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# Monitoring of methotrexate and 7-hydroxy-methotrexate in saliva from children with acute lymphoblastic leukemia receiving high-dose consolidation treatment: relation to oral mucositis

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**The purpose of the study was to find out if saliva concentrations of methotrexate (MTX) and its main metabolite, 7-hydroxymethotrexate (7-OHMTX), can predict oral mucositis in children with acute lymphoblastic leukemia (ALL) after treatment with high-dose consolidation therapy. We have also studied the relationship between the concentrations of MTX and 7-OHMTX in saliva and the unbound concentrations in plasma. Twelve patients (36 infusions) were studied during treatment with high-dose MTX as remission consolidation therapy (5–8 g/m² by 24 h i.v. infusion followed by leucovorin rescue). Plasma and saliva concentrations of MTX and 7-OHMTX were determined concomitantly by HPLC at 20 h and at various times following infusion. Unbound plasma concentrations of MTX and 7-OHMTX were determined after ultrafiltration. Oral toxicity was graded according to the WHO criteria (grade 0–4). The concentrations of MTX and 7-OHMTX in saliva were not directly related to the development of mucositis. In patients with oral mucositis (WHO grade 1 or greater), the ratio to 7-OHMTX and MTX in saliva at 20 h was significantly lower than in patients without symptoms ($p = 0.014$, Mann–Whitney rank sum test), but not at 42 and 66 h after starting the infusion. The salivary concentration of 7-OHMTX at 20 h ranged from undetectable (less than 1 nmol/l) to 1.6 μmol/l. No significant correlation was found between the unbound and total plasma concentrations of MTX and 7-OHMTX and the drug concentrations in saliva at different points in time. The concentrations of 7-OHMTX in saliva were 11, 23 and 13% of the unbound plasma concentrations at 20, 42 and 66 h, respectively, after starting the infusion. The respective median corresponding values for MTX were 1.6, 16.1 and 61.6%. The results suggest that determinations of saliva concentrations of MTX and 7-OHMTX may predict oral mucositis. This opens up the possibility of early identification of patients at high risk of developing oral mucositis in order to intensify topical or systemic treatment of these patients.**

*Key words*: 7-Hydroxymethotrexate, methotrexate, oral mucositis, saliva.

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## Introduction

Intravenous high-dose methotrexate (HD-MTX, 5–8 g/m²) can be safely administered as remission consolidation therapy to children with acute lymphoblastic leukemia (ALL) when it is followed by leucovorin rescue guided by plasma MTX monitoring.[1,2]

MTX exerts its cytotoxic effect by inhibiting dihydrofolate reductase, a key enzyme in the formation of nucleic acid precursors. The major metabolite in plasma, 7-hydroxymethotrexate (7-OHMTX), is not usually determined during routine plasma concentration monitoring. Soon after infusion of the drug its plasma concentration exceeds that of MTX.[3] The significance of the metabolite concentration for the therapeutic and toxic effects of HD-MTX is not clear. It is a much weaker inhibitor of dihydrofolate reductase than the parent compound,[4] but may influence the effects of MTX by interaction with carrier-mediated transport processes. Indeed, a low plasma concentration of 7-OHMTX or a low 7-OHMTX/MTX plasma concentration ratio has been reported to be a risk factor for the development of MTX toxicity.[5,6]

Mucositis is a major toxicity during HD-MTX treatment[1,7] and we have found that oral mucositis occurred in 52% of the infusions according to the NOPHO-ALL protocol (5–8 g/m² for 24 h).[6] The plasma and salivary concentrations of MTX and their relation to oral mucositis has been investigated extensively.[8–13] It has been reported that there is no significant difference between MTX plasma levels in patients with and without oral mucositis.[9,13] In our study, oral mucositis occurred in 39% of the infusions with no delayed MTX elimination (below 1 μmol/l at 42 h). The development of mucositis in this group correlated significantly with low systemic clearance and a low plasma 7-OHMTX/MTX concentration ratio at 66 h after the start of the infusion.[6]

The MTX concentration in saliva was found to be a predictor of the risk of developing mucositis in one study,[9] but not in others.[10,11,13] We have now investigated whether the simultaneous determination of MTX and 7-OHMTX in saliva could improve the possibility of predicting oral mucositis.

## Materials and methods

### Chemicals

MTX, (+)-amethopterin, for HPLC analysis, was obtained from Sigma (St Louis, MO) and 7-OHMTX was generously donated by Dr WE Evans, St Jude Children's Research Hospital, Memphis, TN. Other chemicals used were of analytical purity and were obtained from standard commercial sources.

### Patients

Twelve children were studied during 36 HD-MTX courses according to the 1992 protocol of the Nordic Association for Paediatric Haematology and Oncology (NOPHO) (5–8 g/m$^2$, depending on the risk group, as an i.v. infusion during 24 h). Ten percent of the dose was administered during 1 h and the remainder during a constant rate infusion for 23 h. Leucovorin rescue (15 mg/m$^2$) was administered i.v. at 36, 42, 48 and 54 h after starting the infusion. In the case of delayed MTX elimination (plasma concentration greater than 1 $\mu$mol/l at 42 h or greater than 0.2 $\mu$mol/l at 66 h), the leucovorin rescue administration was intensified. All patients were hydrated with 3000 ml/m$^2$ of 5% glucose with the addition of 40 mmol/l sodium bicarbonate and 20 mmol/l potassium chloride. Mouthwash with chlorhexidine 0.1% or hibitane jelly was administered during all courses. Topical treatment with leucovorin (3 mg/dose three times daily) was administered in 72.7% of the infusions due to the development of early symptoms of oral mucositis or the occurrence of mucositis in previous courses.

### Collection of blood and saliva samples

Blood samples were collected at 20 ($C_{ss}$), 28, 36, 42, 54, 66, 120 and 168 h after the infusion. All blood samples were drawn from a central venous catheter except the steady-state sample which was taken from a peripheral vein. Blood was collected in heparinized glass tubes and plasma was prepared by centrifugation (3500 r.p.m., 15 min) at 4°C. Two samples of mixed non-stimulated saliva were collected simultaneously for 5 min on filter paper from each side of the glandulae parotis ductal orifice at 20, 42 and 66 h after starting the infusion. All samples were stored at −80°C until analyzed. Mucositis was assessed by oral inspection by one of the investigators (CR) on days 0, 3 and 7 after starting the MTX infusion according to the WHO criteria (grades 0–4).

### Drug analysis

MTX and 7-OHMTX were analyzed in plasma using solid-phase extraction and liquid chromatography with fluorometric detection.[14] After dilution (10–200 times) saliva was injected directly into the column without sample preparation. Binding of MTX and 7-OHMTX to plasma proteins could only be determined in 16 infusions (from 12 patients), at 20, 42, 66 and 168 h after starting the infusions. Ultrafiltration of 0.5–1 ml of plasma was carried out at 37°C using the ultrafiltration technique of the Amicon Centrifree Micropartition System (WR Grace, Beverly, MA) according to the manufacturer's instructions. The extent of unbound fraction was calculated as: % unbound = $(C_f/C_p) \times 100$, where $C_p$ is the initial concentration of the drug in plasma and $C_f$ is the concentration in the filtrate.

### Statistical evaluation

Statistical calculations of unpaired data were based on the Mann–Whitney rank sum test and paired data on Wilcoxon's signed rank test. Correlation between total plasma, unbound and saliva concentrations of MTX and 7-OHMTX was analyzed by Pearson's correlation coefficient and linear regression analysis. Statistical calculations were done using StatView software (Abacus Concepts, Berkeley, CA). A probability value of less than or equal to 0.05 was considered statistically significant.

## Results

The pharmacokinetic profiles of MTX and 7-OHMTX in plasma and saliva are shown in Figure 1(A–C). The detailed pharmacokinetic parameters of MTX and 7-OHMTX in plasma and of MTX in saliva of these patients have been reported earlier.[6] In all infusions, the salivary concentration of MTX was

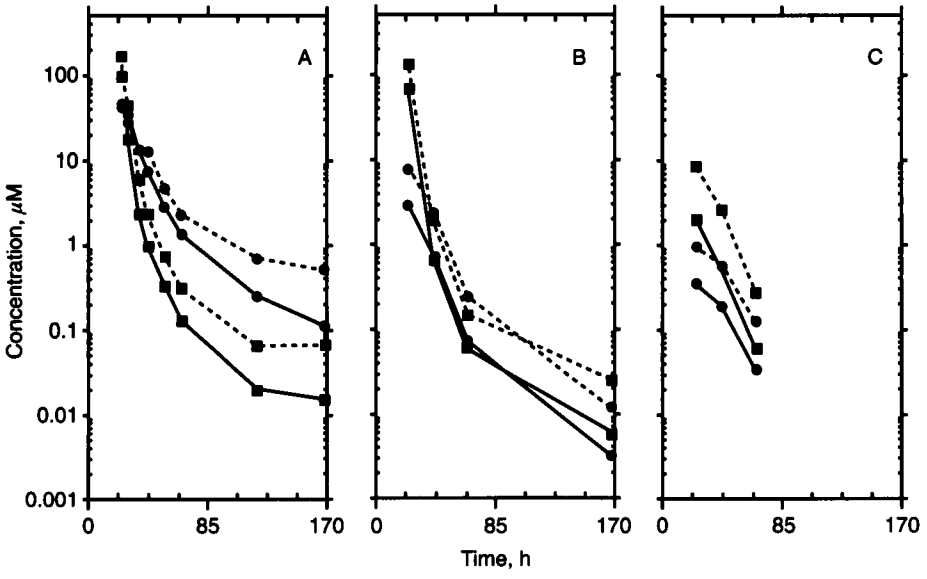


**Figure 1.** Total plasma (A), unbound plasma (B) and salivary (C) MTX (■) and 7-OHMTX (●) profiles of 16 HD-MTX infusions. Dashed lines show 2SD of the respective compounds.

detectable 66 h after starting the infusions. In 14 of 36 infusions, salivary concentration of MTX exceeded 1 μmol/l 20 h after starting of infusion. The overall correlations between salivary concentrations compared to unbound and total plasma concentrations are $r = 0.38$ ($p = 0.016$) and $r = 0.39$ ($p = 0.014$) for MTX and $r = 0.46$ ($p = 0.003$) and $r = 0.47$ ($p = 0.003$) for 7-OHMTX, respectively (Figure 2A and B). At different points in time, the total or unbound plasma concentration was not significantly correlated with salivary concentrations of MTX or 7-OHMTX (data not shown). The median ratio between the saliva and unbound plasma concentrations of MTX increased with time from 0.016 at 20 h to 0.62 at 66 h after starting the infusion (Table 1). The corresponding median ratio for 7-OHMTX was 0.11 and 0.13, repectively. The median unbound plasma MTX fraction at 20 h was 0.69 for MTX and 0.06 for 7-OHMTX (Table 1). The unbound MTX plasma fraction at 66 h was significantly different from the unbound MTX fraction at 20 h ($p = 0.008$, Wilcoxon's signed rank test) and 42 h ($p = 0.04$) (Table 1).

Oral mucositis was found to be significantly related to a low ratio between 7-OHMTX and MTX concentrations (WHO grade 1 or above, median ratio 0.15, and WHO grade 0, median ratio 0.35, $p = 0.014$, Mann–Whitney rank sum test) in saliva

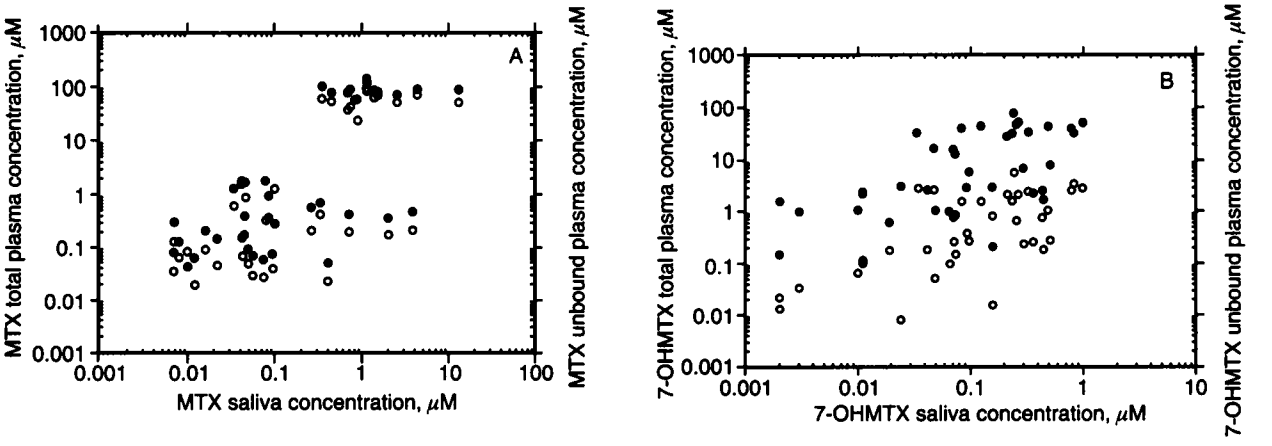


**Figure 2.** Correlation between salivary concentration and unbound plasma (○) and total plasma (●) concentration of MTX (A, r = 0.38 and r = 0.39) and 7-OHMTX (B, r = 0.46 and r = 0.47), respectively, n = 49.

**Table 1.** Median ratio of salivary (S) MTX and 7-OHMTX to total (P) and unbound (F) plasma concentrations during MTX infusion (20 h), 42 and 66 h after starting the infusion in 16 courses

| Substance | Time (h) | S/P | S/F | Fraction unbound in plasma |
|---|---|---|---|---|
| MTX | 20 | 0.009 (0.00–0.15)[a] | 0.016 (0.00–0.25) | 0.69 (0.42–0.98) |
| | 42 | 0.072 (0.02–8.09) | 0.161 (0.02–18.07) | 0.49 (0.18–1.1) |
| | 66 | 0.240 (0.01–7.97) | 0.616 (0.02–17.42) | 0.45 (0.23–0.57) |
| 7-OHMTX | 20 | 0.006 (0.00–0.03) | 0.112 (0.00–0.47) | 0.07 (0.01–0.12) |
| | 42 | 0.024 (0.00–0.26) | 0.234 (0.00–2.44) | 0.12 (0.02–0.31) |
| | 66 | 0.007 (0.00–0.75) | 0.132 (0.00–9.65) | 0.06 (0.03–0.18) |

[a]Range.

**Table 2.** Median ratio between salivary MTX and 7-OHMTX concentrations during (20 h) and after HD-MTX treatment in 12 patients (36 infusions)

| | | 7-OHMTX/MTX | | | |
|---|---|---|---|---|---|
| | WHO grade | 20 h | 42 h | 66 h | No. of courses |
| Mucositis | ⩾ 1 | 0.15 (0–0.53)[a] | 0.92 (0–2.18) | 1.05 (0–7.40) | 16 |
| Mucositis | 0 | 0.35 (0.05–0.88) | 1.03 (0.18–5.82) | 0.96 (0–4.10) | 20 |
| *p* values[b] | | 0.014 | 0.856 | 0.631 | |

[a]Range
[b]Mann–Whitney rank sum test.

at 20 h after the start of the 24 h infusion (Table 2). The individual concentrations of MTX at 20 h (WHO grade 1 or above, median 0.694 μmol/l and WHO grade 0, 0.814 μmol/l, $p = 0.444$) and 7-OHMTX (WHO grade 1 or above, mean 0.115 μmol/l and WHO grade 0; 0.248 μmol/l, $p = 0.07$) in saliva were not related to the development of mucositis. No difference in the elimination rates of MTX and 7-OHMTX was observed in saliva samples from patients with and without oral mucositis (data not shown).

## Discussion

Mucositis is one of the major problems associated with HD-MTX treatment. In a previous study we reported that the plasma MTX concentration at 20 h and a low 7-OHMTX/MTX concentration ratio at 66 h after the start of the infusion are significantly related to the degree of oral mucositis.[6] There was, however, no significant correlation between the occurrence of oral mucositis and the concentration of MTX in saliva. The present investigation constitutes the first detailed study, to our knowledge, of the 7-OHMTX level in saliva and its relation to oral toxicity.

The study reported here demonstrates the benefit of simultaneous measurements of the saliva concentrations of MTX and 7-OHMTX in the prediciton of oral mucositis. We found no significant correlation between the individual concentrations of MTX and 7-OHMTX in saliva, and they were not related to the development of mucositis.

The considerable variation in the elimination of MTX from saliva and increasing saliva/plasma MTX ratios after terminating the infusion are in accord with previous reports.[10,13] Since only the unbound

drug is able to cross biological membranes and is biologically active, we also investigated the protein binding of MTX and 7-OHMTX. No significant correlation was found between the unbound and total concentration of MTX and 7-OHMTX with salivary concentrations. The drug concentrations in saliva were much lower at 20 h than the unbound plasma concentrations, showing that unbound concentrations of MTX and 7-OHMTX are not in equilibrium with the drug in saliva. This is probably due to the occurrence of active transport processes.

The major finding of the present study was that the formation of 7-OHMTX from MTX, as determined in saliva, influences the oral toxicity of MTX. 7-OHMTX has much lower affinity to dihydrofolate reductase than MTX,[4] but 7-OHMTX polyglutamates are more potent inhibitors of dihydrofolate reductase than the non-polyglutamated compound and may also inhibit other enzymes involved in the interconversion of tetrahydrofolates and thereby affect the cytotoxicity of MTX. Lankelma *et al.* have shown that 7-OHMTX interferes with the accumulation of MTX in Ehrlich ascites tumor cells[3] and also with the formation of MTX polyglutamates in human lymphoblastic cells.[15] These findings suggest that 7-OHMTX might protect cultured cells from the toxic effect of MTX by preventing the cellular uptake and accumulation of MTX. Our results are in accord with this hypothesis.

Topical oral leucovorin has been reported to reduce MTX-induced oral mucositis.[13,16,17] In the present study topical leucovorin (3 mg/dose thrice daily) was administered in two-thirds of the infusions starting at 66 h after the infusion due to the development of early symptoms of mucositis or to the occurrence of mucositis in previous courses. In spite of the topical leucovorin treatment, many patients developed mucositis. We do not think that this treatment has biased our results. On the contrary, it may have weakened the correlations observed between the pharmacokinetics and the symptoms provided it was effective. Payet *et al.* also reported an interaction between leucovorin and 7-OHMTX in Raji cells.[18] They found that the rate of 7-OHMTX influx was competitively inhibited and also that the metabolism of 7-OHMTX to its cellular polyglutamyl derivatives was depressed by 90% by the presence of an equimolar leucovorin concentration. This might partly explain why Oliff *et al.* did not observe any significant effect on the development of mucositis even thought leucovorin mouthwash was given during the MTX infusion.[13]

The results suggest a re-evaluation of the saliva concentrations of MTX and 7-OHMTX as possible predictors of the oral toxicity in patients treated with high-dose MTX. This could open up the possibility of early identification of patients at high risk of developing oral mucositis. Studies of the use of leucovorin mouthwash introduced at an early point in time should be made to evaluate the efficacy of this treatment on mucositis. However, the data presented here obtained in a small number of patients do not allow us to distinct a cut-off point where the salivary 7-OHMTX/MTX ratio could be regarded as a risk factor. Further studies are needed to evaluate the clinical role of the saliva concentration of MTX and 7-OHMTX in patients with ALL.

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