A Fully Automated, Continuous-Flow Radioimmunoassay for Methotrexate

Reyad Kamel,¹ John Landon,¹ and Gordon C. Forrest²

We describe a fully automated continuous-flow radioimmunoassay for methotrexate. [125I]Histamine-labeled methotrexate was used as tracer. Anti-methotrexate serum was coupled to a magnetizable solid-phase and the bound and free fractions were separated with an electromagnetic field. The assay is precise (CV <2.5%) and rapid (30 samples per hour), incubation volume is small (about 160 μL), and incubation brief (10 min). The accurate timing inherent in the system obviates the need to attain equilibrium, so that assay of each sample takes only 15 min. The assay is sensitive (1–100 μg/L). There is no significant carryover between samples of high and low concentration. Results by the automated method correlated well with those by both a manual assay in which the same reagents and separation technique are used (r = 0.99) and a competitive protein-binding assay (r = 0.96).

Methotrexate (MTX) currently is the drug most widely used in treating various kinds of cancer (1), and it is also used extensively for some non-neoplastic diseases such as psoriasis. Toxicity and effectiveness are related to its concentration in serum and the duration of administration (2). A 6% incidence of MTX-related mortality has recently been reported (3), and Salasoo et al. (4) suggested that high doses of MTX not be administered if concentrations in serum are not being monitored.

The wide range of serum values found and the need to monitor both clearance and peak concentrations of MTX adds to the already increasing demand for MTX monitoring. To meet this demand, we developed a fully automated continuous-flow radioimmunoassay (RIA) for use on the Technicon automated system (5). Anti-MTX serum is coupled to magnetizable particles, and the bound and free fractions are separated by applying an external electromagnetic field. The results obtained for patients' samples were correlated with those by other methods (6, 7).

Materials

Aminopterin, folic acid, dihydrofolic acid, tetrahydrofolic acid, folicin acid, and bovine serum albumin (type V) were from Sigma (Poole, Dorset, U.K.), vimblastine sulfate and vincristine sulfate from Lilly (Basingstoke, U.K.), cytarabine from Upjohn (Sussex, U.K.), fluorouracil from Roche Products (Welwyn Garden City, U.K.), and cyclophosphamide from W. B. Pharmaceuticals (Bracknell, Berks, U.K.).

Anti-MTX serum: Antisera to MTX were raised in sheep against a MTX–polyalanyl–polysine conjugate prepared according to the method described by Jaton and Ungar-Waron (8).

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Magnetizable solid-phase antibody: Anti-MTX serum (2 mL) was covalently coupled by the cyanogen bromide method of Axen et al. (9) to 1 g of cellulose/iron oxide particles, prepared by the method of Robinson et al. (10). The product was suspended in 20 mL of sodium phosphate buffer (100 mmol/L, pH 7.5), containing, per liter, 2.5 g of bovine serum albumin, 2.5 mL of Tween 20, and 1 g of sodium azide (diluent buffer). Stored at 4 °C, it remained stable for at least six months.

Tracer: The 125I-labeled histamine derivative of MTX, prepared as described earlier (6), was diluted in diluent buffer to give about 1000 cpm/100 μL (∼150 pg/100 μL).

MTX standards: Standards were prepared by diluting MTX (25 g/L) injectable MTX; Cyanamid of Great Britain, Gospot, Hampshire, U.K.) in pooled normal human serum to give concentrations of 1, 2.5, 5, 10, 25, 50, and 100 μg/L. These were aliquoted and stored at −20 °C until required, remaining stable for at least six months.

The drug and analogs used for cross-reactivity studies were added to normal human serum to give final concentrations of 1, 5, 10, 25, 50, 100, 250, 500, and 1000 μg/L.

Methods

Automated Assay

Automated equipment previously described (5) was used with a standard glass mixing-coil, giving a 10-min incubation, and a plastic counter coil providing 80 s of counting time. Tracer and solid-phase volumes were each about 50 μL, and the experimental system operated at a rate of 30 samples/h with a 1/2 sample/wash ratio.

Manual Assay

For correlation studies we used both a manual RIA (6) and a competitive protein-binding assay (7).

Developmental Aspects

Essentially the same tracer and solid-phase antibody reagents proved applicable to the automated and both of the manual assays. Because MTX concentrations can vary greatly, depending on the type of sample and the time of sampling after medication, we selected to use a standard curve covering the concentration range of 1–100 μg/L.

Sample: A sample volume of about 60 μL was adopted, giving a total incubation volume of about 160 μL.

Tracer: 125I-labeled MTX concentration was selected to give a recorded count of 8000 to 10 000 counts per 80 s for the highest standard, thereby minimizing statistical counting errors.

Antibody-linked magnetizable particles: We chose a concentration of solid-phase antibody to give a standard curve spanning the selected range (1–100 μg/L). The weight of solid phase used (0.62 mg in 50 μL) was insufficient to activate the instrument's detector system, and so we added normal sheep
IgG-linked particles (1.5 mg in 50 μL), to increase particle density.

**Incubation time:** Figure 1 illustrates the kinetics of binding of tracer in the presence of the zero- and highest-concentration standards. Incubations of 2 to 30 min were studied; steady state was reached after 5 min. For compatibility with other assays run on the system (digoxin, thyroxine, triiodothyronine, triiodothyronine uptake, human placental lactogen, and cortisol) we used a standard 10-min incubation coil, but a shorter incubation could be used if desired.

**Assay Procedure**

A series of standards was aspirated at the beginning and end of each assay, and quality-control sera were inserted in duplicate after every 10 samples. At the beginning of each run a sample of MTX-free normal human serum sample was aspirated, in at least duplicate, to determine the binding of the 125I-labeled MTX to antibody in the absence of unlabeled drug (C0). Counts obtained for the standards were expressed as a percentage of C0 and plotted vs MTX concentration in the standards (Figure 2). Patients' samples were assayed either undiluted or diluted in normal human serum. Because MTX concentrations during therapy may vary greatly, the samples often must be diluted. Table 1 shows appropriate sample dilutions consistent with various doses and routes of administration.

**Table 1. Typical Sample-Dilution Factors as a Function of MTX Dose, Time of Administration, and Sampling Time after Administration**

<table>
<thead>
<tr>
<th>Total dose of MTX, mg</th>
<th>Route of administration *</th>
<th>Time after dosage, h</th>
<th>Concentration of MTX, μg/L serum or plasma</th>
<th>Dilution factor</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>Oral</td>
<td>1–4</td>
<td>200–800</td>
<td>10X</td>
<td>our finding</td>
</tr>
<tr>
<td>25</td>
<td>i.m.</td>
<td>12</td>
<td>10–100</td>
<td>undiluted</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>i.m.</td>
<td>1–4</td>
<td>200–1000</td>
<td>20X</td>
<td>our finding</td>
</tr>
<tr>
<td>200</td>
<td>24-h i.v. infusion</td>
<td>Plateau level of infusion</td>
<td>400–700</td>
<td>10X</td>
<td>(15)</td>
</tr>
<tr>
<td>200–400</td>
<td>i.m. or i.v.</td>
<td>6</td>
<td>1000–2500</td>
<td>50X</td>
<td>(15)</td>
</tr>
<tr>
<td>200–1250</td>
<td>i.m. or i.v.</td>
<td>48</td>
<td>1–10</td>
<td>undiluted</td>
<td></td>
</tr>
<tr>
<td>1250</td>
<td>i.v.</td>
<td>6</td>
<td>5720</td>
<td>100X</td>
<td>(15)</td>
</tr>
</tbody>
</table>

* i.m. = intramuscular, i.v. = intravenous.

**Results**

**Carryover:** This was assessed by repeatedly assaying the zero and highest (100 μg/L) standards alternately, in duplicate. Thus, we obtained data for the zero standard preceded by both a 100 μg/L and a zero standard and likewise data for the 100 μg/L standard preceded by both a zero and a 100 μg/L standard. The results (Table 2) demonstrate that carryover is insignificant.

**Precision:** Two serum specimens from patients receiving MTX were each assayed 24 times, to assess within-assay precision. Mean results were 32.6 and 14.7 μg/L, with CVs of 2.4 and 2.1%, respectively.

**Accuracy:** Both analytical recovery and correlation studies were performed. MTX was added to give a 50 μg/L concentration in 15 individual samples of normal human serum and five lipemic sera with triglyceride concentrations between 1.9 and 4.0 mmol/L. The average analytical recovery of MTX from the normal sera was 101.4% (SD 5.4%), from the lipemic specimens 102.2% (SD 3.8%). The correlation coefficients (r) for the fully automated RIA vs manual RIA (6) and fully automated RIA vs competitive protein binding assay were 0.99 and 0.96, respectively (Figure 3).

**Specificity:** We determined cross reactivity of a series of MTX analogs (aminopterin, folic acid, folinic acid, dihydrofolic acid, tetrahydrofolic acid), and of drugs that may be given in combination therapy (vincristine, vinblastine, cytarabine,
fluorouracil, and cyclophosphamide), using concentrations of these up to 1 mg/L. Only aminopterin showed any significant cross reaction (18%).

$^{125}$I-labeled MTX: Labeled MTX showed no significant loss of immunoreactivity after storage for five months at $-20$ °C, or in either liquid form or freeze-dried when stored at 4 °C. The tracer had a high specific activity (230 Ci/g) as calculated by a self-displacement technique (11), and its immunoreactivity was similar to that of unlabeled MTX.

**Discussion**

Measurement of MTX concentration in the serum of patients who are being treated for cancer is becoming increasingly important in effective management. Our automated assay provides a rapid means of measuring many such sera with high accuracy and precision, and represents the only fully automated assay for MTX yet described. Any automated assay for MTX, whether based on direct or continuous-flow principles, must be capable of handling the wide range of concentrations commonly encountered in patients' sera. In the present system, known high-concentration samples are pre-diluted before assay, so that concentrations $>100$ μg/L are not encountered. Fortunately, samples can be readily diluted into the appropriate range if data on time and dosage are available. Carryover—a major problem with earlier continuous-flow automated systems involving long incubation periods (12-14)—is not a problem here, and indeed the sampling rate of 30 samples per hour could be increased in view of the absence of carryover and the use of tracer with high specific activity.

The relatively high concentration of bovine serum albumin in the assay buffer was used to minimize the possible effects of inter-sample variation in protein content.

A key feature of the automated system (5) is the discrete, synchronized addition of both tracer and solid-phase to sample-containing segments only. During the wash cycle, these reagents are re-circulated and not pumped into the assay stream, which ensures that they are utilized with maximal economy.

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