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Assay of methotrexate and 7-hydroxymethotrexate by gradient-elution high-performance liquid chromatography and its application in a high-dose pharmacokinetic study

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Abstract: A paired-ion high-performance liquid chromatographic method is described for the simultaneous determination of methotrexate (MTX) and its major metabolite, 7-hydroxymethotrexate (7-OH-MTX), in plasma and urine. In addition, this technique permits the separation of other known metabolites of MTX, such as DAMPA and MTX-polyglutamates. After selective extraction on an anion-exchange resin column, both compounds and the internal standard, aminopterin, were separated on a reversed-phase octadecylsilane column with UV-detection at 313 nm. The detection limits for plasma and urine samples were approximately 40 ng/ml (8.8×10^{-8} M) for MTX and 100 ng/ml (2.1×10^{-7} M) for 7-OH-MTX. This method was applied in pharmacokinetic studies following 24-h infusion of high-dose MTX in four patients during two successive treatments. After the end of the infusion, the mean apparent half-life for the metabolite was 19.1 h, while that for MTX was 8.8 h. A stepwise increase in the plasma concentrations of both MTX and its metabolite was observed during the second MTX infusion. This increase was reflected in the cumulative urinary excretion of both drug and its metabolite.

Keywords: *Methotrexate (MTX); 7-hydroxymethotrexate; high-dose pharmacokinetics; gradient-elution HPLC; ion-pair reversed-phase HPLC.*

Introduction

Methotrexate (MTX)† is widely used in the treatment of several human tumours, either

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† *Abbreviations used*: MTX, methotrexate (2,4-diamino-N^{10}-methyl-pteroylglutamic acid); 7-OH-MTX, 7-hydroxy-methotrexate; DAMPA, 2,4-diamino-N^{10}-methyl-pteroic acid; aminopterin, 2,3-diaminopteroylglutamic acid; MTX-G1, 2,4-diamino-N^{10}-methyl-pteroylglutamylglutamic acid; MTX-G2, 2,4-diamino-N^{10}-methyl-pteroylglutamyl glutamylglutamic acid.

as a single agent or in combination with other antineoplastic drugs. Therapeutic doses of MTX have been increased from 30 mg/m^2 to 100–200 mg/m^2 in the treatment of acute leukemia. This was proposed by Djerassi *et al.* [1] on the assumption that high concentrations of MTX in extracellular fluid should potentiate transport of the drug into cells. More recently, doses as high as 500 mg/kg, in conjunction with folinic acid (to reduce toxicity related to folate stress) have been used in the treatment of solid tumors [2, 3].

A major factor limiting high-dose MTX administration is the nephrotoxicity, arising from its insolubility in acid urine, which necessitates maintenance of an alkaline diuresis [4]. Hepatotoxicity, myelosuppression and changes in the digestive mucosa have also been reported [4].

In 1976, Jacobs *et al.* [5] reported that the level of the primary plasma metabolite, 7-hydroxymethotrexate (7-OH-MTX), is significant after high-dose MTX therapy, and represents 1–11% of the administered dose [6, 7]. Recent studies report serum concentrations of 7-OH-MTX exceeding MTX serum concentrations, following high-dose therapy. This was attributable to the longer elimination half-life of the metabolite [8–13], which is three to five-fold less water-soluble than MTX. Since urine concentrations of the metabolite may exceed the concentration of the parent compound, this could contribute to the observed nephrotoxicity [5, 7, 13]. Thus, there is a clinical need to monitor the levels of MTX and its principal metabolite in blood and urine during high-dose therapy.

Radioimmunoassay and enzyme immunoassay [14–16] have been used for MTX, but cross-reactivity with metabolites may limit their applicability. Several methods based on high-performance liquid chromatography (HPLC) have been proposed [7–10, 12, 13, 17–30]. Some of these methods may show poor resolution of MTX and 7-OH-MTX or may offer limited sensitivity. Moreover, several of these methods employ lengthy extractions with analytical recoveries ranging from 70 to 90% [20]. The present work reports a modification of the ion-exchange extraction proposed by Donehower *et al.* [20], coupled with a gradient-elution ion-pair HPLC method for MTX and all of its known metabolites. Although some methods allow good resolution of MTX, 7-OH-MTX and DAMPA [10, 14, 28], until now no method permits perfect resolution of all these derivatives and of the intracellular MTX-polyglutamyl derivatives with long chainlength, such as MTX-G_2. The application of the method to a study of MTX and 7-OH-MTX pharmacokinetics is discussed.

Materials and Methods

Clinical data

The revelant clinical information for this study, which involved two men and two women, is summarized in Table 1. The various malignant tumors required therapy with high-dose MTX alone or in combination with other antineoplastic drugs.

Since there are large inter-subject variations in MTX clearance, the dose to be

Table 1
Summary of clinical data for patients examined

Patient	Age	Sex	Weight (kg)	Clinical diagnosis	Other drugs administered Before treatment	During treatment
M.	62	F	81	Head and neck carcinoma	None	None
G.	34	M	60	Colon adeno-carcinoma	None	5-Fluorouracil
V.	63	F	48	Colon adeno-carcinoma	None	5-Fluorouracil
S.	62	M	71	Head and neck carcinoma	Vindesin, bleomycin, cis-platin	Vindesin

administered for i.v. perfusion was calculated according to a test-dose protocol previously described [31], using the pharmacokinetic data of each patient.*

Sample protocol

Blood samples were collected in heparinized tubes prior to the beginning of infusion (blank sample) and at the following intervals during infusion: 0.08, 0.5, 1, 4, 6, 12, 20 and 24 h. After the end of the infusion (at 24 h), further samples were collected at 0.5, 1, 2, 6, 12, 18, 24, 30, 36 and 48 h.

Urine specimens were collected prior to the infusion and then at hourly intervals during MTX infusion for the first 6 h, after which specimens were collected at 6-h intervals until the infusion was completed. Then a specimen was collected hourly for 6 h, followed by samples at 6-h intervals for at least two days.

According to the clinical protocol, the second infusion was administered at least three weeks after collection of the last sample following administration of the first infusion.

Reagents and standard solutions

Commercially available MTX was used as supplied by Lederlé (Oullins, France). The metabolite 7-OH-MTX was isolated from rabbit urine as previously described by Jacobs *et al.* [5]. The purity of the compound was confirmed by ultraviolet spectroscopy [5, 20] and also by HPLC, by comparison with an authentic reference standard kindly supplied by Dr B. Chabner (National Cancer Institute, Bethesda, Md. USA) who also supplied standard DAMPA. MTX-G_1 and MTX-G_2 were a kind gift of Dr C. M. Baugh (Department of Biochemistry, University of South Alabama, Mobile) and Dr J. Montgomery (Southern Research Institute). The internal standard aminopterin and the ion-pair reagent tetrabutylammonium nitrate (TBAN) were purchased from Fluka (Buchs, Switzerland).

* This drug monitoring [31] rested on preliminary pharmacokinetic examination of each patient after i.v. bolus of low doses (50 mg of MTX per square meter).

The 24-h infusion dose (q_0) to be administered to obtain a desired plasma level (P) was calculated by the formula:

$q_0 = P \times Cl \times 24 \times$ molecular weight, where the plasma clearance (Cl) of MTX was calculated according to the formula:

$$Cl = \frac{\text{Dose}}{\text{AUC}_{0\rightarrow t}}$$

where $\text{AUC}_{0\rightarrow t}$ denotes area under the curve from t_0 to t_w.

A 50 mM phosphate buffer was prepared from NaH_2PO_4, $2H_2O$ (7.8 g/l), adjusted precisely to pH 7.4 with 0.1 M sodium hydroxide and diluted to 5 mM with distilled water prior to use. Standard solutions of 1.00 mg/ml aminopterin, MTX and 7-OH-MTX were prepared in 5 mM phosphate buffer, stored at 4°C and renewed every 15 days. The dilutions required were prepared using the same solvent. Methanol was HPLC-grade as supplied by Merck (Darmstadt, FRG). All other chemicals used were of analytical reagent grade. A 50 mM stock solution of TBAN was prepared in distilled water.

Extraction procedure

The extraction method proposed by Donehower *et al.* [20] was modified as described below. To 0.5–10 ml of heparinized plasma and to 0.1–1 ml of urine was added 10–50 µl of internal standard solution (1 mM); 10 µl for 8.8×10^{-8} M to 2×10^{-6} M; and 50 µl for 2×10^{-6} M to 5×10^{-5} M).

The sample was applied to a 50 × 5 mm i.d. column of AG_1-X_4 anion exchange resin (BioRAD laboratories, Richmond, Ca., USA), previously prepared by washing with 10 ml of double-distilled water followed by 5 ml of methanol. The column was then eluted with 8 ml of 1 M acetic acid in methanol and 4 ml of 2 M acetic acid in methanol and the eluates combined before evaporating to dryness under a stream of nitrogen at 45°C. The residues were stored at 4°C pending analysis, then each sample was dissolved in 100–200 µl of phosphate buffer (5 mM, pH 7.4) and a 10–20 µl aliquot injected on to the HPLC column.

Chromatographic system

A Waters HPLC system (Waters Associates Inc., Milford, USA) was employed. This comprised a model M 45 and a model 6000 A pump, controlled by a model 660 solvent programmer with a model U6K injector, a model 440 absorbance detector and a C_{18}-µ-Bondapack reversed-phase column (300 × 2.9 mm i.d., 10 µm) at ambient temperature. Detection was carried out at 313 nm with a sensitivity of 0.04 a.u.f.s. A Hewlett–Packard model 3390 A data system was used for digital integration of peak areas. The mobile phases consisted of 5 mM sodium phosphate buffer (pH 7.4)–2.5 mM TBAN (Solvent A) and methanol–2.5 mM TBAN (Solvent B).

The solvent programmer was set to deliver 20% of B isocratically for 10 min, followed by a linear gradient to 30% of B during a period of 25 min at a constant flow rate of 2 ml/min.

Calibration data

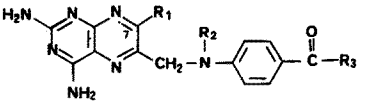
Standard calibration curves for MTX and 7-OH-MTX over the range 2×10^{-7}–4×10^{-5} M were prepared using drug-free serum and 1 mM aminopterin as internal standard. The ratio of analyte to internal standard peak area, calculated as a function of analyte concentration, was linear over the therapeutic concentration range tested, the regression data being: $y = 0.593\,x + 0.019$ for MTX ($n = 8$) and $y = 0.236\,x + 0.026$ for 7-OH-MTX ($n = 8$). The correlation coefficients were respectively 0.994 and 0.999. On the basis of those results, the routine calibration curves were performed on three points. All determinations were carried out at a sensitivity of 0.04 a.u.f.s.

Results and Discussion

Chromatography

The retention times of aminopterin, DAMPA, MTX and 7-OH-MTX were 10.6, 15.7, 20.2 and 23.2 min respectively (Fig. 2c). The intracellular polygammaglutamyl

metabolites of MTX were also resolved at 25.3 min (MTX-G_1) and 29.7 min (MTX-G_2) respectively. Aminopterin was chosen as the internal standard since it is structurally similar to MTX (Fig. 1), their extraction properties being comparable, and their maximum UV-absorption wavelengths being approximately the same (285 nm for aminopterin and 302 nm for MTX and 7-OH-MTX). Aminopterin is not used as a drug, nor is it a metabolite of MTX in man. The internal standard and all the compounds examined were well resolved from each other and from endogenous peaks (Figs 2a–2c).



Methotrexate	R_1 = H	R_2 = CH_3	R_3 = Glu*
7-OH-MTX	R_1 = OH	R_2 = CH_3	R_3 = Glu
D.A.M.P.A.	R_1 = H	R_2 = CH_3	R_3 = OH
Aminopterin	R_1 = H	R_2 = H	R_3 = Glu

*Glu = –NH–CH(COOH)–CH_2–CH_2–COOH

Figure 1
Structure of methotrexate, aminopterin (internal standard) and metabolites of methotrexate.

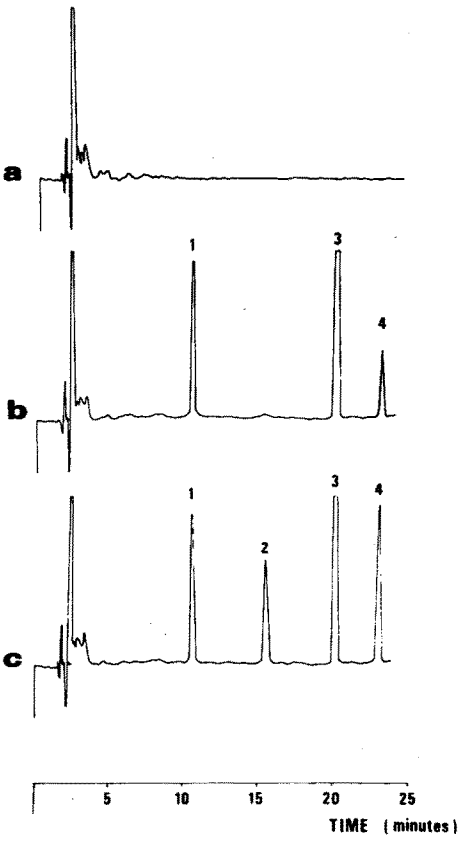


Figure 2
Chromatograms of: (a) blank plasma of a patient before administration of methotraxate; (b) plasma of the same patient after a single high-dose methotrexate infusion; (c) plasma blank spiked with aminopterin (1), DAMPA (2), methotrexate (3) and 7-hydroxymethotrexate (4). For details of chromatography see text.

Specificity

Although many HPLC methods have been reported for MTX and its metabolites, some do not include an internal standard [7, 16–18], while others are characterized by poor resolution [7, 12, 16, 18, 19]. Although Lawson *et al.* [10], Buice *et al.* [14] and Farid *et al.* [28] have reported HPLC methods showing good resolution of the parent compound and its plasma metabolites, Farid *et al.* [28] and Watson *et al.* [12] reported low resolution of the different polyglutamyl derivatives. The ion-pair method proposed in the present work allows complete resolution of all known metabolites of MTX examined. Moreover, the anticancer drugs 5-fluorouracil, adriamycin, vinblastine, vincristine, cyclophosphamide, bleomycin and carmustine (BCNU) were injected to determine whether they would interfere with MTX or its metabolites. At the detection wavelength employed (313 nm), only carmustine and vinblastine interfered. However, these drugs were not included in the treatment protocol of patients examined in the present study. MTX analogues, such as folinic acid, do not interfere in the assay. Potential interference by concurrently administered antibiotics, analgesics, sedatives or other drugs co-prescribed with MTX should, however, be borne in mind [28].

Extraction procedure and recovery

Most of the extraction methods previously described include protein precipitation and/or extraction and yield poor recoveries for MTX and 7-OH-MTX respectively: 46%, 26% [12, 19]; 78%, 70% [17]; and 75%, 75% [24]. However, Lawson *et al.* [10] used simple protein precipitation and reported recoveries of 82% for MTX and 98% for 7-OH-MTX. Moreover, Farid *et al.* [28] used a modified method of Donehower and obtained 94.5 and 72.4% respectively. In the present modification of the Donehower method [20], MTX and 7-OH-MTX are eluted from the anion-exchange resin column, using the more volatile eluent methanol–acetic acid (instead of water–acetic acid), thereby reducing the time required for evaporation. The mean absolute recoveries for MTX and 7-OH-MTX added to plasma at different concentrations, are summarized in Table 2.

Table 2
Absolute recovery (%) of methotrexate and 7-hydroxymethotrexate from plasma

Compound	Molar concentration	Recovery (%)	± S.D.	RSD (%) ($n = 2$)
MTX	2×10^{-5} M	77.5	±1.15	1.48
MTX	2×10^{-6} M	84.1	±5.65	6.72
MTX	5×10^{-7} M	88	±12	13.8
7-OH-MTX	3×10^{-5} M	97.3	±0.55	0.57
7-OH-MTX	4×10^{-6} M	77.3	±7.05	9.13
7-OH-MTX	1×10^{-6} M	94.9	±1.4	1.48

Sensitivity and reproducibility

The sensitivity, defined as twice the signal-to-noise ratio, was *ca* 40 ng/ml in plasma for MTX (8.8×10^{-8} M) and *ca* 100 ng/ml for 7-OH-MTX (2.1×10^{-7} M). The relative standard deviations for repeated analyses of plasma sample ($n = 6$) at different concentrations are detailed in Table 3.

Table 3
Reproducibility of methotrexate and 7-hydroxymethotrexate determination from plasma

Compound	Molar concentration	RSD (%) ($n = 6$)
MTX	2×10^{-5} M	8.66
MTX	2×10^{-6} M	4.67
MTX	5×10^{-7} M	17.2
7-OH-MTX	3×10^{-5} M	6.95
7-OH-MTX	4×10^{-6} M	8.99
7-OH-MTX	1×10^{-6} M	13.6

Plasma data

The doses for administration in the 24 h infusion were calculated [31] to attain steady-state MTX plasma levels between 2.5×10^{-5}–1×10^{-4}M (Table 4); the corresponding high-dose regimen ranged from 1.52 to 4.69 mg/kg.h. Plasma levels of MTX and 7-OH-MTX at the end of each 24-h infusion are shown in Table 4; a typical plasma profile is illustrated in Fig. 3. In all patients the MTX plasma levels at the end of the first infusion were reasonably in agreement with the predicted values. Moreover, for the three patients M., V. and S. who received the same dose successively, a stepwise rise in the plasma level of MTX at the end of the second infusion was observed, compared with MTX levels after the first infusion. During the 24-h infusion period, 7-OH-MTX levels remained lower than MTX levels, but after the end of the infusion, 7-OH-MTX was excreted more slowly than MTX (Fig. 3), consistent with the longer apparent elimination half-life value of the metabolite (Table 5) [8–13]. In three of the four cases (Table 4), the 7-OH-MTX concentrations reached higher values during the second infusion. It should, however, be noted that the estimated elimination half-life values for 7-OH-MTX have not been corrected for concurrent MTX metabolism (Table 5).

Mean half-life values for the terminal phase of MTX have been reported to be in the range of 8–15 h in patients with normal total body clearance [13, 18, 32–35]. The present results indicate a mean terminal phase half-life of 8.78 h, comparable with data reported by Stoller *et al.* [35] and Isacoff *et al.* [34]. The apparent half-life value of MTX fell within the range 2.8–16.1 h.

Table 4
Dosage of methotrexate, predicted plasma levels, and plasma levels observed at the end of infusion

Patient	Infusion No.	Dose administered: Total dose (g)	Dose administered: Rate of infusion (mg/kg/h)	Plasma levels predicted for the first infusion: MTX (10^{-5} M)	Plasma levels observed at the end of infusion: MTX (10^{-5} M)	Plasma levels observed at the end of infusion: 7-OH-MTX (10^{-5} M)
M.	1	2.96	1.52	2.5	2.92	1.47
	2	2.96	1.52		5.67	2.74
G	1	5.94	4.12	5	4.39	1.66
	2	6.27	4.35		7.15	3.29
V.	1	2.48	2.15	5	4.76	0.86
	2	2.48	2.15		5.20	0.78
S.	1	8.00	4.69	10	11.5	1.69
	2	8.00	4.69		19.0	2.51

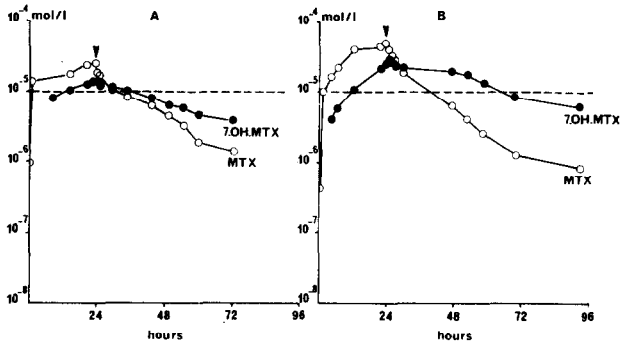


Figure 3
Typical plasma profile of methotrexate (○—○) and its 7-hydroxy-metabolite (●—●) in patient M during two successive treatments (A and B).

Urine data

The urinary excretion of MTX and 7-OH-MTX was studied in the four patients during the two successive courses of treatment (Table 6).

During administration of the infusion (0–24 h), urinary excretion of MTX was 20.7–58.5% of the administered dose; from 24 to 48 h, a relatively small proportion of the given dose was excreted. The cumulative urinary excretion varied between 25.3 and 66.4% for the first infusion and 57.2 and 83.7% for the second. These results are broadly comparable with data reported elsewhere [6, 24, 31, 32].

Urinary excretion of 7-OH-MTX ranged from 0.48 to 1.4% of the MTX administered dose in the first 24 h (Table 6), the total urinary excretion varying between 1.33 and 8.21%. These results confirm previously reported data [11, 13, 16, 20]. The total percentage of metabolite excreted during the second treatment was greater than that during the first (Table 6).

Interpretation of results

An attempt has been made to examine whether a significant difference exists between the plasma and urine data of two successive courses of MTX. The analysis of variance procedure was used to break down the overall observed measurement error in three components (Table 7): (i) errors due to the between-course variability; (ii) errors due to the inter-individual variability; and (iii) random errors.

This procedure allows the estimation of between-course variability, which is the only factor of interest in a statistical study of time dependency. Application of the Westlake test using the confidence interval approach further confirmed that the differences in the MTX and 7-OH-MTX characteristics observed in the second infusion treatment were significant [37].

Conclusions

The present HPLC method enables the simultaneous determination of MTX and its major metabolite 7-OH-MTX in plasma and urine. This method has been applied to a study of MTX and 7-OH-MTX plasma profiles. In a first approach, the study of some parameters such as AUC, $t_{1/2}$ and quantities excreted, demonstrated a time-dependency of the kinetics of these compounds. Thus, in order to study more precisely the time-dependence of MTX kinetics, a model which integrates biochemical data was elaborated.

Table 5
Pharmacokinetic parameters for methotrexate and 7-hydroxymethotrexate in plasma

Patients	Infusion No.	Course dose (g)	MTX $t_{½}$* (h)	MTX AUC_1† (μg/ml × h)	MTX Cl ‡1.h^{-1}	7-OH-MTX $t_{½}$ (h)	7-OH-MTX AUC_2 (μg/ml × h)§	$\frac{AUC_2}{AUC_1}$
M.	1	2.96	13.5	522	5.7	24.8	364	0.70
	2	2.96	16.1	637	4.6	26.7	701	1.15
G.	1	5.94	9.2	493	12.1	14.4	188	0.38
	2	6.27	10.2	794	7.8	17.3	655	0.83
V.	1	2.48	2.8	519	4.8	14.1	135	0.26
	2	2.48	1.2	658	3.8	27.8	107	0.16
S.	1	8.00	6.9	1089	7.3	16.1	154	0.14
	2	8.00	7.3	2011	4.0	11.9	264	0.13
Mean values	1		8.10			17.3		
	2		9.45			20.9		

* $t_{½}$ = half life.
† AUC = area under the curve.
‡ *Cl* = clearance.
§ = concentration units equivalent to methotrexate.

Table 6
Cumulative urinary excretion of MTX and 7-OH-MTX

Patient	Infusion No.	Total dose	0–24 h MTX mg	0–24 h MTX %	0–24 h 7-OH-MTX mg	0–24 h 7-OH-MTX %	24–48 h MTX mg	24–48 h MTX %	24–48 h 7-OH-MTX mg	24–48 h 7-OH-MTX %	48–84 h MTX mg	48–84 h MTX %	48–84 h 7-OH-MTX mg	48–84 h 7-OH-MTX %	Total excretion MTX mg	Total excretion MTX %	Total excretion 7-OH-MTX mg	Total excretion 7-OH-MTX %
M.	1	2.96	842	28.5	39	1.32	167	5.64	16	0.54	63	2.13	14	0.47	1072	36.2	69	2.33
	2	2.96	1647	55.6	32	1.08	652	22.0	78	2.64	179	6.05	133	4.49	2478	83.7	243	8.21
G.	1	5.94	1228	20.7	31	0.52	272	4.58	83	1.39	5	0.08	12	0.20	1505	25.3	126	2.12
	2	6.27	2647	42.2	82	1.31	925	14.7	86	1.37	13	0.21	4	0.06	3585	57.2	172	2.74
V.	1	2.48	1287	51.9	12	0.48	316	12.7	13	0.52	24	0.96	8	0.32	1627	65.6	33	1.33
	2	2.48	1452	58.5	35	1.41	522	21.1	24	0.96	24	0.96	30	1.21	1998	80.6	89	3.59
S.	1	8.00	2098	26.2	47	0.58	215	2.69	75	0.94	22	0.27	18	0.23	2335	29.2	140	1.75
	2	8.00	3027	37.8	94	1.17	1605	20.1	106	1.32	0	0	0	0	4632	57.9	200	2.50

Table 7
Statistical study

	Estimated variances				Westlake's test*		
	Overall	Due to courses	Due to subjects	Random	Mean difference due to courses	Symmetrical limits	Confidence interval (%)
AUC(UP)/D	4.086×10^{3}	8.051×10^{3}	6.227×10^{3}	6.226×10^{2}	63.5	106	70.1
AUC (M)/AUC (UP)	1.454×10^{-1}	7.801×10^{-2}	2.700×10^{-1}	4.318×10^{-2}	0.197	0.568	153.5
(UP %)	5.031×10^{2}	1.870×10^{3}	4.574×10^{2}	9.331×10^{1}	30.6	46.6	118.7
(UP %) + (M %)	5.692×10^{2}	2.172×10^{3}	4.874×10^{2}	1.170×10^{2}	33.0	50.9	123.7
(M %)/(UP %)	6.091×10^{-4}	3.920×10^{-6}	8.691×10^{-4}	5.510×10^{-4}	0.0014	0.0528	92.7
$t_{1/2}$ (MTX)	2.007×10^{1}	3.695	4.519×10^{1}	4.317×10^{-1}	1.35	2.5	30.5
$t_{1/2}$ (7-OH-MTX)	3.953×10^{1}	2.556×10^{1}	5.614×10^{1}	2.770×10^{1}	3.58	13.3	76.4

AUC = Area under the curve.
UP = unchanged product.
D = dose administered.
M = metabolite.
* Applied to between-course variability only.

This pharmacokinetic model takes into account both plasma and urine kinetics of the unchanged compound and its hydroxylated metabolite. The intracellular metabolic pathway including the polyglutamation of MTX and 7-OH-MTX (as shown by the authors [38]) has also been taken into account. On the other hand, in previous *in vitro* studies, it has been shown by the present authors that 'inactive' 7-OH-MTX might influence the transport and the active MTX-polyglutamate formation.

The present results permit the assumption to be made (taking into account the high 7-OH-MTX plasma levels found after the end of infusion, i.e. up to 10-times higher than MTX levels) that 7-OH-MTX might modulate the MTX biochemical response, and consequently the antifolate activity, *in vivo*. Thus, considering the inter- and intra-subject variations in the kinetics of MTX and 7-OH-MTX, it would be interesting to try to examine a larger set of clinical and analytical data, to establish a correlation between efficacy, therapeutic failure and toxicity, with respect to MTX and 7-OH-MTX pharmacokinetics.

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