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An assay for methotrexate and its metabolites in serum and urine by ion-pair high-performance liquid chromatography

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Abstract: A high-performance liquid chromatographic assay for methotrexate and its metabolites, 7-hydroxy-methotrexate, 4-amino-4-deoxy-N^{10}-methyl-pteroic acid and 7-hydroxy-4-amino-4-deoxy-N^{10}-methyl-pteroic acid in the range 10 μg/l to 50 mg/l (2.2×10^{-8} to $1.1. \times 10^{-4}$M) has been developed using L-tryptophyl-L-glutamic acid as internal standard. Extraction was performed using an anion exchange resin (Dowex 1-X2) with subsequent ion-pair chromatography of the appropriate eluent fraction. The method has been found to be sensitive and precise for the analysis of both serum and urine, and may also be used for the quantitation of polyglutamyl metabolites.

Keywords: *Ion-pair chromatography; methotrexate; metabolites; homogeneous enzyme immunoassay; therapeutic drug monitoring.*

Introduction

Methotrexate (MTX)† (Fig. 1) has found wide application in oncology, since its first clinical trial in 1953 [1].

Currently, 'high-dose' MTX up to 200 mg/kg [2] is being used in the treatment of osteogenic sarcoma [3], acute lymphocytic leukemia [4], cancer of the head and neck [5] and other malignancies [1]. However, high dose MTX therapy is accompanied by acute folate stress, which may be life threatening [5]. The toxic effects include bone marrow depression [6], hepatotoxicity [7], nephrotoxicity [8], pulmonary complications [9] and neurotoxicity [10]. Citrovorum factor (folinic acid) is used to protect the patient from these effects [11].

The major metabolite of MTX in man is 7-hydroxymethotrexate (7-OHMTX) [12]. This is much less water-soluble than the parent compound and may cause renal damage due to crystallization in the tubules [12]. MTX may also be converted to 4-amino-4-deoxy-N^{10}-methyl-pteroic acid (DAMPA) following cleavage of the pteroate–glutamate peptide bond by the carboxypeptidases of some intestinal bacteria [13]. A further metabolite, 7-hydroxy-DAMPA (7-OH DAMPA), may also be found.

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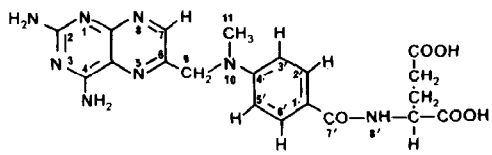
† Non-standard abbreviations used in text: MTX, methotrexate; 7-OHMTX, 7-hydroxymethotrexate; DAMPA, deoxyaminomethyl-pteroic acid (4-amino-4-deoxy-N^{10}-methyl-pteroic acid); 7-OHDAMPA, 7-hydroxydeoxyaminomethyl-pteroic acid.

Figure 1
Structure of methotrexate and metabolites.

Compound	Position 7	7′
Methotrexate (MTX)	H	CO-NHR
7-hydroxymethotrexate (7-OHMTX)	OH	CO-NHR
4-amino-4-deoxy-N^{10}-methyl-pteroic acid (DAMPA)	H	COOH
7-hydroxy-4-amino-4-deoxy-N^{10}-methyl-pteroic acid (7-OHDAMPA)	OH	COOH



Following passage into cells, methotrexate is converted to a series of polyglutamates by the addition of glutamyl residues. These polyglutamates may be retained within the tissues for up to three months, before being degraded by pteroylglutamyl-γ-glutamyl carboxypeptidases and excreted by both the intestinal and renal routes [14].

A knowledge of MTX concentration in serum and urine can assist in avoiding toxicity. Several types of assay are currently in use, including fluorimetry [15], radioimmunoassay [16] and enzyme multiplied immunoassay (EMIT) [17]. Interest in the pharmacological and oncological role of the metabolites is growing, for which separation by high-performance liquid chromatography (HPLC) is the most appropriate. Earlier HPLC methods [18–20] used either trichloracetic acid or perchloric acid as protein precipitants, although poor recovery and lack of sensitivity have been observed by the present authors using this approach.

We have therefore investigated and modified the extraction procedure of Donehower *el al.* [13]. In conjunction with a separation based on reversed-phase ion-pair HPLC, a rapid, sensitive and precise assay for the estimation of serum MTX and its metabolites has been developed.

Materials and Methods

MTX, DAMPA and calcium leucovorin were donated by Lederle (Gosport, Hants, UK). Di- and triglutamates were kindly provided by Professor C. M. Baugh (University of S. Alabama, USA). The anion exchange resin Dowex 1-X2 was obtained from Bio-Rad Laboratories (Watford, Herts, UK). Hexanesulphonic acid was HPLC grade (Fisons Scientific Apparatus, Loughborough, Leics, UK). L-Tryptophyl-L-glutamic acid, used as internal standard, was obtained from the Sigma Chemical Co. (Poole, Dorset, UK). The 7-OHMTX was a gift from Dr David G. Johns (National Cancer Institute, Bethesda, MD, USA). The 7-OHDAMPA was synthesized *in vitro* by a procedure previously described for 7-OHMTX [21] and the identity confirmed by nuclear magnetic resonance and mass spectrometric analysis (Farid *et al.*, in preparation). Methanol was obtained from James Burrough (London); all other reagents were obtained from British Drug Houses (Poole, Dorset, UK). MTX analysis by EMIT was carried out following the manufacturer's protocol (Syva, London, UK).

Apparatus

A Pye-Unicam HPLC system (Cambridge, UK) consisting of an LC-HPS single piston

reciprocating pump, a LC-UV variable wavelength detector and a PM 8251 single-pen recorder were used. The column (100 × 5 mm ID) was obtained from Shandon Southern (Runcorn, UK) and packed in our own laboratory with 5 μm ODS-Hypersil using a Shandon slurry packer and fitted with a valve adaptor.

An 8050 autosampler (Varian Associates, Walton-on-Thames, UK) with a 20 μl loop was used for urine injection according to the manufacturer's instructions. Because of the limited amount of analyte available from serum extracts and the sample line dead volume, the operation of the autosampler was modified in order to reduce the dilution factor, by fitting a small gas pressure gauge and adjusting the outlet pressure to 50 N/m^2. Thus it was possible to inject 20 μl of sample from a 100 μl aliquot. A wash vial between each sample reduced carry-over to less than 2%. Manual injection when required was made using a Rheodyne 7125 injection valve (Scotlab Instrument Sales Ltd, Scotland). The data generated was handled by an Infotronic CRS 304–40 integrator (Belmont Instruments Ltd, Glasgow, UK). A Stasar spectrophotometer (Syva, London, UK) was used for EMIT assays.

HPLC conditions

The mobile phase consisted of 0.1% v/v hexanesulphonic acid in 50 mM phosphoric acid–methanol (72:28 v/v), the operating conditions being: ambient temperature; flow rate 1 ml/min, detector wavelength 307 nm; chart speed 0.5 cm/min; absorbance range 0.16 a.u.f.s. for urine samples, or as shown in Table 1 for the serum extracts. Samples were automatically injected every 30 min after a 1–min flush cycle.

Table 1
Conditions for assay of methotrexate at various concentrations

MTX concentration range (μg/ml)	(M)	Stock IS* (mg/ml)	Detector a.u.f.s.
0–0.09	$0–2 \times 10^{-7}$	0.25	0.04
0.09–0.9	$2 \times 10^{-7}–2 \times 10^{-6}$	1	0.04
0.9–50	$2 \times 10^{-6}–1.1 \times 10^{-4}$	5	0.16

* Internal standard: L-tryptophyl-L-glutamic acid.

Procedure

Serum. A 100 μl aliquot of internal standard (as in Table 1) was added to 1 ml serum and the pH adjusted to 8 with 50 μl 0.02 M potassium carbonate solution. The sample was then applied to an ion-exchange column of Dowex 1-X2 anion exchange resin, previously mixed with deionized water for 5 min and packed in a Pasteur pipette to a height of 2 cm. Columns were first washed with 10 ml deionized water followed by 4 ml methanol. The methotrexate and metabolites were eluted using 2 ml of 5% v/v acetic acid in methanol, followed by 4 ml of 25% v/v acetic acid in methanol. The combined fractions were evaporated to dryness under vacuum at 35°C. The residue was dissolved in 100 μl of HPLC mobile phase, mixed by vortex mixer for 15 sec, centrifuged for 1 min and 20 μl injected onto the column using the autosampler. For low concentrations of MTX the residue was dissolved in 25 μl HPLC solvent and 20 μl of this was injected manually on the column.

Urine. Direct injection of urine (20 μl) yielded satisfactory chromatography of MTX and its metabolites.

Quantitation

The concentrations of methotrexate and metabolites in sera were calculated by the peak area ratio of analyte to internal standard and comparison with the standard curves. The urine concentrations were determined by using an external standardization procedure as previously described, [22] using a target standard of 50 mg/l (1.1×10^{-4}M).

Application

The kinetics of MTX and 7-OHMTX were studied in a patient with lymphoma following a continuous infusion of 375 mg/hr of MTX over a period of 24 hr. Serum samples were obtained at 1.7, 2, 2.5, 4, 5, 6, 13 and 24 hr after administration. Urine was collected at 2-hourly intervals for the first 22 hr and then every 6 hr for the next 18 hr. The elimination half-life was derived from a plot of logarithmic serum concentration against time.

Results

Chromatography

The capacity ratios for leucovorin, the internal standard, MTX, 7-OHMTX, DAMPA and 7-OHDAMPA were 1.3, 5.4, 8.3, 10.4, 21.1 and 24.9 respectively. The diglutamate and triglutamate metabolites had capacity ratios of 6.8 and 6.3 (Fig. 2).

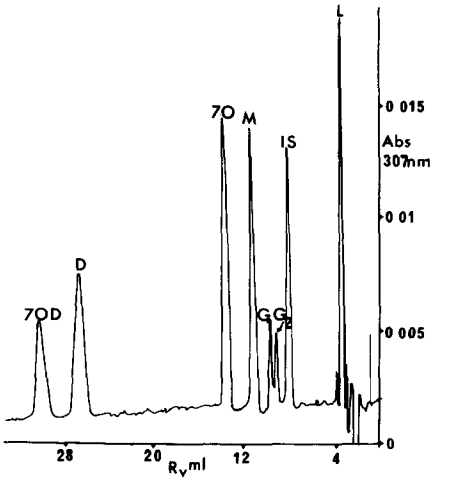


Figure 2
Chromatogram of a standard injection of MTX and its metabolites with L-tryptophyl-L-glutamic acid as internal standard. 1, Leucovorin (L); 2, L-tryptophyl-L-glutamic acid (internal standard, IS); 3, triglutamyl MTX (G2); 4, diglutamyl MTX (G1); 5, MTX (M); 6, 7-OHMTX (7O); 7, DAMPA (D); 8, 7-OHDAMPA (7OD).

Analytical recovery

Absolute recoveries of MTX, 7-OHMTX and DAMPA from serum are detailed in Table 2. Recovery relative to L-tryptophyl-L-glutamic acid as internal standard, calculated by the ratio of peak areas, was found to be 96.1 ± 3.8% (n = 16) for MTX.

Linearity and sensitivity

Serum and urine. The response to MTX was linear over the range 0–10 μg on-column sample weight for extracts using the internal standard or for directly injected aqueous

Table 2
Absolute recovery of methotrexate and its metabolites 7-OHMTX and DAMPA from serum ($n = 16$)

Compound	Absolute recovery (%)		
	X	± S.D.	RSD (%)
MTX	94.5	4.8	5.1
7-OHMTX	72.4	5.8	8.1
DAMPA	89.5	5.8	6.4

standards, this was equivalent to a linear range of 0–50 mg/l (0–1.1 × 10^{-4}M) and 0–500 mg/l (0–1.1 × 10^{-2}M) respectively.

Sensitivity

The lowest detectable concentration, defined as twice the signal-to-noise ratio, was 12.5 μg/l (2.75 × 10^{-8}M) for serum or 500 μg/l (1.1 × 10^{-6}M) for urine MTX (equivalent to 10 ng on-column).

Precision

MTX was measured at specified levels in serum, while both MTX and metabolites were measured repetitively in urine. The precision studies are summarized in Table 3.

Table 3
Precision studies on methotrexate in serum and on methotrexate and its metabolites in urine ($n = 16$)

Compound	Matrix	Concentration		CV (%)
		Mass units	Molar units	
MTX	Serum	162 μg/l	3.56 × 10^{-7}M	4.2
MTX	Serum	9.6 mg/l	2.1 × 10^{-6}M	4.1
MTX	Serum	50 mg/l	1.1 × 10^{-5}M	5.1
MTX	Urine	42.4 mg/l	9.3 × 10^{-5}M	2.6
7-OHMTX	Urine	15.8 mg/l	3.35 × 10^{-5}M	5.6
DAMPA	Urine	71.1 mg/l	2.10 × 10^{-4}M	2.7
7-OHDAMPA	Urine	6.68 mg/l	1.98 × 10^{-5}M	6.4

Accuracy

Serum. The possibility of interference by a number of drugs, especially anti-cancer drugs, was examined by the addition of aqueous solution to the same pooled serum used for the preparation of MTX solutions. Drugs studied were: cyclophosphamide, dacarbazine, fluorouracil, vincristine, daunorubicin, adriamycin, cis-platinum, cytarabine, trimethoprim, paracetamol and diazepam. The last two were examined because they are frequently co-prescribed with methotrexate in this hospital. Trimethoprim was studied in view of its reported interference with other methods for determining MTX

[23]. None of the drugs examined interfered with the assay of MTX and its metabolites. Over 200 MTX-free sera have been assayed with no evidence of interference.

Serum MTX concentrations were determined by EMIT and compared with the values obtained by HPLC. A less biased correlation is found between serum MTX plus 7-OHMTX concentrations than for MTX alone (Fig. 3).

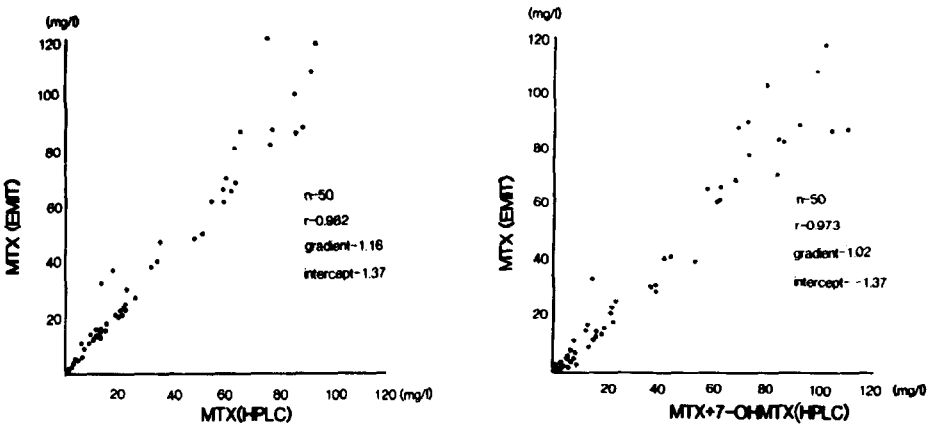


Figure 3
Correlation of serum EMIT MTX concentrations with: (i) serum MTX measured by HPLC; and (ii) the sum of serum MTX and 7-OHMTX measured by HPLC.

Urine. Although no extraction was used, no interference has been observed in over 200 MTX-free urine samples.

Measurement in biological fluids

The chromatogram obtained from an extract of serum from a patient treated with MTX is shown in Fig. 4a. An extract of serum from a subject taking no known medication is shown in Fig. 4b. The chromatograms of the corresponding urine samples are shown in Figs. 5a and 5b respectively.

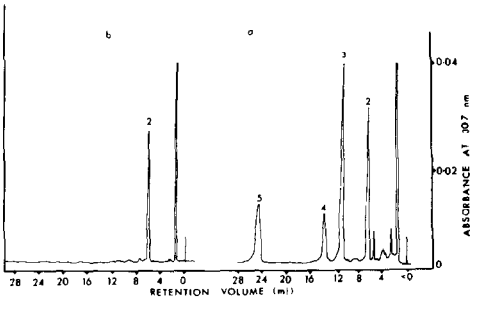


Figure 4
Chromatogram of serum extract from a subject after MTX therapy: (a) 1 g i.v. bolus followed by 2 g over 24 hr; (b) drug-free subject. Peak identities: (1) IS, (3) MTX, (4) 7OHMTX, (5) DAMPA.

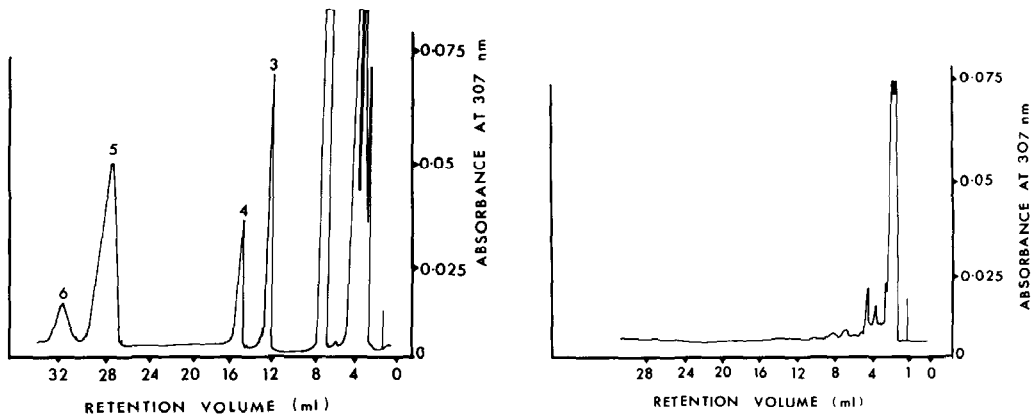


Figure 5
Chromatogram of 20 μl of urine direct injection from: (a) subject after MTX therapy (as above); and (b) urine from drug-free subject. Peak identities as in Fig. 4. (6) 7OHDAMPA.

Application

The elimination of MTX and the increase and decline of 7-OHMTX following i.v. dosage with MTX is shown in Fig. 6. The calculated elimination half-life ($t_{1/2}$) of MTX was 2.6 hr, while for 7-OHMTX it was 21.6 hr. As would be expected from the half-life, the 7-OHMTX concentration 24 hr after the dose is greater than that of MTX. The MTX and 7-OHMTX concentrations in urine are shown in Fig. 7. There is a consistent decline in MTX concentrations, whereas the appearance of 7-OHMTX is rapid, although its concentration fluctuated from collection to collection.

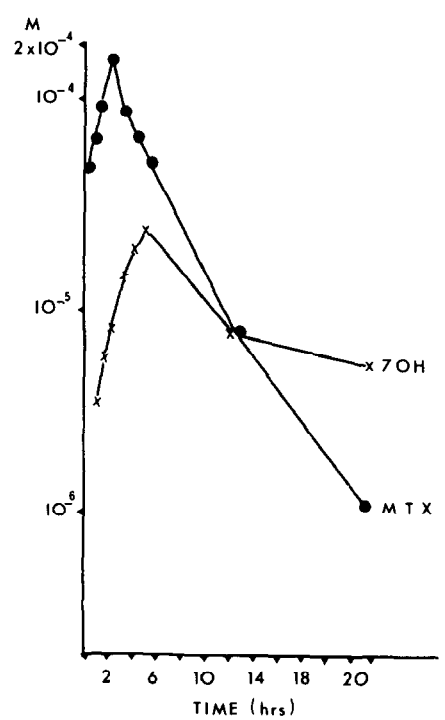


Figure 6
Serum concentrations of MTX and 7-OHMTX following a dose of 9 g MTX (i.v.) over 24 hr.

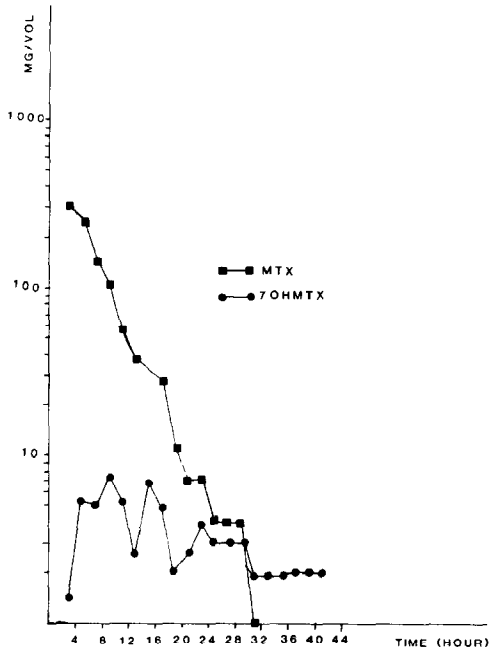


Figure 7
Urine concentrations of MTX and 7-OHMTX following a dose of 9 g MTX (i.v.) over 24 hr.

Discussion

The extraction procedure is a modification of an earlier method [13] which did not report the absolute recovery of 7-OHMTX. The adjustment of the serum to pH 8 was necessary to ensure that MTX and its metabolites would effectively interact with the ion-exchange resin; failure to perform this step led to reduced recoveries of 7-OHMTX. The methanol wash yielded cleaner blank samples than the water wash of Donehower *et al.* An acid eluent was required to elute MTX and its metabolites. Methanol–acetic acid was used since it formed an azeotropic mixture, with a consequent improvement in the speed of the evaporation step.

Any HPLC method which requires an extraction step is slower than a one-step assay such as an optical immunoassay. However, for pharmacokinetic studies it is necessary to measure the unchanged drug and also its major metabolites in plasma and urine. It has been reported that 7-OHMTX cross-reacts with the immunoassay to an extent of about 10% [18]. This is borne out in the present work. Since 7-OHMTX concentration may be higher than MTX concentration when serum levels are assessed, this is a potential source of error.

The accuracy and sensitivity of the HPLC assay make this technique preferable to the immunoassay procedure at low concentration. The immunoassay procedure may be preferred where rapid sample turn-round is required during high-dose MTX therapy. The procedure we describe is sensitive and linear over a wide range. It compares well with optical immunoassay, which is an order of magnitude less sensitive and linear over a much narrower range. The HPLC method of Lawson *et al.* [20] is also an order of magnitude less sensitive than the proposed procedure. The internal standard L-tryptophyl-L-glutamic acid exhibited similar physiochemical behaviour to MTX in the analytical system and was well resolved from MTX and its metabolites.

The elimination half-life reported for MTX is 2.1 hr [24] and for 7-OHMTX it is 28 hr

[25] in agreement with the present results. These results confirm the observation [25] that significant 7-OHMTX levels are attained in high-dose MTX therapy, exceeding MTX concentrations from about 12 hr after dosage. It may be that the persistence of 7-OHMTX could be of significance in the continuation of side-effects, particularly renal damage.

The proposed method provides a simple, reliable extraction procedure for the rapid determination of MTX, 7-OHMTX, DAMPA and 7-OHDAMPA in serum and in urine by HPLC. It is applicable to the study of the kinetics of MTX and metabolites following either high- or low-dose therapy. In view of the ability of the assay procedure to provide a quantitative measurement of the di- and triglutamated metabolites, longer term studies on the kinetics of these metabolites are being pursued.

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