7-Hydroxymethotrexate as a Urinary Metabolite in Human Subjects and Rhesus Monkeys Receiving High Dose Methotrexate

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ABSTRACT Human subjects and rhesus monkeys receiving the antitumor agent methotrexate at the high dose levels recently introduced into clinical use (> 50 mg/kg) excrete significant amounts of the metabolite 7-hydroxymethotrexate. The metabolite is not detected in these species after methotrexate therapy at conventional dose levels. The evidence indicates that in primates, the in vivo conversion of methotrexate to 7-hydroxymethotrexate is a dose-dependent phenomenon, with the enzyme system(s) catalyzing the reaction having a low affinity for the drug.

INTRODUCTION

The folate antagonist methotrexate (MTX; 4-amino-4-deoxy-N^{10}-methylpteroylglutamate) [1] is in general clinical use as a single agent or in combination with other antineoplastic agents in the treatment of choriocarcinoma, Burkitt's lymphoma, acute leukemia of childhood, epidermoid carcinoma of the head and neck, and breast cancer (1, 2). Recently, "high-dose" MTX therapy (> 50 mg MTX/kg) with leucovorin rescue has been shown to be of value in the treatment of osteogenic sarcoma (3, 4). The toxicity of this regimen is significant, with renal failure having been observed, in addition to the gastrointestinal ulceration and bone marrow depression seen with conventional MTX therapy. Earlier studies of the clinical pharmacology of MTX administered at conventional dose levels indicate that in man and other primates, the compound is not metabolized but is excreted unchanged in the urine (5–7). We now report evidence that at the high dose levels presently in clinical use, MTX undergoes quantitatively significant conversion both in man and the rhesus monkey to a metabolite with extremely limited aqueous solubility, 7-hydroxymethotrexate.

Received for publication 11 September 1975 and in revised form 17 November 1975.

[1] *Abbreviations used in this paper:* MTX, methotrexate; 7-hydroxyMTX, 7-hydroxymethotrexate.

METHODS

Five human male subjects with osteogenic sarcoma or with epidermoid carcinoma of the lung, ranging in age from 11 to 48 yr, were given high dose MTX (50–200 mg/kg) [2] in 6-h i.v. infusions. Three normal mature female rhesus monkeys were given either MTX or [3′,5′,9-^{3}H]MTX (8) (sp act 148 μCi/mmol) in 6-h i.v. infusions at a dose-level of 200 mg/kg. Beginning with the start of the infusion, urine was serially collected over time periods of 0–6, 6–12, 12–18, and 18–24 h for the human subjects, and 0–6, 6–12, and 12–24 h for the monkeys. 3-ml aliquots of the collected urines were adjusted to pH 8.3 and chromatographed on columns of DEAE-cellulose with ammonium bicarbonate gradient elution over a concentration range of 0.1–0.4 M (9). 100 6-ml fractions were collected. The position of elution of MTX in this chromatographic system (fractions 20–26) was determined from ultraviolet and visible absorption spectra of the individual fractions, by assay of the fractions for dihydrofolate reductase inhibitory activity (10), and in the experiments with tritium-labeled MTX, by liquid scintillation counting. Monkeys were killed 24 h after the start of the infusion and kidney tissue slices were prepared for histological and autoradiographic studies. Samples of the same kidneys were also stored at −8°C for later determination of MTX content by dihydrofolate reductase inhibition assay and by measurement of tritium levels.

[2] The purity of MTX used in the high dose clinical studies (Lederle Laboratories, Pearl River, N. Y.) was assessed by chromatography on DEAE-cellulose. The material was found to be homogeneous except for a small amount (< 3%) of the previously reported compound N^{10}-methylpteroylglutamate (7), a by-product of the manufacturing process.

TABLE I

Urinary Excretion of 7-HydroxyMTX by Human Subjects in Early (6–12 h) and Late (18–24 h) Time Periods after the i.v. Administration of MTX in the High Dose Range (>50 mg/kg)

Patient	Age	Diagnosis	Dose	Total dose	6–12-h Collection period*		18–24-h Collection period*	
					MTX plus 7-OH-MTX excreted	Percentage as 7-OH-MTX	MTX plus 7-OH-MTX excreted	Percentage as 7-OH-MTX
	yr		*mg/kg*	*g*	*mg*	%	*mg*	%
R. F.	11	Osteogenic sarcoma	200	8.0	1,550	2.1	112	33
S. J.	29	Osteogenic sarcoma	100	9.3	1,830	4.0	152	30
F. K.	31	Osteogenic sarcoma	100	7.0	1,029	9.6	78	22
G. R.	48	Epidermoid cancer of lung	50	3.0	652	0.7	179	7
W. C.	35	Osteogenic sarcoma	50	2.8	1,348	0.9	9	10

All patients studied were male.

* The difference between percentage 7-OH-MTX excreted in the 6–12-h collection period and in the 18–24-h collection period is significant at $P < 0.05$, utilizing Student's two-tailed t test.

RESULTS

Of unusual interest in the chromatographic studies was the unexpected finding of a slowly migrating MTX-derived compound (fractions 59–69) which was present at detectable levels in the monkey urines after 6 h and in all the human urines examined (Table I). Typical elution patterns for this compound and for MTX in the human and monkey urines are shown in Fig. 1. As a percentage of total excreted drug, the excretion of the metabolite was greater in the later time periods, ranging from 7 to 33% of excreted drug in the 18–24-h human urine samples vs. only 0.7–9.6% in the 6–12-h samples (Table I). The slowly migrating metabolite was identical to authentic 7-hydroxymethotrexate (7-hydroxyMTX) in its chromatographic behavior on DEAE-cellulose, its ultraviolet and visible absorption spectra, its infrared absorption spectrum (Fig. 2), and its weak activity as an inhibitor of mammalian dihydrofolate reductase (11). The aqueous solubility of 7-hydroxyMTX was determined over the pH range 5.0–7.0 and found to be three- to fivefold less than that of MTX (Table II).

Histological sections and autoradiographs of tissue slices from the kidneys of monkeys which had received tritium-labeled MTX 24 h previously revealed crystalline-like deposits of MTX or MTX-derived material within the lumen of the renal tubules. The glomeruli appeared normal. The autoradiograph (Fig. 3) shows the crystalline-like deposits within the tubules, with exposed silver grains in the emulsion overlying the deposits. On DEAE-cellulose chromatography of the protein-free supernatant fractions from homogenates of the same kidney tissue used in the autoradiographic studies, it was found that only 23% of the tritium radioactivity in the kidney was present as MTX, while the remainder was present as 7-hydroxyMTX.

DISCUSSION

The consistent presence of 7-hydroxyMTX in urines of human subjects and rhesus monkeys receiving MTX, and in renal tissues from monkeys receiving MTX, was unexpected in view of previous reports from this and other laboratories (5–7), that MTX in these two species is excreted essentially unchanged. These early studies were carried out at conventional dose levels, while the present studies were carried out at dose levels some 5- to 2,000-fold higher in man and 600-fold higher in the monkey. From the early report by Henderson et al. (7), it is apparent from the chromatographic and spectrophotometric data cited that 7-hydroxyMTX was detected as a minor metabolite, although not identified, at the highest dose of MTX used by these workers in studies with human subjects (10 mg/kg); since the compound was not seen at lower dose levels, its presence was attributed to an artifact arising from bacterial metabolism of the drug after collection of the urine samples. That the metabolite is indeed generated in vivo, however, is clear from the present studies, in which 7-hydroxyMTX was detected in urine samples chromatographed immediately after

TABLE II

pH Dependence of Aqueous Solubility of 7-HydroxyMTX and MTX

Compound	Aqueous solubility, *mg/ml*		
	pH 5.0	pH 6.0	pH 7.0
7-HydroxyMTX	0.13	0.37	1.55
MTX	0.44	1.60	8.90

Concentrations of dissolved 7-hydroxyMTX and MTX were determined spectrophotometrically after a 2-h equilibration period in sodium phosphate buffer, 0.1 M, at 37°C.

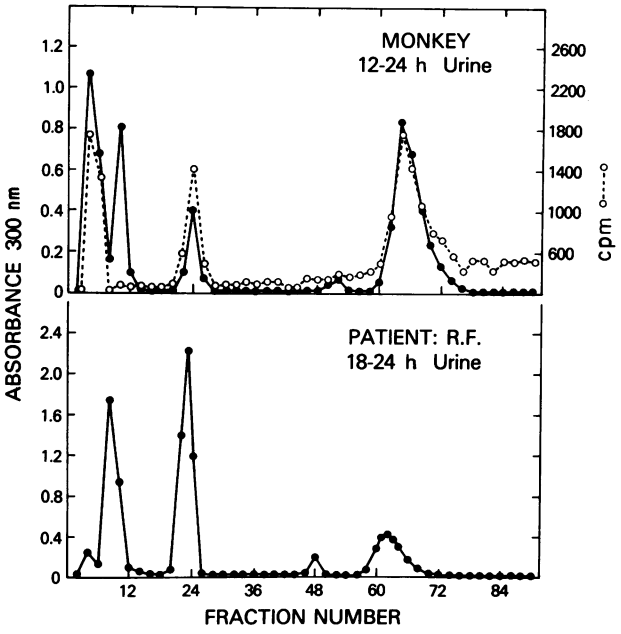

FIGURE 1 DEAE-cellulose chromatographic elution patterns of MTX and its metabolite in monkey and human urine after i.v. administration of MTX at a dose level of 200 mg/kg. (*Upper panel*) [3′,5′,9-^{3}H]MTX (Dhom Products, North Hollywood, Calif.; 10 Ci/mmol) was purified by column chromatography on DEAE-cellulose and diluted with unlabeled MTX to give a final sp act of 148 μCi/mmol. A female rhesus monkey (6 kg) was given 200 mg/kg (546 μCi) of the labeled drug in a 6-h i.v. infusion, and total urine collection was carried out over the time periods 0–6, 6–12, and 12–24 h after the start of the infusion. For the experiment recorded above, a 3-ml aliquot of the 12–24-h urine sample was applied to a DEAE-cellulose column (1.3 × 16 cm) and gradient elution carried out with ammonium bicarbonate over a concentration range of 0.1–0.4 M. 100 6-ml fractions were collected. 93% of ^{3}H radioactivity applied to the column was recovered. (●), Absorbance at 300 nm; (○), tritium radioactivity, cpm/0.2 ml eluate. The early peak of radioactivity (fractions 2–8) has been previously shown to be made up of cleavage products of MTX (7). The identities of the MTX and 7-hydroxyMTX peaks (fractions 20–26 and 59–69, respectively) were established from the site of elution of standard reference compounds, from the ultraviolet and visible absorption spectra of the peak fractions, and, in the case of MTX, from dihydrofolate reductase inhibition assay. The peak at fractions 46–54 is N^{10}-methylpteroylglutamate, a previously reported by-product of the manufacturing process for methotrexate (7). The quantity of MTX and 7-hydroxyMTX was determined by integration of the area under each peak. (*Lower panel*) A male patient, R. F. (40 kg), was given MTX, 200 mg/kg, in a 6-h i.v. infusion, and a 3-ml aliquot of the 18–24-h pooled urine chromatographed as described above. The results shown are from a single representative experiment; data from five patients are summarized in Table I.

collection, and in samples of renal tissue which were kept frozen until immediately before extracts were prepared for chromatography.

The unusual dose dependence for the appearance of 7-hydroxyMTX in the urine is most readily explained by postulating that the enzyme catalyzing the oxidation has only a low affinity for the parent drug. Other possibilities exist as well (e.g. saturation of an alternate route of excretion via the biliary tract), but are less

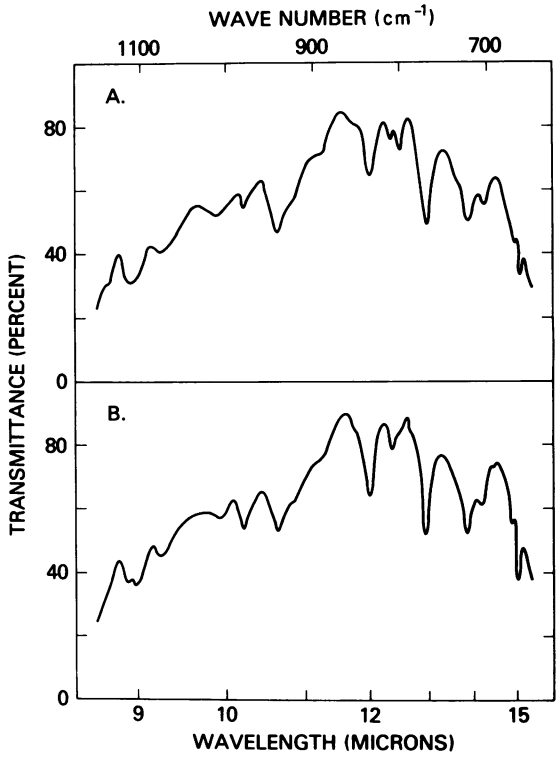

FIGURE 2 Infrared absorption spectra of (A) authentic 7-hydroxyMTX and (B) of the MTX metabolite isolated from monkey urine after the administration of MTX, 200 mg/kg. A female rhesus monkey (6 kg) was given MTX, 200 mg/kg in a 6-h i.v. infusion and the 12–24-h urine (110 ml) pooled and stored overnight at 5°C. The lemon-yellow precipitate which formed was collected by centrifugation and washed once with cold acetone. The precipitate was redissolved in sodium hydroxide solution, 0.05 N, and the insoluble residue discarded. Glacial acetic acid was added dropwise to the supernatant solution until a pH of 2.0 was reached. The solution was stored overnight at 5°C. The precipitate was washed twice with cold acetone and dried *in vacuo* over phosphorus pentoxide for 48 h. Yield was 80 mg. On chromatography on DEAE-cellulose, the material was found to be homogeneous and to migrate identically with authentic 7-hydroxyMTX. Spectra were recorded with a model 621 infrared spectrometer (Perkin-Elmer Corp., Norwalk, Conn.); compounds were suspended in a paraffin oil mull.

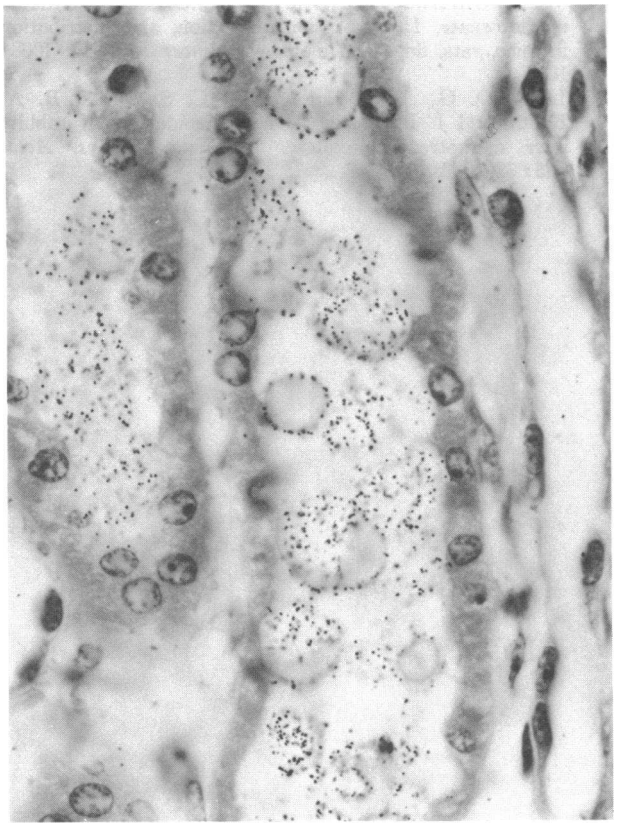

FIGURE 3 Kidney autoradiograph, × 860, from a rhesus monkey killed 24 h after administration of [3',5',9-^{3}H]MTX (546 μCi) at a dose of 200 mg/kg. The section is from the renal medulla. The tissue was immediately fixed in absolute alcohol. Tissue sections of 5 μm thickness were cut. The slides were dipped in Kodak Nuclear Track Emulsion NTB2 (Eastman Kodak Co., Rochester, N. Y.), heated to 45°C, exposed for 14 days, developed, and stained with hematoxylin and eosin.

likely since previous studies of biliary excretion of MTX administered at conventional dose levels in the monkey have not shown the presence of the 7-hydroxy metabolite (12). In studies of the 7-hydroxylation of MTX by the rabbit, in which species the reaction is quantitatively significant even at lower dose levels, the hydroxylation has been shown to be catalyzed by the hepatic metalloflavoprotein aldehyde oxidase (EC 1.2.-3.1); the Michaelis constant for the reaction with the rabbit enzyme is of the order of 0.1 mM (13). The latter concentration would be considerably exceeded in vivo after the administration of high-dose MTX (assuming equal distribution of the drug throughout body water), but would not be achieved after the administration of MTX in the conventional dose range. Whether hepatic aldehyde oxidase is responsible for the conversion in primates has not, however, been established, and is presently under investigation in this laboratory.

7-HydroxyMTX has previously been shown to be some two orders of magnitude less effective as an inhibitor of mammalian dihydrofolate reductase than is MTX (11). The metabolite would not therefore contribute significantly to the folate antagonist activity of MTX in vivo, and in this respect the 7-hydroxylation reaction can be considered a detoxication mechanism. In other respects, however, these findings are of possible clinical and toxicological significance. A frequent complication of high-dose MTX therapy is impairment of renal function, as evidenced by a rising serum creatinine level and a falling creatinine clearance (3). This syndrome can readily be reproduced in the monkey, and is associated in the latter species with the presence of deposits of MTX-derived crystalline material in the renal tubules (Fig. 3). Since both MTX and 7-hydroxy-MTX are limited in aqueous solubility, particularly at acid pH, both compounds may contribute to the formation of this crystalline material, although as noted above, the aqueous solubility of the 7-hydroxy metabolite is some three- to fivefold less than that of the parent compound.

ACKNOWLEDGMENTS

We thank Dr. Ti Li Loo for providing 7-hydroxyMTX prepared by chemical synthesis, Dr. John A. Beisler for the infrared absorption spectra, and Mr. C. Jerry Derr for skillful technical assistance.

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