Evaluation of Enzyme Immunoassay, Radioassay, and Radioimmunoassay of Serum Methotrexate, as Compared with Liquid Chromatography

Robert G. Bulce,1 William E. Evans,2 James Karas,2 Charles A. Nicholas,1 Paramjeet Sidhu,1 Arthur B. Straughn,1 Marvin C. Meyer,1 and William R. Crom2

We evaluated three commonly used clinical methods for measuring serum methotrexate: enzyme immunoassay (EMIT5), radioassay, and radioimmunoassay. Because potentially interfering compounds can be resolved by liquid chromatography, this method was selected as the comparison method. Patients' serum samples, taken during 24 h after 6-h high-dose infusions, contained methotrexate in concentrations ranging from 10⁻⁷ to 10⁻⁴ mol/L. Chromatograms revealed substantial amounts of 7-hydroxymethotrexate in all samples, actually exceeding methotrexate by 12 and 24 h. Nevertheless, analysis of variance revealed no significant differences in results by any of the four methods, nor did results by the three test methods differ significantly after being adjusted by analysis of covariance with liquid chromatography as the covariate. Evidently any of the four techniques is suitable for monitoring serum methotrexate for 24 h after high-dose therapy.

The advent of high-dose methotrexate therapy with leucovorin “rescue” created a need for analytical methods specific for methotrexate (MTX). The ensuing wide variety of analytical techniques (ref. 1 is the most recently reported), each possessing its own advantages and restrictions, initiated the present concern for how results by each method might intercompare. Particularly disconcerting is the potential interference from MTX metabolites, especially the less-soluble and less-pharmacologically active 7-hydroxymethotrexate (7-OH MTX), which is present in substantial concentrations in the serum of patients undergoing high-dose therapy with MTX (2). We evaluated the accuracy of three commonly used clinical methods for the quantitation of serum MTX: enzyme immunoassay (EMIT5), radioassay, and radioimmunoassay. Because MTX and 7-OH MTX can be readily resolved by “high-pressure” liquid chromatography, with no interfering bands appearing in the serum blank, we used this technique as the comparison method.

Materials and Methods

Reagents and test-method kits. All solvents used for liquid-chromatographic analyses were “HPLC” grade and all chemicals were reagent grade. 7-OH MTX was obtained from Dr. Y. M. Wang, M.D. Anderson Hospital and Tumor Institute, Houston, TX. The internal standard, N-[4(2,4-diamino-6-quinazolyl)methylamino]benzoyl aspartic acid, was supplied by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, NCI, Bethesda, MD. MTX was obtained from Sigma Chemical Co., St. Louis, MO 63178. EMIT5 and radioassay4 kits were used as received from the suppliers. Radioimmunoassay kits were used according to the manufacturer’s instructions except for the preparation of fresh MTX calibration standards and a modification of the dilution method to assure that determinations would be read from a linear portion of the calibration curve.

Clinical samples. Thirty serial blood samples were obtained from 16 patients (ages 12 to 16 years). The series was incomm-
Table 1. Conditions Used in Preparing Each Calibration Curve

<table>
<thead>
<tr>
<th>Curve</th>
<th>MTX concn (stock)</th>
<th>Internal std concn</th>
<th>Extraction method</th>
<th>Reconstitution vol, µL</th>
<th>Injection volume, µL</th>
<th>Detector sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>2.2 × 10^{-5}–2.2 × 10^{-4}</td>
<td>50.0</td>
<td>ion pair</td>
<td>200</td>
<td>75</td>
<td>0.1</td>
</tr>
<tr>
<td>b</td>
<td>2.2 × 10^{-6}–2.2 × 10^{-5}</td>
<td>5.0</td>
<td>ion pair</td>
<td>200</td>
<td>100</td>
<td>0.01</td>
</tr>
<tr>
<td>c</td>
<td>2.2 × 10^{-7}–2.2 × 10^{-6}</td>
<td>0.5</td>
<td>SEP PAK</td>
<td>100</td>
<td>80</td>
<td>0.005</td>
</tr>
</tbody>
</table>

*mg/L; 200 µL of this solution was added to each serum sample before the extraction.

Complete in some of these patients, but in general samples were
taken at 0.5, 6, 12, and 24 h after completion of 6-h intravenous
infusions of MTX (3500–5000 mg/m² body surface). Serum,
separated by centrifugation, was stored at −70 °C until assayed. Every serum sample was assayed by each test method
and by the comparison method.

Comparison method. For the reversed-phase liquid chromatography we used a Waters Associates system consisting
of a µBondapak C18 column (30 cm × 4 mm), a U6K injector,
a Model 6000A solvent delivery system, and a Model 440 absorbance detector (313 nm). The mobile phase, acetate buffer
(0.2 mol/L, pH 5.5 with 0.03 mol of added EDTA per liter),
methanol, and acetonitrile (85.3/8.4/6.3 by vol), was passed
through the column at a flow rate of 1.5 mL/min. Serum
samples obtained at 12 h or earlier were processed by ion-pair
extraction as described by Watson et al. (2). Because the 24-h
samples required better assay sensitivity, we replaced the
ion-pair extraction by the following procedure. To each
200-µL serum sample we added 200 µL of internal standard
(see Table 1), 400 µL of Tris (10 mmol/L), and 1 mL of water.
This mixture was vortex-mixed for 60 s and passed through
a reversed-phase “SEP PAK” cartridge (Waters Associates)
that had been pretreated with 10 mL of methanol and 10 mL
of acetate buffer (0.2 mol/L, pH 5.5). The SEP PAK was then
treated with an additional 5 mL of acetate buffer, 1 mL of 0.1
mol/L sodium hydroxide, and another 1 mL of acetate buffer,
dried under reduced pressure, and the compounds of interest
were eluted with 2 mL of methanol. The methanol was evaporated
under nitrogen and the residue reconstituted in the
mobile phase for injection. Three separate calibration curves
were required to cover the entire MTX concentration range.
Table 1 lists conditions used in preparing each of these.

Analytical recovery for the ion-pair extraction exceeded
40%; the SEP PAK extraction increased it to 90%. Neither
extraction technique resulted in interfering bands from serum,
and reproducibility was adequate with each method (ion-pair
±5%, SEP PAK ±3%). Representative chromatograms are
presented in Figure 1.

Statistical methods. We used a two-way analysis of variance for unbalanced data (3), with time as the blocking factor
and method as the main effect. Grouping the data in this
manner removed any potential effect of having used a different
extraction method prior to chromatographic analysis of the 24-h samples. Duncan’s New Multiple Range Test (4)
was applied for pairwise comparison of clinical methods. We
also did an analysis of covariance (5) comparing the three
evaluated test methods after adjusting for the covariate,
liquid-chromatography.

Results and Discussion

Serum MTX concentrations as determined by each test
method are plotted vs those determined by the comparison
method in Figure 2. Interpretations of linear regression
parameters have been reported (6) and limitations of this
method in method comparisons have been discussed (7). Thus
we did not use linear regression analysis in the present studies
because of the wide range of MTX concentrations being
measured. With such a range, the lower values (10⁻⁷ mol/L)
would exert an insignificant weight on the linear regression
analysis as compared with the higher values (10⁻⁴ mol/L).
Therefore we substituted analysis of variance and analysis of
covariance for traditional linear regression analysis.

Analysis of variance revealed no significant deviation of test
method values from those determined by the comparison
method in the concentration range studied (10⁻⁷ to 10⁻⁴
mol/L). Moreover, results by the three methods did not differ
significantly from each other after being adjusted by analysis
of covariance, with chromatography as the covariate. The 12-
and 24-h samples contained substantial amounts of 7-OH
MTX (Figure 1), suggesting that 7-OH MTX cross reacts
negligibly in the test methods. A recent study (1) comparing

Fig. 1. Representative chromatograms of (1) serum blank, (2)
serum calibration sample containing MTX (8.80 × 10⁻⁷ mol/L)
and 7-OH MTX (8.50 × 10⁻⁷ mol/L), and (3) patient’s sample
(24 h) containing MTX (9.86 × 10⁻⁷ mol/L) and 7-OH MTX (un-
quantitated).
a dihydrofolate reductase binding assay with radioimmunoassay reports higher MTX determinations in samples taken at 48 and 72 h after MTX administration and attributes the error to cross reactivity from 4-deoxy-4-amino-N10-methylpteroyl acid, a MTX metabolite. Although our chromatographic analyses of samples through 24 h reveal none of this metabolite, their findings (1) suggest that this metabolite, if present in later samples, might influence results of the test methods. To check this, we supplemented pooled human serum with either 7-OH MTX or the pteroic metabolite in concentrations of $10^{-6}$ mol/L and did three separate analyses of duplicate aliquots of each such sample by each method. Figure 3 summarizes the mean cross reactivity of the three immunological or enzymic methods. Cross reactivity, calculated as (MTX equivalent concn/metabolite concn) $\times$ 100, was substantial for all three methods. The radioimmunoassay and EMTT procedures cross reacted by about 100% with the pteroic metabolite; the radioassay method cross reacted about 20%.

Cross reactivity with 7-OH MTX, the major metabolite present in serum after intravenous therapy with MTX, was less in all three methods, averaging <4% with each method. These data suggest that the equivalence of the four methods is at least partly ascribable to the relatively low cross reactivity with 7-OH MTX and the low concentrations of the pteroic metabolite present in serum samples obtained within 24 h of a 6-h high-dose infusion of MTX. Thus the presence of interfering metabolites in clinical samples obtained later (48 h), samples taken after MTX administration by other routes (oral), or samples obtained after longer MTX infusions (24 h) may influence the accuracy of the three test methods evaluated in this work. However, any of the four analytical methods would appear to be adequate when previously described criteria are applied (8): to monitor high-dose MTX therapy, measure MTX concentrations within 24 h after its infusion.

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References
5. Ibid., pp 305–331.