Evaluation of a Methotrexate Chemiluminescent Microparticle Immunoassay

Comparison to Fluorescence Polarization Immunoassay and Liquid Chromatography–Tandem Mass Spectrometry

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ABSTRACT

Objectives: For most laboratories, methotrexate (MTX) concentrations are routinely monitored by fluorescence polarization immunoassay (FPIA). In anticipation of an announced withdrawal of the FPIA reagent on the Abbott TDxFLx (Abbott Diagnostics, Abbott Park, IL), we have evaluated a new reagent kit developed by Abbott on the Architect i1000, based on chemiluminescent microparticle immunoassay (CMIA).

Methods: Precision, inaccuracy, and selectivity were assessed. Interassay variability was established using 75 plasma patient samples treated with MTX and analyzed by two methods: FPIA and liquid chromatography–tandem mass spectrometry (LC-MS/MS).

Results: For MTX, the intraday inaccuracy was between –6.37% and +3.52%, while interday performance was between –3.70% and 7.90%. Intraday and interday imprecision was less than 2.65% and less than 2.22%, respectively. The correlation coefficient between CMIA and FPIA or LC-MS/MS was 0.9969 and 0.9985, respectively.

Conclusions: These results comparing CMIA vs FPIA and LC-MS/MS indicate that CMIA is a suitable alternative to the FPIA method.
HDMTX TDM, but it was gradually substituted by various assays because of its progressive marketing cessation. In recent years, different immunoassays have been marketed for routine hospital assays. ARK Diagnostics provides an immunoassay for Roche Diagnostics, Abbott Diagnostics (Abbott Park, IL), Siemens, and Beckman systems; Ortho Clinical Diagnostics provides an immunoassay (EMIT) using a Syva Siemens system. More recently, Abbott Diagnostics has provided an MTX assay kit for the Architect i1000 system.

The purpose of this study was to evaluate the characteristics of this new chemiluminescent microparticle immunoassay (CMIA) immunoassay by comparison with both the Abbott TDxFLx analyzer and a liquid chromatography–tandem mass spectrometry method (LC-MS/MS).

Materials and Methods

Patients

All plasma patient samples (n = 75) tested in this work were derived from an ongoing drug monitoring program and were reported in accordance with ethical guidelines. Characteristics of patients (n = 27) are summarized in Table 1. Informed consent was not required, but all samples were deidentified and anonymized. Only K3EDTA plasma samples were used in this study.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Patient Demographics (n = 27)</th>
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</thead>
<tbody>
<tr>
<td>Characteristic</td>
<td>Value</td>
</tr>
<tr>
<td>Age, mean (range), y</td>
<td>21.71 (3.01-70.34)</td>
</tr>
<tr>
<td>Male, No. (%)</td>
<td>18 (67%)</td>
</tr>
<tr>
<td>Number (range) of samples per patient, mean</td>
<td>2.96 (1-9)</td>
</tr>
<tr>
<td>Diseases, No.</td>
<td></td>
</tr>
<tr>
<td>Lymphoma</td>
<td>9</td>
</tr>
<tr>
<td>Acute lymphoblastic leukemia</td>
<td>12</td>
</tr>
<tr>
<td>Osteosarcoma</td>
<td>6</td>
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</table>

Chemicals, Reagents, and Standard Solutions

All solvents and reagents were of high-performance liquid chromatography (HPLC) grade and purchased from VWR International (Fontenay-sous-Bois, France). MTX solution (25 mg/mL, NaCl 0.9%) was purchased from Mylan (Saint Priest, France), DAMPA was purchased from Schircks laboratories (Jona, Switzerland), and 7-OH-MTX and 13C2H3-MTX, used as the internal standard, were purchased from Alsachim (Illkirch, France). Abbott Diagnostics provides two different MTX assay kits, one for FPIA used on the Abbott TDxFLx and one for CMIA used with the Architect i1000 system. For these two immunoassays, plasma multilevel calibrators and quality controls (QCs) were provided by Abbott Diagnostics. In-house prepared QCs and plasma multilevel calibrators containing MTX were used for LC-MS/MS assays.
The assay was performed according to the manufacturer's instructions (Abbott Diagnostics). Calibrators, controls, or patient plasma samples (100 μL) were transferred into reaction cells and submitted to the Abbott TDxFLx instrument for automated analysis. The analytical measurement range was 0.03 to 1.00 μmol/L, and samples containing MTX at higher concentrations were diluted with reagent provided by Abbott Diagnostics. The lower limit of quantitation was 0.03 μmol/L.

CMIA

The assay was performed according to the manufacturer’s instructions (Abbott Diagnostics). Calibrators, controls, or patient plasma samples (100 mL) were transferred into reaction cells and submitted to an Architect i1000 system for automated analysis. The analytical measurement range was 0.04 to 1.50 μmol/L, and samples containing MTX at higher concentrations were diluted with free drug plasma reagent provided by Abbott Diagnostics. The lower limit of quantitation (LLOQ) was 0.04 μmol/L.

LC-MS/MS Assay

The method used was previously published. Briefly, 50 μL of plasma, calibrator, QC, or patient sample was precipitated by 100 μL methanol/0.2 mol/L ZnSO₄ (80:20, vol/vol) containing [¹³C₂]H₃-MTX (internal standard). The mixture was vortex mixed and centrifuged. Then, 20 μL of the supernatant was injected into the chromatographic system, consisting of Agilent 1200 Series components (Agilent Technologies, Palo Alto, CA) and an ABSciex API 3200 (ABSciex, Toronto, Canada) equipped with a turboionspray source. Online solid-phase extraction enrichment was performed (Poros column [Thermo Scientific, Illkirch France], R1/20, 2.1 mm; 30 mm) and then a back-flush elution to the separative chromatography toward a 4-minute runtime (2- to 50-mm column; Phenomenex [Le Pecq, France] Luna 5-mm Phenyl Hexitl). Samples were analyzed using an HPLC Agilent 1200 Series and ABSciex API 3200. The electrospray was operated in positive ionization mode monitoring the following mass transitions: m/z 455.11/308.3 for MTX and m/z 459.1/312.3 for internal standard [¹³C₂H₅-MTX]. The method was linear up to 50 mmol/L, and intraday and interday QC coefficients of variation (CVs) were below 8.3%. The lower limit of quantitation was 0.025 μmol/L, and samples containing MTX at higher concentrations were diluted with free drug plasma. The standard line slope CV percentage was less than 3%, and the slope difference was less than 6%. The method was validated in human plasma over the concentration range of 0.05 to 50 μmol/L for MTX.

Validation Procedures

Inaccuracy and Imprecision

Inaccuracy and imprecision were evaluated by analyzing QC samples at low, medium, and high concentrations. For intraday validation, 15 samples of each low and high control were analyzed on the same day. For interday validation, concentrations of the QC samples were determined on 30 separate days. Inaccuracy was defined as the percentage of deviation from the nominal level and imprecision as the percent CV within a single run (intra-assay) and between different days (interassay). The performance was judged acceptable when observed error was less than allowable error and not acceptable when the observed error was greater than allowed error. The allowable errors used were the company claims for CMIA (<10%) or FPIA (<10%) methods and 15% for the LC-MS/MS method.

Carryover Effects

Carryover effects were assessed by testing successively three high levels QCs (H1, H2, and H3) and three low levels QCs (L1, L2, and L3). This step was reproduced five times. If no statistical difference between L1 and L3 averages using a Student test was found, the procedure was judged free of carryover effects.

Cross-Reactivity and Interference Assessment

The antibody cross-reactivity between MTX metabolites and related products was determined. MTX-free plasma was spiked with three levels of DAMPA (0.03 μmol/L, 1.5 μmol/L, and 3 μmol/L) or 7-OH-MTX (1 μmol/L, 5 μmol/L, and 25 μmol/L). Potential of interference with hemolytic, lipemic, and icteric plasma samples was evaluated by spiking MTX-free hemolytic, lipemic, or icteric plasma samples to a final MTX concentration of 1 μmol/L. Three different hemolytic, lipemic, or icteric plasma samples were studied.

Limit of Detection, Limit of Quantification, Linearity, and Stability

These data were provided by Abbott Diagnostics laboratory. Samples must be analyzed within 3 hours after loading on the Architect system. The method was linear from 0.04 to 1.5 μmol/L. The lower limit of quantification was 0.04 μmol/L. The stability of plasma samples stored at –20°C for at least 3 months, at +4°C for 7 days, or at room temperature for 3 days was checked. The mean concentration at each level should be within ±15% of the nominal concentration.

Data Analysis, Interpretation, and Statistics

Linear regression analyses, statistical analyses, sensitivity, and specificity were performed with Prism 6.0f software.
Method Comparison

We have compared the CMIA method with two validated methods used in the laboratory. The first comparison concerned the widespread FPIA method on the Abbott TDxFLx system. The second one was done with the LC-MS/MS method. The same 75 plasma samples were analyzed with the three methods. The range of MTX plasma concentration samples, monitored by LC-MS/MS, was 0.029 to 62 μmol/L.

Results

Inaccuracy and Imprecision

The inaccuracy and imprecision, determined for both intraruns and interruns, are summarized in Table 2. Intraday and interday QC CVs were below 3.68% for the CMIA method. For MTX, the intraday inaccuracy was between –6.37% and +3.52%, while interday performance was between –3.70% and 7.90%.

Carryover Effects, Cross-Reactivity Assessment, Interference, and Stability

Carryover effects proved to be moderate and acceptable, and any statistical difference between L1 and L3 could not be established using a Student test. The means ± SEMs of L1 and L3 were 0.0732 ± 0.0007348 μmol/L and 0.0722 ± 0.0008602 μmol/L, respectively.

Table 3 indicates antibody cross-reactivity obtained with three concentrations of DAMPA and 7-OH-MTX. Whatever the concentration tested, 7-OH-MTX did not cross-react with the antibody used in the CMIA kit, while DAMPA systematically cross-reacted with the antibody even for concentrations less than 0.05 μmol/L. In-house evaluation of potential interference posed by icteric, lipemic, and hemolyzed samples showed stable values Table 4.

Stability was guaranteed in plasma at –20°C for 3 months and in plasma stored at +4°C for 7 days or at room temperature for 3 days.

Comparison to FPIA and LC-MS/MS Methods

A good correlation was found between CMIA and FPIA with a correlation coefficient of $r = 0.9969$ (95% confidence interval [CI], 0.995-0.998), as well as between CMIA and LC-MS/MS with a correlation coefficient of $r = 0.9985$ (95% CI, 0.997-0.999). The Passing-Bablok method comparison test showed no significant difference between the two methods with no significant deviation from linearity Figure 2A and Figure 2C. Figure 2B and Figure 2D show the Bland-Altman systematic difference for the two method comparisons.

To complete the method comparison, we have monitored the ability of the new method to lead to identical clinical decisions as reference methods. The threshold of 0.15 μmol/L or less was used to decide whether the patient would stay hospitalized. Regarding this cutoff, we determined agreement, the
Concordance coefficient (named the $\kappa$ coefficient), and, more specifically, sensitivity and specificity, which are useful to compare a new method with a reference method. Whatever the reference method considered (FPIA or LC-MS/MS), CMIA allowed us to obtain very similar results, with percentage of agreement up to 97% and a $\kappa$ coefficient up to 94%. The CMIA method had similar sensitivity and specificity with the threshold used. Results are reported in Table 5.

Discussion

We describe the performance of a new automated assay based on the CMIA principle developed on the Abbott Architect i1000 system to determine MTX plasma concentrations. The main objective of this work was to complete an evaluation of this new reagent to ensure, as simply as possible, the continuity of MTX plasma concentration monitoring when the Abbott TDxFLx system is definitively stopped. Intraday and interday imprecision did not exceed 4% for all concentrations tested (Table 2). Method accuracy was within $-6.37\%$ and $+4.57\%$ for intrarun and interrun. This method achieved an LLOQ of 0.04 $\mu$mol/L, and the signal in the blank matrix remained inferior to the LLOQ (data not shown). These performances are consistent with TDM and pharmacokinetic studies of MTX.

The second purpose was to evaluate the performance at MTX concentrations less than 0.1 $\mu$mol/L. This was an
important matter because, according to the established protocols, plasma concentrations less than 0.1 to 0.15 μmol/L determine an effective elimination of MTX dose and allow the discharge of the patients from the hospital. Two recently published articles reported problems with two reagent kits. In 2012, a team published a contravance to “drop” the LLOQ of MTX with the EMIT reagent kits used on the Siemens Viva-E instrument.\(^7\) In 2014, a French study reported an overestimation of the plasma concentration of MTX with the EMIT Siemens reagent kit.\(^\text{10}\) With the CMIA reagent kit, whatever the reference method considered (FPIA or LC-MS/MS), CMIA obtains very similar results, with percentage of agreement up to 97% and a κ coefficient up to 94%. The CMIA method presents similar sensitivity and specificity with the threshold used. Unfortunately, because of antibody cross-reactivity between DAMPA and MTX, neither the Abbott CMIA reagent nor the Abbott FPIA kit for the Abbott TDxFL\(_x\) or ARK Diagnostics reagent can be used to monitor MTX concentrations up to 48 to 72 hours after carboxypeptidase administration (Table 3).\(^\text{14 - 16}\) After 48 hours, because the DAMPA half-life ranges from 5 to 9 hours, the immunoassay can be used again. In this context, the only way to determine MTX plasma concentrations is to use a chromatographic separation, like our LC-MS/MS method.\(^\text{11,14}\)

**Table 51**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>CMIA vs FPIA</th>
<th>CMIA vs LC-MS/MS</th>
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</thead>
<tbody>
<tr>
<td>Agreement, %</td>
<td>98.67</td>
<td>97.33</td>
</tr>
<tr>
<td>k Coefficient, mean ± SE</td>
<td>0.971 ± 0.028</td>
<td>0.945 ± 0.04</td>
</tr>
<tr>
<td>95% CI</td>
<td>0.915 to 1.00</td>
<td>0.943 to 1.00</td>
</tr>
<tr>
<td>Sensitivity, %</td>
<td>96.4</td>
<td>93.1</td>
</tr>
<tr>
<td>Specificity, %</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

\(^\text{a}\)Both FPIA and LC-MS/MS were considered reference methods.

**Conclusion**

These results comparing CMIA and FPIA or LC-MS/MS methods are consistent and indicate that therapeutic drug monitoring of MTX with the CMIA reagent kit on the Abbott Architect i1000 system is a suitable and welcome alternative to the FPIA method on the Abbott TDxFL\(_x\). Nevertheless, cross-reactivity with DAMPA remains a limitation, whatever the immunoassay used.

**References**