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High-performance liquid chromatography method for serum methotrexate levels in children with severe steroid-dependent asthma

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ABSTRACT

Monitoring of low-dose methotrexate (MTX), as used in severe steroid-dependent asthma, requires a sensitive and reproducible technique which has hitherto not been available. A high-performance liquid chromatographic method for the determination of MTX in serum is reported. The method involves deproteinization with acetone followed by addition of butanol and diethyl ether. The percentage recovery with this method compared with others was high (90 *versus* 70%). The samples were chromatographed on a reversed-phase ODS column and monitored at 313 nm. The retention time for MTX was 14.7 min. Pharmacokinetics of MTX was studied in five patients (age 3–15 years) with severe asthma who received a weekly oral dose of 10 mg/m^2 body surface area. Following administration, the serum disappearance was monophasic with a half-life of 5 h. A metabolite, 7-hydroxymethotrexate was detected in serum after 2 h and reached a maximum concentration after 6 h. This new method will facilitate monitoring of asthmatic patients on methotrexate and allow for dose response and toxicity studies to be conducted.

INTRODUCTION

Methotrexate (MTX) is an antimetabolite with therapeutic value in a variety of clinical conditions. It was first introduced in 1948 by Farber *et al.* [1] for the treatment of acute leukemia. Since then it has been used in low doses in the management of psoriasis and several rheumatic diseases, as a corticosteroid sparing drug [2–11]. MTX is thought to exert its action in these conditions as an anti-inflammatory agent [12–14].

Based on evidence that inflammation plays a significant part in airway hyperresponsiveness which is characteristic of bronchial asthma, Mullarkey and coworkers [15,16] have reported using a low oral dose of MTX in the treatment of steroid-dependent asthma. Their study concluded that low-dose weekly pulses of MTX permitted a significant reduction of steroid usage in asthmatics without deterioration in objective or subjective assessments of pulmonary function.

The choice of dose and dose interval has been made predominantly to avoid toxic side effects rather than with any knowledge of dose–response relationships.

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Clearly, greater understanding of the anti-inflammatory properties of MTX in relation to serum levels is required to rationalise this therapy. With high-dosage regimens of MTX, blood level analysis did not necessitate high sensitivity and several analytical methods have been employed in these situations including fluorometry [17], ultraviolet techniques [18,19], radioimmunoassay [20] and enzyme inhibition assays [21].

High-performance liquid chromatography (HPLC) is the only technique which employs separation steps capable of resolving and quantitating MTX metabolites which offers both selectivity and sensitivity. We have, therefore, developed a sensitive and reliable HPLC method for the quantitation of MTX in serum following low-dose oral administration in children with severe steroid-dependent asthma.

EXPERIMENTAL

Equipment

A Model 114M pump was used for solvent delivery (Beckman Instruments, High Wycombe, U.K.). Samples were injected through a syringe loading 210A sample injector. Chromatography was performed on an Ultrasphere ODS RP18 (5 μm) stainless-steel 250 mm × 4.6 mm I.D. column (Beckman Instruments). Detection was performed using a 160 absorbance detector at a fixed wavelength of 313 nm. Peak integration was carried out using an SP4290 integrator (Spectra Physics, San Jose, CA, U.S.A.).

Mobile phase

The mobile phase was 5% tetrahydrofuran in 0.05 *M* sodium dihydrogen orthophosphate buffer (pH 4.85). After degassing on a vacuum pump for 10 min the mobile phase was pumped through the column at a flow-rate of 0.6 ml/min. The chromatographic separation was carried out at ambient temperature with detection at 313 nm.

Reagents

MTX and aminopterin, used as internal standard, were obtained from Sigma (Poole, U.K.). Both sodium dihydrogen orthophosphate and tetrahydrofuran were obtained from BDH (Poole, U.K.). All solvents used for chromatography were of HPLC-grade purity (Rathburn Chemicals, Walkerburn, U.K.).

Sample pretreatment

Two extraction procedures were studied. The first method (A) consisted of a simple deproteinization step, and the second (B) provided more extensive purification and preconcentration.

Method A. Internal standard (40 μl of 40 mg/l aminopterin) was added to 460 μl of serum. To this solution 500 μl of 0.8 *M* trichloroacetic acid (TCA) were

added. The sample was vortex-mixed for 2 min, quite vigorously, and then centrifuged at 1000 *g* for 10 min. The supernatant was directly injected onto the HPLC column (10 μl).

Method B. (Based on Brimmell and Sams technique [22]). A 25-μl volume of internal standard (40 mg/l aminopterin) was added to 225 μl of serum. Acetone (250 μl) was added and the solution was vortexed for 5 min quite vigorously. The mixture was then centrifuged at 1000 *g* for 10 min. A 300-μl volume of the supernatant was transferred to another tube containing a mixture of butanol (300 μl) and diethyl ether (400 μl). Following mixing and centrifuging as described before, the supernatant was discarded. The lower aqueous portion was directly injected onto the column (10 μl).

Recovery

For recovery assessment, spiked samples which had been put through sample pretreatment at two concentrations (1 and 10 μg/ml) were used. When the sample pretreatment procedure was carried out the supernatants were evaporated or freeze-dried to dryness. The resulting solid was then reconstituted in the mobile phase and its concentration was obtained by absorbance measurements. Consequently the amount of MTX in this layer was calculated as a percentage of total drug.

Reproducibility study

A reproducibility study was carried out at two concentrations for MTX and aminopterin (internal standard). Six replicate analyses of serum samples from healthy volunteers spiked with stock solutions of both compounds to final concentrations of 0.01 and 10 μg/ml were carried out.

Samples

Blood samples were obtained from five patients receiving weekly MTX, in a dosage of 10 mg/m^2 for high-dose oral steroid-dependent asthma. Blood samples were obtained at 0, 1, 2, 3, 4, 5, 6, 8, 12, 16, 20 and 24 h and allowed to clot.

Clotted blood was centrifuged for 10 min at 1200 *g* before analysis. The volume of extracted serum samples injected into the column was 10 μl. Samples which could not be chromatographed immediately were stored frozen at −20°C.

Peaks were identified by using retention times compared with the internal standard. MTX concentrations were determined by peak-area ratios of the analyte to internal standard and comparisons with a standard curve, prepared daily by diluting a stock MTX standard with drug-free serum to give a standard range from 0.01 to 10 μg/ml.

RESULTS

Calibration, linearity and reproducibility

Calibration curves of MTX dissolved in mobile phase were linear (typical correlation coefficient of 0.995 and intercept not significantly different from zero) in the range 0.01–10 μg/ml injected. The detection limits based on a signal-to-noise ratio of 3 was 4 pmol for the 250 mm x 4.6 mm I.D. column with 5-μm packing.

The within-day relative standard deviation was 1.2%. This was determined by spiking pooled serum with MTX and assaying, ten times, during a single run. The day-to-day relative standard deviation ($n=6$ repeated two weeks apart) was 4.2%.

A standard curve (MTX aminopterin peak-area ratio *versus* concentration of MTX injected) generated in the range 0.01–10 μg/ml MTX on-column (40 mg/l aminopterin) was linear with an intercept of zero. The area under the curve (AUC) for MTX-spiked serum, extrapolated to infinity, had a strong correlation with the peak concentration: $r=0.79$.

Excellent reproducibility was obtained for MTX and aminopterin as shown in Table I.

Method A. The recovery for this method was below 70% for the highest concentration of 10 μg/ml and even less for all other lower concentration down to 5 μg/ml.

Method B. The recovery for this method was above 90% for all concentration ranges investigated (*i.e.* 0.01–10 μg/ml). Due to the superior recovery rate of method B all the patients data reported here are obtained by employing the method B.

Serum concentration of MTX

Chromatograms from blank human serum, serum spiked with MTX and in-

TABLE I

REPRODUCIBILITY DATA FOR KNOWN CONCENTRATIONS OF MTX AND AMINOPTERIN (n = 6)

Concentration (μg/ml)	Absorbance	S.D.	C.V. (%)
MTX			
0.01	$1.035 \cdot 10^{-4}$	0.079	7.0
10	$5.525 \cdot 10^{-4}$	0.15	2.6
Aminopterin			
0.01	$0.750 \cdot 10^{-4}$	0.038	4.0
10	$4.480 \cdot 10^{-4}$	0.10	2.2

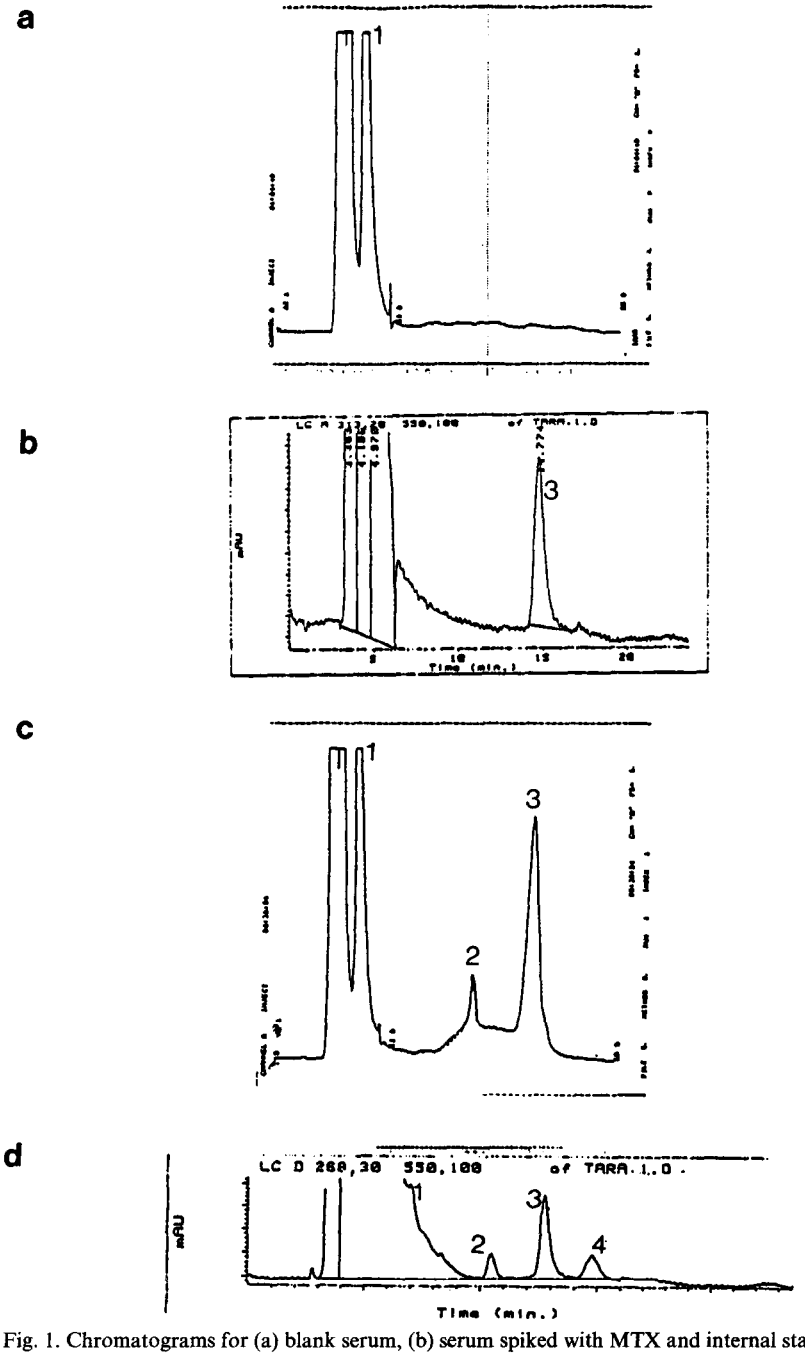

Fig. 1. Chromatograms for (a) blank serum, (b) serum spiked with MTX and internal standard, (c) serum from a patient on MTX therapy (1 h after administration) with the internal standard (injection volume, 10 μl) and (d) serum from the same patient 6 h after MTX administration (injection volume, 10 μl). Peaks: 1 = serum front; 2 = internal standard (aminopterin); 3 = MTX; 4 = 7-OH-MTX. Detector sensitivity setting, 0.01 a.u.f.s.

ternal standard, and serum from a patient on MTX therapy (after 1 and 6 h) are shown in Fig. 1. 7-Hydroxymethotrexate (7-OH-MTX) was undetectable in serum of the patients in the first hour after administration (Fig. 1c), but thereafter its concentration increased rapidly, reaching its maximum after 6–8 h (Fig. 1d). Following this the concentration decreased slowly with time. Retention times obtained from six different HPLC analyses were 14.7 ± 0.7 min for MTX and 10.2 ± 1.1 min for the internal standard, aminopterin.

The results of interference studies with steroids dexamethasone, prednisone and prednisolone showed no interference with the analysis of MTX since these steroids do not absorb at 313 nm and they all have an absorbance maximum of 240 nm.

A summary of five patients serum level profiles when receiving 10 mg/m^2 MTX by oral administration is shown in Fig. 2. Absorption following this oral dose was fairly rapid, with peak serum MTX concentration (1200 ng/ml) being achieved by 2 h. A monophasic disappearance was noticed, with a mean elimination half-life of 5 h.

The between-patient variation in MTX absorption measured by peak serum levels and AUC was not significant. As we did not consider it ethically justified to repeat the profiles a second time on children, we have no information on intra-individual variation.

DISCUSSION

There are a number of techniques for measuring serum MTX, but the methodologies employed in these studies had inherent difficulties that limit their value. They lack specificity due to the interference of metabolites such as 7-OH-MTX.

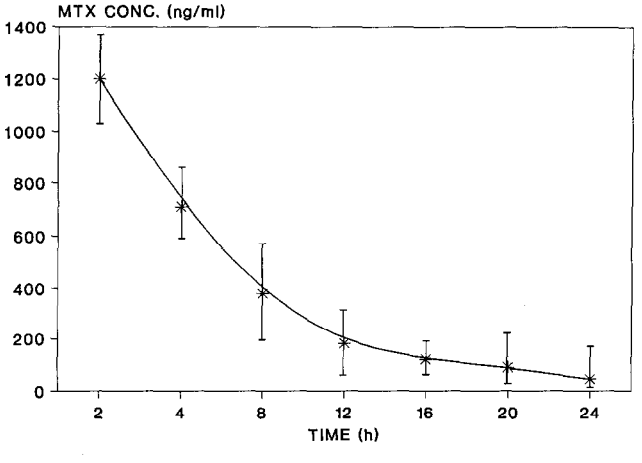

Fig. 2. Mean serum methotrexate concentration following an oral dose of 10 mg/m^2 body surface area (n = 5). Error bars represent the range in concentration of MTX at each time in the five patients studied.

Furthermore, these methods were not capable of quantitating the metabolite and are susceptible to interference by other drugs including steroids. Finally and most important the sensitivity associated with these assays was low and therefore not adequate to monitor low-dose therapy as employed in asthma.

We believe that the HPLC technique provides a method for measuring MTX and its metabolite, which is technically straightforward and economical with a high degree of specificity and sensitivity. Of several solvent systems investigated the system in this paper (method B) proved to be convenient and ideal for the routine biochemistry laboratory. This method also has the advantage of having a relatively short chromatography time with peaks which are well separated and can easily be isolated for further investigations.

With regard to serum extraction procedures, a simple deproteinization of serum (such as that reported in method A) is desirable because of its ease of performance and reduction of analysis time. However, as described in the Results, the percentage recovery following this method was low. Although there are a number of reports in literature where extraction procedures with recoveries less than 70% are used, we feel that when monitoring low-dosage drug therapy a high percentage recovery after extraction is required. Method B was more time consuming but provides more extensive sample purification. In this way, detection limits were considerably lowered. Thus we prefered this method in monitoring low concentrations and/or extended pharmacokinetics studies.

Following oral administration of MTX, the drug is metabolised in the liver by a mixed function oxidase, aldehyde-oxidase, into 7-OH-MTX which is more polar than MTX (partition coefficient of MTX = 2.7, partition coefficient of 7-OH-MTX = 1.3). 7-OH-MTX is thought to be the major metabolite causing nephrotoxicity and hepatic dysfunction [23]. Even at low dose (10 mg/m^2) there is significant hydroxylation of MTX to the metabolite 7-OH-MTX (Fig. 1). The study does not support the theory that the hydroxylation of MTX is a dose-dependent phenomenon seen only at high dosage. The data reported are in general agreement with those described by Stewart *et al.* [24]. We are currently investigating the pharmacokinetic profile of this metabolite since we feel it would be of considerable value for metabolic and toxic studies.

In summary, we have reported a sensitive and reproducible method which will form the basis for dose–response and side-effect studies of low-dose oral MTX *in vitro* and *in vivo*. We would suggest that sampling is preferred 2–4 h after a dose for MTX and 6–8 h for 7-OH-MTX monitoring.

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