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Methotrexate Test-Dose Protocol in the Presence of 7-Hydroxy-Methotrexate*

N. LENA,†‡ A.M. IMBERT,† R. FAVRE,§ J.P. CANO¶ and Y. CARCASSONNE§

Departments of †Biochemical and Clinical Pharmacokinetics Laboratory, §Clinical Oncology Division and ¶INSERM U.278, Institute J.Paoli-I.Calmettes, 232 bd Ste Marguerite, 13273 Marseille Cedex 9, France.

Abstract—*Methotrexate (MTX) and 7-hydroxy-methotrexate (7-OH-MTX) plasma concentration–time curves (AUC) have been analyzed in 24 patients after different routes of MTX administration. After an i.v. bolus (50 mg/m^2), the AUC for 7-OH-MTX is correlated with that for MTX and inversely correlated with the MTX plasma clearance. When MTX is administered with plasma steady level standardization, using the test-dose protocol, at a level of 10^{-5} M over 36 hr (10^{-5}, 36 hr), 7-OH-MTX-AUC is still correlated with the i.v. bolus pharmacokinetic parameters. The dose prediction using the classical test-dose protocol provides a less efficient MTX dose adjustment at 5×10^{-4}M over 8 hr (5.10^{-4}, 8 hr) and the hydroxylation process is no more correlated with the i.v. parameters.*

On the opposite, upon 6 successive infusions with 10^{-5}, 36 hr or 5.10^{-4}, 8 hr protocols, the plasma concentrations of 7-OH-MTX are not significantly modified. This suggests that the hydroxylation process is not inducible.

INTRODUCTION

SINCE Jacobs *et al.* [1] first observed the hydroxylated metabolite of methotrexate (MTX) in plasma and urine of patients treated by this antifolate, studies have been undertaken to determine the implication of the 7-hydroxy-methotrexate (7-OH-MTX) in the MTX pharmacokinetics [2–6].

Concomitantly, a mathematical model established by Reich [7], based upon the knowledge of MTX plasma pharmacokinetics led to the development of a test-dose protocol [8, 9]: the determination of the MTX plasma clearance after the administration of a low, non-toxic dose (10–50 mg/m^2) allows the computation of infusion doses to reach a predetermined plasma steady-state level. This model does not take account of the MTX hydroxylation process because of the simultaneity of the two approaches and the poor sensitivity of the 7-OH-MTX measurement. It has been claimed that the hydroxylation occurred only after high-dose MTX treatments [2, 5, 10, 11]. The development of more specific assays, increasing the sensitivity for the measurement of 7-OH-MTX, have shown that the MTX hydroxylation takes place even after doses as low as 15 mg [6]. This biotransformation set the problem of the accuracy of the test-dose protocol based on MTX levels only, when large amounts of metabolite are present.

Previous results [12] have shown a good prediction of the MTX plasma levels after the test-dose protocol in the range of predetermined levels comprised between 10^{-5} M and 10^{-4} M over 24–36 hr. But the prediction was not so efficient when this protocol was applied to 5×10^{-4} M regimen. Then, in this study, we have been interested in the MTX hydroxylation to determine how it could influence the dose prediction. We compared the kinetics of 7-OH-MTX after an i.v. bolus injection and during 2 different high-dose MTX protocols.

PATIENTS AND METHODS

Patients

Twenty-four patients treated in the Institut J.Paoli-I.Calmettes for different tumor localizations entered the study. Ages ranged between 9 and 74 years. Only patients with serum creatinine < 130 μM, granulocyte count $> 1500/mm^3$ and

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Correspondence and requests for reprints should be addressed to N. Lena, Institut J.Paoli-I.Calmettes, 232 bd Ste Marguerite, 13273 Marseille Cedex 9, France.
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platelet count > 100,000/mm^3 were eligible to take part into the study.

Therapeutic protocol

Patients were treated once a month by continuous i.v. infusion with a predetermined MTX plasma steady-state level of 10^{-5} M over 36 hr (10^{-5}, 36 hr) or 5×10^{-4} M over 8 hr (5.10^{-4}, 8 hr). The choice of the treatment was subordinated to the tumor localization. Patients with head and neck carcinoma (7 patients) and patients considered as high-risk patients received 10^{-5}, 36 hr treatment. The other patients, most of them with osteosarcoma, received 5.10^{-4}, 8 hr protocols.

All the patients underwent hydratation and urine alkalinization pretreatment [13] 12 hr prior to the MTX infusion. Blood samples were taken at specified time intervals until MTX plasma levels were lower than 10^{-7} M. Folinic acid rescue started 36 hr after the beginning of the MTX infusion, following 2 different routes of administration: an i.v. continuous infusion (200 mg/m^2 over 12 hr) for 10^{-5}, 36 hr treatment or 15 mg i.v. every 6 hr for 5.10^{-4}, 8 hr protocol.

Infusion dose determination

To determine the MTX infusion dose for each patient [8], the individual MTX plasma clearance (Cl) was estimated after an identification i.v. bolus (50 mg/m^2). For a constant rate infusion, the MTX infusion dose (q_o) necessary to achieve a predetermined steady-state level (C_{ss}) is computed by:

$$q_o = C_{ss} \times T \times Cl$$

where T represents the infusion duration.

MTX and 7-OH-MTX assay

MTX assay. MTX plasma concentrations were measured by an enzymatic assay based on the dihydrofolate reductase inhibition [14] which has been adapted on a Cobas Bio centrifugal analyzer [15]. The useful concentration range was between 9×10^{-9} M and 1.6×10^{-7} M with coefficient of variation (c.v.) lower than 4.8%. The 7-OH-MTX interference on the MTX measurement was lower than 1%.

7-OH-MTX assay. We used a liquid phase radioimmunoassay developed by Boré *et al.* [16] who kindly supplied us with antisera against 7-OH-MTX. We confirmed that MTX did not interfere significantly (cross-reactivity factor = 2.5×10^{-4}). Concentrations of unknown samples were calculated by interpolation on linearized logit (B/BO) vs. log of concentration standard reference curve. With this procedure 7-OH-MTX was measured between 8.6×10^{-10} M and 2.15×10^{-9} M (c.v. lower than 7.5%). All samples were run in duplicate.

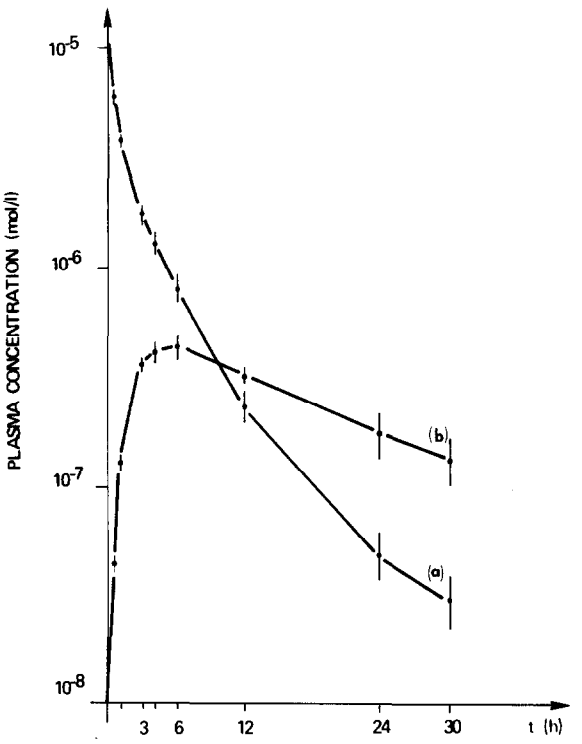

Fig. 1. MTX (a) and 7-OH-MTX (b) plasma concentration–time curves after a MTX i.v. bolus (50 mg/m^2). Each point represents the mean value of 24 determinations (± standard deviation).

Statistical analysis

Areas under the concentration vs. time curves (AUC) were estimated by trapezoidal integration. To give an estimation of the variability in the experimental data, the standard deviation (S.D.) or the coefficient of variation (c.v.) was indicated with the mean values.

The prediction of the infusion dose was evaluable by the comparison between the predicted and achieved plasma levels (C_{ss}). The bias of the prediction was measured by an estimation of the mean prediction error [12, 17].

$$\text{bias} = \frac{\text{achieved } C_{ss} - \text{predicted } C_{ss}}{n}$$

A two-way analysis of variance was carried out with standard methodology to analyze the source of variability (inter-individual or between courses) upon repeated treatments.

Unless specifically mentioned, the level of significance was set at $P = 0.05$.

RESULTS

MTX and 7-OH-MTX kinetics after i.v. bolus

The median plasma concentration curves for MTX and 7-OH-MTX from 24 patients following i.v. bolus administration (50 mg/m^2) are shown in Fig. 1.

Fifteen min after the MTX injection, the MTX plasma level was $(1.036 \pm 0.06) \times 10^{-5}$ M, and decreased until $(3.1 \pm 0.8) \times 10^{-8}$ M at the 30th hr. The sensitivity of the radioimmunoassay used for measuring 7-OH-MTX concentrations allowed us to demonstrate the presence of this compound as soon as 15 min after the MTX i.v. bolus with a mean value of $(1.47 \pm 0.24) \times 10^{-8}$ M. The 7-OH-MTX concentration increased to reach, after 4 hr, a maximum value of $(4.63 \pm 0.46) \times 10^{-6}$ M and then decreased slowly to a concentration of $(1.36 \pm 0.33) \times 10^{-7}$ M, 30 hr after the infusion. The median areas under the plasma concentration–time curves were respectively 2.31×10^{-5} M × hr (c.v, = 39.8%) for MTX which corresponds to a median plasma clearance of 8.59 l/hr, and 9.56×10^{-6} (c.v. = 69.9%) for 7-OH-MTX. Because of a slower disappearance from the plasma (median half-life time of 15 hr 15 compared to 8 hr 30 for the parent drug between 24 and 30 hr after administration), the median 7-OH-MTX exceeded that of MTX as soon as 10 hr after the i.v. push and the median ratio of AUC for 7-OH-MTX to that of MTX was 0.414. These results denote an active hydroxylation process after 50 mg/m^2 MTX i.v. push with a great inter-individual variation (c.v. = 69.9%).

There was a highly significant correlation between the AUC of MTX and 7-OH-MTX ($r = 0.747$; $P = 0.001$) and an inverse correlation between the MTX clearance and the area under the 7-OH-MTX curve ($r = 0.554$; $P = 0.01$) after the MTX i.v. push.

MTX and 7-OH-MTX kinetics during infusion

Figure 2 shows the median MTX and 7-OH-MTX plasma concentration–time curves after 10^{-5}, 36 hr protocol (11 patients, 21 infusions, Fig. 2A) and after 5.10^{-4}, 8 hr treatment (9 patients, 22 infusions, Fig. 2B). In all cases, MTX infusion doses have been computed from MTX clearance determined after the i.v. bolus. As previously described [12], the MTX predicted steady state level was achieved with a good precision for 10^{-5}, 36 hr infusions: the estimated bias was -6.1×10^{-7} M (the 95% confidence interval including zero). On the opposite, during 5.10^{-4}, 8 hr treatment, the estimated bias ($+ 7 \times 10^{-5}$ M) was signifcantly positive leading to an over-estimation of the MTX dose (+ 14%).

MTX hydroxylation characteristics

While the MTX steady state level during 10^{-5}, 36 hr infusions was achieved at the 12th hr, the 7-OH-MTX concentration was increasing from 1.86 to 3.16×10^{-6} M which corresponds to an increase of the ratio between the 7-OH-MTX level to that of MTX from 0.196 to 0.329. This ratio

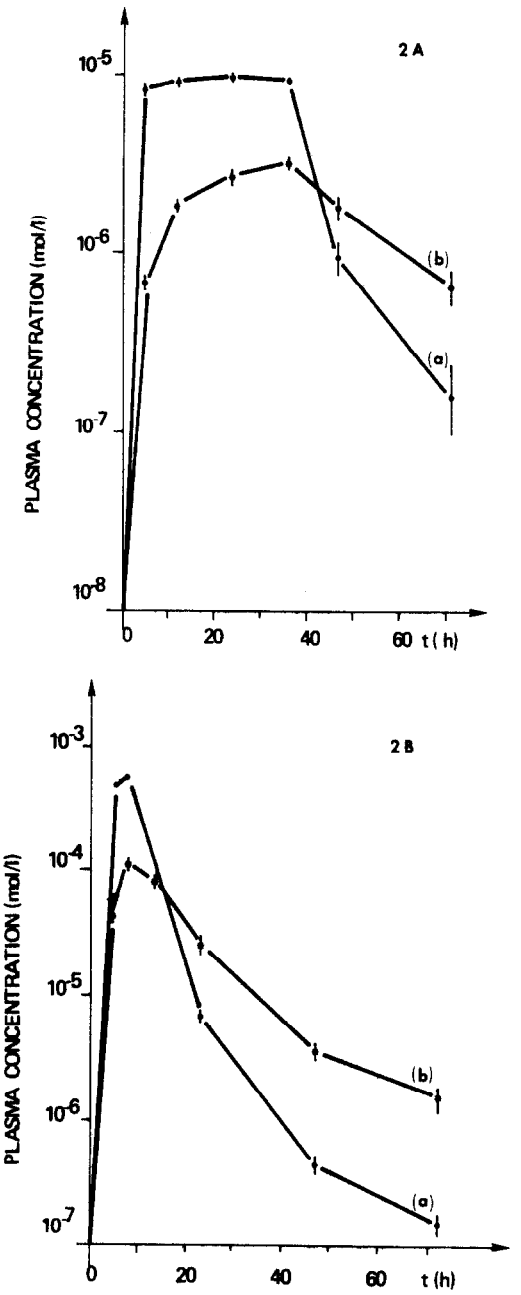

Fig. 2. MTX (a) and 7-OH-MTX (b) plasma concentration–time curves during 10^{-5}M, 36 hr (*Fig. 2A*) *and* 5.10^{-4}M, 8 hr (*Fig. 2B*) *infusions. Each point represents the mean value of* 21 *determinations* (*Fig. 2A*) *and* 22 (*Fig. 2B*) (± *standard deviation*).

was only of 0.198 at the end of the 5.10^{-4}, 8 hr infusions.

The ratio of AUC during the infusion phase for 7-OH-MTX to that of MTX can be an approach to appreciate the hydroxylation phase. The comparison of these ratios shows that they are significantly different and lower during 5.10^{-4}, 8 hr infusions (mean ratio = 0.097) than after 10^{-5}, 36 hr protocols (mean ratio = 0.220).

The AUC of MTX and 7-OH-MTX have been compared between the first infusion therapy and the intravenous bolus injection for each patient. The results showed a significant positive correlation

Table 1. Statistical analysis of 7-OH-MTX AUC after courses 1, 2, 4 and 6

Origin of variation	Estimated variances	Degree of freedom	*F*
Overall variability	183.8	35	
Inter-individual variability (*A*)	151.33	8	6.9 S
Variability due to courses (*B*)	10.60	3	0.5 N.S.
Random	21.87	24	

Two-way analysis of variance (*F*-test): the overall observed measurement of variability was separated into 3 components: inter-individual variability (*A*), variability due to the successive courses (*B*) and random variability. S = Significant; N.S. = non-significant. *F* = Ratio between the estimated variances (*A* or *B*) and the random variability. The level of significance was $P = 0.01$.

after 10^{-5}, 36 hr infusions for MTX ($r = 0.714$) and 7-OH-MTX ($r = 0.665$). In contrast, in the case of 5.10^{-4}, 8 hr treatment, the correlation was not so close for MTX ($r = 0.627$; $P = 0.1$) and there is no correlation for 7-OH-MTX ($r = 0.269$).

To study the MTX hydroxylation upon repeated treatments, we analyzed courses 1, 2, 4 and 6 for 9 patients. The comparison of the areas under the curves for 7-OH-MTX (expressed as 10^{-8} M × hr/mg of injected drug) during these 4 courses was performed using the analysis of variance procedure. This test allowed us to study the between-course variability independently of the inter-individual variability. No significant difference in MTX hydroxylation was associated with repeated treatments (Table 1).

DISCUSSION

Most of the previous studies on MTX pharmacokinetics did not take into account its hydroxylation process. The sensitivity of the radioimmunoassay used [16] for measuring 7-OH-MTX allowed us to detect this compound as soon as 15 min after the MTX i.v. bolus. The use of highly specific assays for these 2 compounds (enzymatic assay for MTX and radioimmunoassay for 7-OH-MTX) has made possible the monitoring of the parent drug and its metabolite, even when the 7-OH-MTX concentrations exceeded those of MTX at the end of the plasma elimination phase. In the 24 patients who received a MTX i.v. push (50 mg/m^2), 7-OH-MTX was always measurable.

The large inter-individual variability of the hydroxylation after the i.v. push (c.v. = 69.9%) is in contrast with the constancy of the 7-OH-MTX levels during repeated infusions in the same patient. These results confirm the data described by Brienhaupt [2] and Fabre [18] who have shown a great inter-individual variability in the MTX hydroxylation, but they contrast with the data reported by Erttman *et al.* [3]: they have indicated a decrease in the hydroxylation process during repetitive weekly infusions, probably due to a cumulative hepatic toxicity, sustained by the weekly treatments. The discrepancy between these results and our data may reflect the difference in the interval between the infusions: the one-month interval may lead to the recovery of normal hepatic functions and permit a constant level of hydroxylation for each patient. All these results do not support the hypothesis of the inductibility of the hydroxylation process [5].

The knowledge of the MTX pharmacokinetic comportment allowed the determination of individual infusion doses to reach a predetermined steady-state level [7, 8, 19]. This test-dose protocol is currently applied, and has been for more than 6 years, in the Institut Paoli-Calmettes. In routine clinical practice it was used for a predetermined plasma level of 10^{-5} M over 36 hr and more recently it was adapted to reach 5×10^{-4} M over 8 hr. As previous results [12] have shown, the dose prediction is efficient for the 10^{-5}, 36 hr protocol, but there is a significant positive bias for 5×10^{-4} M over 8 hr treatment, leading to an overestimation of the MTX dose. The modification noted in the 7-OH-MTX formation could explain this discrepancy: indeed, as there is a close correlation between the 7-OH-MTX AUC obtained after the i.v. push and the first 10^{-5}, 36 hr infusion, this correlation does not exist for the 5×10^{-4}, 8 hr protocol, and we showed that the 7-OH-MTX formation is proportionally decreased during these high-dose infusions. The slackening of the hydroxylation leads to higher MTX plasma levels and then to a shift in the metabolic clearance of the parent drug. This could provide from a modification of the hydroxylation process due to a saturation of the hepatic enzymatic system. Nevertheless, our studies do not allow us to omit the hypothesis of a decrease in the exchange between hepatic cells and plasma according to the shorter time of the infusion.

Until now, 7-OH-MTX was considered as nephrotoxic and without any antitumor activity, contributing to the detoxification of high-dose therapy [3]. But recent *in vitro* results have shown that this compound can be metabolized to 7-OH-MTX polyglutamyl derivatives [19, 20]. These derivatives are better inhibitors for both the dihydrofolate reductase [21, 22] and the folyl polyglutamate synthetase [21]. They also inhibit the thymidylate synthetase and the phosphoribosylanimoimidazolecarboxamide transformylase [23].

Further studies are required to determine whether the 7-OH-MTX activity could (or not) represent a benefit in the clinical practice. As the level of

hydroxylation is different according to the infusion protocol, it could represent a further parameter in the choice of the MTX protocol administration. If high-dose MTX therapy ($> 5 \times 10^{-4}$ M) has to be chosen, it would be necessary to determine a model which takes the hydroxylation process into account to predict MTX doses more efficiently.

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