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Interference of 7-hydroxymethotrexate with the determination of methotrexate in plasma samples from children with acute lymphoblastic leukemia employing routine clinical assays

Kambiz Fotoohi[a,*], Tor Skärby[b], Stefan Söderhäll[c], Curt Peterson[d], Freidoun Albertioni[a,d]

[a] *Department of Oncology and Pathology, Cancer Center Karolinska (CCK), Karolinska University Hospital, S-17176 Stockholm, Sweden*
[b] *Department of Clinical Pharmacology, Lund University Hospital, Lund, Sweden*
[c] *Department of Childhood, Cancer Research Unit, Karolinska University Hospital, Stockholm, Sweden*
[d] *Department of Medicine and Care, Division of Clinical Pharmacology, Faculty of Health Sciences, Linköping, Sweden*

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Abstract

The accuracy of two clinical assays, the enzyme-multiplied immunoassay (EMIT) and fluorescence polarization immunoassay (FPIA2), universally employed for measurement of plasma levels of methotrexate (MTX) in children administered a high dose of this drug for treatment of acute lymphoblastic leukemia was evaluated here. Because of its superior specificity, sensitivity, and precision, high performance liquid chromatography (HPLC) was selected as the reference method with which the other two procedures were compared using approximately 420 different plasma samples for method comparison. 7-Hydroxymethotrexate (7-OHMTX), the major plasma metabolite of MTX, that can be detected in plasma at relatively high concentrations for long periods following infusion of a high dose of MTX, was also quantitated by HPLC. Forty-two and 66 h after infusion, the plasma level of MTX was overestimated in 2% and 3% of the samples by the FPIA2 procedure in 5% and 31% by the EMIT assay. The overall correlation coefficients (r^2) for the values obtained by FPIA2 or EMIT versus those based on HPLC were 0.989 and 0.663, respectively. The presence of 7-OHMTX exerted a highly significant influence ($p = 0.0007$ as determined by the unpaired *t*-test) on MTX measurement by the EMIT assay. We conclude that the rapid automated procedures routinely used at present and in particular EMIT, suffer from cross-reactivity with metabolites of MTX. Thus, the relatively high percentage of samples in which the level of MTX is overestimated at check-points by EMIT may result in longer periods of hospitalization, higher costs and prolonged administration of elevated doses of "rescue" leucovorin with an increased risk for relapse.

Keywords: Methotrexate; 7-Hydroxymethotrexate; Enzyme-multiplied immunoassay; Fluorescence polarization immunoassay; High performance liquid chromatography; Leukemia

1. Introduction

Methotrexate (MTX, 4-amino-N^{10}-methylpteroylglutamic acid) has been used worldwide to treat a broad spectrum of malignant and non-malignant diseases during the last half-century. Today this chemotherapeutic agent is commonly used in the treatment of leukemias and lymphomas, as well as of certain solid tumors. For treatment of several types of leukemia, including acute lymphoblastic leukemia (ALL), a high dose of MTX (HDMTX) (i.e., >1 g/m^2 body surface) is administered intravenously, followed by rescue with leucovorin. The most common side-effects associated with HDMTX therapy without such rescue are myelosuppression and mucositis, the severity of both of which is related to the dose and duration of administration. On the other hand, the low plasma levels of MTX obtained using a dose of 1 g/m^2 are

Abbreviations: MTX, methotrexate; 7-OHMTX, 7-hydroxymethotrexate; EMIT, enzyme-multiplied immunoassay; FPIA2, fluorescence polarization immunoassay; HPLC, high performance liquid chromatography; NOPHO, Nordic Association for Paediatric Haematology and Oncology

* Corresponding author. Tel.: +46 8 517 756 55; fax: +46 8 517 703 68.
E-mail address: kambiz.fotoohi@onkpat.ki.se (K. Fotoohi).

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associated with an increased risk for relapse of the disease [1].

In order to identify patients with a high risk of developing toxicity despite adequate rescue, the plasma level of MTX must be monitored frequently both during and after administration, which is a unique approach among antineoplastic drugs. Accordingly, administration of leucovorin, alkalization and hydration can be adjusted individually in such a way as to minimize and, in most patients, even prevent the side-effects [2]. According to the protocol recommended by the Nordic Association for Pediatric Hematology and Oncology (NOPHO) in 1992, the plasma level of MTX should be determined at least after infusion has been completed and at regular time intervals thereafter until this level falls to below 0.2 μM [3].

Leucovorin rescue is initiated at 36 h after administration of MTX. Higher doses of leucovorin is recommended when the plasma concentration of MTX is ≥1 μM 42 h after administration. A recent study suggests that higher leucovorin doses during HDMTX therapy increases the risk of relapse in children being treated for ALL (Dr. Tor Skärby, personal communication). Since choice of the dose of leucovorin is based on the plasma concentrations of MTX, this concentration must be determined accurately.

Several methods can be employed to measure MTX in plasma, including enzyme inhibition radio-immunoassay (RIA), fluorescence polarization immunoassay (FPIA2), enzyme-multiplied immunoassay (EMIT) and high performance liquid chromatography (HPLC), each of which has its own advantages and limitations [4–6]. The automated procedures utilized world-wide for routine monitoring of MTX during HDMTX therapy, e.g., FPIA2 and EMIT, require relatively little pre-treatment of the sample and produce rapid results.

The major metabolite of MTX detected in plasma is 7-hydroxymethotrexate (7-OHMTX), the concentration of which exceeds that of MTX itself a few hours after high-dose administration. For instance, when the plasma level of MTX is around 0.2 μM, the simultaneous level of 7-OHMTX can be more than 20-fold higher [7,8]. Cross-reactivity with metabolites of MTX is a serious disadvantage of immunoassays [4–6]. Indeed, with the exception of HPLC, none of the assay procedures routinely employed can distinguish readily between MTX and 7-OHMTX. Recently, two other methods capable of detecting and resolving MTX and 7-OHMTX in human plasma have been described, i.e., a 384-well solid-phase procedure [9] and an assay based on capillary zone electrophoresis [10]. Since HPLC coupled with ultraviolet or fluorescent detection allows sensitive, accurate and selective measurement of both MTX and its metabolites in biological fluids, the present study was designed to compare two widely used automatic and routine clinical assay procedures with HPLC as a sort of the "golden standard". Frequent over-estimation of MTX concentrations in plasma at check-points, due to cross-reactivity with metabolites, provides an unreliable basis for effective therapy, as well as possibly increasing the risk for relapse by causing clinicians to administer excessively high doses of leucovorin for rescue.

2. Materials and methods

2.1. Chemicals

MTX and 7-OHMTX (>98% pure) (Schircks Laboratories, Jona, Switzerland), non-endcapped C_8 Isolute solid-phase extraction cartridges (100 mg) (International Sorbent Technology, United Kingdom), methanol and acetonitrile of HPLC grade (J.T. Baker, Deveuter, Holland), and sodium dihydrogen phosphate monohydrate, sodium dihydrogen phosphate dihydrate, hydrogen peroxide and trifluoroacetic acid (TFA) (Merck, Darmstadt, Germany) were purchased from sources indicated.

2.2. Plasma samples

Four hundred and twenty plasma samples were obtained from 49 ALL patients treated intravenously with MTX according to the NOPHO protocol of 1992 [3]. As the project was a part of the routine clinical care, a full research ethical evaluation was not demanded. Briefly, as part of their treatment, these patients were received a total of 5–8 g MTX per m^2 in two phases: 10% of the total dose was administered i.v. during the first hour and the remainder was infused over a period of 23 h. In conjunction with the 5- and 8-g/m^2 doses, leucovorin was administrated at doses of 15 and 50 mg/m^2, respectively 36 h after beginning the infusion of MTX. Additional leucovorin (15 mg/m^2) was given 39 h (to patients receiving 8 g MTX per m^2) and 42 h (to all patients who have received either 5 or 8 g doses) after starting the treatment.

The dose of leucovorin administered was increased if the patient's plasma level of MTX was >1 μM after 42 h. Following the protocol, the same dose of leucovorin given after 42 h was administered once every 6 h thereafter until the plasma concentration of MTX fell below 0.2 μM. All of the patients were hydrated (3 L/m^2/24 h) with glucose solution (50 g/L) containing sodium bicarbonate (42 mM) and KCl (20 mM). Unless the plasma level of MTX 36 h after initiation of infusion was >3 μM, in which case the rate of hydration was stipulated to be increased to 4.5 L/m^2/24 h.

After initiation of MTX infusion, blood samples were collected 20–23 h later (to obtain the steady-state concentration), 36 h later and once every 6 h thereafter until the plasma concentration of MTX had fallen to <0.2 μM. The steady-state samples, were taken from a peripheral vein and all other samples were drawn from a central venous catheter. These blood samples were collected in heparin-coated glass containers and kept refrigerated and protected from light for as long as 4 h prior to centrifugation (900 × *g*, 15 min, 4 °C). The plasma samples thus obtained were

stored at −20 °C in polypropylene tubes until analyses were performed.

2.3. The HPLC assay procedure

The HPLC assay used in this work has been described in details and evaluated previously [11]. Briefly, the C_8 cartridges (Isolute, UK) were activated with 3 ml methanol and 3 ml 0.1 M phosphoric acid, followed by addition of the plasma samples diluted with phosphoric acid. The cartridges were subsequently washed with 5% aqueous methanol and were eluted with 2% TFA, after which the elute was evaporated to dryness under a steam of nitrogen at 40 °C. The residue was then redissolved in 0.1 M phosphoric acid with mixing for 5 m and centrifuged, following which the supernatant was subject to HPLC analysis.

The HPLC system consisted of a CM 4000 pump (Milton Roy, LDC Division, USA); a CMA-240 auto sampler (Carnegie Medicine, Stockholm, Sweden); a beam boost photochemical reactor (ICT HG, Frankfurt, Germany); equipped with a 254-nm UV-lamp and a 10 m × 0.3 mm reaction coil; and a Shimadzu RF-10 AXL fluorescence detector (Shimadzu Corp., Kyoto, Japan) with excitation and emission wavelengths of 350 and 435 nm, respectively. The mobile phase consisting of 100 mM phosphate buffer, pH 6.5, acetonitrile, and 30% hydrogen peroxide (92:6:2, v:v:v) was passed through a 0.22 μm filter (Millipore, Ireland) and degassed prior to use. The column contained Chromtech RP-C18 and the flow rate was 1 ml/min. The chromatograms were processed by Chromatography Station for Windows version 1.5 (Watrex International Inc. CA).

2.4. Automated assays

EMIT assay, based on separation employing antibody-antigen binding and quantitation with an enzyme reaction, was performed on an automated drug analyzer (Behring Diagnostic, Syva Business, San Jose, CA, USA). The FPIA assay is based on measurement of the polarization of fluorescence probes attached to antigens which occurs upon specific binding to an antibody. Monoclonal antibody is used in the FPIA2 procedure. This procedure was performed on an FLx TDx analyzer (Abbott Scandinavia AB, Diagnostic Division, Stockholm, Sweden) and both assays were carried out according to the instructions in the manuals.

2.5. Statistical analysis

The data obtained were analyzed utilizing the graphic principle, described in detail previously [12]. Briefly, in order to visualize deviations in lower values more clearly, we plotted the relative values obtained by the automated methods against the values provided by the HPLC procedure. The influence of 7-OHMTX on MTX measurements were evaluated employing an unpaired *t*-test. In all cases, a *p*-value <0.05 was considered to be statistically significant. All statistical analyses were performed by using StatView software (Abacus Concepts, Berkeley, CA, USA).

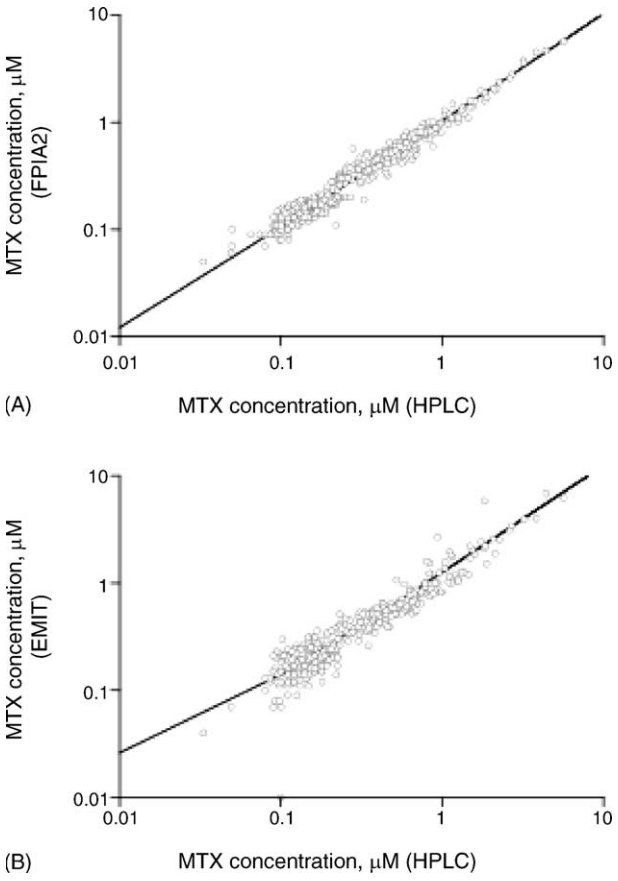

Fig. 1. Blood samples of 49 children with ALL have been taken as part of their treatment at the indicated time controls up to 66 h following HDMTX therapy. All 420 samples were analyzed using HPLC as standard method and two routine laboratory assays, FPIA2 and EMIT assays. Scatter-plots of the correlation between the MTX concentrations obtained by the HPLC and FPIA2 (A) or EMIT (B) assay procedures. The Pearson correlation coefficient (r^2) was 0.989 for FPIA2 vs. HPLC, and 0.633 for EMIT vs. HPLC ($n = 418$) and 0.635 for EMIT vs. FPIA2.

3. Results

Since MTX and 7-OHMTX can be resolved readily by HPLC, and no interfering peaks were observed in plasma devoid of these substances, we chose this approach as the reference procedure. The sensitivity, precision and accuracy of all three assay procedures utilized the present study have been described and evaluated in an earlier study [6]. Analysis of plasma samples by EMIT, FPIA2 and HPLC involved lower limits of detection of 0.300, 0.050 and 0.0001 μmol/L, respectively. The same day and day-to-day precision of the HPLC method in measuring 7-OHMTX at all concentrations was <9.4%.

In Fig. 1A and B, the concentrations of MTX as determined by EMIT and FPIA2 were plotted against the corresponding concentrations obtained by HPLC. The Pearson

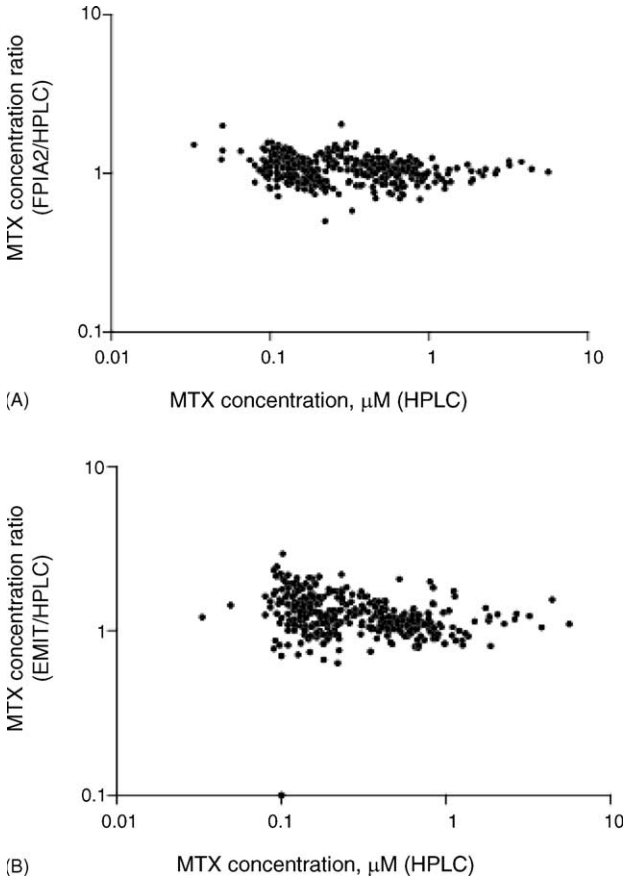

Fig. 2. Scatter-plots of the relationship between the ratio of the MTX concentrations as determined by FPIA2/HPLC (A) and EMIT/HPLC (B) and the actual MTX concentrations as measured by HPLC.

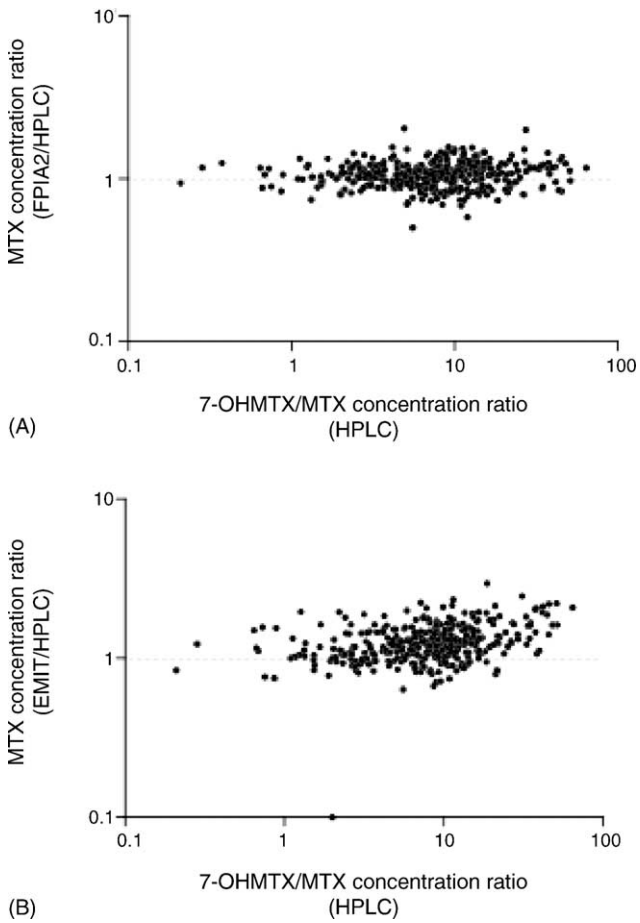

Fig. 3. Influence of the presence of 7-OHMTX on determination of MTX by the FPIA2 (A) and EMIT (B) assay procedures. The results obtained with the FPIA2 and EMIT procedures were plotted against the ratio of 7-OHMTX/MTX concentrations as measured by HPLC. The influence of the presence of 7-OHMTX on the values provided by the FPIA2 and EMIT assays become more pronounced as this ratio increased.

correlation coefficients (r^2) were 0.989 for the plot of FPIA2 versus HPLC; 0.633 for EMIT versus HPLC, and 0.635 for EMIT versus FPIA2.

The relationship between the concentration of MTX detected by FPIA2 or EMIT and the corresponding concentration obtained by HPLC are presented in Fig. 2A and B, respectively. As shown, the discrepancies were more pronounced at the lower concentrations of MTX present in plasma samples, taken 42 h post-infusion, especially in the case of the EMIT assay (Fig. 2B). We performed a more detailed analysis at two check-points 42 and 66 h post-infusion, the former being an important check-point for choosing the dose of leucovorin to be administered according to the NOPHO-92 protocol.

Among 196 samples analyzed by HPLC, FPIA2 and EMIT after 42 h, 26, 30 and 32 samples, respectively, were found to contain concentrations of MTX greater than 1 μM; and among 114 samples examined in a similar manner after 66 h, 7, 7 and 18 samples, respectively contained >0.2 μM MTX. In comparison to the HPLC values, the FPIA2 assay overestimated the MTX concentration in 2% of the plasma samples after 42 h and 3% after 66 h overestimated; While the corresponding values for the EMIT procedure were 5% and 31%.

In order to investigate the possible influence of 7-OHMTX, the major metabolite of MTX found in plasma, on the measurements performed here, the results obtained with the FPIA2 and EMIT procedures were plotted against the ratio of 7-OHMTX/MTX concentrations as measured by HPLC. The influence of 7-OHMTX on the values provided by the FPIA2 and EMIT assays becomes more pronounced as this ratio increased (Fig. 3A and B). The mean concentration of MTX in the samples taken 66 h after infusion obtained by HPLC was 0.15 ± 0.5 μM, while the corresponding value for 7-OHMTX was 1.7 ± 1.67 μM. At this time-point, the concentrations of 7-OHMTX varied from 2 to 51-fold higher than those of MTX.

In order to test the hypothesis that the presence of 7-OHMTX may interfere with determination of MTX, the unpaired *t*-test was used to compare the ratio of 7-OHMTX/MTX concentrations for two groups of samples one in which the EMIT procedure gave on overestimation of plasma MTX levels compared to HPLC and another group for which these procedures resulted in similar values (Fig. 4).

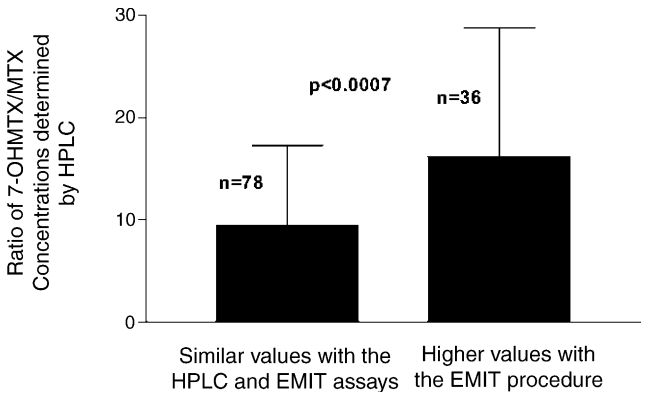

Fig. 4. Discrepancies in plasma concentrations of MTX in 196 samples measured by automated methods at 42 and 66 h. The ratio of 7-OHMTX/MTX concentrations (as determined by HPLC) for two groups of plasma samples, one for which similar MTX concentrations were obtained by EMIT and HPLC (left) and the other in which the MTX concentration was overestimated by the EMIT assay (right). The difference between these groups was highly significant ($p = 0.0007$) using the unpaired *t*-test. No such difference was observed in the case of FPIA2 assay ($p > 0.05$).

The mean ratio of 7-OHMTX/MTX concentrations in these two groups of samples were 9.5 ± 7.8 and 16.2 ± 12, respectively, the difference being highly significant ($p = 0.0007$) using the unpaired *t*-test. No such difference was observed in the case of the FPIA2 assay procedure ($p > 0.05$).

4. Discussion

Intensified chemotherapy of children with ALL has improved their outcome, increasing the rate of event-free survival to 80% in the case of non-mature B-cell [3]. According to the 1992 NOPHO protocol for treatment of ALL, a high-dose of MTX should be administered by infusion in two phases, i.e., 10% of the total dose (5–8 g) during the first hour, followed by infusion of the remaining dose over a 23-h period. Higher concentrations of MTX can result in subsequent administration of higher Leucovorin doses, which can increase the risk for relapse in children treated for ALL (Dr. Tor Skarby, personal communication).

Many different procedures for monitoring plasma MTX levels have been recommended by various investigators, including: relatively complex microbiological, fluorometric, enzymatic and HPLC approaches. However, routine measurement of MTX is performed most frequently by EMIT and FPIA2. Although rapid and simple, these routine methods are based on immunoassays that are unable to distinguish between MTX and its metabolites. Furthermore several endogenous and exogenous substances have been widely reported to interfere with the EMIT assay. For instance, Oellerich et al. [13] reported that bilirubin, hemoglobin and triglycerides influence the EMIT assay in such a manner as to cause deviations of less than 10% from the expected MTX values.

MTX is extensively oxidized by aldehyde oxidase in the liver to produce 7-OHMTX [14]. This metabolite demonstrate markedly lower affinity (>100-fold) for binding to dihydrofolate reductase compared to MTX, but its polyglutamate derivatives inhibit thymidylate synthase and dihydrofolate reductase with a potency comparable to that of MTX polyglutamates [7,15]. 7-OHMTX can attenuate the therapeutic effect of MTX as a consequence of its slow rate of elimination in combination with competition with MTX for cellular uptake and intracellular polyglutamylation. As we have shown recently, 7-OHMTX can even give rise to resistance to MTX, at least in vitro [16].

In the present study, we have shown that 7-OHMTX can lead to overestimation of the plasma level of MTX by EMIT procedure after MTX-infusion (Fig. 4A), which could result in subsequent over-dosage with leucovorin [2]. Buice et al. [4] also found cross-reactivity with 7-OHMTX of less than 4% in EMIT, MRA and RIA assay procedures for MTX. These authors suggested that these methods could nonetheless be reliable for rapid determination of plasma MTX within 24 h after a 6-h infusion, when the concentration of the pteroic metabolite is low.

Donehower et al. [17] reported that RIA assay of MTX 48 and 72 h after MTX administration resulted in values that were too high because of cross reactivity with metabolites such as 2,4-diamino-N^{10}-methylpteroic acid (DAMPA). However, in contrast to our findings here, Chandra and coworkers [5] reported a good correlation between the values obtained by HPLC and EMIT, with no cross-reactivity in connection with the EMIT assay. One explanation for this discrepancy could be that these other investigators collected plasma samples only up to 48 h after the start of MTX infusion, whereas we found the major discrepancies after 66 h, when the concentration ratio of 7-OHMTX/MTX is much higher. In our previous investigation we also observed a greater degree of cross-reactivity in the EMIT than in the FPIA2 assay, in good agreement with the present findings [6]. Furthermore, in agreement with Seidel et al. [18] we were also unable to detect any pronounced difference in the MTX concentration as determined by FPIA2 and HPLC. This concentration was overestimated by FPIA2 in only 2–3% of the samples which was not statistically significant. In addition, Najjar and co-workers [19] reported a generally good correlation between plasma MTX levels determined by HPLC and FPIA, although the differences were somewhat more pronounced for samples collected during the elimination phase than during infusion of MTX. These investigators speculated that such differences could be due to the presence of 7-OHMTX and the correlation coefficient they observed versus HPLC was 0.633 which is similar to our present correlation coefficient between EMIT and FPIA2. Thus, these earlier findings support the present conclusion that the major metabolite of MTX does not influence the accuracy and precision of plasma MTX determination by FPIA2. Also noteworthy is the fact that the lower degree of accuracy of the EMIT compared to FPIA2 is also associated with lower sensitivity, the lower limits of detection for these assays being approximately 300 nM, and 50 nM, respectively.

Carboxypeptidase-G_2, a novel agent for "rescuing" MTX can be used to convert >98% of plasma MTX to inactive compound, DAMPA. Although use of this agent is presently limited to patients who exhibit delayed clearance of MTX as a consequence of MTX-induced renal damage, the success of this approach in this setting may eventually lead to more widespread use of CPDG2 as a rescue agent in other settings. Since the cross-reactivity of DAMPA with MTX is 41% in the case of the FPIA2 procedure and almost 100% for the EMIT assay [6], HPLC may be the clear method of choice for determining plasma MTX in patients receiving this agent. Dihydrofolate reductase enzyme inhibition assay has been proposed as the method of second choice, after HPLC, under these conditions [20].

Our recently reported data indicated that 7-OHMTX can provoke resistance in leukemic cells by a different mechanism than that of MTX [16]. This finding may provide clinical indications to measure concentration of 7-OHMTX in plasma as well, which demands a method that is capable of detecting 7-OHMTX without cross-reactivity with MTX.

In summary, we conclude that the rapid automated procedures routinely used at present suffer from cross-reactivity with metabolites of MTX where patients with overestimated plasma MTX concentrations may thus be hospitalized for an unnecessarily long period of time, as well as receiving an overdose of leucovorin that jeopardizes the antileukemic effect of MTX and increases the risk for relapse.

Acknowledgements

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