

## HPLC Method of Determination of Methotrexate in Human Serum

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This study described a simple and fast HPLC method for the determination of methotrexate (MTX) in serum samples were collected from adult cancer patients receiving 60 mg/m<sup>2</sup> dose of methotrexate at Mahatma Gandhi Memorial Hospital (Warangal, India) at various time intervals after the end of each infusion. Serum was deproteinized with trichloroacetic acid and the supernatant was injected into a 250 × 4.6 mm octadecylsilane column. Mobile phase was made of tris-phosphate buffer (pH 5.7):methanol:acetonitrile (70:20:10) with a flow rate of 1 mL/min. Ultraviolet detection was done at 313 nm at ambient temperature. *p*-Aminoacetophenone was used as internal standard. Methotrexate and internal standard retention times were 4.6 and 9.5 min, respectively. Results showed that reproducibility (precision) of method within a day was 2.6 to 6.0 % and between days was 5.5 to 9.5 %. The recovery of the method was between 61.5 and 72.7 %. The quantitation limit of the method for methotrexate was 0.1 μM. This method is suitable for quantitation of methotrexate after infusion of 60 mg/m<sup>2</sup> doses of this drug and has good accuracy, precision and quantitation limit.

**Key Words: Methotrexate, HPLC, Serum concentration.**

### INTRODUCTION

Methotrexate (MTX) formerly known as amethopterin, is an antimetabolite and antifolate drug used in treatment of cancer and autoimmune diseases. It acts by inhibiting the metabolism of folic acid. Methotrexate is a weak dicarboxylic acid with pKa 4.8 and 5.5 and thus it is mostly ionized at physiologic pH. Methotrexate is metabolized by intestinal bacteria to the inactive metabolite 4-amino-4-deoxy-N-methylpteroic acid (DAMPA) and accounts for less than 5 % loss of the oral dose. It can be taken orally or administered by injection (subcutaneous, intramuscular, intravenous or intrathecal).

Methotrexate concentration in plasma and other biological fluids is determined to study its pharmacokinetics and also to predict and prevent its toxicity when administered in high dose intravenous infusion of the drug by modification of the calcium folinate administration schedule according to the plasma concentration of methotrexate<sup>1-3</sup>. Different methods have been described for the determination of

methotrexate in plasma and other biological fluids including enzyme inhibition methods, radiometry and radio immunoassay, enzyme multiple immunoassay technique (EMIT), fluorescence polarization immunoassay (FPIA)<sup>1,4</sup> and high performance liquid chromatography (HPLC)<sup>4-14</sup>.

The aim of present study is to describe a simple, fast, accurate and precise method for the determination of methotrexate in serum for pharmacokinetic studies and routine therapeutic drug monitoring in 60 mg/m<sup>2</sup> dose intravenous infusion of this drug.

## EXPERIMENTAL

Methotrexate(+)-amethopterin (> 98.5 % purity for HPLC) and *p*-aminoacetophenone were purchased from Sigma Aldrich(USA). Methanol and acetonitrile (Merck, Germany), trichloroacetic acid (Himedia, India), TRIS (trishydroxymethyl-aminomethane (Merck, Germany), sodium dihydrogen phosphate, 2H<sub>2</sub>O (Himedia, India) were all analytical grade.

**Chromatographic conditions:** A Shimadzu liquid chromatography system equipped with a LT 10AT VP pump, a SPD 10A VP variable wavelength UV visible spectrophotometric detector and a Rheodyne 20  $\mu$ L loop injector system was used (Shimadzu, Kyoto, Japan). INERTSIL ODS-3V C-18, 4.6  $\times$  250 mm [GL Sciences Inc, Japan] chromatography column was used for analysis.

The mobile phase consisted of phosphate-tris buffer (0.1 M dihydrogen phosphate and 0.01 M tris; pH 5.7):methanol:acetonitrile, with the ratio of 70:20:10, respectively. The flow rate was 1 mL/min and the eluent was monitored spectrophotometrically at 313 nm at room temperature.

**Solutions of external and internal standards:** Due to the possible instability of methotrexate in aqueous phosphate buffer solutions<sup>13</sup>, a stock standard solution of methotrexate (10000  $\mu$ M/mL) was prepared using 0.1 N sodium hydroxide. Stock solutions of internal standard (*p*-aminoacetophenone) were also prepared in the same solvent at the concentrations of 5  $\mu$ g/mL. Further dilution of methotrexate stock solution was done with drug free plasma to prepare different concentrations of methotrexate (5, 10, 30, 50 and 70  $\mu$ M/mL)<sup>15</sup>.

**Sample collection and preparation:** Received methotrexate at doses of 60 mg/m<sup>2</sup> as small infusions, as part of protocols for the treatment of various cancer diseases at Mahatma Gandhi Memorial Hospital (Kakatiya Medical College, Warangal, India). Blood samples were collected at various times after the end of each infusion. To each 100  $\mu$ L of patient's or standard sample, 100  $\mu$ L of stock solutions of internal standard 5  $\mu$ g/mL was added to samples with methotrexate concentration of above and less than 5  $\mu$ M, respectively. After complete mixing of samples with internal standard, 40  $\mu$ L of trichloroacetic acid (2 M in ethanol) was added and vortex mixed for 2 min, then centrifuged at 3000 rpm for 15 min, 10 or 20  $\mu$ L aliquots of the supernatant was directly injected into the chromatography column. Each sample was analyzed in duplicate. All samples or standard solutions were stored at -80  $^{\circ}$ C until analyzed.

**Recovery and precision:** The recovery was studied by preparation of the various amounts of methotrexate in blank plasma (spiked blank). Methotrexate was determined according to the described method. The recovery was calculated by comparison of the found amounts with the added ones.

## RESULTS AND DISCUSSION

Under the conditions used for the chromatography, the retention times for methotrexate and the internal standard were  $4.6 \pm 0.03$  and  $9.5 \pm 0.98$  min, respectively. Fig. 1 shows the chromatograms of human blank plasma used for preparation of different concentrations of methotrexate standard solution (2) and patients samples (1 and 5) withdrawn from patients who received 16 mg/sqm of methotrexate at 4 h infusion. The chromatographic condition employed was quite specific for methotrexate and paminacetophenone.

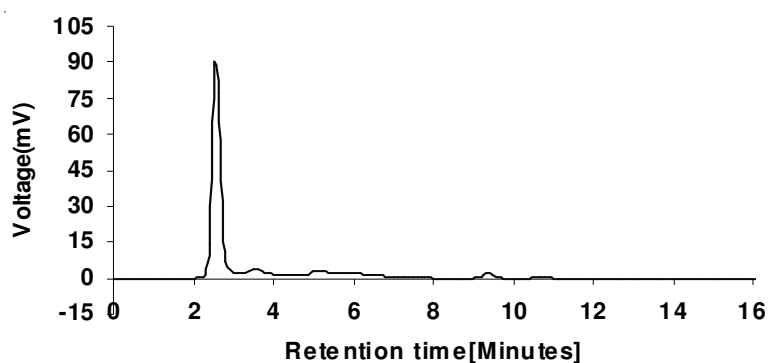


Fig. 1. Human blank serum

Other drugs that might be administered concomitantly with methotrexate such as leucovorin, adriamycin, bleomycin, cyclophosphamide, mercaptopurine, vincristine, metocloperamide, prednisolone, diazepam, aspirin and trimethoprim-sulfamethoxazole could not interfere with methotrexate peaks because either they have no significant absorption at 313 nm or their retention times are quite different. This fact was proved in this study by adding each of these drugs to plasma samples containing methotrexate and analyzed by chromatography.

According to the previous reports, the major and most important metabolite of methotrexate is 7-hydroxymethotrexate but its pharmacological effect is not significant<sup>5-7,9-12</sup>. We did not have any pure standard of this metabolite and unable to obtain it from elsewhere, therefore it was not possible to identify its retention time positively and to quantitate its concentration in plasma.

In order to determine plasma concentration of methotrexate, internal standardization method was used. After preparation of various concentrations of methotrexate and analyzing chromatography each standard solution, two standard curves were

prepared by plotting the ratio of peak area of methotrexate to internal standard (*p*-aminoacetophenone) *versus* concentration of methotrexate. A good linearity was seen for both the standard curves (Figs. 1 and 2).

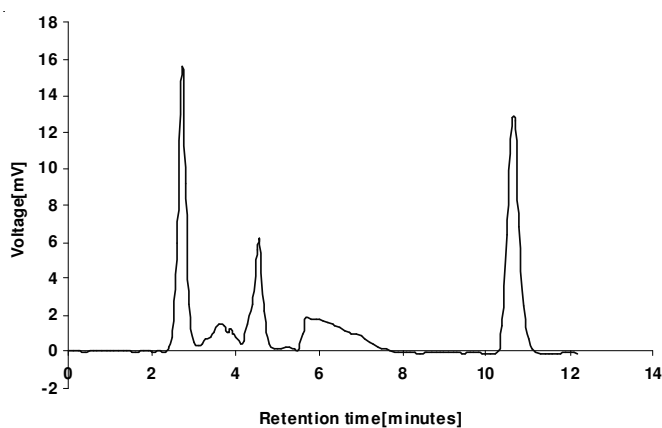


Fig. 2 Serum sample spiked with  $\mu\text{M}/\text{mL}$  MTX [methotrexate] and PAAS [*p*-amino acetophenone]

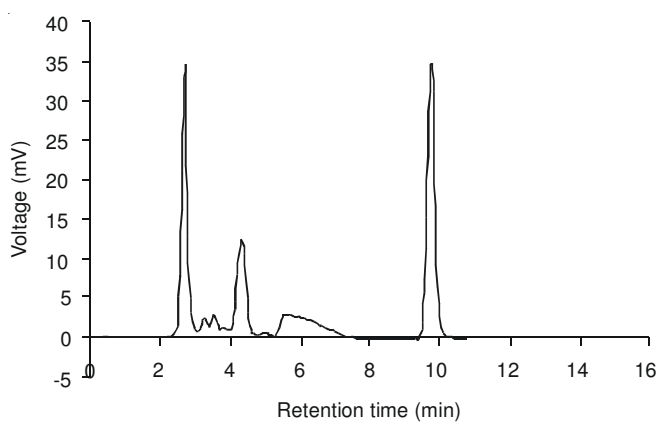


Fig. 3. MTX and IS [PAAS] in patient serum sample, at 10 min after 4 h infusion

To assess the accuracy of the method, recovery of methotrexate from plasma samples with known concentrations was compared with the solutions of methotrexate at the same concentrations as shown in Table-1. For the assessment of method precision, reproducibility of the results obtained for different concentrations of methotrexate was determined at 5 different days and 5 times in 1 d. The results of reproducibility study are shown in Table-2 as recovery and accuracy determination.

The limit of quantitation of methotrexate in plasma with the above sample pretreatment method was  $5 \mu\text{M}$ .

TABLE-1

Spiked concentration ( $\mu\text{M}/\text{mL}$ )	Day	Measured concentration		
		Mean ( $\mu\text{M}/\text{mL}$ )	SD	RSD
Inter-day variation				
5	1	4.80	0.080	1.667
	2	4.90	0.100	2.041
	3	5.10	0.096	1.882
	4	5.21	0.030	0.576
30	1	31.20	0.650	2.083
	2	29.80	0.120	0.403
	3	30.36	0.050	0.165
	4	32.60	0.960	2.945
70	1	69.32	0.960	1.385
	2	70.15	0.069	0.098
	3	69.99	0.870	1.243
	4	68.95	0.050	0.073
Intra-day variation				
5		5.10	0.15	2.941
30		29.60	0.97	3.277
70		70.26	0.12	0.171

SD = Standard deviation, RSD = Relative standard deviation and Mean value of 4 determinations.

TABLE-2  
ASSESSMENT OF RECOVERY AND ACCURACY  
DETERMINATION OF MTX IN HUMAN SERUM

Substance	Concentration	Recovery (%) Mean $\pm$ SD	Accuracy (%) Mean $\pm$ SD
MTX	5 ( $\mu\text{M}$ )	98.00 $\pm$ 2.80	100.06 $\pm$ 1.90
MTX	30 ( $\mu\text{M}$ )	98.08 $\pm$ 4.04	97.70 $\pm$ 1.07
MTX	70 ( $\mu\text{M}$ )	99.98 $\pm$ 0.80	99.80 $\pm$ 0.77
Internal Standard	5 ( $\mu\text{g}/\text{mL}$ )	96.35 $\pm$ 1.02	96.69 $\pm$ 1.03

SD = Standard deviation and mean value of 5 determinations.

Various methods of high-performance liquid chromatography for the determination of methotrexate in biological fluids have been described so far which differ in chromatography type (reverse phase or ion-pair chromatography) or detection system (UV or fluorescence)<sup>5-12</sup>. Reverse-phase high performance liquid chromatography with UV detection has been most recommended<sup>4-8,10,11,14</sup>. But, an important point is that most of them are tedious and expensive because they use more material and have many stages of experiment. Besides, they are not suitable for a routine and quick therapeutic drug monitoring (TDM) test which is necessary for a child cancer patient in a high dose infusion therapy at hospital, or reference laboratories.

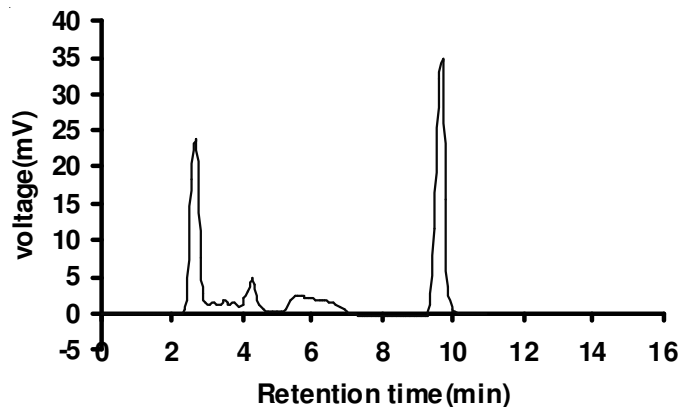


Fig. 4. MTX and IS [PAAS] in patient serum sample, at 30 min after 4 h infusion

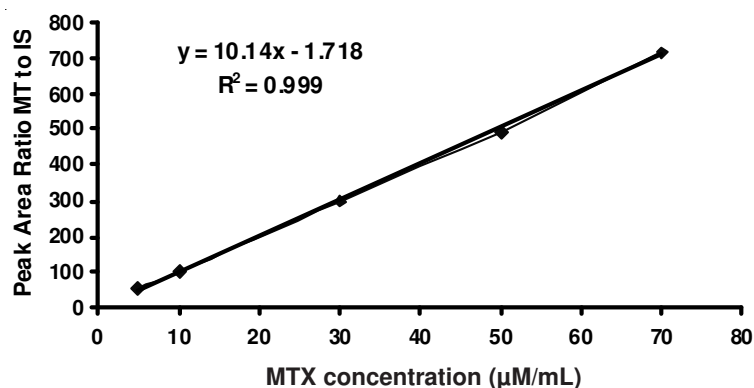


Fig. 5. Linear standard curve determination of methotrexate in serum (concentration range 5-70 µM/mL)

In present studies, a simple and fast method for the determination of methotrexate in serum is described that has equal precision and accuracy to other similar methods<sup>6-8,10,11,14</sup>. A full chromatography takes 10 min. To include sample preparation time it needs 25 min for the whole of each analysis, which is comparatively a short time. The short duration of assay time is of quite importance in routine monitoring of the drug in serum to predict and prevent future toxicity in high-dose methotrexate intravenous infusion. On the other hand this method has a satisfactory quantitation limit that makes it ideal for pharmacokinetic studies and therapeutic drug monitoring of methotrexate after the administration of high doses of this drug. To improve the quantitation limit further we could use solid phase extraction technique along with fluorescence detection after post column derivitization of the methotrexate to fluorescent compounds so that the method become suitable for determination of methotrexate after the administration of lower doses of the drug but such methods are more tedious, time consuming and expensive.

## Conclusion

A one step extraction procedure for methotrexate from serum and an improved method for determination of methotrexate are reported. Compared to previously published methods, the suggested extraction procedure is considerably more simple, rapid, reliable and sensitive. The HPLC technique based on UV detection is suitable for determination of small amounts of methotrexate with good accuracy and reproducibility. Simple sample preparation procedure and a relatively short chromatographic time make this method suitable for processing of multiple samples in a limited amount of time for pharmacokinetic studies

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