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Note

Rapid and simple method for the measurement of methotrexate and 7-hydroxymethotrexate in serum by high-performance liquid chromatography

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Methotrexate (MTX, amethopterin, 4-amino-N^{10}-methylpteroylglutamic acid), a folate antagonist which acts by inhibition of dihydrofolate reductase, is used as an anti-neoplastic agent alone or in combination with other anti-neoplastic drugs. Toxic reactions have become more likely with the recent introduction of high dose MTX therapy [1], one study [2] quoting a 6% mortality rate using this regimen. Many of the side-effects are due to delayed excretion of MTX and adverse reactions depend upon the duration of exposure to, as well as the magnitude of, excessive MTX concentrations [3]. The monitoring of serum MTX concentrations is essential to detect those patients at risk of developing toxicity and allow appropriate action to be taken [4]. Measurement of the less soluble major metabolite 7-hydroxymethotrexate (7-OH MTX) may also be important as it has been implicated in the development of renal toxicity [5].

Methods for measuring MTX concentrations have been reviewed [6]. Techniques using high-performance liquid chromatography (HPLC) include ion-exchange [7—10] and reversed-phase chromatography after prior extraction and concentration [11, 12] or pre-column derivatisation [13]. Some of these also measure the major metabolite 7-OH MTX [8—12]. This paper

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describes a reversed-phase technique which allows direct injection of supernatant after protein precipitation.

MATERIALS AND METHODS

Equipment

An Applied Chromatography Systems reciprocating pump was used with a Cecil CE 212 variable-wavelength UV monitor fitted with an 8-μl chromatography cell. A 120 mm × 4 mm I.D. stainless-steel analytical column and a 45 mm × 4 mm I.D. pre-column were packed with the 5-μm particle size stationary phase Hypersil—octadecylsilane (Shandon, London, Great Britain), using a slurry technique. Injections were performed with a Specac loop injector, fitted with a 50-μl loop.

Reagents

Methanol (HPLC grade) was obtained from Rathburn Chemicals (Walkerburn, Great Britain). The perchloric acid (PCA) used was 2 *M* in distilled water.

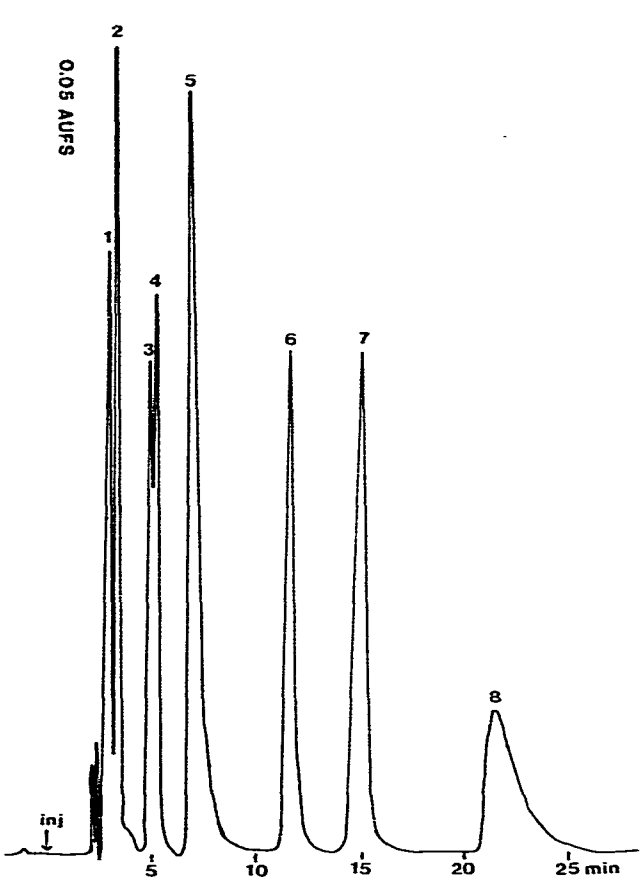
Non-formulated (USP) methotrexate (94.1% nominal purity) and analogues were obtained from Lederle Labs. (Hampshire, Great Britain). Stock standards of 1 mg/ml in 0.1 *M* sodium hydroxide were prepared (stored at 4°C and found to be stable over several months). Subsequent dilutions for working standards were prepared in either mobile phase buffer or pooled serum. 7-OH MTX (90% pure) was kindly donated by Dr. D.G. Johns (N.C.I., Bethesda, MD, U.S.A.). The internal standard (I.S.), N-[4[[(2,4-diamino-6-quinazolinyl) methylamino]benzoyl]] aspartic acid, was a gift from Dr. L.H. Kedda (N.C.I.), and was used at a concentration of 10 μg/ml in 2 *M* perchloric acid. All other chemicals were of AnalaR grade.

Procedure

Serum (500 μl) and 300 μl I.S. (10 μg/ml in 2 *M* PCA) were vortex mixed and centrifuged for approximately 5 min at 2000 rpm (approximately 1300 *g*). A 50-μl aliquot of the supernatant was injected onto the column. The eluting solvent was 20% methanol in Tris—sodium dihydrogen phosphate (both 0.1 *M*), pH 6.7, at a flow-rate of 1 ml/min, corresponding to a pressure drop of approximately 75 bars. Chromatography was performed at ambient temperature with detection at 305 nm. The ratios of peak heights of MTX and 7-OH MTX to I.S. in chromatograms from serum samples were compared with those obtained from a working standard. Results obtained with HPLC were compared with those obtained by radioimmunoassay (RIA) [14] using ^{125}I-labelled MTX [15].

RESULTS

Fig. 1 shows the separation of MTX, 7-OH MTX, I.S. and five related compounds chromatographed using this sytem. Chromatography of serum taken from a patient 4 h after a 6-h infusion of 3.3 g MTX is shown in Fig. 2. A blank sample of pooled serum showed no significant interference (Fig. 3).



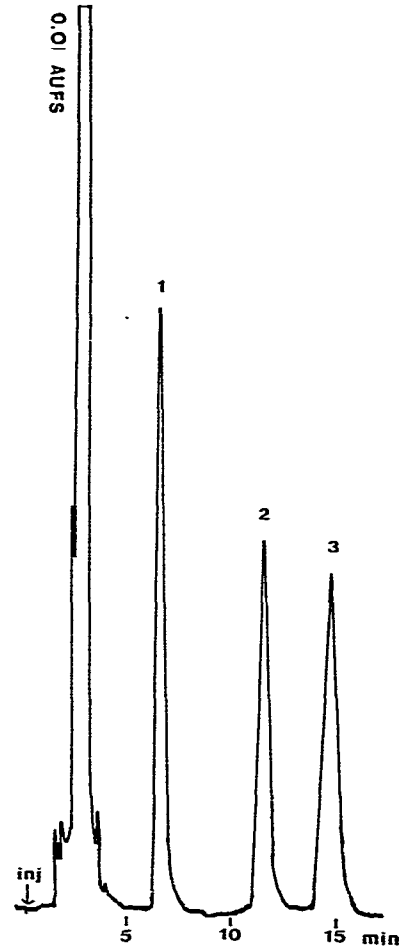


Fig. 1. Separation of (1) folinic acid, (2) folic acid, (3) aminopterin, (4) N^{10}-methylfolic acid, (5) internal standard, (6) MTX, (7) 7-OH MTX, (8) 2,4-diamino-N^{10}-methylpteroic acid. Conditions as in text.

Fig. 2. Chromatogram of sample from patient 4 h after termination of a 6-h infusion containing 3.3 g MTX. (1) Internal standard; (2) MTX; (3) 7-OH MTX.

The recoveries of MTX, I.S. and 7-OH MTX from pooled serum estimated by comparison with external standards were 82.2%, 87.5% and 98.2%, respectively. Standard dilutions of MTX and 7-OH MTX in Tris—phosphate buffer and pooled serum were assayed in duplicate over the range 0.5—20 μg/ml ($1.1 \cdot 10^{-6}$—$4.4 \cdot 10^{-5}$ *M*). The standard curves obtained were linear. MTX and 7-OH MTX were added to serum at two different levels and assayed five times during a single run, and on five different days. The within- and the between-batch variations are shown in Table I.

Table II lists those drugs that might commonly be administered with MTX.

TABLE I

PRECISION DATA

Concentration (μg/ml)		Coefficient of variation (n = 5)	
		Within-batch (%)	Between-batch (%)
MTX	1	3.4	10.3
	10	1.9	7.3
7-OH MTX	1	3.2	12.6
	10	2.9	8.6

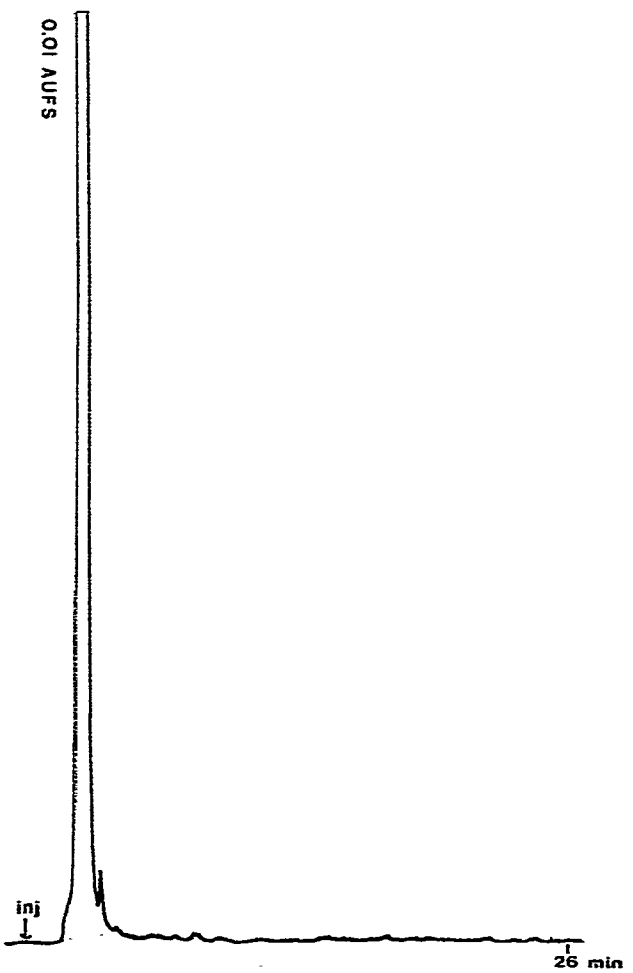


Fig. 3. Chromatogram of blank sample from patient not receiving therapy.

TABLE II

DRUGS TESTED FOR CHROMATOGRAPHIC INTERFERENCE

Caffeine	Imipramine
Salicylate	Desipramine
Cortisol	Trimipramine
Prednisone	Diazepam
Prednisolone	Nitrazepam
Dexamethasone	Chlordiazepoxide
Morphine	Phenobarbitone
Cytosine arabinoside	Hydrallazine
Vincristine	Folinic acid
Daunomycin	

Only cytosine arabinoside produced an interfering peak ($k' = 4.8$, retention relative to MTX = 1.12). The others were either retained indefinitely or not at all.

The lowest measurable levels of MTX and 7-OH MTX were considered to be 100 ng/ml ($2.2 \cdot 10^{-7} M$) and 200 ng/ml ($4.2 \cdot 10^{-7} M$) respectively at which concentrations the peak heights were five and three times the noise level of the system respectively.

Fifty-two samples were obtained from patients during the 48 h following high dose MTX (approximately 3 g per square metre body surface area infused in 6 h). The samples were analysed by HPLC and RIA for MTX and 32 of the samples were analysed for 7-OH MTX by HPLC. The concentrations of MTX measured by HPLC ranged from 0.1—216 μg/ml ($0.2—476 \cdot 10^{-6} M$) and correlated well with the results obtained by RIA ($r = 0.99$, $y = 0.77x + 0.99$, where x and y are RIA and HPLC values respectively measured in μg/ml). RIA gave overall higher values ($p < 0.01$, Wilcoxon Matched-pairs Signed-Ranks test [16]).

7-OH MTX concentrations ranged between 0.2—56 μg/ml ($0.4—119 \cdot 10^{-6} M$). Up to 2 h post infusion the 7-OH MTX concentrations were always less than the MTX concentrations but after 4 h post infusion 7-OH MTX always exceeded MTX, being up to ten times greater at 24 h post infusion. Serial samples from four patients showed that the maximum concentration of 7-OH MTX occurred 2 h post infusion and was 35—56 μg/ml.

DISCUSSION

This reversed-phase system gives good baseline resolution of MTX, 7-OH MTX and I.S.; a similar reversed-phase system using 10-μm RP-8 [13] failed to achieve this baseline separation although the authors did not illustrate the improved performance claimed for 7-μm particles. The method has a high degree of specificity since these compounds are well separated from endogenous compounds, N^{10}-methylfolic acid and aminopterin (contaminants of commercial MTX preparations and likely to be measurable after high dose infusions), folinic acid (citrovorum factor, used as antidote in high dose regimens) and the minor metabolite 2,4-diamino-N^{10}-methylpteroic acid (DAMPA).

Of nineteen drugs likely to be administered simultaneously, only cytosine arabinoside interfered, although its presence would be detected by a shoulder on the MTX peak; however when co-administered its much shorter half-life and lower dosage diminish the likelihood of significant interference.

There was a close correlation between results for MTX obtained using HPLC and RIA although the latter gave overall higher values; this was not due to a lower recovery as the use of the I.S. compensates for this but suggests that RIA measures some substance not present in the chromatography peak. Discrepancy between the RIA and competitive dihydrofolate reductase binding assay has been reported [17], and this may be due to the nominal 30% cross-reaction of the antibody with DAMPA. Further studies are in progress using HPLC to determine the effects of DAMPA on RIA.

Sample preparation is simple and rapid and gives a high recovery of MTX, 7-OH MTX and I.S.; a batch of samples can be prepared for chromatography in about 10 min and a single sample can be chromatographed in about 20 min. The sensitivity of the assay is 100 ng/ml ($2.2 \cdot 10^{-7} M$) for MTX which enables toxic levels to be measured up to 48 h post infusion.

Methods using an organic solvent extraction step [8, 10—12] are considerably more time-consuming and have only a modestly increased sensitivity, their overall recovery being often less than 50%. Methods offering about a tenfold increase in sensitivity use either pre-column fluorescence derivatisation [13] or an on-line column concentration step [7, 9].

Later than 4 h post infusion, 7-OH MTX concentrations are greater than those of MTX and 24 h post infusion we have found them up to ten times higher, similar to those reported by Lankelma and Van der Klein [9]. Cohen et al. [12] state that their 7-OH MTX concentrations measured in MTX equivalents due to lack of an authentic standard were probably only about one quarter of the "true" concentrations, and if their MTX/7-OH MTX time course relationship is corrected accordingly it agrees well with our findings. Measurement of 7-OH MTX may be clinically important due to its probable effect upon renal function [5] and because it competes with MTX for entry into cells thereby decreasing the therapeutic efficiency of MTX [9].

This method would be particularly useful in a department where the workload is small but the results are required quickly. It is simple to operate and cheap to run.

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