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Improved high-performance liquid chromatography determination of methotrexate and its major metabolite in plasma using a poly(styrene-divinylbenzene) column

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Abstract

A sensitive high-performance liquid chromatographic assay has been developed for measuring plasma concentrations of methotrexate and its major metabolite, 7-hydroxymethotrexate. Methotrexate and metabolite were extracted from plasma using solid-phase extraction. An internal standard, aminopterin was used. Chromatographic separation was achieved using a 15-cm poly(styrene-divinylbenzene) (PRP-1®) column. This column is more robust than a silica-based stationary phase. Post column, the eluent was irradiated with UV light, producing fluorescent photolytic degradation products of methotrexate and the metabolite. The excitation and emission wavelengths of fluorescence detection were at 350 and 435 nm, respectively. The mobile phase consisted of 0.1 *M* phosphate buffer (pH 6.5), with 6% *N*,*N*-dimethylformamide and 0.2% of 30% hydrogen peroxide. The absolute recoveries for methotrexate and 7-hydroxymethotrexate were greater than 86%. Precision, expressed as a coefficient of variation ($n=6$), was $<10\%$ at each of five methotrexate concentrations in the range 2.5–50 ng/ml. The limits of quantitation of methotrexate were 1 and 2.5 ng/ml for methotrexate and 7-hydroxymethotrexate, respectively (using 1 ml plasma). A robust HPLC method has been developed for the reproducible quantitation of methotrexate in plasma of patients taking a weekly dose of methotrexate for rheumatoid arthritis.

Keywords: Methotrexate; 7-Hydroxymethotrexate

1. Introduction

Low-dose methotrexate (4-amino-*N*10-methylpteroglutamic acid, Fig. 1) is well established for the treatment of rheumatic diseases such as rheumatoid arthritis and psoriatic arthritis [1]. Methotrexate acts as an antimetabolite by inhibiting dihydrofolate reductase. Methotrexate is metabolised by hydroxylation at the seven position to 7-hydroxymethotrexate which is thought to be active but inhibits dihydrofolate reductase with only 1/10–1/100 the potency of methotrexate [2–4].

There are data to suggest that the kinetics of methotrexate exhibit within- and between-patient variability [5]. Such variability leads to an inconsistent relationship between dose and concentration. It is common for patients receiving rheumatological

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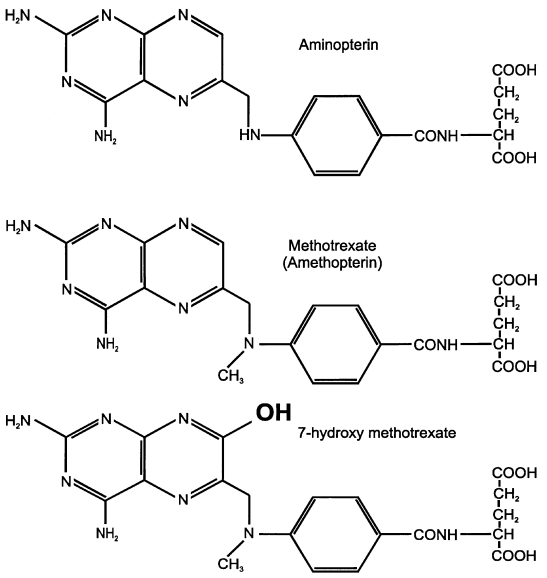

Fig. 1. Chemical structures of methotrexate and its major metabolite 7-hydroxymethotrexate. Aminopterin (4-amino folic acid) is the internal standard.

doses of methotrexate to require numerous dosage adjustments to optimise effect. By analogy with other drugs (for example cyclosporin-A [6], hydroxychloroquine [7], digoxin [8]), concentration is likely to be more closely related to effect than dose is. Methotrexate may be a good candidate for dosing according to specific concentration targeting. This hypothesis is worthy of further investigation to optimise patient outcomes. Successful therapeutic drug monitoring needs to be based on a sensitive and specific assay. There is a need for a robust methotrexate assay that has enhanced sensitivity, as the doses used in rheumatic disease are considerably lower than oncological doses and the dosage interval is extended to a week.

Methotrexate is also used in high doses as an anti-cancer drug. Such high-dose therapy requires close monitoring of the plasma methotrexate concentration to avoid toxicity [2]. Methotrexate has been quantified in plasma by fluorescence polarisation immunoassay (TDx®, Abbott Diagnostics), radioimmunoassay [9–13], enzyme immunoassay [11–12], capillary zone electrophoresis [14] and a dihydrofolate reductase inhibition assay [15]. These methods, especially those based on immunological techniques, are at least to some degree prone to interference from drug metabolites, endogenous factors, haemolysis of the sample and various disease states in the patient [2].

There are several HPLC assays, some which are suitable for use after high doses such as those used for cancer treatment [16–21]. Others which are available for low doses of methotrexate are complicated and involve column switching [20–21] or non-standard equipment [16–22].

The most sensitive assay published to date is an HPLC assay combining solid-phase extraction with fluorescence detection to achieve a reported sensitivity of 0.05 ng/ml in plasma [22]. An internal standard was not used in this assay.

This manuscript describes the development of a sensitive HPLC assay with fluorescence detection following a sample clean up and concentration procedure using solid-phase extraction based on the method of Beck et al. [23]. In our hands, rapid degradation of the silica-based column also occurred using this assay. This current method incorporates an internal standard, to enhance reproducibility. A polymer-based stationary phase was used to increase assay robustness.

2. Materials and Methods

2.1. Chemicals and reagents

Methotrexate and aminopterin (4-aminofolic acid/internal standard) were obtained from Sigma (Sigma–Aldrich Pty, Castle Hill, NSW, Australia). Other chemicals used were of analytical grade, and were obtained from commercial sources. Bond Elut Certify II solid-phase extraction cartridges were used (Varian Australia Pty, Frenchs Forest, Australia).

The methotrexate metabolite, 7-hydroxymethotrexate, was prepared enzymatically by the method described by Cairnes and Evans [24], in which methotrexate is incubated in a Tris–HCl rabbit liver homogenate to produce 7-hydroxymethotrexate. Lyophilization, and re-crystallization gave chromatographically pure 7-hydroxy/methotrexate.

2.2. Instrumentation and HPLC analysis

The HPLC system consisted of a Shimadzu LC 10AS pump (Shimadzu Corporation, Japan) and an ICI LC1610 automatic injector (ICI Instruments, Victoria, Australia). The injection volume was 50 μl. A fluorescence spectrophotometer (Shimadzu RF 535; Shimadzu Corporation, Japan), (excitation monochromator slit width, 13 nm) with excitation and emission wavelengths set at 350 and 435 nm, respectively, was used. A beam boost photochemical reaction unit equipped with a 254-nm lamp and a 10 m×0.3 mm reaction coil (ICT Handel, Frankfurt, Germany) was also used post-column (before the detector). The column was a 15-cm reversed-phase PRP-1® (5 μm diameter particle size poly (styrene divinylbenzene) polymer, Hamilton Company, Reno, NV). The integrator was a Shimadzu chromatopac C-R5A (Shimadzu Corporation, Japan).

The mobile phase consisted of 0.1 *M* phosphate buffer (pH 6.5), with 6% *N*,*N*-dimethylformamide and 0.2% of 30% hydrogen peroxide. The mobile phase was degassed and filtered before use and pumped at a constant flow rate of 1.0 ml/min.

2.3. Biological samples

The specificity of method was verified by analyzing six plasma samples from hospital blood bank. The chromatograms of these blank plasma samples were compared with chromatograms obtained after spiking blank plasma samples with internal standard, methotrexate and 7-hydroxymethotrexate to ascertain that endogenous substances did not interfere with the analytes of interest.

Blood samples were also obtained from patients with rheumatoid arthritis receiving methotrexate as therapy as part of a larger study investigating the pharmacokinetics and pharmacodynamics of methotrexate. Research Ethics Committee approval has been granted for this larger study. Samples were collected by venipuncture into heparinised 10-ml Vacutainer collection tubes (Becton Dickinson Pty, Lane Cove Australia). Plasma was prepared by centrifugation at 6300 *g* for 10 min as soon as possible after the time of blood collection. For those patients whose blood was collected as part of a home visit, the blood was stored on ice, transported and centrifuged immediately on return to the hospital in less than 3 h. Plasma samples were stored at −20°C until assayed.

2.4. Solid-phase extraction procedure

A sample of 1.0 ml of plasma was mixed with l.0 ml of 0.10 mol/l phosphoric acid and 50 μl of internal standard (125 ng/ml) and applied on Bond Elute solid-phase extraction cartridges (pre-conditioned before use with 2.5 ml of methanol and 2.5 ml of 0.10 mol/l phosphoric acid) and allowed to drain under gravity. A slight vacuum was applied if necessary to start flow. The cartridges were then washed sequentially with 2.0 ml of 5% methanol in 0.10 mol/l phosphoric acid, 2.0 ml of 0.10 mol/l sodium phosphate buffer (pH 8.0), and 2.5 ml of 25% methanol in water. The methotrexate was finally eluted with l.0 ml of freshly made 2% formic acid in methanol. The eluent was collected in glass test tubes and evaporated to dryness under air in a water bath (40°C). The residue was redissolved in 200 μl of 0.10 mol/l phosphoric acid and injected into the chromatographic system.

2.5. Quantitation

Quantitation of methotrexate and 7-hydroxymethotrexate was based on peak-area ratio [methotrexate/internal standard] referenced to the five-point standard curve, which was assayed with each analytical run.

Acceptance criteria [25] for analytical runs were: (1) a correlation co-efficient of $r>0.99$ for the standard curve; (2) less than ±10% deviation of the concentrations calculated from the standard curve samples from the known spiked concentrations back extrapolated; and (3) four out of six independently prepared quality controls (two each at low, medium and high concentrations) were within 15% of the target concentrations. That is, a bias of <15% for the back-calculated concentrations for the quality controls samples was considered acceptable. Bias was calculated as:

$$\text{Bias} = \left[\frac{(\text{observed concentration-nominal concentration})}{\text{nominal concentration}}\right] \times 100. \quad (1)$$

2.6. Assay validation

A 3-day validation of the analytical method was carried out over the concentration range 2.5 ng to 50 ng/ml. The validation run included a set of duplicate calibration standards (i.e., two standard curves), and three independently prepared quality control samples at three concentrations.

The intra- and inter-day precision of the method was calculated as the coefficients of variation. The precision of the assay was calculated by analysing spiked plasma samples six times on 1 day to determine the intra-day variability and on 3 different days to determine the inter-day variation.

Accuracy was calculated by back calculation of the calibrators and comparison to the known (spiked) concentration.

2.7. Recovery

Absolute recoveries were determined by comparing the peak area of extracted samples to those of unextracted solutions prepared in 0.1 *M* Na_2HPO_4.

3. Results and discussion

Fig. 2 shows a typical chromatograph acquired from the analysis of a plasma sample spiked with methotrexate and 7-hydroxymethotrexate (50 ng/ml). Typical retention times were 10, 13 and 5.5 min for methotrexate, metabolite and internal standard, respectively. Baseline resolution was achieved between all three compounds. There was no interference from endogenous compounds.

3.1. Recovery

The recovery of methotrexate from plasma was 86±5% (2.5 ng/ml, $n=6$), 90±8% (10 ng/ml, $n=5$), and 111±6%, (50 ng/ml, $n=6$). Recovery of metabolite was 102±16% (2.5 ng/ml, $n=3$), 101±3.5% (10 ng/ml, $n=3$) and 91±0.3% (50 ng/ml, $n=3$). The recovery was high, but did vary for methotrexate, indicating the internal standard would assist with accurate quantitation.

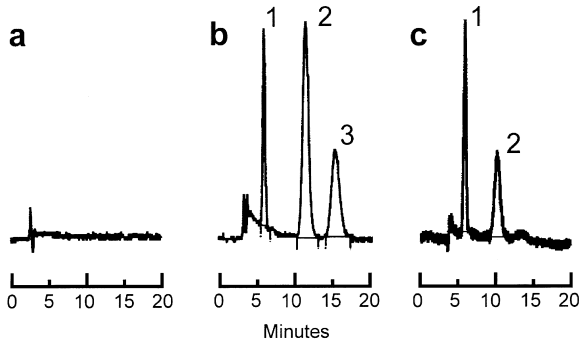

Fig. 2. Typical chromatographs obtained from the analysis of (a) 'blank' human plasma; (b) blank human plasma spiked with 50 ng/ml methotrexate and 50 ng/ml 7-hydroxymethotrexate; and (c) plasma from a patient containing 32 ng/ml of methotrexate, 8 h after 7.5 mg of methotrexate orally. Peak 1 is the internal standard, 2 is the methotrexate and 3 is the 7 hydroxymethotrexate.

3.2. Accuracy and reproducibility

The intra- and inter-day assay variability of the methotrexate determination was determined from six spiked plasma samples (Table 1). The calibration curves were linear over the concentration range, $r>0.995$. The accuracy of the method was acceptable and the back calculated concentrations of methotrexate were within 10% of the nominal value.

The limits of quantitation were 1 and 2.5 ng/ml (reproducibility with a co-efficient of variation (intra-day) less than 10%) for methotrexate and 7-hydroxymethotrexate respectively. The lowest measurable concentrations were 0.25 and 1 ng/ml for methotrexate and 7-hydroxymethotrexate, respectively, from analysis of 1 ml of plasma.

Recently, others have improved the photo-reaction procedures of the post column photo-degradation of methotrexate by UV irradiation to further enhance the sensitivity of this assay [22,26], using shorter,

Table 1
Intraday and interday variation of methotrexate and metabolite quantitation

Methotrexate (ng/ml)			7-Hydroxymethotrexate (ng/ml)		
Known concentration (ng/ml)	Back calculated concentration ±SD	% CV	Known concentration (ng/ml)	Back calculated concentration ±SD	% CV
Intraday	$n=6$		Intraday	$n=6$	
2.5	2.5±0.1	4.4	2.5	2.3±0.1	7.4
5	5.4±0.2	4.4	5	5.6±0.2	3.3
10	11.5±0.9	8.0	10	9.6±0.9	8.2
30	32.5±0.6	1.8	30	32.5±0.6	1.8
50	54.4±3.4	6.3	50	54.4±3.4	6.3
Interday			Interday		
2.5	2.6±0.1	4.8	2.5	2.1±0.6	6.8
5	5.2±0.6	10.0	5	5.7±0.5	4.3
10	10.0±0.2	3.5	10	9.3±0.2	4.0
30	33.4±0.5	6.5	30	33.4±0.3	4.1
50	54.6±0.4	5.6	50	54.5±0.4	4.2

non-standard photo-reaction coils or altering the excitation and emission wavelengths of the fluorescence detection. However, others continue to publish with the same excitation and emission wave lengths (350 and 435 nm, respectively), and flow rate (1 ml/min), with a reaction coil of the same length (10 m) [17,27]. The shorter non-standard polyethylene reaction coil may produce an even more enhanced sensitivity. The present assay has the strengths of robustness (polystyrene column) and reproducibility.

The advantage of the PRP-1® column relates to its ability to with stand the contents of the mobile phase (hydrogen peroxide is an oxidising agent and is corrosive). The damage to silica-based columns was evidenced by a black particulate matter that accumulated around the top of the column. The polymer-based column has lasted to date for more than a year, during which 3000 plasma samples have been analysed. In our hands, silica-based columns of several different brands lost resolution quickly and the black particulate matter formed. Hydrogen peroxide, a component of the mobile phase is an oxidising agent and is the likely cause of this corrosion. Poly(styrene divinylbenzene) (PRP-1®) appears to be resistant to this damage. No loss of resolution has occurred and no black material has formed on the column.

4. Conclusion

In conclusion, a sensitive HPLC method for methotrexate and its major metabolite, combining the selectivity of a solid-phase extraction procedure with the sensitivity of fluorimetric detection, the robustness of a polymer stationary phase and the reliability of an internal standard, was established.

The method offers the sensitivity to monitor plasma methotrexate for up to 1 week after the low, weekly doses used therapeutically for rheumatic diseases.

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