Inhibition of Renal Tubular Transport of Methotrexate by Probenecid

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SUMMARY

The mechanism of excretion of methotrexate (MTX) has been investigated in the monkey. Under steady-state conditions of varied plasma levels of MTX, it was determined that MTX was excreted by renal tubular transport as well as by glomerular filtration. The maximum rate of renal tubular transport of MTX (81 μg/min) was attained at plasma levels of MTX from 6 to 8 μg/ml. Correspondingly, the rate of clearance of MTX from plasma was shown to diminish from a value that was 3-fold greater than the glomerular filtration rate at plasma levels of MTX of 0.04 to 1.7 μg/ml to values approximating glomerular filtration rate at plasma levels of MTX from 6 to 32 μg/ml.

Pretreatment of animals with probenecid (700 mg/sq m) totally inhibited renal tubular transport of MTX when MTX was administered in doses from 1.8 to 621 mg/sq m. Following inhibition of renal tubular transport of MTX by probenecid, steady-state plasma levels of MTX were seen to be double corresponding levels determined in non-probenecid-pretreated controls given comparable doses of MTX. The total urinary excretion of MTX in animals pretreated with probenecid (700 mg/sq m) was reduced by a factor of 2.6 from values determined in non-probenecid-pretreated control animals receiving similar varied doses of MTX (1.8 to >600 mg/sq m).

The mode of i.v. injection of MTX was seen to affect the concentration of MTX in plasma. Initial loading followed by continuous sustaining infusion of MTX provided stable and higher levels of MTX in plasma than was determined in controls or in experimental animals pretreated with probenecid and receiving identical doses of MTX by single bolus injection.

INTRODUCTION

Intensive use of high doses of MTX has been of demonstrated effectiveness in the treatment of various cancers (9, 10). It would be of considerable clinical value, therefore, to establish methods by which consistently high and stable levels of MTX could be maintained in plasma in the clinical setting. A major factor militating against achievement of these ends is the rapid excretion of MTX determined in man as well as in experimental animals.

We undertook this experimental study to develop a systematic approach to the maintenance of high plasma levels of MTX by modification of mechanisms responsible for rapid renal excretion of MTX and by providing a mode of i.v. injection of the drug that would further augment the long-term maintenance of desired plasma levels of MTX.

MATERIALS AND METHODS

Animals and Materials Adult monkeys (Macaca mulatta, 3.5 to 4.7 kg body weight), as supplied commercially to Roswell Park Memorial Institute, were used in all studies. Experiments were performed under phenylcyclohexide hydrochloride anesthesia (0.25 mg/kg body weight i.m.; Sernylan; Parke, Davis & Co., Detroit, Mich) and atropine sulfate (0.1 mg i.m.). The following isotopically labeled compounds were used: [3,5-3H]MTX (21 to 25 mCi/mg), obtained from Amersham/Searle Corp., Arlington Heights, Ill., and Monsanto Research Corp., Dayton, Ohio; [methoxy-3H]inulin (0.11 mCi/mg), [carboxyl-14C]inulin (0.005 mCi/mg), and [1-14C]PAH (0.09 mCi/mg), obtained from New England Nuclear, Boston, Mass. Human radioiodinated 1111-serum albumin (0.005 mCi/mg) was obtained from E. R. Squibb & Sons, Inc., New Brunswick, N. J. Prior to use isotopically labeled MTX was purified according to the column chromatographic method of Oliverio (18) and was checked by radiochromatography to assure >98% radiopurity. Nonlabeled MTX for injection was obtained from Lederle Laboratories, Pearl River, N. Y. Nonlabeled PAH as a sterile 20% (w/v) aqueous solution was obtained from Merck, Sharp & Dohme, West Point, Pa. Nonlabeled inulin as a 10% (w/v) aqueous solution was obtained from Arnarn-Stone Laboratories, Inc., Mount Prospect, Ill. Probenecid as a white powder to be brought into solution with a minimum volume of 1 N NaOH was obtained from Merck, Sharp & Dohme. Solubilized probenecid was brought to a concentration of 50 mg/ml in 0.9% NaCl solution for i.v. injection. Balanced physiological salt solutions for injection were obtained as Ringer's injection from Travenol Laboratories, Inc., Morton Grove, Ill. All other drugs and...
Renal Excretion of MTX in the Monkey

Procedures for Injection and Sampling. The femoral artery on one side and both femoral veins were cannulated to a length of 7.5 cm with 30-cm segments of polyethylene tubing (inner diameter, 0.045 inch; outer diameter, 0.062 inch; PE 160; Clay-Adams, Inc., Boston, Mass.) to provide access for i.v. infusion and arterial sampling. The femoral artery on the other side was similarly cannulated for the recording of arterial blood pressure by connection to a calibrated Statham transducer, Model P23AA, and a Sanborn recording system. A size 8 French pediatric feeding tube (C. R. Bard, Inc., Murray Hill, N. J.) was transurethrally inserted to serve as a bladder catheter. In order to ensure consistent hydration and urine flow (19), 20 ml of Ringer's solution were infused i.v. at a rate of 0.97 ml/min prior to the start of indicator solute of MTX infusion. Thereafter, a continuous i.v. infusion of Ringer's solution was maintained at a rate of 0.365 ml/min by means of a Harvard infusion pump throughout the entire experimental period. This method of hydration of experimental animals permitted a constant urine production of 0.08 to 0.11 ml/min during the period of renal solute clearance study. An inlying rectal temperature probe was connected to a recording thermistorometer (Model 425C; Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio). Under all conditions of use, probenecid was given as a bolus i.v. infusion approximately 30 min prior to the infusion of MTX.

For the determination of renal clearance of MTX, inulin, or PAH, 0.1 mCi of the corresponding isotope was added to specified amounts of the appropriate nonlabeled moiety and brought to a volume of 10.5 ml. Of this volume, 0.5 ml was saved for determination of specific activity (cpm/µg); the remaining 10 ml were injected i.v. by means of a Harvard infusion pump as a loading dose (5 ml at a rate of 1.086 ml/min; total injection time, 4.6 min) and a following sustaining infusion (5 ml at a rate of 0.021 ml/min; total injection time, 235 min). Immediately prior to and during the period of i.v. infusion of the isotope with nonlabeled solute, arterial blood (1 ml) was drawn into heparinized tubes every min for the 1st 7 min and at 10 min. Thereafter, blood samples were drawn every 30 min during a total experimental period of 240 min. Plasma was rapidly separated as previously described and saved for analyses (6). Coincident with selected times of blood sampling, the inlying urinary bladder catheter was permitted to drain into calibrated tubes. Thus, urine was sampled immediately prior to the start of drug infusion and at 10 min after the start of isotope injection. Thereafter, the urinary bladder was permitted to drain every 30 min during the total experimental period. To ensure total emptying of the urinary bladder, gentle digital suprapubic pressure was applied. By 60 min after the start of isotope injection, the plasma levels of each of the 3 agents used in renal clearance studies had reached steady-state concentrations and urinary output had become constant. Both parameters remained relatively unchanging throughout the ensuing six 30-min sampling periods constituting the total experimental period for a single animal study.

Monitoring of Vital Signs and Vital Function. During experimentation, arterial blood pressure monitored as already described was found to be maintained at 120 to 180/70 to 90 mm of mercury. Plasma pH, pCO₂, and pO₂ were determined on periodic samples of arterial blood at the start and near the end of the total experimental period by microgasometric analysis (5). The mean values ± S.D. for these parameters in 19 animals were as follows (in mm mercury or pHe units): pCO₂: start, 44.8 ± 1.0; end, 44.9 ± 0.9; pO₂: start, 82.7 ± 1.2; end, 80.3 ± 1.9; pH: start, 7.41 ± 0.01; end, 7.45 ± 0.03. The values for microhematocrits from these 19 animals at the start and end of the total experimental period were: start, 39.7 ± 3.9%; end, 34.5 ± 3.3%. Body temperature was maintained at 39° by warming blankets.

Binding of MTX to Plasma Proteins. The extent to which MTX was bound to monkey plasma proteins was determined on plasma from blood of normal controls and from animals given injections of probenecid (700 mg/sq m) 1 hr previously. Binding was determined according to the methods of Toribara et al. (24) and Spector et al. (23). The range of concentrations of MTX in plasma studied for binding was 0.002 to 0.15 µg/ml. Under all conditions the binding of MTX to plasma proteins was determined to be 46.3 ± 6.2% for n = 14. The calculated portion of MTX not bound to plasma proteins was 53.7%.

Determination of the Molecular Integrity of Plasma-borne MTX and Urinary MTX. During the course of experiments on control monkeys and on animals pretreated with probenecid (200 to 700 mg/sq m) and receiving loading followed by sustaining infusions of MTX (2.9 to >600 mg/sq m), samples of blood and urine were obtained at 1 and 4 hr after the start of injection of MTX for the determination of the metabolic integrity of MTX. It was determined that the folic acid analogs determined in these fluids represented at least 90% pure MTX when analyzed according to the methods of Oliverio (18) or Kates and Tozer (15).

Counting of Samples. Portions of blood plasma and urine (0.1 ml) containing ¹³C- or ¹⁴C-labeled isotopes were placed in glass counting vials (Wheaton Glass Co., Millville, N. J.). Appropriate blanks containing urine or plasma were similarly prepared. Thereafter, 10 ml of liquid scintillation phosphor (4) were added and the samples were counted with a Packard Model 3320 liquid scintillation spectrometer. Quenching was determined by the channel ratio method and appropriate corrections were made. Samples containing ¹³¹I-albumin were counted in glass vials in a Packard Auto Gamma spectrometer, Serial No. 11265, for determination of systemic plasma volume (7).

In order to convert cpm/ml to µg/ml of each radioactive solute injected and subsequently determined in samples of plasma and urine, 0.1 ml of a 1:100 dilution of the primary injection material (isotopically labeled MTX, inulin, or PAH with known amounts of nonlabeled carrier) were counted. Appropriate conversion of cpm/ml to µg/ml in plasma and urine was based on the specific activity of the primary injectate. All fluid samples containing MTX were assumed to contain 100% pure MTX because studies of the metabolic integrity of MTX in plasma and urine following
initial i.v. injection indicated that molecular integrity of MTX approximated this high order.

Calculations. The calculations used in the determination of various parameters of renal function were those presented by Smith (21) and are as follows:

\[
\text{Clearance} = \frac{U \cdot V}{P}
\]

where \(U\) is urine concentration of the indicator solute (\(\mu g/ml\)), \(V\) is urine flow (ml/min), and \(P\) is plasma concentration of the indicator solute (\(\mu g/ml\)). Clearance is given in units of ml/min.

Renal tubular transport of MTX = \(U_{\text{MTX}} \cdot V - f \cdot P_{\text{MTX}} \cdot C_f\)

where \(f\) is nonprotein-bound fraction (\%) of plasma-borne MTX and \(C_f\) is filtration rate (ml/min) as determined by inulin clearance. Renal tubular transport of MTX is given in units of \(\mu g/min\).

The dose of MTX, PAH, or insulin administered was expressed as dose/sq m body surface to assist in interspecies comparison as suggested by Freireich et al. (12). To compute the surface area of monkeys the following formula was used:

\[
\text{Surface area} (\text{sq m}) = \frac{(K \cdot W^{2/3})}{10^4}
\]

The value for the conversion factor (\(K\)) was taken to be 11.8 as suggested by Freireich et al. (12). \(W\) is body weight in g.

RESULTS

In order to provide points of reference for the studies of renal clearance of MTX, the values for renal plasma flow as determined by PAH clearance and the values for glomerular filtration rate as determined by inulin clearance are presented in Table 1. In 2 of the experiments, simultaneous \(^{[14}C\)PAH and \(^{[3}H\)inulin clearances were determined in a single animal. There being no significant difference between these results for each parameter and results obtained from studies in which inulin and PAH clearance values were determined separately in different animals, all values for a single parameter were combined. The renal clearance of PAH and inulin were experimentally determined under conditions of phenylcyclidine hydrochloride and atropine anesthesia as were all clearance data in this study. Steady-state plasma levels of the indicator solutes were varied over a 90-fold or a 3.8 \(\times 10^3\)-fold range for inulin and PAH, respectively. Despite the wide variance in values for plasma levels of these solutes, the derived clearance data for each moiety were quite consistent, respectively, and are similar to data previously reported for these determinations in the monkey (17, 19, 22, 25). As shown in Chart 1, the renal clearance of MTX diminished with increasing plasma levels of the drug over the range 1.7 to 6 \(\mu g/ml\). Increase of the steady-state plasma levels of MTX by increments from a plasma level of 6 to 32 \(\mu g/ml\) was not associated with further change in the apparent renal clearance of MTX, which at these high plasma levels of MTX approximated the range of values for glomerular filtration. In contrast, at low steady-state plasma levels of MTX (0.04 to 1.7 \(\mu g/ml\)) the renal clearance of MTX observed exceeded the glomerular filtration rate by a factor of 3. This pattern of reduced renal clearance of MTX associated with raised plasma levels of the drug suggested that a saturable renal excretory transport mechanism for MTX might be operant. This possibility was confirmed (Chart 2). It was seen that the renal tubular transport of MTX reached a maximum of 81 \(\mu g/min\) at

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**Table 1**

Average inulin and PAH clearances in the monkey

<table>
<thead>
<tr>
<th>No. of animals</th>
<th>Body wt (kg)</th>
<th>Surface area (sq m)</th>
<th>No. of periods</th>
<th>Clearance (ml/min/kg)</th>
<th>Clearance (ml/min/sq m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inulin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>4.13 ± 0.55</td>
<td>0.302 ± 0.026</td>
<td>66</td>
<td>2.1 ± 0.6</td>
<td>29.0 ± 8.3</td>
</tr>
<tr>
<td>PAH</td>
<td></td>
<td>0.327 ± 0.021</td>
<td>36</td>
<td>22.4 ± 4.6</td>
<td>333.3 ± 63.8</td>
</tr>
</tbody>
</table>

**Chart 1.** Renal clearance (ml/min/kg) of MTX (ordinate) plotted as a function of the concentrations (\(\mu g/ml\)) of MTX in arterial plasma (abscissa). Each plotted point represents the mean in a single animal for 6 consecutive determinations. Each of the 6 sampling periods was for 30 min, during which the plasma level of MTX was held constant at the concentration indicated. The total dose of MTX administered i.v. to a single animal by loading followed by sustaining infusion varied from 0.03 to 500 mg/sq m. Animals pretreated with probenecid received 700 mg/sq m.
plasma levels of MTX of 6 to 8 μg/ml. Increasing plasma levels of MTX by increments to a maximum plasma level of 33 μg/ml resulted in no further increase in the renal tubular transport.

Probenecid, a benzoic acid derivative, is a well-known and clinically useful inhibitor of renal tubular transport of other organic acids (1, 3). It seemed reasonable to study its action on renal excretion of MTX. Pretreatment of animals with probenecid was associated with the reduction in the renal clearance of MTX to levels approximating the glomerular filtration rate, regardless of the concentration of MTX in plasma over the range 2 to 21 μg/ml (Chart 1). The reduction in the rate of clearance of MTX from plasma following the use of probenecid was related to inhibition of renal excretion of MTX. Chart 3 demonstrates that the rate of urinary excretion of MTX was reduced by a factor of 2.6 (p < 0.001) following pretreatment of animals with probenecid compared with untreated control animals. Moreover, probenecid (700 mg/sq m) provided consistent inhibition of renal excretion of MTX over a wide range in values for the plasma concentration of MTX (0.06 to 21.3 μg/ml) following infusion of MTX in doses from 2.9 to 621 mg/sq m. Increased levels of MTX in plasma reflected the reduced renal excretion of the drug following the use of probenecid (Chart 4). Thus, over a wide range of dosages of MTX investigated (1.8 to more than 600 mg/sq m), the resultant steady-state plasma level of MTX was twice (p < 0.001) that determined in non-probenecid-treated controls given comparable dosages of MTX.

**Effect of Dose and Mode of i.v. Injection on MTX.**

The rate that plasma is cleared of MTX by renal mechanisms depends in large measure on the plasma concentration of MTX as already described. It was not surprising to find that the fraction (%) of a single dose of MTX that remained in the systemic plasma compartment following initial bolus injection depended on the dose of the drug initially administered (Chart 5). The integrated arithmetical average fraction (%) of each different dose that remained in the systemic plasma compartment [as determined by 111In-albumin dilution (7)] over the 4-hr experimental period was as follows: 506 mg/sq m, 2.0%; 150 mg/sq m, 0.9%; 0.12 mg/sq m, 0.6%; 0.03 mg/sq m, 0.4%. It remained to determine whether the mode of i.v. injection (bolus versus loading followed by sustaining infusion) of a constant dose of MTX affected the resultant plasma levels of the drug.

MTX (175 mg/sq m) was injected as a single bolus or as a loading followed by a continuous sustaining infusion (Chart 6, “bolus control” versus “infusion control”). The integrated arithmetical average concentration (μg/ml) of MTX in plasma representing values for the entire 4-hr experimental period following both modes of i.v. injection was quite similar (“bolus control,” 3.9 ± 0.6 μg/ml; “infusion control,” 3.2 ± 0.1 μg/ml). However, following the 1st hr of the experimental period and continuing for the remaining 3 hr studied, the actual plasma levels of MTX following loading and continuous sustaining infusion (2.6 ± 0.4 μg/ml) exceeded the corresponding plasma levels of
of the 4-hr experiment in animals pretreated with probenecid and receiving loading and sustaining i.v. infusions of MTX exceeded comparable plasma levels of MTX in non-probenecid-pretreated control animals receiving bolus injections by a factor of 3 (Chart 6). We found that varied doses of probenecid (200, 500, or 700 mg/sq m) were equally effective in maintaining similarly high levels of MTX in plasma after injection of MTX (175 mg/sq m). However, doses of probenecid (13 or 133 mg/sq m) that were less than the dose of MTX injected were less effective in maintaining high levels of MTX in plasma.

DISCUSSION

Studies in the mouse, rat, dog, and man have shown that normalized data relating the plasma concentration of MTX to time after initial injection demonstrated a striking interspecies correlation (8). This correlation would be expected if the principal excretory mechanism for MTX was similar in all species studied. Indeed, the kidney has been shown to be the principal route of excretion of MTX in all these mammalian species (13). Furthermore, studies of renal clearance of MTX in man have indicated that MTX is excreted by renal tubular transport as well as by passive glomerular filtration (11, 13, 14). The present study in the

MTX following bolus injection (1.7 ± 0.5 μg/ml) by a factor of 1.5 (p < 0.001). The high plasma levels of MTX that were achieved rapidly following initial bolus injection tended to offset the low plasma levels of MTX determined during the latter three-quarters of the experimental period following bolus injection of MTX, compared with corresponding values that were determined following loading and sustaining infusion. The result was equalization of integrated average plasma values of MTX, which reflected values for the entire experimental period following both modes of i.v. injection.

Pretreatment of animals with probenecid dramatically altered the levels of MTX in plasma following injection of a constant dose (175 mg/sq m) of MTX (Chart 6). The integrated arithmetical average concentrations (7) of MTX in plasma of probenecid-pretreated animals were similar following bolus injection and loading followed by sustaining infusion of the drug. The values were 7.0 ± 1.4 and 6.4 ± 1.2 μg/ml, respectively. These values were double (p < 0.001) comparable values determined in non-probenecid-pretreated control animals already described. After the initial 90 min following the start of injection of MTX, the plasma levels of MTX that were determined following loading and sustaining infusion exceeded by as much as a factor of 2 comparable values for MTX determined following bolus injection over the remaining 150 min of observation. After injection of a standard dose of MTX (175 mg/sq m), the levels of the drug in plasma determined for the last 3
probenecid blockade of renal tubular transport of MTX, dosages (2.2 to 621 mg/sq m). Furthermore, following totally inhibited the renal tubular transport of MTX in the plasma levels of MTX were double comparable levels of the monkey when MTX was administered i.v. in a wide range of doses (2.2 to 621 mg/sq m). Therefore, the rapid disappearance of the drug from plasma (20) is undoubtedly related to rapid renal excretion of MTX. It has been shown that little of an i.v. dose of MTX escapes into the intracellular fluid compartment (11). Therefore, the rapid disappearance of the drug from plasma (20) is undoubtedly related to rapid renal excretion of MTX. It would be of significant practical value safely to inhibit renal tubular transport of MTX in the monkey was attained at relatively high levels of MTX (6 to 8 µg/ml) in plasma.

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drug in plasma of control animals given similar dosages of MTX.

Companion studies presented in this report indicated that long-term maintenance of stable and high plasma levels of MTX were influenced not only by inhibition of renal tubular excretion of the drug but also by the mode of i.v. injection of MTX. A priming injection followed by sustaining infusion of MTX together with pretreatment of the experimental animals with probenecid provided more constant and 2- to 3-fold higher plasma levels of MTX over the majority of the 4-hr experimental period than was determined in animals receiving identical dosages of MTX by i.v. bolus injection with or without pretreatment with probenecid. These findings deserve evaluation in the clinical setting.

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