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Pharmacokinetics of methotrexate and 7-hydroxy-methotrexate in plasma and bone marrow of children receiving *low*-dose oral methotrexate*

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Summary. The absorption, distribution, and elimination kinetics of low-dose p.o. methotrexate (MTX) were repeatedly studied in 19 children during maintenance treatment of childhood acute lymphoblastic leukemia. Plasma concentrations, urinary elimination, and bone marrow concentrations of MTX and 7-hydroxymethotrexate (7-OH-MTX) were monitored during 24 h following a routine p.o. dose (30 mg/m^2) using high-pressure liquid chromatography. Significant interindividual variability was found in time to peak concentration (30–180 min), peak concentration (0.41–2.77 μM), and to a lesser extent the half-lives ($t_{½}\alpha$: 32.8–86.1 min; $t_{½\beta}$: 43.6–350.0 min; $t_{½}$ absorption: 25.2–60.3 min) and plasma area under the concentration-time curve from zero to infinity (195.6–818.5 μM.min). Significant amounts of 7-OH-MTX were detected in plasma, with a mean area under the concentration-time curve from zero to infinity of 208 μM.min compared with 365.6 μM.min for MTX. High concentrations of 7-OH-MTX were present in bone marrow 24 h after oral MTX (15/19 patients) and were at least five fold those in plasma and three fold the concentration of MTX in bone marrow. In four patients occasionally neither MTX nor metabolite could be detected. Repeated examination of these pharmacokinetic parameters in plasma and bone marrow showed that the intraindividual variability was small.

Introduction

The efficacy of methotrexate (MTX) in maintenance treatment for childhood acute lymphoblastic leukemia (ALL) is well established. A wide variability in the absorption of orally administered MTX and unpredictable serum concentrations have been observed in several studies [1, 2, 5, 8, 15, 18, 19, 20]. Fast systemic clearance of MTX following an intermediate dose is associated with a higher probability of relapse [8]. Also, patients with lower serum concentrations of this drug may be at greater risk of central nervous system relapse [7].

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Likewise, metabolic conversion of MTX to 7-OH-MTX which is a less potent inhibitor of dihydrofolate reductase, may affect the therapeutic effectiveness of MTX [10]. In man, conversion of MTX to 7-OH-MTX has been observed following high-dose infusion [6, 11, 12, 13, 16, 24] and after intermediate-dose infusion [4, 23]. Balis et al. [2] reported low concentrations of 7-OH-MTX at 6 h after low-dose MTX.

We report here the pharmacokinetics of MTX and 7-OH-MTX in plasma and bone marrow following 59 courses of low-dose oral MTX (30 mg/m^2) in 19 children treated with an MTX-containing regimen of maintenance therapy during complete remission of ALL.

Materials and methods

Patients. Nineteen patients (11 male, 8 female) in complete remission of ALL were included in the study with informed (parental) consent. Ages were 3–14 years, with a mean age of 6.3 years. Patients were treated according to the current protocols of the Dutch Childhood Leukemia Study Group or institutional protocols. In these regimens, induction treatment consisted a.o. of prednisone (40 mg daily), vincristine, and L-asparaginase, followed by cranial irradiation and five intrathecal injections of MTX. No MTX other than these intrathecal injections was given before the maintenance therapy, which consisted of 5 weeks of daily 6-mercaptopurine together with weekly MTX (30 mg/m^2 p.o.) alternating with 2 weeks of prednisone and 2-weekly injections of vincristine, for 110 weeks. Pharmacokinetic studies were first done after 3 or 4 weeks of 6-mercaptopurine and always 7 days after the last oral MTX dosage. Pharmacokinetic profiles were obtained during 59 consecutive courses in 19 patients. In these patients 49 bone marrow punctures were performed. Thirteen children also received cotrimoxazole daily.

Sample collection. The pharmacokinetic studies were performed under clinical observation at weeks 9, 30, 58, 86, or 116 after the start of treatment. All children received MTX as 2.5 mg tablets after an overnight fast. No food was allowed for 90–120 min after MTX administration. The dosage of MTX was 30 mg/m^2 in all children, while the absolute dose was 17.5–52.5 mg. Blood samples were obtained at −5, 30, 60, 90, 120, 150, 180 min and 4, 5, 6, and 24 h, the time of MTX administration being regarded as the zero time point. The samples were centrifuged and plasma was

stored at −20 °C until analysis. Except at week 9 a bone marrow aspirate was obtained by crista puncture 24 h after MTX and stored at −20 °C. Urine was collected during the 24-h period following p.o. MTX.

Drug assay. All plasma, bone marrow, and urine samples were stored at −20 °C and analyzed after thawing with high-pressure liquid chromatography (HPLC) [22]. The lower limit of detection of MTX with this assay is 2×10^{-8} *M*, while it is capable of separating MTX from its major metabolites 7-OH-MTX and 4-amino-4-deoxy N_{10} methyl pteroid acid (DAMPA) using aminopterin as an internal standard. Also, this assay is capable of separating and detecting the MTX polyglutamates MTX-PG_1, MTX-PG_2, and MTX-PG_3. Screening for the presence of these MTX polyglutamates was performed in the bone marrow samples using HPLC separation only and pure MTX-PG_1, MTX-PG_2, and MTX-PG_3 standards provided by Dr J. R. Piper from the Southern Research Institite, Alabama, USA [21].

Aliquots of plasma and urine were treated with 500 µl 10% trichloracetic acid (TCA) in 0.1 *N* HCl after addition of 10 µg aminopterin in 1 ml. Bone marrow was extracted as 10% homogenate in 0.9% sodium chloride with 10% TCA in 0.1 *N* HCl. After centrifugation, 100 µl of the supernatant was injected into the column. Separation was performed with a reverse-phase Lichrosorb 5RP18 column and a M440 UV detector (Waters, USA) fitted with a 313 nm interferential filter, using an isocratic mobile phase of 67 m*M* sodium acetate, 5% acetic acid/acetonitrile (88 : 12) (pH = 2.56), at a flow rate of 0.8 ml per min. With this assay retention times of MTX, 7-OH-MTX, DAMPA, MTX-PG_1, MTX-PG_2, and MTX-PG_3 were 12 min 51 s, 15 min 00 s, 18 min 00 s, 8 min 37 s, 6 min 95 s and 6 min 14 s, respectively.

Pharmacokinetic calculations. The MTX absorption curve was calculated using a two-compartment open model described by

$$C(t) = A.e^{-\alpha t} + B.e^{-\beta t} + F.e^{-kXt},$$

where C(t) is the plasma concentration at time t, kx is the absorption rate constant, and F is the fraction of the dose absorbed. The plasma area under the curve was calculated with this model. Plasma clearance was derived from

$$Cl_{pl} = \frac{\text{Dose}}{AUC_{0 \to \infty}} \quad (2)$$

where $AUC_{0 \to \infty}$ is the area under the plasma concentration-time curve as calculated from fitted data extrapolated to infinity. The $AUC_{0 \to \infty}$ for 7-OH-MTX was determined by the trapezoidal method [24].

Correlation coefficients were calculated with Spearman correlation analysis. The Wilcoxon signed rank test for nonparametric data was used to determine the significance of differences.

Results

With the standard dose of 30 mg/m^2 the peak concentration of MTX in plasma showed a considerable variation between patients. The mean peak concentration was 1.37 ± 0.22 µ*M* (mean ±2SE of 59 curves) with an absolute range of 0.41 µ*M*–2.77 µ*M*. The time to peak concentration in plasma varied from 30 to 180 min with a mean time of 100 min. One patient had a double peak on four occasions. No correlation was observed between the time of the peak concentration and the peak concentration of MTX. Table 1 shows the results of pharmacokinetic analysis of first courses only. The postabsorptive phase plasma levels were best fitted to a monoexponential or first-order decay

Table 1. Summary of results obtained in 19 children after first course of oral methotrexate (30 mg/m^2)

Patient	Age (years)	Sex	Week of treatment	Time to peak (min)	Peak concentration (µ*M*)	Model fit	$t_{1/2\alpha}$ (min)	$t_{1/2\beta}$ (min)	$t_{1/2\,abs}$ (min)	$AUC_{0-6\,h}$ µ*M*·min	$AUC_{0-\infty}$ µ*M*·min	Plasma clearance (ml·min^{-1}·m^{-2})	Renal clearance (ml·min^{-1}·m^{-2})	Maximum level of 7-OH methotrexate (µ*M*)
1	3	M	9	120	2.25	0.97	78.1	82.1	48.3	406.1	433.5	150.0	91.2	0.56
2	3	M	30	60	0.99	0.98	86.1	350.0	58.1	316.1	818.5	173.3	46.4	0.28
3	5	F	30	30	2.07	0.92	36.5	165.0	34.7	251.1	311.0	184.8	75.8	0.20
4	8	M	9	90	1.47	0.99	45.3	105.0	39.8	293.8	334.3	202.4	97.3	0.26
5	14	F	9	150	1.35	0.99	32.5	192.5	25.2	302.6	501.2	129.9	76.6	0.26
6	3	F	9	180	1.80	0.97	49.9	330.1	38.7	319.4	483.3	131.3	59.1	0.22
7	3	M	30	120	0.94	0.99	38.7	108.3	38.1	196.4	236.3	269.8	157.7	0.23
8	6	M	9	90	0.89	0.98	39.2	130.8	37.5	166.6	195.6	335.5	88.7	0.12
9	5	F	9	180	1.39	0.99	57.6	170.1	43.9	395.3	492.9	130.4	41.9	0.23
10	6	M	9	90	1.29	0.97	52.3	60.4	36.1	175.7	203.4	408.1	169.5	0.36
11	3	F	9	60	1.82	0.97	34.8	43.6	34.3	241.3	244.3	260.7	54.1	0.24
12	2	M	9	90	1.86	0.99	47.8	111.8	40.1	367.5	426.0	156.9	9.5	0.21
13	3	F	9	120	1.02	0.98	52.1	123.8	39.2	317.9	347.5	192.4	62.0	0.31
14	6	M	9	90	2.44	0.97	47.2	119.5	38.3	481.8	519.0	148.6	79.1	0.13
15	12	M	30	90	1.66	0.98	46.3	75.3	60.3	246.3	267.4	247.7	ND[a]	0.29
16	2	F	30	60	1.20	0.99	46.3	60.2	32.1	204.7	251.3	267.2	ND[a]	0.25
17	14	F	30	90	1.59	0.97	36.3	147.5	34.8	268.7	334.6	124.5	28.1	0.36
18	14	M	9	180	1.08	0.99	34.1	>200	35.0	201.4	565.0	118.0	46.6	0.25
19	8	M	30	60	1.15	0.97	36.5	187.3	34.7	220.7	293.8	226.1	194.5	0.20

[a] Not done

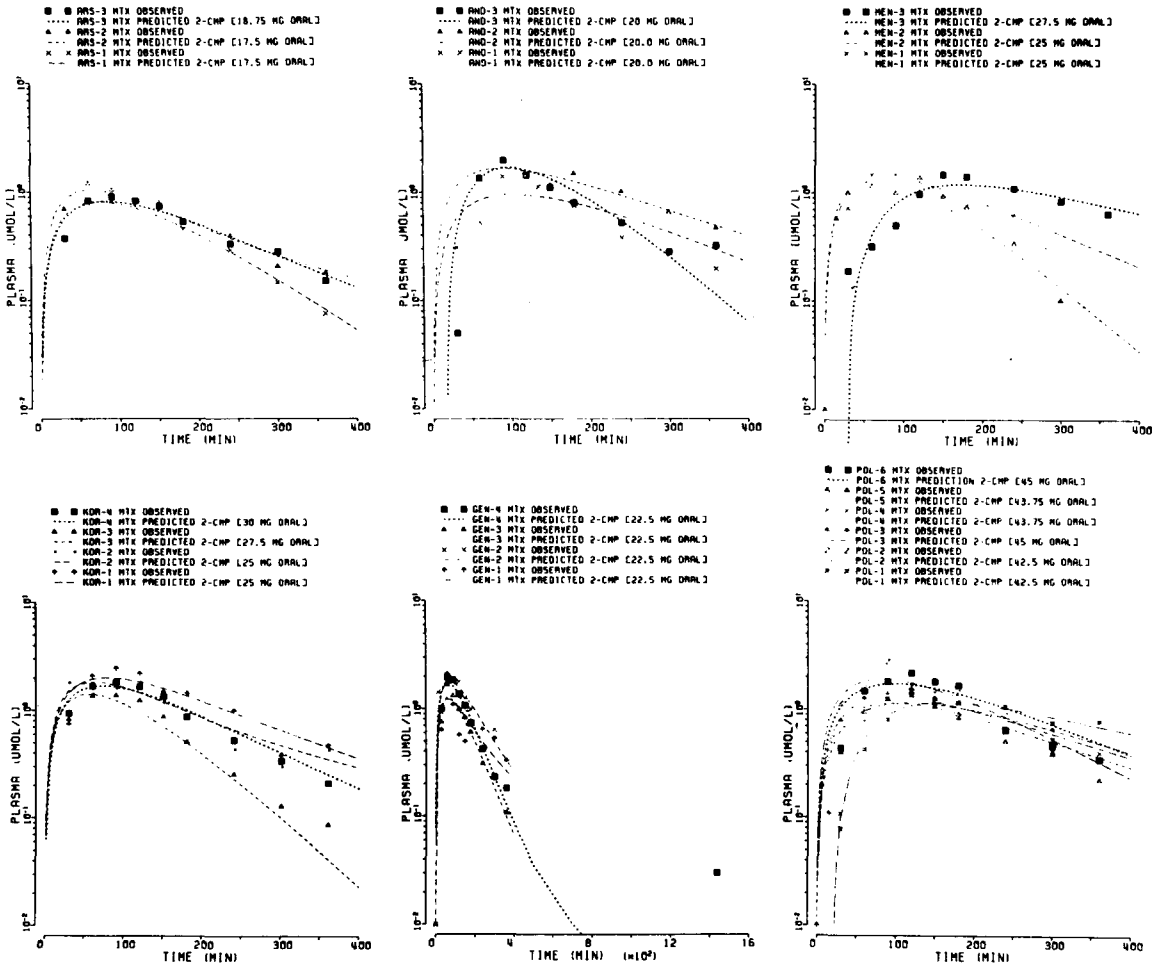

Fig. 1. Plasma concentration-time profiles fitted to a two-compartment model in six patients after three or more consecutive courses of oral methotrexate (30 mg/m^2). Numbers indicate the order of the studies in each patient

curve. The goodness of fit was greater than 0.91 in all cases. The intraindividual variation of the pharmacokinetic parameters was small and generally less than 20% of the values obtained at the first course, except for the time to peak concentration in plasma.

Figure 1 shows the model fit in six patients, who were studied during three or more consecutive doses of MTX. These curves demonstrate the small intraindividual variations compared with the interindividual differences. The curves of patient are shown because this patient consistently had early peak levels and a short terminal half-life compared with the others.

The plasma area under the curve, extrapolated to infinity, had a strong correlation with the peak concentration ($R = 0.79$). In Fig. 2 the peak concentrations obtained from the single patient with double peaks of MTX have not been included. Examination of the plasma samples revealed that in all patients 7-OH-MTX was present. The time of appearance of this metabolite was between 1 and 3 h after the administration of MTX. The mean area under the curve ($0 \rightarrow \infty$) was 208 μM.min for 7-OH-MTX, as against 365.6 μM.min for MTX.

As can be seen in Fig. 3a, the levels of 7-OH-MTX were close to the MTX concentration in plasma at 6 h after

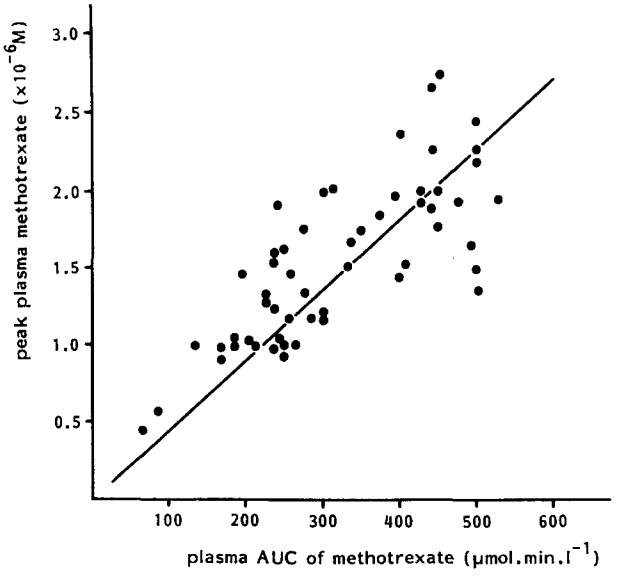

Fig. 2. Area under the curves from time zero extrapolated to infinity ($AUC_{0\rightarrow\infty}$) according to the two-compartment model after the first studied dose of oral methotrexate (30 mg/m^2) compared with the peak concentration attained in plasma

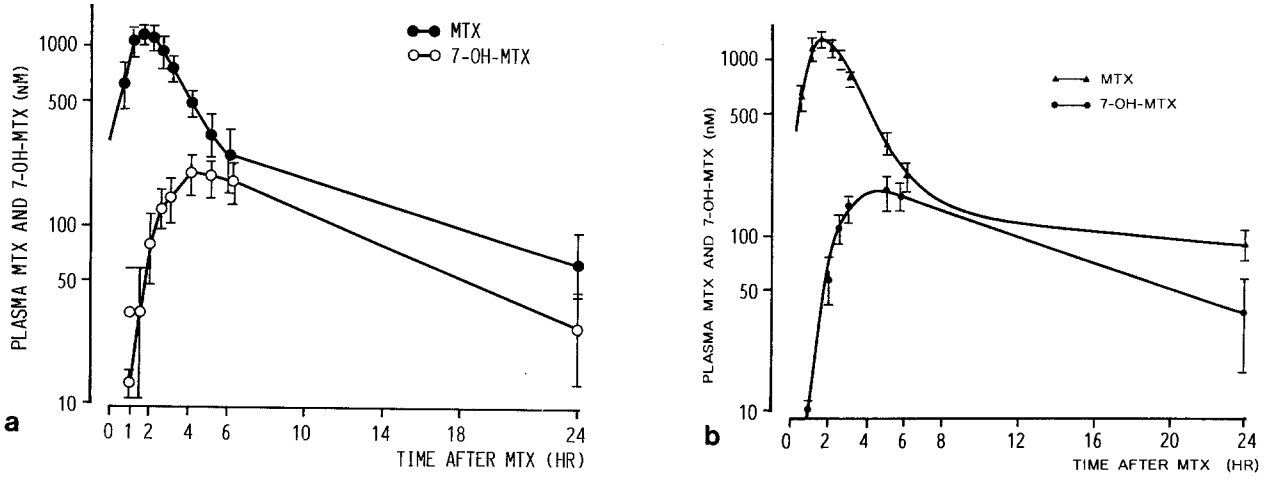

Fig. 3a, b. Plasma concentrations of methotrexate and 7-hydroxymethotrexate after 30 mg/m^2 p.o. in 19 patients. **a:** Data obtained after first dose of methotrexate ($M \pm 2SE$); **b:** cumulated data of 59 plasa concentration-time profiles after 30 mg/m^2 p.o. of methotrexate in 19 children ($M \pm SE$)

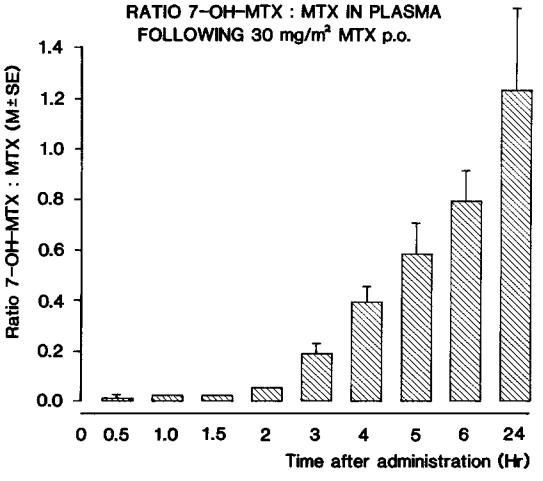

Fig. 4. Ratio of 7-hydroxymethotrexate and methotrexate in plasma after 30 mg/m^2 p.o. methotrexate ($M \pm SE$)

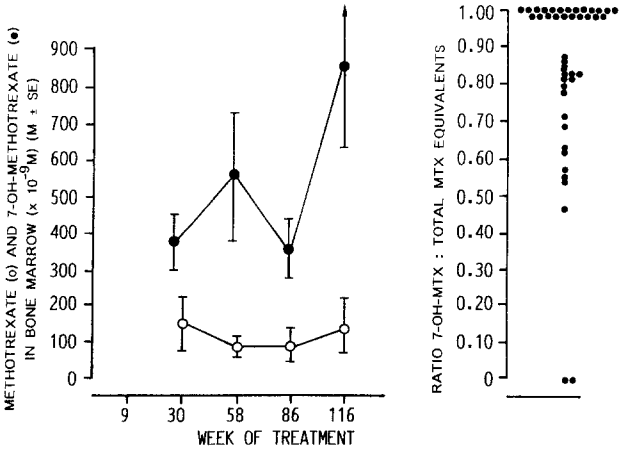

Fig. 5. Concentrations of methotrexate and 7-hydroxymethotrexate in unfractionated bone marrow aspirates obtained 24 h after methotrexate (30 mg/m^2 p.o.) ($M \pm 2$ SE of 49 aspirates). The data are presented according to the week of treatment

the first course of MTX p.o. In Fig. 3b, the plasma curves of all courses ($n=59$) in these patients are combined. The amount of 7-OH-MTX as compared to the parent compound significantly increased with time (Fig. 4). The proportion of metabolite present in individual patients studied during consecutive courses of MTX was consistent, indicating that hydroxylation of MTX after low-dose oral administration is a reproducible process. DAMPA was not detected in the plasma of any patient at a detection limit of 0.1 μM.

Analysis of unfractionated bone marrow cells obtained 24 h after MTX surprisingly showed that the majority of drug is present as 7-OH-MTX. At 24 h the concentrations of 7-OH-MTX per ml bone marrow were fivefold those in plasma (Fig. 5). In some patients (4/49 samples from four patients) neither MTX nor metabolite could be detected in the bone marrow. However, this was not a specific characteristic of these patients, since at subsequent course(s) of MTX, quantitative levels of 7-OH-MTX and/or MTX were determined. In 28 of 49 bone marrow samples no MTX was present, while 7-OH-MTX was detected in those samples at concentrations ranging from 0.1 to 20.7 μM.

Discussion

The absorption of low-dose MTX administered p.o. has been reported to be nearly complete [25]. However, Balis et al. [2] demonstrated that saturation of the absorption mechanism may occur at dosages as low as 12 mg/m^2. Moreover, a significant interindividual variability of plasma (serum) levels may occur following a dose of MTX p.o. [2, 5, 15, 18, 19, 20].

We report here on the plasma bioavilability of MTX following a fixed dose of 30 mg/m^2 p.o. The interindividual variability of plasma levels of MTX following this dose is similar to that reported previously. The time to peak concentration and the peak concentration itself are evidently different between these patients. The other parameters, such as half-lives, plasma clearance, and to a lesser extent renal clearance, show a moderate interindividual variation. However, these pharmacokinetic parameters re-

mained constant in individual patients during consecutive identical administrations, as is also evidenced by the plasma profiles. Apparently, the variability in drug levels should be attributed to individial differences of resorption and renal clearance rather than to differences of the plasma clearance.

In this group of patients, extensive metabolization of MTX to 7-OH-MTX was observed, following a low-dose oral administration. Previous studies of the plasma pharmacokinetics of MTX after a low dose given p.o. did not use HPLC analysis [5, 15, 19] or used a less sensitive HPLC assay [2], only capable of detecting concentrations greater than 0.1 μM.

All 19 children studied with the present HPLC analysis had considerable quantities of 7-OH-MTX in plasma with a range of 0–0.4 μM at 2 h, to 0.09–1.1 μM at 4 h and 0.1–0.4 μM at 6 h after MTX. In seven patients 7-OH-MTX could be detected as early as 1 h after the oral dose was given.

Previous reports of the occurrence of 7-OH-MTX following high-dose infusion indicate that the metabolite appears from 6 to 12 h after start of the infusion [3, 17]. Since the addition of the 7-hydroxy-group is mediated by aldehyde oxidase in the liver, the possibility of a first-pass effect responsible for the early appearance of 7-OH-MTX in case of an oral dose must be considered.

The degree to which MTX is metabolized to 7-OH-MTX may also differ between patients, as can be concluded from the variable ratio of MTX to 7-OH-MTX in plasma. Thus metabolic conversion may significantly add to the variability of plasma concentrations observed after low-dose MTX.

High levels of 7-OH-MTX were also detected in bone marrow at 24 h after MTX. In these patients unfractionated bone marrow was studied, because literature data and data obtained in a pilot study in 3 of our 19 patients in this study indicate that contaminating erythrocytes contain 0.2 nmol MTX per ml erythrocytes, as against 100–100000 nmol MTX + 7-OH-MTX per ml bone marrow [14]. Thus, the amount of MTX present in eryhtrocytes does not significantly add to the concentration in total bone marrow. No attempt was made to separate blast cells, because the target cells of MTX, i.e., the leukemic lymphoblastic cells, are below the limit of physical detection and separation during complete remission of ALL. The large proportion of 7-OH-MTX in bone marrow at 24 h may certainly have implications for the effect of this treatment. It may be that 7-OH-MTX competes with MTX for cellular uptake or transport [9, 17], while its inhibitory activity towards dihydrofolic acid reductase is approximately 200-fold less than that of MTX itself [10]. This is even more important in those patients in whose bone marrow no MTX could be detected.

Polyglutamates of MTX and its 7-OH-MTX were not found in these bone marrow samples. It is possible that these metabolic products were present at quantities below the detection limit of HPLC separation, since measurable amounts have been detected in vivo mostly following medium- or high-dose MTX. We are presently exploring other techniques in order to lower the detection limit of polyglutamates in bone marrow samples of these patients.

This study confirms previous reports on the variability of plasma levels following low-dose oral MTX, and it demonstrates the occurrence of in vivo conversion of MTX to 7-OH-MTX in these patients, which may contribute to the above-mentioned interindividual variation. This variation necessitates careful monitoring of plasma curves for the determination of a therapeutic range.

The fact that 7-OH-MTX is the predominant metabolite of MTX in bone marrow indicates the need for in vivo studies regarding the time dependency of levels of this drug in target tissues.

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