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INCREASED METHOTREXATE TOXICITY DUE TO CONCURRENT PROBENECID ADMINISTRATION*

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Abstract—The toxicity of a single dose of methotrexate in the rat was observed to be substantially increased when probenecid was concurrently administered. The increased toxicity was associated with a marked inhibition of the elimination of methotrexate from the blood. The effects of probenecid on the renal and biliary secretion of methotrexate were determined using steady state conditions. Inhibition of biliary secretion accounted for most of the decreased elimination of methotrexate.

Methotrexate, a folic acid antagonist, has been used in the treatment of a variety of neoplastic diseases. Like most anticancer drugs, methotrexate is a nonspecific agent which prevents cell replication in normal as well as in malignant tissues. The response of a tissue to the effect of methotrexate is greatly dependent on the length of time for which the tissue is exposed to the drug after the initial insult [1]. If a tissue is exposed to methotrexate for a prolonged time, recovery from the initial insult is prevented, and complete tissue destruction may occur. The nonmalignant tissues most susceptible to the effect of methotrexate are those with rapid cell turnover rates, most notably the mucosa of the gastrointestinal tract and the bone marrow [2].

The importance of the length of exposure to methotrexate was first reported by Ferguson *et al.* [3]. They observed that methotrexate was more toxic to mice and rats when administered in divided doses over a period of time than when given as a single dose. They reported an LD_{50} for a single intraperitoneal dose of 94 ± 9 mg/kg in mice, but when the total intraperitoneal dose was divided into five equal consecutive daily fractions, the LD_{50} decreased to 9.7 ± 1.5 mg/kg. Increased toxicity, even death, can occur after prolonged exposure to methotrexate resulting from either continuous or frequent administration, or as a result of impairment of an individual's ability to eliminate the drug [4]. The problem of dosing methotrexate in patients with impaired renal function has been investigated [5, 6], and though no firm guidelines have been established for administering methotrexate to such patients, the problem is recognized and caution can be exercised.

The problem of inhibition of the elimination of methotrexate by concurrently administered drugs, however, has not received very much attention. Leigler *et al.* [7] have reported that salicylate and high concentrations of *para*-aminohippurate reduce the renal clearance of methotrexate to below the glomerular filtration rate, and that sulfisoxazole also has a slight inhibitory effect on methotrexate's renal excretion. More recently Bourke *et al.* [8] have reported that probenecid inhibits the renal secretion of methotrexate in dogs. Because of the relatively nonspecific nature of the acid transport systems which are involved in the elimination of methotrexate, numerous drugs of an acidic nature have the potential for interfering with methotrexate's elimination.

Both active renal secretion and biliary secretion of organic acids are known to be inhibited by probenecid. Methotrexate is eliminated from the blood by both these processes [9]. Because of the possible toxic consequences resulting from current administration of methotrexate and probenecid, an investigation of this potential drug interaction was undertaken.

MATERIALS AND METHODS

Animals. Male Sprague–Dawley rats, weighing 225–275 g, obtained from Simonsen Laboratories were used for these studies.

Drugs. Sodium methotrexate parenteral solution, marketed by Lederle Laboratories, Division of American Cyanamid, was administered in the initial toxicity study. Tritiated methotrexate was used in the blood level and clearance studies. The tritiated drug, obtained from Amersham/Searle Corp., was diluted with nonlabeled methotrexate and purified by the method of Oliverio [10]. The nonradiolabeled methotrexate was obtained from Nutritional Biochemicals Corp. A [^{3}H]methotrexate solution for injection was prepared by dissolving a weighed amount of the purified drug in a few drops of pH 8.3, 0.1 M ammonium bicarbonate buffer and then diluting with normal saline. The final concentration was 12.5 mg/ml. Probenecid, obtained as a powder from Merck Sharp & Dohme Research Laboratories, was prepared by dissolving a weighed amount of the powder in a few drops of 1.0 N NaOH, and the pH was adjusted to 7.4 with a pH 7.0, 0.1 M phosphate buffer. This solution was then diluted to the desired volume with normal saline. The final concentration of the probenecid injectable solution was 20 mg/ml.

Assay procedures. The concentration of radiolabel in blood, bile and urine samples was determined by scintillation counting techniques. All samples were collected in dialysis casing sacs, dried, and oxidized

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on a Packard model 305 sample oxidizer in preparation for scintillation counting. Blood samples were weighed immediately after they were obtained. To estimate the blood volume, the weight of each sample was divided by 1.05, the average specific gravity of rat blood [11]. The amount of radioactivity in each sample was determined by counting on a Packard model 3375 Tri Carb scintillation spectrometer. The automatic external standard feature was utilized for all quench corrections.

One sample each of bile, blood and urine was also examined for the presence of nonmethotrexate labeled species by a previously reported paper chromatographic method [12].

The concentration of probenecid in plasma samples was determined by the method of Dayton *et al.* [13].

Toxicity study. Twenty rats were injected intraperitoneally with 12.5 mg/kg of methotrexate. Ten rats also received intraperitoneal injections of 100 mg/kg of probenecid 20 min before and 20 min after the methotrexate was administered. A total of 200 mg/kg of probenecid was given.

Another 20 rats were injected intraperitoneally with 25 mg/kg of methotrexate. Ten of these were injected with 200 mg/kg of probenecid in the same manner as described above.

All rats were observed for signs of toxicity for 30 days. Feces were collected daily and assayed for occult blood by the Benzidine test [14] to determine if any gastrointestinal hemorrhaging was occurring.

Blood level study. Since these studies required frequent sampling of blood from rats, the right jugular vein was cannulated to facilitate blood collection. The rats were anesthetized with pentobarbital for the cannulation procedure, but were allowed to recover from anesthesia prior to commencement of the study. The rats were kept in restraining cages [15] throughout the study.

Five rats were injected intravenously with 25 mg/kg of [^{3}H]methotrexate, and blood samples were withdrawn at specified times. Another five rats were injected intravenously with 200 mg/kg of probenecid in addition to the 25 mg/kg of [^{3}H]methotrexate. Probenecid was administered in two equal doses as in the toxicity studies. Blood samples were drawn at specific times and the concentration of radiolabel was determined.

Clearance study. In order to measure the effect of probenecid on the renal and biliary clearance of methotrexate, the bile duct and the urinary bladder were cannulated. As described previously, the right jugular vein was cannulated for the sampling of blood. Another cannula, used for maintaining a constant infusion of the two drugs, was inserted into the aorta via the left cartoid artery.

Methotrexate was administered by infusion at a constant rate of 0.5 mg/hr throughout the study after a loading dose of 0.5 mg. Five hr into the methotrexate infusion probenecid was administered in a loading dose of 15 mg and by infusion at the constant rate of 5 mg/hr to the end of the study.

Bile and urine samples were collected over intervals of 1 hr. Blood samples were obtained at the midpoint of each sampling period. Plasma samples to be assayed for probenecid were obtained at 8.5, 12.5 and 15 hr after the initiation of the study.

RESULTS AND DISCUSSION

The results of the toxicity study are presented in Table 1. While administration of a dose of 12.5 mg/kg of methotrexate alone did not produce any signs of toxicity, the concurrent administration of probenecid produced mild transient diarrhea on days 2 and 3 after the injection. Fecal examinations for occult blood were negative. All rats appeared healthy after the brief episode of diarrhea, and no deaths were noted during the 30 days of observation.

A dose of 25 mg/kg of methotrexate produced moderate to severe diarrhea from about day 2 through day 5 after the injection, and all rats suffering from diarrhea appeared to be dehydrated. The observed course of intoxication corresponded very closely to the description of methotrexate intoxication presented by Ferguson *et al.* [3]. All the rats receiving only methotrexate at this dose level survived, and appeared to have fully recovered by day 10 after administration.

Concurrent administration of probenecid with a dose of 25 mg/kg of methotrexate produced very severe intoxication. Eight of the ten rats in this group died. The rats which died all appeared extremely dehydrated and suffered from severe diarrhea. Due to the destruction of the intestinal mucosa, sodium and water reportedly leak into the lumen, and the animal dies from dehydration [16]. After the initial destruction of the intestinal mucosa, dehydration occurs fairly rapidly and death may occur as soon as 3 days after the injection of a lethal dose [3]. In this study, of the ten rats injected with both methotrexate and probenecid, four died between day 3 and day 5 and four more died between day 5 and day 10. The two surviving rats in this group recovered from the diarrhea syndrome by day 7.

Examination of the blood concentration-time course of radiolabel in the presence and absence of probenecid reveals that elimination of methotrexate from the blood is substantially inhibited by probenecid. Figure 1 illustrates a semilogarithmic plot of the blood concentration-time course of radiolabel in rats after an intravenous injection of a dose of 25 mg/kg of methotrexate. The blood concentrations of radiolabel in the probenecid-treated rats, between 1 and 8 hr, were markedly greater than the levels at the corresponding times in the nonprobenecid-treated rats. It

Table 1. Effect of probanecid on the toxicity of methotrexate in male Sprague Dawley rats

Drugs administered*	N	Cases of diarrhea	Deaths†
Methotrexate (12.5 mg/kg)	10	0	0
Methotrexate (12.5 mg/kg) + probenecid (200 mg/kg)	10	10	0
Methotrexate (25 mg/kg)	10	10	0
Methotrexate (25 mg/kg) + probenecid (200 mg/kg)	10	10	8

* Methotrexate was administered as a single i.p. injection. Probenecid was administered as two i.p. injections of 100 mg/kg each. One was administered 20 min before and the other 20 min after the methotrexate was administered.

† Rats were observed for 30 days.

should be noted that plasma levels reported are for total radiolabel, and may reflect the presence of both methotrexate- and nonmethotrexate-labeled species. As reported previously [12], after an i.v. bolus of methotrexate to rats, the contribution of nonmethotrexate species to total plasma radiolabel may become significant at the later time points. No attempt was made, however, to separate methotrexate from other labeled species in this study. As will be discussed, this refinement of the plasma concentration data was not necessary to satisfy the intended objectives.

An estimate of the effect of probenecid on the total body clearance of methotrexate can be made by comparing the areas under the blood concentration-time curves for methotrexate in the presence and absence of probenecid. The time-averaged total body clearance of a drug is related to area by the equation [17]

$$\text{dose/area} = \text{total body clearance}$$

Using the trapezoidal rule, the areas under the two blood concentration-time curves were determined for the time period 15 min–8 hr. When methotrexate was administered alone, the area for the 25 mg/kg dose was 6.4 μg-hr/ml; when administered in the same dose with probenecid, the area was found to be 27.5 μg-hr/ml. From these data it can be established that probenecid retards the removal of methotrexate from the plasma. The estimated reduction of the total body clearance of methotrexate due to the administration of probenecid was approximately 75 per cent.

No calculation of the actual total body clearance of methotrexate was made, as the area under the curve was only determined for the period 15 min–8 hr. It should also be pointed out that the presence of nonmethotrexate-labeled species in the plasma does not significantly interfere with this estimation, as their presence only becomes important beyond 4 hr, whereas 95 per cent of the measured area in the non-probenecid-treated rats occurred prior to this time.

In an effort to further elucidate and quantitatively assess the processes involved in this interaction, a study of the effect of probenecid on the renal and biliary clearance of methotrexate was conducted. Figure 2 illustrates the biliary and urinary clearance data obtained from this study. The clearance values are plotted at the midpoint of each collection interval. The values for renal and biliary clearance for hr 1 are unreliable estimates; they are much higher than the actual clearances. The reason for this can be readily explained. As shown in Fig. 1, blood concentration of methotrexate, after a bolus injection, decreases by at least a factor of ten during hr 1. Despite the initiation of a constant infusion of the drug, the blood concentration measured 30 min after the start of the study was too low to be representative of the average blood concentration during hr 1. Thus, the calculated value of clearance was too high.

Ignoring the values for hr 1 of the control, and of the probenecid-treated periods, the blood concentration and the biliary, renal and total body clearances were averaged (Table 2). Averaging was felt to be justified as the levels of methotrexate and probenecid were each virtually at steady state. The concentrations of probenecid in the plasma at 8.5, 12.5 and 15 hr after the start of the study were 86, 100 and 120 μg/ml respectively. The concentration of methotrexate during the probenecid treatment period averaged 2.12 μg/ml with a range of 1.7 to 2.6 μg/ml.

The average renal and biliary clearances of methotrexate during the control (1–5 hr) period were 4.3 and 5.5 ml/min respectively. From these data it can be seen that the biliary excretion of methotrexate was significantly inhibited by probenecid. The average biliary clearance of methotrexate, between hr 6 and hr 15, was 1.1 ml/min. This was a decrease of 80 per cent from the control value. The inhibitory effect of probenecid on the renal secretion of methotrexate was much less than the effect on biliary secretion. The average renal clearance during the period of probenecid administration was 2.8 ml/min, a decrease of 35 per cent from the control value. The total body clearance of methotrexate, as determined by dividing the infusion rate by the averaged steady state blood concentration, was accounted for, within experimental error, by the sum of renal and biliary clearances.

As with the single-bolus studies, total radioactivity was used to determine the concentration of methotrexate in the blood. In a previous report [12] a fraction of the radioactivity in the blood was shown to be due to nonmethotrexate species after a single bolus injection of methotrexate to rats, and the fraction of total label representing this nonmethotrexate component increased with time. In this study, however, it was found that the fraction of nonmethotrexate radiolabel, in blood, bile and urine samples, collected during hr 10 of the infusion was undetectable. There

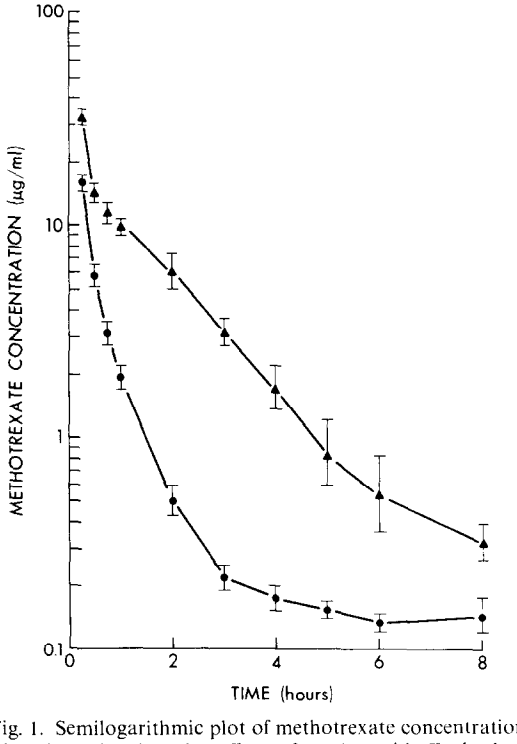

Fig. 1. Semilogarithmic plot of methotrexate concentration with time showing the effect of probenecid. Each data point represents the geometric mean (±S.E.M.) of five studies. A dose of 25 mg/kg of methotrexate was administered at $t = 0$. Doses of 100 mg/kg of probenecid were administered 20 min before and 20 min after methotrexate administration. Key: methotrexate alone (●); methotrexate plus probenecid (▲).

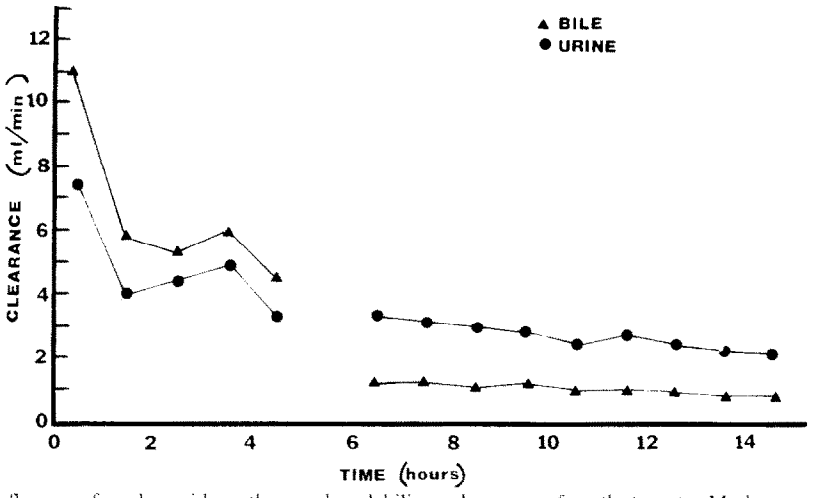

Fig. 2. Influence of probenecid on the renal and biliary clearance of methotrexate. Methotrexate was infused throughout the study at a rate of 0.5 mg/hr after an initial loading dose of 0.5 mg. Probenecid was infused from hr 5 on at a rate of 5 mg/hr after a loading dose of 15 mg. Key: biliary clearance (▲); renal clearance (●).

are two possible explanations for a significant nonmethotrexate fraction of the radiolabel in rat plasma after a bolus injection but not in the current studies in which methotrexate is administered by constant infusion. After a bolus injection, methotrexate is rapidly eliminated from the blood, while the nonmethotrexate component, being very slowly removed, becomes the increasingly dominant species present. The nonmethotrexate component after a single bolus gains significance with time due to its retention in the blood. Under conditions in which a constant blood level is maintained, the concentration of the nonmethotrexate component is insignificant in relation to the concentration of the unchanged drug.

Another possible explanation arises from the fact that methotrexate has been shown to be metabolized by the bacteria of the lower intestine [18, 19]. The nonmethotrexate species present in the blood could be an absorbed bacterial metabolite. In the current studies, the bile ducts of the rats were cannulated, and therefore methotrexate was not available in the intestinal lumen for bacterial metabolism. The radiolabel in the urine and bile was found to only be associated with methotrexate.

Table 2. Summary of average methotrexate clearance data

	Control (1–5 hr)	Probenecid treatment (6–15 hr)
Average blood concentration (μg/ml)	0.83 (0.71–1.00)†	2.12* (1.7–2.6)†
Biliary clearance (ml/min)	5.5	1.1*
Renal clearance (ml/min)	4.3	2.8‡
Biliary plus renal clearance (ml/min)	9.8	3.9
Total body clearance, (ml/min)	10.1	3.9

* $P < 0.001$.
† Range of concentrations.
‡ $P < 0.005$.
, Total body clearance is calculated by dividing the infusion rate by the average blood concentration.

In conclusion, the data obtained in this study demonstrate that probenecid substantially increases the toxicity of methotrexate in the rat. This interaction primarily appears to be a result of the inhibition of the biliary secretion of methotrexate.

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