Monitoring the Administration of Methotrexate in Antimetabolite Therapy

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ABSTRACT

An enzymatic method for the measurement of methotrexate (MTX) in serum is presented in which the inhibition of the enzyme dihydrofolate reductase by MTX is measured. Reduction of the substrate dihydrofolate by the enzyme and cofactor NADPH is lessened in direct proportion to the amount of MTX present. Measurements can be made in the “therapeutic range” of MTX which corresponds to the $10^{-7}$ to $10^{-8}$ M concentration of MTX in serum.

Introduction

Methotrexate (amethopterin, 4-amino-4-deoxy-N10-methyl folic acid, MTX) is a recognized antineoplastic medication used in treatment of acute leukemia, choriocarcinoma, osteogenic sarcoma, head and neck carcinoma, breast cancer and others. In addition, it is used in treatment of nonneoplastic diseases such as psoriasis, Wegener’s granulomatosis and sarcoidosis. The accepted mechanism of action is that MTX competes with folic acid for the enzyme dihydrofolate reductase, (5,6,7,8-tetrahydrofolate:nicotinamide adenine dinucleotide phosphate [NADP] oxidoreductase, EC 1.5.1.3.) prevents the formation of reduced folic acid and thus interferes with purine synthesis, thymidylate formation, DNA synthesis and protein synthesis. Methods for the determination of MTX in serum are microbiological isotopic, fluorometric and enzymatic. The method presented here is enzymatic and is based on a recent paper by Overdijk et al after the original method of Bertino and Fischer.

Principle

MTX is a competitive inhibitor in the conversion of dihydrofolate to tetrahydrofolate by action of the enzyme dihydrofolate reductase. If MTX is added to a reaction mixture of buffered dihydrofolate (FH$_2$), dihydrofolate reductase and nicotinamide adenine dinucleotide phosphate, reduced (NADPH), the reaction will be inhibited and the change in absorbance at 340 nm will be less than if MTX were not included in the reaction.
mixture. The reaction without MTX termed the blank, is as follows:

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5,6,7,8\text{-tetrahydrofolate} + \text{NADP} = 7,8\text{-dihydrofolate} + \text{NADPH}
\]

MTX addition may be in aqueous solution, serum, plasma or urine.

**Reagents**

*Sodium citrate buffer, pH 5.9, 1.0 M.* Exactly 294 g of trisodium citrate (2 H₂O) are dissolved in 200 ml of water and the pH is adjusted to 5.9 with concentrated HCl. The solution is adjusted to 1L and the pH checked once again and adjusted to pH 5.9 with either 0.1 N HCl or 0.1 N NaOH if required.

*Potassium chloride, 1.5 M.* Exactly 11.19 g of reagent grade KCl are dissolved in pure, deionized water to a volume of 100 ml.

*Dihydrofolate reductase.* Exactly 5 mg of powdered enzyme preparation* are weighed and dissolved in 50 ml of pure water. Two ml portions are aliquoted into clean test tubes, capped and placed in a freezer. The excess powdered enzyme in its container is kept in a jar in which is placed indicating Drierite. The jar, tightly capped, is placed in a freezer until ready for use. The jar and its contents are allowed to reach room temperature before sampling.

*Dihydrofolate (FH₂)*† 0.5 mM. Exactly 22.2 mg of FH₂ are mixed with about 50 ml of pure water in a 100 ml volumetric flask. A 1.0 ml portion of 1 N NaOH solution is added, the contents of the flask are swirled to dissolve the FH₂, and the volume is made up to 100 ml with pure water. Aliquots of 3 ml each are transferred to clean test tubes and the tubes are placed in a freezer.

*β-Mercaptoethanol, 0.1 M.* Exactly 0.43 ml of β-mercaptoethanol is diluted to 50 ml with pure water. Two ml aliquots are placed in test tubes and placed in a freezer.

*NADPH, 0.5 mM.* NADPH commercially available as the tetrasodium salt is usually associated with water (about 4.5 molecules per mole) and about three percent ethanol owing to its method of preparation. The manufacturer provides a corrected theoretical molecular weight based upon these adducts. Thus for a preparation having a corrected molecular weight of 941.8 (Lot #15C-7320), 47.0 mg of NADPH Na₄ are diluted to 100 ml with pure water. Aliquots of 3 ml each in test tubes are placed in a freezer. It is recommended that the enzymatically reduced preparation be used.

**Standard Solutions**

*Stock solution of MTX,*‡ 1 × 10⁻⁴ M. Exactly 4.54 mg of methotrexate are diluted to 100 ml with 0.05 N NaOH.

A 1 × 10⁻⁶ M concentration is prepared by diluting 1 ml of stock solution to 100 ml with pure water.

Concentrations of 0.5, 1, 3, 5, 7.5 and 10 × 10⁻⁸ M standards are prepared by diluting 0.5, 1, 3, 5, 7.5 and 10 ml of the 1 × 10⁻⁶ M MTX solution to 100 ml with pure water. Aliquots of 2 ml each of these prepared standards in test tubes are placed in the freezer.

**Special Apparatus**

A high quality spectrophotometer is needed with capabilities of (1) an 8 nm or less spectral band pass, (2) a 340 nm analytical wave length, (3) a thermostatted cell compartment and (4) a cell volume of about 0.3 ml. Preferable is the fixed cuvet type of instrument into which

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* Obtainable from R. L. Kisliuk, Ph.D., Department of Pharmacology, Tufts University School of Medicine, Boston, MA.
† Obtainable from the Sigma Chemical Co., St. Louis, MO.

‡ The methotrexate used in this study was kindly supplied by E. W. Cantrell, Ph.D., of the Lederle Laboratories.
sample is drawn by suction and drawn to waste before the following sample is drawn.

**Procedure**

The spectrophotometer is allowed to warm up and the cell compartment operating temperature is set at 37°. The necessary frozen reagents and standards are allowed to defrost and the contents of each tube are mixed well before using. The contents of the tubes containing FH₂, β-mercaptoethanol and NADPH are mixed together with the admixture of 2 ml each of citrate buffer and 1.5 M potassium chloride and 4 ml of pure water.

To 0.8 ml of the mixed reagents (step 3) in a small test tube is added 0.1 ml of standard. (All the standards are run before samples and a calibration curve is drawn in the usual way. A blank is also run substituting water for the standard). As a final step, to start the reaction, 0.1 ml of enzyme solution is added, the contents mixed and aspirated into the spectrophotometer cell compartment. Absorbances are recorded for five minutes after allowing 20 seconds to elapse.

The serum, plasma or urine is treated in a similar way. With all samples, the liquid is drawn into a cell, exhausted of previous sample or wash water, in a single draw of about 0.7 ml. Readings are started 20 seconds after sampling.

**Calculations**

If a printing readout is used, the absorbance at five minutes is subtracted from the absorbance at 20 seconds. If a recorder is used, the linear portion of the regression line is used, and the delta-absorbance for five minutes is derived. It is a matter of choice how the calibration line is drawn. Either delta-absorbance of blank (zero MTX) and MTX sample delta-absorbances are graphed versus MTX concentration, or delta-absorbances of MTX samples are subtracted from the blank delta-absorbance and the resulting figures are graphed versus MTX concentrations. The latter method gives an ascending calibration line with zero as the origin.

The unknown solution concentration is derived from the calibration line in the usual way. If a dilution of unknown sample must be made, a correction for dilution is made in the usual way. The necessity for dilution is seen from a lack of absorbance change. A calibration line is made for each individual run.

**Discussion**

The procedure described can be readily performed in any well-equipped and staffed clinical laboratory where manual, spectrophotometric enzyme analyses are performed. Although the procedure is more involved than microbiological and fluorometric methods, the enzymatic method seems to be the best suited for the purpose. Fluorometric methods give acceptable answers for MTX at a level at the $1 \times 10^{-6}$ M level but therapeutic levels are roughly 10 times less and therefore the fluorometric method lacks the capability of fulfilling the needs of the clinician. Microbiological methods suffer from the lengthy period needed to complete an assay, while radioisotope methods cannot be performed in all laboratories and, further, are complicated procedures. Fortunately, for the laboratory performing the enzymatic analysis for MTX, all the reagents necessary for the analysis are readily available.

NADPH is available as a salt prepared by reduction of NADP by either chemical or enzymatic means. While the NADPH, chemically reduced, is probably suitable, it has been reported by Hillcoat et al.⁹ that the chemically reduced salt contains sulfite which may cause decomposition of the NADPH. Therefore, the enzymatically reduced NADPH (98 percent pure) was used in the method reported here.
The original Overdijk et al\textsuperscript{18} article presented a procedure in which the individual reagents were mixed in a sequential manner. This stepwise mixing procedure results in a tedious assay method when many standards and samples are to be run. There is also the possibility of a multiplication of volumetric errors in the pipeting of several reagents rather than one or two. In the method reported here the mixing of the several reagents into a single reagent presented no disadvantage in terms of readings or absorbance changes. Also, the procedure of adding the enzyme last (suggested by Kisliuk\textsuperscript{16}) to start the reaction was of definite advantage in that greater delta-absorbances were achieved. Lastly, the use of $37^\circ$ rather than the temperature of $20^\circ$ called for by Overdijk et al\textsuperscript{18} resulted in a more sensitive assay than the original method. It is also noted that the pH maximum in the Bertino\textsuperscript{2} method is on the acid side, pH 5.9 being chosen for operation, whereas the \textit{L. Casei} enzyme prepared by Kisliuk and used in this method\textsuperscript{16} has a pH maximum at 7.4. Further work will be done on a method based on the higher pH conditions.

Sources of Error

Sources of possible error are the amount of enzyme used in the test, the NADPH, the FH$_2$ and the water used in all phases of the procedure. Too much enzyme will cause the reaction to come to completion too rapidly, while too little enzyme will result in low readings. The FH$_2$ and NADPH preparations may be suspect when the reaction shows a faulty (slow) reaction. These solutions may be made up fresh if difficulty is encountered. FH$_2$ also shows a strong substrate inhibition, for that reason the amount of FH$_2$ should be carefully monitored. Contamination from the water used should be considered when the blank reaction is unsuitably slow. An unstable instrument will render a test invalid so maintenance of the instrument and the temperature control mechanism must be carefully maintained. Delivery pipets should be carefully monitored and maintained since small volumes of reagents are used in the reaction.

Normal Ranges

Since MTX is not expected to exist in the untreated patient, normal ranges do not exist and thus only therapeutic ranges will be discussed here. Because of the wide diversity of dosages given by different investigators expected amounts of MTX in serum may vary by magnitudes of difference.

In the heavily dosed patient with whom "leucovorin rescue" is to be performed, serum levels may rise to 50 $\mu$g per ml or more.\textsuperscript{10} The levels that are to be encountered may be expected to be from about $1 \times 10^{-8}$ M (0.00454 $\mu$g per ml) to about $1 \times 10^{-4}$ M (45.4 $\mu$g per ml) depending on the time after infusion that the test is conducted. It is to be noted that the useful calibration range is from $1 \times 10^{-8}$ M to $1 \times 10^{-7}$ M so some sera may have to be diluted 10,000 fold to be measured by this method. Dilutions are best made by serial 10 fold dilutions to a total of four dilutions.

Where "therapeutic doses" are administered the approximate serum levels to be expected are: (1) for a 0.1 mg per kg dose I.V., 0.2 $\mu$g MTX per ml about 20 minutes after dosage, and 0.03 $\mu$g per ml at six hours after dosage; (2) for a 0.4 mg per kg dose, the corresponding figures are approximately four times as much. Chabner and Young\textsuperscript{5} have reported that ($^3$H)-deoxyuridine was incorporated into deoxyribonucleic acid (DNA) (50 percent of pre-treatment levels) in bone marrow when the plasma MTX fell to $10^{-8}$ M or less. In intestinal mucosa, to achieve 50 percent of pre-treatment level of incorporation, plasma MTX had to be $5 \times 10^{-9}$ M.
or less. It should be noted that the analytical method presented here has a limit of sensitivity at about $5 \times 10^{-9}$ M. The recently reported radioassay by Arons et al\(^1\) extends the limit of sensitivity to an order of magnitude below the enzymatic method's sensitivity.

**Résumé of Clinical Interpretations**

Toxic manifestations of MTX may be evidenced by myelosuppression, mucositis and gastrointestinal symptoms.\(^4\) Very high doses may result in lesions of the intestinal tract, bone marrow and liver. Reversible fibrosis is often a result of MTX administration. The usual therapeutic dose in clinical practice is 0.1 mg per kg\(^17\) but as will be discussed, much higher dosage is being used in order to bind the drug as completely as possible within the tumor cells.

Toxicity of MTX has been related to duration of infusion\(^4,11\) even at such high doses as 450 to 20,000 mg (250 mg per m\(^2\) to 12,000 mg per m\(^2\)). In cases of infusions ranging from 24 to 36 hours, plasma MTX reached as high a level as 70 µg per ml except in one case of massive dose infusion (12,000 mg per m\(^2\)) where the plasma level reached 240 µg per ml. Usually with doses of 750 to 8000 mg over 24 to 36 hours, the plasma MTX did not exceed about 20 µg per ml.\(^11\) Such high levels of treatment of course are accompanied by "leucovorin rescue." Exposure to doses of MTX resulting in a prolonged level of $1 \times 10^{-7}$ M (4.5 $\times 10^{-2}$ µg per ml) is considered to be toxic in humans. "In mice exposure to inhibitory plasma levels (2 $\times 10^{-8}$ M) for longer than 48 hours produced fatal toxicity owing to gastrointestinal damage, whereas lower blood levels or shorter durations of exposure were nonlethal . . . these investigations have led to the following important conclusions: the cytotoxic action of methotrexate requires the presence of free intracellular drug above a specific threshold level for each tissue; and the toxic effect of methotrexate on a target organ is primarily a function of the duration of exposure to supra threshold concentrations of drug rather than the peak levels of drug achieved."\(^4\)

Methotrexate levels clear from the blood in an exponential manner with a half-life of four to more than 24 hours,\(^10\) although others\(^14\) have stated that the half-life could extend to as long as 69 hours. Most of administered MTX is excreted unchanged into the urine so renal sufficiency should be monitored during the therapy period. Frei et al\(^10\) cited a case in which citrovorum-factor was administered to 72 hours in increased doses when a patient exhibited an abrupt rise in serum creatinine at 24 hours after dosage. The MTX level at 24 hours was $1.1 \times 10^{-5}$ M. At hour 96, the serum MTX was still at $5.8 \times 10^{-6}$ M, but deoxyuridine incorporation into aspirated bone marrow cells was normal. Delay in this kind of rescue might result in unreversible toxicity.

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