Analysis of Methotrexate in Human Plasma by High-Pressure Liquid Chromatography with Fluorescence Detection

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SUMMARY

Methotrexate in human plasma at a concentration as low as 0.01 μg/ml can be assayed with the use of high-pressure-liquid chromatography and a fluorescence detection system. Methotrexate is oxidized stoichiometrically to 2,4-diaminopteridine-6-carboxylic acid, a fluorescent product that is separable from other fluorescent materials in plasma with the use of an octadecylsilane (reversed phase) column. The detector response is linear over the range of 0.01 to 10 μg/ml. Neither folic acid nor citrovorum factor interferes with the analysis. N-[(4-[(2,4-Dihydroxy-6-pteridyl)methyl]-amino]benzoyl]glutamic acid may be used as an internal standard, since it can be extracted from plasma and oxidized like methotrexate. The procedure is rapid (about 30 min) and should be a useful method for monitoring methotrexate plasma concentrations.

INTRODUCTION

The monitoring of MTX3 in plasma of patients receiving high-dose therapy (8) is essential to avoid drug toxicity. The methods currently used to measure MTX include enzyme inhibition (6, 19, 21), competitive protein binding (15), radioimmunoassay (2, 3, 9, 10, 12, 13, 18), and spectrofluorometry (4, 7, 11). HPLC, particularly with reversed-phase microparticulate columns (14), is effective for separating folate-like compounds. This paper describes a HPLC procedure for MTX analysis and illustrates the potential of fluorescence detection. A preliminary report of this work has been presented (17).

MATERIALS AND METHODS

Materials. Pure samples of MTX and citrovorum factor were kindly provided by Dr. John A. Montgomery of Southern Research Institute, Birmingham, Ala. 2,4-Dihydroxyppteridine-6-carboxylic acid, 2-amino-4-hydroxyppteridine-6-carboxylic acid, and 2-hydroxyfolic acid were prepared with the use of previously described methods (1, 5). Other chemicals were of the highest purity available from Sigma Chemical Co., St. Louis, Mo. Dr. Alexander Wang provided clinical specimens and MTX levels with the use of enzyme inhibition analysis (20).

HPLC. A Waters Associates Model GPC/LC-204 instrument equipped with the Model 6000 pump was used. A Waters Associates μBondapak C18 column (4 mm x 30 cm) and a Model UJX Injector system were also used. A Model SF-770 multiple wavelength UV detector and Model FS-970 fluorescence detector (Schoeffel Instrument Corp., Westwood, N. J.) were connected in series to the outlet of the column. The 10-mV outputs of these detectors were connected to a Houston Instrument 2-pen chart recorder. Two solvent systems were used: Solvent System 1, 0.1 M Tris adjusted to pH 6.7 with phosphoric acid; and Solvent System 2, 0.1 M Tris-PO4, pH 6.7, plus 20% methanol. The flow rate was 1 ml/min, and the excitation wavelength of the fluorescence detector was set at 275 nm. Excitation Filter No. 7-54 and Emission Filter KV-418, with a cutoff near 410 nm, were also used. Sensitivity was 63.0, and the time constant was 4 sec. Other details of the HPLC separations are given in the chart legends.

Procedure. Standard curves for MTX were determined by adding known amounts of drug to pooled human plasma. Protein was precipitated by adding 0.3 ml of 10% trichloroacetic acid to each 1 ml of plasma. Following centrifugation, MTX in the acid extract could be measured with a UV detector at 305 nm. Recovery of MTX in the acid extracts was reproducibly about 40% (range, 38 to 41% in 9 determinations). For each 0.5 ml of trichloroacetic acid extract, 0.05 ml of 5 M acetic acid-sodium acetate buffer, pH 5.0, was added. Oxidation was performed at room temperature in 5 min by the addition of 0.05 ml of 5% potassium permanganate aqueous solution. The samples were decolorized by addition of 0.05 ml of a 3% solution of hydrogen peroxide. This procedure resulted in the quantitative conversion of MTX to 2,4-diaminopteridine-6-carboxylic acid as determined by the loss of MTX and appearance of the fluorescent pteridine-6-carboxylic acid with the use of the HPLC systems described above.

When 2-hydroxyfolic acid is used as the internal standard, it is added to the trichloroacetic acid solution to give a final concentration in plasma equivalent to 0.5 μg/ml. The ratio of peak height for the MTX oxidation product versus peak height of the internal-standard oxidation product is used to determine MTX levels. Alternatively, the area under the peak of interest can be estimated by triangulation (the product of height (mm) multiplied by width (mm) at one-
half height]. Peak area is generally a more reproducible means of quantitation.

RESULTS

Chart 1 illustrates the HPLC with UV detection method for determination of MTX in trichloroacetic acid extracts of plasma. Addition of methanol (circa 20%) is required to elute MTX from the column when 0.1 M Tris-PO₄ buffer, pH 6.7, is used (14). Determination of MTX is facilitated at 305 nm, since there is minimal interference by other components eluting near the retention time of MTX, about 8 min in Chart 1. The UV detector was set at its maximal sensitivity (0.01 AUFS), and 100 μl of acid extract were injected onto the column. Up to 250 μl can be injected before resolution of MTX is lost; therefore, the limit of detection of this method is about 0.4 μg/ml.

Chart 2 illustrates the HPLC determination of MTX in plasma as its fluorescent oxidation product, 2,4-diaminopteridine-6-carboxylic acid. The lower limit of sensitivity is about 0.01 μg/ml when 250 μl are injected. Again, with the use of a single column and the elution conditions described, this represents a near-maximal sample load.

In the range of MTX concentrations from 0.01 to 0.1 μg/ml, the fluorescence response is linear (Chart 3). Although not shown, linearity persists to levels as high as 10 μg/ml. Also, neither folic acid nor citrovorum factor (10 μg/ml) interferes with the MTX analysis, since retention time of the oxidation product is about 4 min. With the use of a standard concentration (1.0 μg/ml) of MTX in 5 separate determinations, the range of values for the area under the MTX oxidation peak was ±10% of the mean.

When 2-hydroxyfolic acid is used as an internal standard,
Chart 4. Internal standard for MTX analysis by HPLC and fluorescence detection. Samples of human plasma were "spiked" with MTX (0.1 µg/ml) and 2-hydroxyfolic acid (0.5 µg/ml). Samples (100 µl) of oxidized plasma extracts were loaded onto the chromatograph. The fluorescence detector range setting was 0.2. A, plasma plus MTX and 2-hydroxyfolic acid; B, plasma blank.

Chart 5. MTX plasma standard curve with the use of an internal standard. Plasma containing 2-hydroxyfolic acid (0.5 µg/ml) and different levels of MTX was extracted and analyzed as shown in Chart 4. Points, average for duplicate samples; bars, range of the 2 values.

Chart 6. Determination of MTX in plasma of a patient receiving MTX (50 mg/kg i.v. over a 6-hr period). Peripheral blood samples were drawn at the end of the infusion period and 1 to 3 days later. MTX analysis by the HPLC method was as shown in Charts 2 and 3. MTX analysis in plasma by enzyme inhibition was performed by Dr. Alexander Wang as previously described (20).

Table 1
Comparison of enzyme inhibition and HPLC methods for MTX analysis in human plasma

<table>
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<tr>
<th>MTX concentration (µM)</th>
<th>Enzyme inhibition</th>
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</table>

* Values determined as described by Wang et al. (20).
* Measurement was made as described herein by HPLC and fluorescence detection without internal standard.

DISCUSSION

A major difficulty in the application of HPLC to routine analysis of drugs in body fluids is lack of sensitivity (16), since virtually any compound can be resolved by some mode of HPLC. The availability of fluorescence detectors specifically adapted to HPLC offers a major step toward achieving the needed sensitivity. As illustrated in Charts 1 and 2, the sensitivity for MTX analysis can be increased about 40-fold when a fluorescent derivative is used. The increased sensitivity makes possible analysis of MTX by HPLC at clinically relevant levels, i.e., 0.01 to 0.1 µg/ml (8). A recent report by Wang et al. (20) reviews the other methods generally used to measure MTX.

2-Hydroxyfolic acid is useful as an internal standard for measuring MTX by HPLC (Charts 4 and 5). The use of an

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internal standard is desirable, since it would correct for day-to-day differences in MTX extraction and oxidation. Without an internal standard, it is necessary to use a working standard that requires additional time and effort.

Theoretically, chemical analysis of MTX such as the HPLC method reported herein has the advantage of being highly specific for MTX. The level of sensitivity, 0.01 μg/ml or about $2 \times 10^{-8}$ M, compares favorably with the sensitivities of other commonly used methods (15, 20).

If the HPLC procedure is to be used clinically, it is necessary to compare MTX concentrations as measured by HPLC with those measured by current methods. Since the other methods used are often less specific for MTX per se (19), the HPLC method may be found to give lower estimates such as is observed in the patient’s samples illustrated in Chart 6. Since very high doses of the drug are sometimes used, minor impurities that are detected as MTX by other methods may have significant clinical effects.

ACKNOWLEDGMENTS

We wish to thank Dr. Alexander Wang, Department of Pediatrics, M. D. Anderson Hospital, for kindly providing the clinical specimens and results of MTX analysis by enzyme inhibition, and Dr. John A. Montgomery, for helpful suggestions.

REFERENCES

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