A Simplified Fluorometric Method for Determination of Plasma Methotrexate

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A method is described for the determination of plasma methotrexate at low concentrations. The method consists of a single fluorescence measurement on plasma protein-free filtrates after oxidation of the drug with KMnO₄ at controlled pH. The complete release of the drug into plasma filtrates is accomplished by heating the reaction mixture in a boiling water bath with occasional stirring.

A fluorometric method for the determination of pteroylglutamic acid (PGA) in nonbiologic material was reported by Alifrey et al. (1). Freeman (2) adapted this method for the determination of amethopterin in plasma. Both procedures oxidized PGA or amethopterin with permanganate to yield a strongly fluorescent substance, 2-amino-4-hydroxypteridine-6-carboxylic acid (3). The fluorescence due to the latter compound is much more intense than that of PGA (1-3). The method involved measuring fluorescence before oxidation and then after oxidation of the compound with permanganate. The difference between the two fluorescence readings was proportional to the concentration of PGA or amethopterin present.

Our laboratory attempted to establish a relationship between chromosome aberration rates in leukocytes and the methotrexate (MTX) level in plasma of psoriatic patients maintained on a MTX regimen (4). For this study, we attempted to duplicate Freeman's method (2) for the determination of plasma MTX levels. The results were not reproducible, especially at concentrations less than 20 mg/ml. However, when the trichloroacetic acid-treated plasma-MTX mixtures were heated at 100° prior to filtering the precipitated plasma proteins, only a single fluorescence reading after oxidation was necessary to obtain linearity between concentration of MTX and fluorescence.

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Received for publication March 15, 1969; accepted for publication June 30, 1969.

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Materials

Instrument

For the quantitative measurement of the drug in plasma a Farrand fluorometer, Model A was used. The filter for incident light was Corning No. 5860 with a wavelength of 360 nm and the filter for the emitted fluorescence was a combination of Corning No. 4308 (462 ± 5 nm) and Corning No. 3389 (391 and 511 nm) with No. 3389 facing the phototube. The combination of secondary filters corresponds to a wavelength of approximately 450 nm.

Reagents

1. 20% (w/v) trichloracetic acid solution (TCA)
2. 3.25% (w/v) sodium hydroxide (NaOH)
3. 5.0 M acetate buffer pH 5.0
4. 3.5% (w/v) potassium permanganate solution
5. 3% hydrogen peroxide, prepared by dilution of 30% H₂O₂
6. 10% (v/v) acetic acid

Reference Methotrexate Solutions

1. Stock solution 10 mg/ml: Dissolve 10 mg methotrexate powder in 100 ml water or dilute the contents of an ampule.
3. Appropriate volume of working reference solution was added to plasma to make a total of 5 ml at each concentration level. Plasma samples made in this fashion were used for preparing a reference calibration curve.

Method

Seven ml of distilled water was added to 1.0 ml of plasma in a test tube and the solution mixed on a Vortex Jr. mixer. Two ml of 20% TCA was then added slowly and the contents of the tube were again mixed thoroughly. The test tubes containing precipitated proteins were then placed in a boiling water bath and heated for exactly 5 min with occasional stirring of the contents with glass rods. After 5 min, the test tubes were removed from the bath, cooled to room temperature, and the contents were filtered through Whatman No. 42 filter paper. To 2.0 ml of filtrate, 0.4 ml of 3.25% NaOH and 0.04 ml of 5.0 M acetate buffer pH 5.0 were added and mixed thoroughly. The pH of the contents of each test tube was then adjusted to pH 5.0 by use of a pH meter by addition of either 3.25% NaOH or 10% acetic acid. To each test tube, 0.01 ml of 3.5% KMnO₄ was then added and mixed by inverting the
tube, which was covered with a parafilm square. After 5 min, 0.01 ml of 3% H₂O₂ was added (to destroy excess KMnO₄) and the solution was mixed again. Fluorescence was measured after an additional 5 min. The instrument sensitivity was set at 2. A filtrate containing 60 μg/ml (600 μg/ml plasma) was set to read 100. The reading due to plasma blank (containing no methotrexate) was subtracted from each reading. The fluorescence readings were found to be stable for a period of 1 hr or more.

**Results**

Fig 1 shows a standard curve of plasma MTX after addition of appropriate amounts of MTX to plasma. These results (Fig 1) indicate that it is possible to measure as little as 5 ng/ml MTX in plasma filtrates.

**Effect of pH**

Fluorescence of protein-free filtrates from a pooled plasma containing 255 ± 2.5 ng MTX/ml was measured in triplicate at pH's ranging from 3 to 6.5. At pH 6 and above a significant amount of precipitate appeared upon the addition of KMnO₄ solution. The fluorescence readings increased with increasing pH up to pH 5. A plateau was obtained between pH 5 and pH 6 (Fig 2).

**Effect of Heat on the Release of Protein-bound MTX**

Poor recovery was encountered when MTX was measured on filtrates without heating in a boiling water bath. Table 1 shows the effect of

![Fig 1. Calibration curve for methotrexate in plasma protein-free filtrate (PFF) using a Farrand fluorometer Model A with sensitivity set at 2. PFF containing 60 μg/ml was used for setting meter reading at 100. The blank readings due to PFF from a plasma containing no MTX was subtracted from each reading. Points represent average of 5 determinations. The vertical bars indicate the distribution of readings.](image-url)
heating and stirring after addition of 20% TCA to the diluted plasma. Complete recovery was achieved only after heating.

**Discussion**

MTX is extensively used for treatment of proliferative diseases such as leukemia (5), carcinoma (6, 7), and psoriasis (8). It may also produce chromosome breaks (4). It is important to have a reliable and reproducible method for the determination of the drug in plasma especially in view of the extremely toxic nature of the drug (9). The method suggested by Allfrey and modified by Freeman (2) for determination of plasma PGA (amethopterin, MTX) is extremely difficult to reproduce when the blood concentration is low. By using permanganate oxidation of the drug in plasma filtrates the resulting fluorescence was found to be proportional to the drug concentration. Plasma MTX levels as low as 50 μg/ml (5 μg/ml filtrate) could be determined if the TCA-treated diluted plasma was heated at 100° prior to separation of the precipitated proteins by filtration. Apparently a variable amount

![Graph](image)

**Fig 2.** Effect of pH on fluorescence due to oxidized MTX. Maximum fluorescence was obtained at pH 5. At pH 4.5 or below fluorescence was significantly less. At pH 6.0 precipitation appeared upon addition of permanganate.

<table>
<thead>
<tr>
<th>Concentration in μg/ml added to plasma</th>
<th><em>Recovery of MTX in μg</em></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Heated at 100° for 5 min.</td>
</tr>
<tr>
<td>100</td>
<td>97 ± 2.0</td>
</tr>
<tr>
<td>200</td>
<td>198 ± 2.6</td>
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<tr>
<td>300</td>
<td>300 ± 4.2</td>
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<tr>
<td>400</td>
<td>398 ± 3.8</td>
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<tr>
<td>500</td>
<td>502 ± 4.0</td>
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</table>

* 3 triplicate analysis.
† 1.0 ml of plasma to which appropriate amount of MTX was added, was diluted to 8.0 ml with distilled water and then 2.0 ml of 20% TCA was added, mixed and heated at 100° for 5 min.
of MTX is absorbed on TCA-precipitated plasma proteins and the heating and stirring causes this absorbed MTX to be released into the supernatant solution.

References