Dihydrofolate Reductase Enzyme Inhibition Assay for Plasma Methotrexate Determination Using a 96-Well Microplate Reader

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Microplate reader assays offer several advantages over conventional spectrophotometric assays. We adapted the dihydrofolate reductase (DHFR) enzyme inhibition assay for use in a 96-well microplate reader to measure plasma methotrexate (MTX) concentrations. The assay is linear from 0.01 to 0.1 μmol/L. The within-run CVs at 0.03 μmol/L and 0.08 μmol/L MTX were 4.0% and 2.7%, respectively, and the interday (total) CVs were 7.6% and 1.8%. Cross-reactivity with the inactive MTX metabolite 2,4-diamino-N¹⁰-methylpteroyl acid (DAMPA) was 3.9%, significantly less than that described with commercial immunoassays; with 7-hydroxymethotrexate cross-reactivity was 1.7%. In addition to sensitivity and specificity, the advantages of this assay are small sample volumes, simultaneous analysis of multiple samples, and rapid turnaround. Because of its greater specificity, the DHFR enzyme inhibition assay may be useful when DAMPA is present in plasma samples and HPLC is not available.

The folic acid analog methotrexate (MTX) inhibits the enzyme dihydrofolate reductase (DHFR; EC 1.5.1.3.), thus depleting cells of chemically-reduced tetrahydrofolates, which are cofactors in the biosynthesis of the purine and pyrimidine nucleotide precursors of DNA. MTX is used to treat a variety of cancers, including osteogenic sarcoma, acute lymphoblastic leukemia, non-Hodgkin lymphomas, breast cancer, and head and neck cancer. Several of these cancers are treated with high-dose methotrexate (HDMTX) regimens (i.e., MTX dose ≥1 gm/m²) followed by leucovorin rescue (1–3). Leucovorin is naturally-occurring folate that alleviates MTX toxicity.

MTX is eliminated primarily by renal excretion, and, to a lesser extent, by hepatic metabolism to the metabolite, 7-hydroxymethotrexate (7-OH-MTX). Occasionally patients who are receiving HDMTX develop MTX-induced nephrotoxicity in spite of adequate intravenous hydration and alkalization. The resulting delayed MTX elimination can exacerbate other MTX toxicities because leucovorin rescue is less effective with persistently increased MTX concentrations. A novel rescue strategy, which entails the administration of an exogenous MTX-metabolizing enzyme, has been developed for patients with HDMTX-induced renal failure. Carboxypeptidase-G₂ (CPDG₂) is a recombinant bacterial enzyme that rapidly hydrolyzes the terminal glutamate from MTX, converting it to the inactive metabolites 2,4-diamino-N¹⁰-methylpteroyl acid (DAMPA) and glutamate. Within 15 min of CPDG₂ administration, >98% of plasma MTX is converted to DAMPA (4–7).

Plasma MTX concentrations are monitored routinely during and after HDMTX administration to determine the dose and duration of leucovorin rescue (8–10). The most commonly used commercial MTX assays are antibody-based immunoassays such as the fluorescence polarization immunoassay (FPIA; Abbott Laboratories) and the enzyme-multiplied immunoassay (Emit; Behring Diagnostics, Syva Business) (11–14). One potential disadvantage of the immunoassays is their low specificity for MTX as a result of cross-reactivity with MTX metabolites. The cross-reactivity with the MTX metabolite 7-OH-MTX is only 0.6% in the FPIA and 4% in the Emit assay. DAMPA, however, is highly cross-reactive in both the FPIA (83% for FPIA1, which uses polyclonal antibodies, and 41% for FPIA2, which uses monoclonal antibodies) and the Emit (100%) methods (11, 13, 15), which leads to significant overestimation of MTX concentration after the administration of CPDG₂ (6, 7).

The DHFR enzyme inhibition assay is a sensitive and
specific method for determining MTX concentrations in biological fluids, but current assay methods are labor-intensive and time-consuming. In this assay, DHFR catalyzes the reduction of dihydrofolate (FH2) to tetrahydrofolate in the presence of NADPH, which is oxidized to NADP+; MTX is quantified by measurement of the decrease in absorbance at 340 nm that occurs when NADPH is converted to NADP+. This assay has a lower limit of quantification, between 0.005 and 0.02 μmol/L (4, 16–18), and has less cross-reactivity with DAMPA (0.7–10%) and 7-OH-MTX (1%) (4, 19) than the standard immunoassays.

We have adapted the DHFR enzyme inhibition assay (4, 17) for use in a 96-well microplate reader. This allows for analysis of 30 plasma MTX samples in duplicate, including calibrators and controls, with one 20-min reading in a microplate reader that measures the absorbance of ultraviolet light. In addition, the assay was evaluated with aqueous calibrators because of the potential usefulness of this assay for tissue culture experiments. The microplate DHFR enzyme inhibition assay was then used to describe the pharmacokinetics of MTX in patients with HDMTX-induced renal failure who received CPDG2.

**Materials and Methods**

**INSTRUMENTATION**
A Biotek EL 340 microplate spectrophotometer (BioTek Instruments Inc.) was interfaced to a Macintosh SE 30 computer running Delta Soft II, Ver. 3.3B software (BioMetallics Inc.). The 96-well flat-bottom microplates were obtained from Costar Corp. A model 1500 automatic pipettor-dilutor was obtained from Cavro Instruments.

**REAGENTS**
FH2, 2-mercaptoethanol, NADPH, Tris-HCl, Tris-base, DAMPA (purity ~90%), and trimethoprim were obtained from Sigma Chemical Co. DHFR from Lactobacillus casei was obtained from Biopure Corp. The MTX was obtained from Immunex Corp. The 7-OH-MTX was kindly provided by Dr. F. Albertioni (Karolinska Institute, Stockholm, Sweden).

**STOCK AND BUFFER SOLUTIONS**
Assay buffer A was 0.5 mol/L Tris buffer, pH 7.5, and buffer B was 0.05 mol/L Tris buffer, pH 7.5. Stock solutions of FH2 (25 mg in 1.5 mL of 2-mercaptoethanol and 6.0 mL of buffer A in 0.25-mL aliquots), NADPH (50 mg in 10 mL of buffer A in 0.4-mL aliquots), and DHFR (2.1 U in 10 mL of buffer B in 0.5-mL aliquots) were stored at −70 °C.

**REACTION SOLUTIONS**
All reaction solutions were prepared fresh daily from stock solutions and were kept on ice. The FH2 reaction solution consisted of one thawed 0.25-mL aliquot of FH2 stock solution in 8.0 mL of buffer B, yielding a final working concentration of 0.104 g/L. The NADPH/DHFR reaction solution consisted of one thawed 0.4-mL aliquot of NADPH stock solution and one thawed 0.5-mL aliquot of DHFR stock solution in 6.0 mL of buffer B, yielding a final working concentration of 0.29 g NADPH/L and 15 U DHFR/L.

**MTX CALIBRATORS**
Aqueous and plasma MTX calibrators were prepared at concentrations of 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, and 0.1 μmol/L and stored at −70 °C.

**MTX MICROPLATE ASSAY**
The microplate DHFR inhibition assay for MTX is described below: FH2 reaction solution (130 μL) was added to each well of the 96-well flat-bottom plate (the outer wells of the plate were not used for sample analysis). MTX calibrators or unknown samples (20 μL of either) were then added to duplicate wells. The microplate was shaken in a plate shaker for 1 min, after which NADPH/DHFR reaction solution (50 μL) was added to each well, and the microplate was again shaken in a plate shaker for 1 min.

The absorbance of each well was read in the microplate reader at room temperature at wavelengths of 340 nm and 490 nm (reference), using the kinetic mode with a reading interval of 20 s for a duration of 18 min. The blank rate (decrease in absorbance measured from 2 to 18 min in the absence of MTX) was verified to be a decrease in absorbance of 0.020–0.025/min. If necessary, the blank rate was adjusted to achieve this range by varying the amount of DHFR in the NADPH/DHFR reaction solution.

The absorbances were downloaded directly into a Macintosh SE 30 computer and analyzed with Delta Soft II software. The linear decrease of absorbance between 2 and 18 min was used for each calibrator and plotted against the MTX concentration to obtain a calibration curve.

**ASSAY TEMPERATURE**
The influence of temperature was evaluated by performing the assay at room temperature and at 30 and 37 °C.

**ASSAY PRECISION AND ACCURACY**
Within-run, intra-, and interday CVs were measured with three replicate assays of aqueous and plasma samples at 0.03 and 0.08 μmol/L MTX, twice daily on 20 days. The accuracy was determined by analyzing aliquots of plasma samples of 0.05, 0.2, and 0.8 μmol/L MTX in triplicate twice daily on 10 days. All MTX samples were stored at −70 °C. Plasma unknowns were serially diluted with plasma from healthy volunteer donors (or with buffer B for aqueous unknowns), using an automatic pipettor-dilutor to fall within the range of the calibration curve.

**ASSAY CROSS-REACTIVITY**
To determine the degree of cross-reactivity of DAMPA, trimethoprim, and 7-OH-MTX with MTX in this assay, multiple dilutions of 1 mmol/L DAMPA (five experi-
ments), 1 mmol/L trimethoprim (five experiments), and 1 mmol/L 7-OH-MTX (three experiments) stock solutions were assayed. The cross-reactivity was determined by comparing the calibration curves that were derived from the known concentrations of MTX, DAMPA, trimethoprim, and 7-OH-MTX.

For six patients with HDMTX-induced renal dysfunction (five patients with osteosarcoma and one patient with non-Hodgkin lymphoma), who were treated with CPDG2 on a compassionate-use protocol of the Cancer Therapy Evaluation Program of the National Cancer Institute (7), plasma MTX and DAMPA concentrations were determined with HPLC using a previously described reversed-phase method (6, 7) and with the microplate DHFR enzyme inhibition assay. The results were compared with MTX concentrations determined with the FPIA (FPIA1 in one patient and FPIA2 in five patients) by the participating institutions.

**Results**

The DHFR enzyme inhibition assay is based on the conversion of FH$_2$ to tetrahydrofolate and the resulting oxidation of NADPH to NADP$^+$, which was monitored on the kinetic plate reader by measurement of the absorbance at 340 nm. At room temperature, a linear decrease in absorption over the time period from 2 to 18 min was observed with the blank solution (no MTX) and with MTX calibrators ranging from 0.01 to 0.1 µmol/L (Fig. 1A). The concentration of MTX from 0.01 to 0.1 µmol/L was linearly related to the decrease in absorption rate measured (Fig. 1B). The slope of this linear calibration curve ranged from $-0.111$ to $-0.144$ absorbance $\cdot$ min$^{-1}$ $\cdot$ µmol$^{-1}$ $\cdot$ L$^{-1}$ for 20 aqueous calibration curves and from $-0.111$ to 0.139 absorbance $\cdot$ min$^{-1}$ $\cdot$ µmol$^{-1}$ $\cdot$ L$^{-1}$ for 20 plasma calibration curves, with the SE ranging from $\pm 4.2$ to $\pm 6.9$ and $\pm 3.6$ to $\pm 6.5$, respectively. The $y$-intercept of the calibration curve ranged from 0.020 to 0.026, with the SE ranging from $\pm 0.2$ to $\pm 0.4$ and $\pm 0.3$ to $\pm 0.4$, respectively. The regression coefficient for the 20 calibration curves ranged from 0.98 to 0.99. At 0.01 µmol/L, the absorbance rate differed from the blank rate by $>5\%$.

**Influence of Temperature**

The assay was performed at room temperature and at 30 and 37 °C. The reaction was complete (absorbance at 340 nm plateaued) after 20, 12, and 8 min, respectively. The temperature throughout the plate, however, did not appear to be evenly maintained in the plate reader at the higher temperatures. The CV for blank rates utilizing the entire plate (excluding the outer wells) at room temperature and at 30 and 37 °C were 1.5%, 7.2%, and 10.1%, respectively. The assay was therefore performed at room temperature.

**ASSAY PRECISION AND ACCURACY**

The mean within-run CVs were $<5\%$ for 0.03 and 0.08 µmol/L plasma MTX calibrators. The intraday CVs were 6.2% and 1.4%, and the interday CVs were 7.6% and 1.8% at 0.03 and 0.08 µmol/L, respectively (Table 1). The mean recoveries of 0.05, 0.2, and 0.8 µmol/L MTX in plasma samples were 111%, 105.2%, and 104.3%, respectively (Table 2).

**CROSS-REACTIVITY**

DAMPA was 3.9% ± 1.3% as effective as an inhibitor of *L. casei* DHFR as MTX under our assay conditions; trimethoprim was 2.0% ± 0.6% as effective, and 7-OH-MTX was 1.7% ± 0.3% as effective as MTX (Fig. 2).

**Table 1. Within-run, intra-, and interday CVs for plasma calibrators assayed in triplicate twice a day for 20 days.**

<table>
<thead>
<tr>
<th>MTX conc., µmol/L</th>
<th>Within-run*</th>
<th>Intraday*</th>
<th>Interday</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.03</td>
<td>4.0 (1.5–7.6)</td>
<td>6.2 (1.4–12.5)</td>
<td>7.6</td>
</tr>
<tr>
<td>0.08</td>
<td>2.7 (1.4–5.6)</td>
<td>1.4 (0–2.8)</td>
<td>1.8</td>
</tr>
</tbody>
</table>

*conc., concentration.

*Mean (range).*

![Fig. 1. Change in absorbance of the reaction mixture at 340 nm for the blank (no MTX) from a representative experiment (A) and calibration curve for MTX with concentrations ranging from 0.01 to 0.1 µmol/L (B).](image-url)
In analysis of plasma samples from six patients who had received CPDG₂ for MTX-induced renal dysfunction, comparison with HPLC revealed that the DHFR enzyme inhibition assay overestimated the plasma MTX concentration to a lesser degree than the FPIA, especially at the earlier time points after enzyme administration when high concentrations of DAMPA were present (Fig. 3) (6,7). In the presence of high DAMPA concentrations (83–411 μmol/L) 15–30 min after the administration of CPDG₂, the microplate assay overestimated the MTX concentration by a median of 4.6-fold (range, 3.8- to 5.0-fold). The FPIA was used for the first time between 4 and 66 h (median, 12 h) after CPDG₂ administration, and in the presence of lower DAMPA concentrations (0.5 to 307 μmol/L), the FPIA overestimated the MTX concentration by a median of 47-fold (range, 17.4- to 78.5-fold). In these patients with renal dysfunction, DAMPA is converted to other metabolites [e.g., 7-OH-DAMPA (20)] that may also interfere with the FPIA and DHFR enzyme inhibition assays. Comparison of MTX concentrations determined for time points between 6 and 77 h after CPDG₂ administration by HPLC, FPIA, and the DHFR enzyme inhibition assay simultaneously demonstrated that the FPIA and DHFR enzyme inhibition assay overestimated plasma MTX concentrations by a median of 32-fold (range, 3.4- to 79-fold) and 2.9-fold (range, 1.5- to 3.9-fold), respectively. As the DAMPA was cleared rapidly from the patients’ plasma, the magnitude of this overestimation in the DHFR microplate assay decreased significantly.

### Discussion

Microplate reader assays offer several advantages over conventional spectrophotometric assays. The requirement for small sample volumes allows for repeated sample analysis, and multiple samples can be analyzed simultaneously within a short time period.

With the microplate DHFR inhibition assay described here, plasma MTX can be monitored accurately until it is safe to discontinue leucovorin rescue (MTX <0.05 μmol/L). HPLC is the most specific method, and it has an acceptable lower limit of quantification (0.02 μmol/L).
The HPLC method can also detect and quantify MTX metabolites. It is, however, a more time-consuming method and requires more sophisticated instrumentation and larger sample sizes than the DHFR inhibition assay and the automated immunoassays.

The interference of trimethoprim with L. casei DHFR has been described previously (23, 24). Average peak plasma concentrations in patients after a standard oral dose of trimethoprim are between 4.1 and 7.2 μmol/L (25). The interference of trimethoprim with the DHFR enzyme inhibition assay described here (2.0 μmol/L trimethoprim gave an apparent value of 0.05 μmol/L MTX) appears to be less than in the DHFR enzyme inhibition assay described by Bock et al. (24), who reported that 1.7 μmol/L trimethoprim gave an apparent value of 0.36 μmol/L MTX. However, therapeutic drug concentrations of trimethoprim may interfere with the determination of low MTX concentrations, and therefore, results using this method should be interpreted with caution in patients who are receiving trimethoprim-containing antibiotics (Bactrim or Septra). The use of DHFR from mammalian sources could potentially overcome this problem because trimethoprim binds to mammalian DHFR with much less affinity compared with DHFR from bacterial sources (26).

After administration of HDMTX, the plasma concentration of 7-OH-MTX typically exceeds that of MTX at later time points (27–29). The interference of 7-OH-MTX in the microplate DHFR enzyme inhibition assay is low, 1.7%, and comparable to 1% cross-reactivity for the single-cell spectrophotometer (19). The microplate DHFR enzyme inhibition assay should, therefore, be suitable for monitoring plasma MTX concentrations after HDMTX administration.

DAMPA plasma concentrations after HDMTX are usually very low (30) unless the patient has received CPDG2. The cross-reactivity of DAMPA is substantially less (3.9%) in the microplate DHFR inhibition assay than in the commercially available FPIA and Emit assays (11–14). In the absence of an HPLC assay, the DHFR enzyme inhibition assay may, therefore, be the method of choice to monitor MTX concentrations and to guide leucovorin rescue after administration of CPDG2.

Although the use of CPDG2 presently is limited to patients who have delayed MTX clearance because of MTX-induced renal dysfunction, the success of this novel rescue approach in this setting may lead to more widespread use of CPDG2 as a rescue agent in other settings. For example, CPDG2 rescue is under investigation in patients with central nervous system lymphoma because systemic MTX can be degraded rapidly without impacting on MTX that has penetrated into the central nervous system (5). As the use of CPDG2 rescue for HDMTX expands, there will be a greater need for alternative methods for monitoring plasma MTX concentrations, such as the microplate DHFR enzyme inhibition assay.

We thank Jill Savitch and W. Archie Bleyer for sharing the method for the dihydrofolate reductase enzyme inhibition assay developed for a single-cell spectrophotometer.

References


