggests that the reduction of 57% (=100−43) and 38% (=43−5) can be attributed to the first-pass effect and incomplete absorption, respectively, i.e., 62% (=100−38) is absorbed from the intestine and 5% (=62−57) circulates systemically (Fig. 5). The high first-pass effect in monkeys is likely to result from the high level of AO activity, since 7-OH-MTX was detected in monkey plasma but not in rat plasma, and the ratio of the cumulative urinary excretion of 7-OH-MTX to MTX after oral dosing was higher than that after i.v. dosing (Fig. 4). It has been reported that the intestinal absorption of MTX involves a carrier-mediated influx and efflux system. However, the intestinal efflux system of MTX is considered to play a minor role in rats, since most of the radioactivity was excreted into bile and urine after i.v. dosing of 14C-MTX (Fig. 3). It has also been reported that the intestinal bacteria of monkeys are similar to those of humans, and that there are many more bacteria in the duodenum, jejunum and ileum of the rat than in the human. The lower contribution of incomplete absorption in monkeys compared with rats, therefore, appears to result from fewer small-intestinal bacteria and/or the higher carrier-mediated influx.

Low oral doses of MTX have been used to treat RA and the plasma concentrations of MTX in RA patients exhibit wide variability. Since the causes of this variability have not been identified, the reduction in the BA of MTX in RA patients was estimated in the same manner. The BA of MTX in RA patients, estimated from Eq. 1, was approximately 100%, based on the pharmacokinetic parameters at 15 mg. Since the actual BA is 73%, the reduction of 27% (=100−73), which is similar to monkeys (38%) compared with rats (76%), can be attributed only to incomplete absorption. This is understandable, considering the low AO activity, low biliary excretion and similar intestinal bacteria to monkeys are shown in humans. The BA of MTX in humans is high at less than 30 mg/m² (corresponding to 44 mg), and falls to 10−20% at over 80 mg/m² (corresponding to 118 mg) due to saturated absorption. The incomplete absorption in humans, therefore, does not seem to be caused by limited absorption. The human data used for the estimation, however, indicate high and uniform BAs (59−83%, n = 7). The factors affecting the wide variability in the plasma concentrations of MTX in RA patients are expected to become clearer when a set of human data with wide variability is obtained.

In conclusion, the BA of MTX exhibits a species difference. The low BAs in rats and monkeys were due to different major factors, i.e., incomplete absorption in rats and an extensive first-pass effect in monkeys. The estimation method used in this report is useful for evaluating the factors affecting the gastrointestinal absorption and BA of MTX.

REFERENCES