Potentiated Decrease of Plasma Folate Levels Caused by the Coadministration of Folic Acid in Rats Treated with Methotrexate

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ABSTRACT. The decrease of plasma 5-methyltetrahydrofolic acid (5-MF) levels, postulated as an indicator of folate status, was studied following the administration of both methotrexate (MTX) alone and MTX with folic acid (FA) using rats as our experimental model. Blood and urine samples were serially collected over a 9 hr period after the administration of MTX, MTX with FA and from a control group to examine the plasma kinetics and the renal clearance of 5-MF. The pharmacokinetics of MTX and the plasma protein binding of 5-MF were also examined. The concentrations of these analytes were assayed using high performance liquid chromatography (HPLC). MTX administration produced decreased plasma 5-MF levels. This observed decrease was potentiated by oral FA administration, suggesting that the folate status was more severely altered by the coadministration of FA. The renal clearance of 5-MF also increased dose-dependently with FA (0.05–5 mg/kg) coadministration. The plasma protein binding of 5-MF was not affected by the FA administration, which indicates that the fraction of 5-MF that was filtered through the glomerular apparatus appeared to be unchanged. In addition, the pharmacokinetic profiles of MTX also appeared not to be affected by the addition of FA. We conclude that the inhibition of reabsorption of 5-MF in the renal tube by concurrent administration of MTX and FA must be one of the causal factors for the demonstrated decrease in the plasma 5-MF levels in rats. — KEY WORDS: folic acid, methotrexate, 5-methyltetrahydrofolic acid, rat, renal clearance.

MATERIALS AND METHODS

Animals: Female rats (Jcl-Wistar, weighing from 190–210 g, 12–14 weeks old) purchased from Clea Japan Inc., Tokyo, Japan were used in this experiment. These animals were fed mashed feed (Clea Japan Inc.) and allowed to consume water ad libitum. Food was withheld for 24 hr prior to the drug administration. A commercial formulation of MTX (Methotrexate®, Lederle Japan, Ltd., Tokyo, Japan) was used. Folic acid (FA) (Sumika Fine Chemicals Co., Ltd., Osaka, Japan) was suspended in 0.5% carboxy methyl cellulose solution orally. The rats were housed in metabolic cages during this experimental period.

Study protocol:

1. Plasma and urine kinetics of 5-MF: The 5-MF levels in the plasma and urine were determined from specimens collected following the intravenous administration of MTX and MTX with oral FA. The experimental rats were randomly divided into 5 groups, with 4 rats in each group. The groups were the control group, MTX (0.3 mg/kg) alone group, and 3 groups given MTX and oral FA (0.05, 0.5, 5.0 mg/kg). The rats of the control group were administered saline intravenously and 0.5% carboxy methyl cellulose solution orally. The rats were housed in metabolic cages during this experimental period.

Blood samples were obtained from the rat tail vein prior to drug administration, then following the drug administration at 1, 3, 6 and 9 hr. These whole blood specimens were centrifuged at 2,000 G for 5 mins to separate the plasma. Sodium ascobate (2%) was added to all of the plasma aliquots (2 mg/ml of plasma) as an antioxidant and these samples were stored at - 80°C until the assays were performed. The assay was performed within 1 week after...
sampling.

All urine produced by each animal, during the experimental period from 0 to 3, 3 to 6 and 6 to 9 hr after drug administration, was collected. After collection, the sample volume of each specimen was measured and sodium ascorbate was added (2 mg/ml of urine) to each sample. These sample aliquots were then placed into test tubes and stored at -80°C until they were assayed. The assay was performed within 1 week after sampling.

The renal clearance of 5-MF was calculated by dividing the excreted amount of 5-MF in the urine sample collected during the sample period by the plasma 5-MF levels from the specimen collected at the middle of the urine collection period.

2. Plasma kinetics of MTX: The plasma MTX levels were determined from specimens collected after the intravenous administration of MTX and MTX with oral FA. The rats were divided into 2 experimental groups, consisting of 4 rats in each group. The 2 groups were the MTX (0.3 mg/kg) alone group and the MTX with oral FA (5 mg/kg) group. Blood samples were obtained from the rat jugular vein at 1, 2 and 3 hr following the drug administration. These whole blood specimens were centrifuged at 2,000 G for 5 min to separate plasma. These plasma specimens were stored at -20°C until they were assayed. The assay was performed within 1 week after sampling.

A one compartment open model was used for the pharmacokinetic analysis. The pharmacokinetic parameters for each rat were obtained utilizing a curve-fitting program ‘MULTI’ [17]. The biological half-life was calculated by dividing 0.693 by the elimination rate constant (kel). The volume of distribution (Vd) was calculated by dividing the MTX dose by the area under the concentration-time curve (AUC). The AUC was determined as the sum of the value obtained by the trapezoidal method and that of the last MTX concentration divided by kel.

3. Protein binding of 5-MF: The plasma protein binding for 5-MF was determined in vivo. The rats were injected MTX intravenously, and MTX with oral FA administration. These rats were divided into 3 experimental groups, with each group consisting of 4 rats. The rats of first group were received MTX (0.3 mg/kg) only, those of second received MTX and oral FA (5.0 mg/kg) and of third were untreated. Blood was obtained from the abdominal aorta, under ether anesthesia, 3 hr after the drugs were given. These whole blood samples were then centrifuged at 2,000 G for 5 min to separate the plasma. Sodium ascorbate solution (4%) was added (2 mg/ml) to each plasma specimen as an antioxidant. The protein binding percentages were determined using an ultrafiltration kit (Centrifree® micropartition system, Grace Japan Co. Ltd., Tokyo, Japan). Each plasma sample (1.0 ml) was ultrafiltered using centrifugation at 2,000 G for 6 min. Then 100 µl of the ultrafiltrate was injected into a high performance liquid chromatograph with an electro-chemical detector (HPLC-ECD) to determine the concentration of unbound 5-MF. The total concentration of 5-MF was determined as described below. The calculation of the protein binding percentage was as follows:

\[
\text{Protein Binding %} = \frac{[\text{total conc.} - \text{unbound conc.}]}{\text{total conc.}} \times 100
\]

Analytical methods and instrumentation:

1. Chemical determination of MTX: The plasma and urine 5-MF levels were assayed using the HPLC-ECD method by Shimada [11] with some modifications. Our apparatus consisted of a pump (Model LC-9A, Shimadzu, Kyoto, Japan), a fixed loop injector (Model 7125, Rheodine, Cotati, CA), a phenyl-bonded phase column (Nova-pak phenyl, 100 x 8 mm, Waters, Milford, MA), an amperometric detector with a glassy carbon electrode (Model LC-4C, Bioanalytical Systems Inc., IN) and a data processor (Chromatopac C-R4A, Shimadzu, Kyoto, Japan). The mobile phase was a mixture of 20 mM acetic acid buffer, containing 0.1 mM EDTA (pH 3.6 for plasma, pH 4.0 for urine) and acetonitrile (97.5:2.5,v/v). The flow rate of the mobile phase was 0.8 ml/min. The applied potential of the ECD was +240 mV. The plasma specimens (100 µl) were each deproteinized by the addition of an equal volume of 0.5 M perchloric acid and centrifuged at 2,000 G for 2 min. The resulting supernatant was filtered through a 0.45 µm filter (Chromatodisk 4A, Biofield Inc., Tokyo, Japan), and a 100 µl aliquot was injected into the HPLC-ECD system.

The urine samples were each diluted with 0.2% sodium ascorbate solution and then filtered through a 0.45 µm filter (Chromatodisk 4A). An aliquot of 100 µl was injected into the HPLC-ECD system.

2. Chemical determination of MTX: The plasma MTX levels were obtained using HPLC with an ultraviolet detector (HPLC-UVD). This HPLC-UVD apparatus consisted of a pump (Model 576, Gasukurokogyo, Tokyo, Japan), a fixed loop injector (Model 7125), an analytical column (SCX, 25 cm x 2.9 mm, Whatman, Clifton, NJ), a UV detector (Model SPD-6A, Shimadzu) and a data processor (Chromatopac C-R4A). The mobile phase was a mixture of 20 mM monobasic ammonium phosphate buffer (pH 1.6) and acetonitrile (9:1, v/v). The flow rate of the mobile phase was 2 ml/min. The plasma specimens (200 µl) were each deproteinized by the addition of 500 µl of acetonitrile and then centrifuged at 2,000 G for 2 min. The supernatant was next filtered through a 0.45 µm filter (Chromatodisk 4A). An aliquot of 100 µl was injected into the HPLC-UVD system.

Statistical analysis: The mean and standard deviation of the 5-MF and MTX values obtained from this experiment were calculated for each collection or collection period. Statistical significance of the results of “plasma kinetics of
RESULTS

Plasma levels of 5-MF: The plasma 5-MF levels profiles of the rats following MTX alone and MTX with oral FA administration are shown in Fig. 1. The plasma 5-MF level decreased significantly 9 hrs after receiving intravenous MTX. The magnified decreases for the 5-MF levels appeared to be dose-dependent, based upon the increasing FA coadministrations.

Urinary excretion of 5-MF: The cumulative urinary excretion of 5-MF following the intravenous injection of MTX are represented in Fig. 2. The cumulative total amount of 5-MF that was excreted, increased slightly, after the MTX injection compared to the control group, but it was not statistically significant. This observed increase attributable to MTX treatment only, was further amplified by the coadministration of FA. However, this increase was not dose-dependent, as the excretory amount was larger for the FA 0.5 mg/kg group than for the FA 5.0 mg/kg group.

The renal clearance values for 5-MF after treatment with MTX and FA are listed in Table 1. These clearances appear to exhibit a FA dose-dependent increase.

Plasma protein binding of 5-MF: The plasma protein binding values of 5-MF were determined from the plasma specimens obtained from rats given MTX (0.3 mg/kg) only, MTX with oral FA (5.0 mg/kg) and a control group. The results are listed in Table 2. No statistically significant differences were detected between these 3 groups. This indicates that the glomerular filtration rate of 5-MF is not affected by the coadministration of FA.
Pharmacokinetic profiles of MTX: The pharmacokinetic profiles resulting from the administration of intravenous MTX (0.3 mg/kg) alone and with oral FA (5.0 mg/kg) were studied in rats. The pharmacokinetic parameters examined are shown in Table 3. No statistically significant differences were discovered between these 2 experimental groups.

**DISCUSSION**

The plasma 5-MF levels of rats decreased following the intravenous administration of MTX. This observed decrease was amplified by the coadministration of FA. This suggests that the folate status was severely affected by the coadministration of FA. There have been some controversial discussions [8] regarding the propriety of the combination of FA with MTX to reduce the severity of or to overcome, the folate deficiency which is frequently encountered during MTX therapy for the treatment of rheumatism. However, from the results obtained in this study, using rats as our experimental model, we propose that the use of both FA and MTX together is inappropriate, because it does not offer any improvement in the folate status over the administration of MTX alone.

The reduced folates are both produced and regenerated in the liver. 5-MF is a major component of the systemic folates which are filtered by the renal glomerular apparatus and nearly all are reabsorbed from the renal tube via the carrier protein in the brush border. The intestinal reduced folates also are absorbed from the small intestine via the carrier protein in the intestinal mucosa. These reabsorptive mechanisms play a vital role in folate homeostasis. The present study demonstrates that the treatment with oral FA potentiates the folate deficiency that was induced by MTX. We believe that this escalation must be attributable to the severe interference by FA on these homeostasis mechanisms. In conclusion, the following 2 primary factors for the potentiated decrease of plasma 5-MF levels should be examined, 1) the increase in the renal clearance of 5-MF, demonstrated in the present study, and 2) an interruption of the entero-hepatic circulation of endogenous folate including 5-MF, which has previously been reported [5].

The plasma concentration of 5-MF is approximately 50 ng/ml and the plasma protein binding rate is 65% (Table 2). Therefore, 17.5 ng/ml of 5-MF (35% of 50 ng/ml) will be subjected to glomerular filtration in the kidneys. As the flow rate of plasma in the rat glomerular apparatus is stated to be 2.5–3.3 L/day [3], then the filtered amount of 5-MF is estimated to be 0.5 mg/day. The amount of renal excretion of 5-MF was less than 1 µg/kg/day [6] or 1.4 µg/kg/day in this study. Therefore, more than 99% of the filtered 5-MF will be reabsorbed in the renal tube. The active transport system for the reabsorption of endogenous folate has been described and documented in the renal tube, for which the carrier protein, folate binding protein (FBP), is involved [9, 10]. The filtered 5-MF in the glomerular apparatus is mostly reabsorbed due to the action of this carrier protein. It has been demonstrated that the affinity of FA to FBP is 6 times higher than for 5-MF [15]. This suggests that FA competes with 5-MF for the transport protein FBD. Consequently, the reabsorption of 5-MF must be significantly reduced by the coadministration of FA and MTX, leading to a conspicuous increase in the urinary excretion of 5-MF.

Large amounts of reduced folates, including 5-MF, tetrahydrofolic acid, 10-formyltetrahydrofolic acid and 5–10-methyltetrahydrofolic acid are produced in the liver of rats [12]. A portion of the folates produced is excreted into the systemic circulation and into the intestinal lumen via bile. Strum [15] has reported that the brush border in the intestinal membrane has a carrier protein, FBP, which absorbs folate derivatives. FA shares the carrier FBP with the reduced folate derivatives, thereby decreasing the reabsorption of the reduced folates. This binding competition for the intestinal carrier FBP between FA and endogenous folates, as was previously described in the renal tube inhibits the entero-hepatic circulation of folates, leading to a significant decrease of plasma 5-MF levels. Kudo et al. [5] have also reported that oral FA competes in the intestinal transport system with endogenous folates for bile. Consequently, the potentiated decrease of plasma 5-MF levels after MTX and FA have been given must be due to the competition for the carrier FBP binding FA with endogenous folates in both the renal tube and intestinal mucosa, inhibition of renal reabsorption, and the inhibition of enterohepatic circulation of reduced folate by FA.

In conclusion, we propose that the utilization of concurrent FA to correct for these side effects of MTX administration is inappropriate, because the folate deficiency initially induced by MTX is severely aggravated by the FA coadministration.

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**Table 3. Pharmacokinetic parameters of MTX (0.3 mg/kg) after intravenous injection of MTX alone or with oral FA (5.0 mg/kg)**

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Volume of distribution Vdext (L/kg)</th>
<th>Total body clearance TBCL (L/hr/kg)</th>
<th>Half life t1/2 (hr)</th>
<th>Area under curve AUC (ng·hr/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTX</td>
<td>1.18 ± 0.17</td>
<td>1.71 ± 0.37</td>
<td>0.90 ± 0.17</td>
<td>284 ± 49</td>
</tr>
<tr>
<td>MTX + FA</td>
<td>1.26 ± 0.26</td>
<td>1.72 ± 0.80</td>
<td>0.84 ± 0.12</td>
<td>258 ± 56</td>
</tr>
</tbody>
</table>

Each value represents mean ± SD (n=4). No statistically significant variations were detected (Student’s t-test).
REFERENCES


