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Note

Reversed-phase high-performance liquid chromatographic analysis of methotrexate and 7-hydroxymethotrexate in serum

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High dose methotrexate (MTX) followed by citrovorum factor as a biochemical rescue has been widely used in the treatment of a variety of human cancers [1—5]. Because of the inherent risk of toxicity from this regimen [6—8], patient management requires the monitoring of serum MTX to allow identification of patients with high, potentially toxic, MTX concentrations and/or delayed MTX elimination. While the pharmacckinetic behavior of MTX has been reported to be quite different at high dose showing biexponential [9, 10] plasma disappearance up to 72 h post dose compared to a triexponential pattern at lower doses [11], this may be attributable to methodologic differences. In addition, the presence of metabolites, particularly 7-hydroxymethotrexate (7-OH-MTX), appears to be significant [12, 13] at high dose after it had been previously thought to be unimportant at low doses [14].

While there are several analytical methods in the literature for plasma methotrexate including enzyme inhibition [15, 16], protein binding [17], radioimmunoassay [18, 19] and fluorescence [20, 21], none of these employ separation steps capable of resolving or quantitating 7-OH-MTX. Since most were developed prior to a recognition of significant metabolite formation in humans the absolute specificity is not known. A high-performance liquid chromatographic (HPLC) assay for MTX using fluorescence detection has been reported [22]; however, this procedure involves oxidation of MTX to a fluorescent species prior to chromatography and does not differentiate 7-OH-

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MTX. Our laboratory first reported an HPLC assay capable of quantitating both MTX and 7-OH-MTX following high dose MTX therapy using a strong anion-exchange column [23]. In the present paper we report a reversed-phase chromatographic procedure with increased sensitivity and markedly improved column life, which is applicable to the measurement of MTX and 7-OH-MTX in plasma samples from patients receiving MTX therapy in both conventional and high dose therapeutic regimens.

EXPERIMENTAL

Non-formulated (USP) and formulated MTX were obtained from Lederle Labs. (Pearl River, N.Y., U.S.A.). The purity of the former was found to be 98.5% and the latter 95% by this HPLC method. 7-OH-MTX was isolated from rabbit liver homogenate as described previously [23] and an authentic specimen was kindly supplied by Dr. David Johns of the National Cancer Institute (Bethesda, Md. U.S.A.). *p*-Aminoacetophenone as the internal standard was obtained from Aldrich (Milwaukee, Wisc., U.S.A.) and was reported >99% pure. All were used without further purification. All other chemicals were of analytical reagent grade and all solvents were HPLC quality grade. Distilled water was purified by passing it through a reverse osmosis four filter system (Millipore, Bedford, Mass., U.S.A.). Standard solutions of MTX and 7-OH-MTX were prepared in purified water at concentrations of 1 mg/ml. Internal standard solutions were prepared in methanol at a 1.0 mg/ml concentration. All standards were refrigerated at 4° and have been found to be stable over several months.

Serum samples were either from patients receiving MTX therapy under protocol or were normal human sera spiked with MTX and/or 7-OH-MTX. The analytical procedure involved the addition of 25 μl of a 0.01 mg/ml solution of *p*-aminoacetophenone as the internal standard and 1.5 ml of 1.0 *N* $HClO_4$ to a 1.0-ml serum standard or patient sample. The denatured protein was separated by centrifugation at 1650 *g* for 10 min and to the clear supernatant was added 5 g of solid $(NH_4)_2SO_4$ and 2.0 ml of ethylacetate—isopropanol (10:1). This was shaken for 20 min on a table top shaker at high speed and the organic layer was transferred to a small disposable tube (12 × 75 mm) and evaporated in a 60° sand bath under a stream of nitrogen. The residue was reconstituted in 100 μl of 0.005 *M* K_2HPO_4, vortexed and 10—50 μl injected into the chromatograph. Standard curves were generated over the range 0.1—8.0 μg/ml ($2.2 \cdot 10^{-7}$—$1.8 \cdot 10^{-5}$ *M*). MTX concentrations were determined by calculating peak height ratios of drug:internal standard. Generally 7-OH-MTX was calculated similarly, in terms of MTX equivalents due to the scarcity of pure standard of the metabolite.

A Waters Assoc. ALC Model 202 liquid chromatograph (Milford, Mass., U.S.A.) equipped with a U6K injector and a Model 440 ultraviolet detector fitted with a 313 nm filter was used for the analyses. Chromatography was performed on a 25 cm × 4.1 mm I.D. stainless steel column packed with either RP-8, 10 μm particle size (Altex, Costa Mesa, Calif. U.S.A.) or RP-8, 7 μm particle size (Knauer, Western Analytical Products, Yorba Linda, Calif., U.S.A.). The mobile phase consisted of 0.1 *M* phosphate buffer (pH 6.8)—

methanol (85:15) at a flow-rate of 1.5 ml/min and a pressure of 1500 p.s.i. The separation was run at ambient temperature.

RESULTS AND DISCUSSION

Typical chromatograms for a patient sample and a serum blank are shown in Fig. 1. Retention times for MTX, 7-OH-MTX and internal standard were 6.6, 7.4 and 8.8 min, respectively under the analytical conditions described using the 10-μm RP-8 column. Peak shape was generally symmetical and peak height ratios were used to calculate MTX and 7-OH-MTX concentrations. Standard curves from spiked pocled serum were linear in the range of 0.1—8 μg/ml ($2.2 \cdot 10^{-7}$ *M*—$1.86 \cdot 10^{-5}$ *M*) for MTX and 7-OH-MTX. The lower limit of sensitivity from a 1.0-ml specimen with a 25-μl injection volume was 50 ng/ml ($1.1 \cdot 10^{-7}$ *M*) on the 10-μm column. Recovery for MTX was 46% and for 7-OH-MTX was 26% based upon injection of unextracted standards. Typical variation in the duplicate analysis of specimens run from the same standard curve was found to be of the order 5—10% and the relative standard deviation of a 2.0 μg/ml spiked MTX plasma sample run repeatedly was 7.4%, which was typical of the between-run variation observed over this concentration range.

The present method was compared to the HPLC strong anion-exchange method published previously [23] by analyzing duplicate patient samples by both procedures using independently prepared standard curves. In general the agreement was quite good for both MTX and 7-OH-MTX as illustrated by the representative comparison for 5 patient specimens in Table I. The present procedure using a 10-μm column is about 2—3-fold more sensitive than the anion-exchange procedure due mainly to improved peak shape and resolution from baseline components. The utilization of the 7-μm average particle diameter RP-8 column further improved peak shape and resolution and resulted in a minimum detectable MTX concentration of 15 ng/ml ($3.3 \cdot 10^{-8}$ *M*). The retention times were slightly longer under these conditions. This increment of improved sensitivity also makes it possible to detect serum MTX and 7-OH-MTX levels following both high dose and conventional dose therapy.

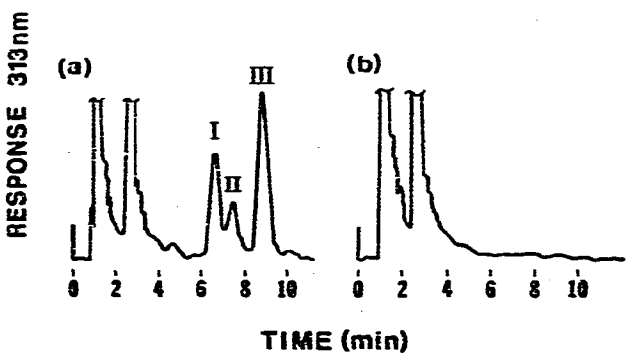

Fig. 1. HPLC chromatograms from (a) a patient receiving high dose MTX intravenous infusion over 6 h and (b) individual patient plasma blanks at 0.01 a.u.f.s. Column was 10 μm RP-8 and conditions are described in the text. I = MTX; II = 7-OH-MTX; III = internal standard.

TABLE I

COMPARISON OF REVERSED-PHASE (RP-8) AND STRONG ANION-EXCHANGE (SAX) HPLC ANALYSIS OF PATIENT METHOTREXATE SAMPLES*

Doses ranged from 2—8 g/m² body surface area administered as a 6-h continuous intravenous infusion.

Patient	MTX concentration (*M*)		7-OH-MTX concentration (*M*)	
	RP-8	SAX	RP-8	SAX
1	$7.0 \cdot 10^{-6}$	$9.8 \cdot 10^{-6}$	$3.9 \cdot 10^{-6}$	$6.6 \cdot 10^{-6}$
2	$3.0 \cdot 10^{-5}$	$3.2 \cdot 10^{-5}$	ND**	ND**
3	$7.6 \cdot 10^{-7}$	$6.8 \cdot 10^{-7}$	$1.5 \cdot 10^{-5}$	$1.2 \cdot 10^{-6}$
4	$2.0 \cdot 10^{-7}$	$4.0 \cdot 10^{-7}$	$9.0 \cdot 10^{-8}$	ND**
5	$2.6 \cdot 10^{-7}$	$3.0 \cdot 10^{-7}$	$6.7 \cdot 10^{-6}$	$8.8 \cdot 10^{-6}$

*See ref. 23.

**ND represents concentrations less than $1.0 \cdot 10^{-7}$ *M* for the SAX method and $7.5 \cdot 10^{-8}$ *M* for the RP-8 method.

In addition to improved sensitivity, the reversed-phase procedure appears to be more reproducible and durable than the ion-exchange procedure over time. The anion-exchange column utilized in the previous assay [23] did not withstand the required pH 7.4 buffer mobile phase over long periods of time and an increased pressure and decreased resolution was noted after 100—400 injections on most columns. Attempted clean-up or separation procedures did not reverse this and a costly column inventory was required. Under the described analytical conditions, the present reversed-phase columns appear stable and remain functional after more than 2000 injections with no appreciable loss of resolution or increase in pressure. In addition, the presence of perchlorate as an ion pair with MTX and 7-OH-MTX, and the difficulty associated with any clean-up extraction steps with amphoteric molecules like the folates, makes the use of the more stable reversed-phase system much more appealing than the less stable ion-exchange systems.

Due to the scarcity of authentic 7-OH-MTX, metabolite levels were generally expressed from a MTX standard curve in terms of MTX equivalents. In one series of samples a 7-OH-MTX standard curve was run and the resultant 7-OH-MTX levels were compared to those calculated from the MTX standard curves. The results indicated that actual 7-OH-MTX levels were four times greater. Generally 7-OH-MTX levels were insignificant at early times (up to 10 h) then increased and were equal to or greater than those of MTX after 24 h. The plasma time course of drug and metabolite for a typical patient is illustrated in Fig. 2. Due to the probable higher levels as described above, it is clear that 7-OH-MTX may be as important or more important in the clinical response and toxicity seen in high dose therapy. These data coupled with the recent suggestion that 7-OH-MTX may be implicated in renal toxicity in the rhesus monkey [12] suggests that the plasma levels of both MTX and 7-OH-MTX be monitored in managing high dose MTX patients. The sensitivity of the present procedure is adequate for the measurement of MTX serum levels with most protocols [10, 12, 24] requiring monitoring until the MTX level drops below

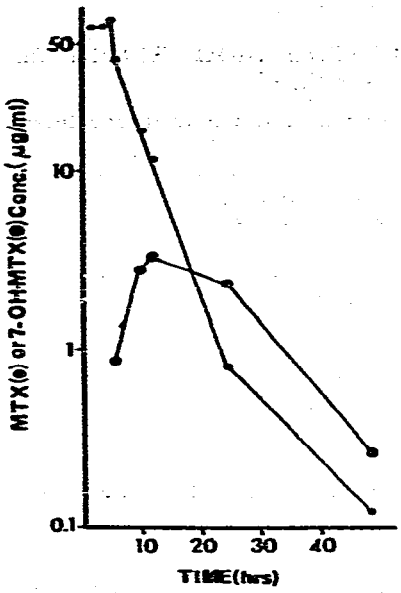

Fig. 2. Plasma time course for MTX (●) and 7-OH-MTX (⊖) in a typical patient receiving 2.5 g MTX by intravenous infusion over 4 h.

$1 \cdot 10^{-7}$ *M*. In addition, while many of the previously published non-chromatographic methods are suitable for detection of parent MTX, none of these methods allow direct measurement of metabolite levels.

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