# HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF METHOTREXATE IN SERUM

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*SUMMARY*

***Purpose :*** *Methotrexate (MTX), which is commonly used as an anticancer drug, is a toxic compound when used in high doses . Therefore, we have planned to establish a new precise method to measure serum MTX levels in patients who are followed at the Pediatric Oncology Department.* ***Methods :*** *The quantitive determination of methotrexate was performed by High Performance Liquid Chromatographic ( HPLC ) technique in serum. The method involves deproteinization with acetone followed by addition of butanol and diethyl ether. Reconstituted residue is analyzed using reverse phase C - 18 column.* ***Results :*** *The retention time for MTX was 9.09 min. Calibration curve of MTX was linear in the range of 2.5 - 10µg MTX/ml injected ( r : 0.999 ). The recovery for this method was found to be 97.9 %. The reproducibility study was carried out at two concentrations of MTX and the coefficients of variation were obtained as 6.05 % and 5.77 %.* ***Conclusion :*** *The method is selective and reproducible, and it covers a wide range of MTX concentrations in serum. So, this method might facilitate monitoring of children treated with MTX and allow for dose response and toxicity studies to be conducted.*

***Key Words:*** *Methotrexate, Aminopterin, High Performance Liquid Chromatography.*

## INTRODUCTION

Methotrexate (MTX) (amethopterin) is a widely used anticancer drug (1-3 ). It binds very tightly and rapidly to dihydrofolate reductase (DHFR). MTX, a folinic acid antagonist, combines irreversibly with dihydrofolate reductase, preventing the synthesis of tetrahydrofolic acid. Tetrahydrofolic acid is converted to several co-factors that take part in important reactions, involving the transfer of one carbon units which are required for the synthesis of thymidylate and purine nucleotides. Thus, MTX inhibits a number of important biochemical pathways, and the final result is inhibition of DNA, RNA and protein synthesis, which eventually leads to cell death (4, 5).

MTX was first introduced in 1948 by Farber et al. for the treatment of acute leukemia; since then it has been used against malignant diseases, such as non-Hodgkin's lymphoma (NHL), choriocarcinoma, breast cancer, osteosarcoma, small cell lung cancer, or head and neck cancer (2, 6).

MTX may also be applied effectively in non-malignant conditions such as psoriasis, asthma, several rheumatic disease (7, 8). The toxicity of the drug is very high, so the serum levels of it should be determined during administration (5).

There are many analytical methods in the literature for the analysis of plasma MTX levels including enzyme inhibition, competitive protein binding, enzyme multiplied immunoassay, radio-immunoasssay, microbiological, fluorometry, or High Performance Liquid Chromatography (HPLC) (9-13). Of these techniques, only HPLC has the potential to readily measure both MTX and its metabolites. It also offers a sensitive and fast analytical result. The higher sensitivity of HPLC method allows the use of smaller amounts of samples for MTX determinations in the serum of pediatric patients.

## MATERIALS AND METHODS

Serum MTX concentrations were determined by the method suggested by Brimmell et al. (6), including some modifications.

*Equipment*

An isocratic HPLC system (Millipore, Waters Division) was utilized . It consisted of a model 510 pump, a model 486 variable wavelength detector set at 313 nm. Chromatography was performed on an Ultrasphere ODS RP C18 (5 µm) stainless-steel 250 mm x 4.6 mm ID column. Injections were performed with a 10 µl Hamilton syringe.

*Reagents*

MTX and aminopterin, used as internal standards, were obtained from Sigma. Tetrahydrofuran was HPLC grade (Sigma). Sodium dihydrogen ortophosphate was obtained from Merck. All solvents used for chromatography were of HPLC grade purify . Deionized water was further purified with vacum pump using membrane filters (type HA, 0.45µM, Millipore).

*Mobile Phase*

The mobile phase was 5 % tetrahydrofuran in 0.05 M sodium dihydrogen orthophosphate buffer (pH : 4.85). The mobile phase was filtered using membrane filters (0.45 µM ); then degassed by immersion in an ultrasonic bath for 30 min. It was pumped through the column at a flow rate of 0.6 ml/min. Chromatography was performed at ambient temperature with detection at 313 nm.

*Blood Samples*

Blood samples were obtained from patients of NHL receiving 1 $g/m^2$ MTX. MTX was administed by a 24 hour iv infusion and blood samples were obtained at 24, 36, 48 and 72 h and allowed to clot. Clotted blood was centrifuged for 10 min at 1200 rpm before analysis . Samples which could not be chromatographed immediately were stored frozen at - 20 °C in polypropene tubes.

*Extraction Procedure*

A 50 µl of internal standart (40 mg / L aminopterin) was added to 450 µl serum in a glass tube. The mixture was vortexed after adding 500 µl aceton and was centrifuged at 1000 rpm for 10 min. A volume of 600 µl of the supernatant was transferred to a second tube containing 600 µl n-butanol and 800 µl diethyl ether. This was mixed using the vortex mixer, then the mixture was transferred to a second microtube sizing 5 mm x 100 mm.

This was centrifuged again at 1000 rpm for 10 min. The supernatant was discarded. The lower aqueous portion was directly injected onto the column (10 µl). Peaks were identified by using retention times compared with the internal standard. MTX concentrations were determined by peak area ratios of the MTX to internal standard and comparisons with a standard curve.

MTX stock solution (100 µg/ ml ) was prepared by dissolving in 0.1 N NaOH. Working standard solutions were prepared daily by diluting a stock MTX standard with drug free serum to give a standard range of 2.5-10 µg/ml .

## RESULTS

Calibration curves of MTX were linear in the range of 2.5-10 µg MTX/ml injected (r : 0.999) (Fig. 1).

All compounds were well separeted within 15 min. Retention times were 9.09 min for MTX and 6.32 min for internal standard, aminopterin (Fig. 2).

A reproducibility study was carried out at two concentrations of MTX . Ten replicate analyses of serum samples from healthy persons were spiked with MTX solutions to final concentrations of 0.1 or 7.5 µg / ml . The coefficient of variation (CV) were found to be 6.05 % and 5.77 %, respectively (Table 1).

Serum MTX levels of the 10 patients receiving 1 $g/m^2$ iv MTX are given in Table 2.

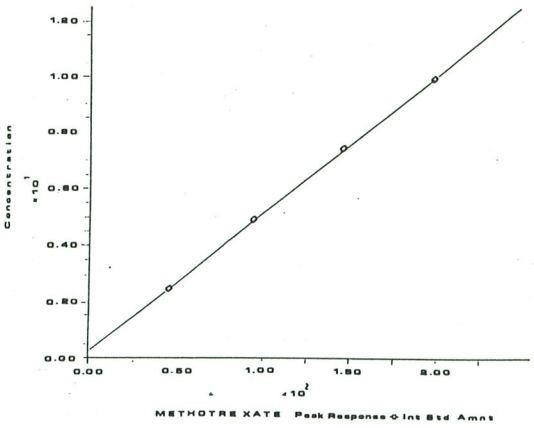

Fig - 1 : Calibration curve for MTX.

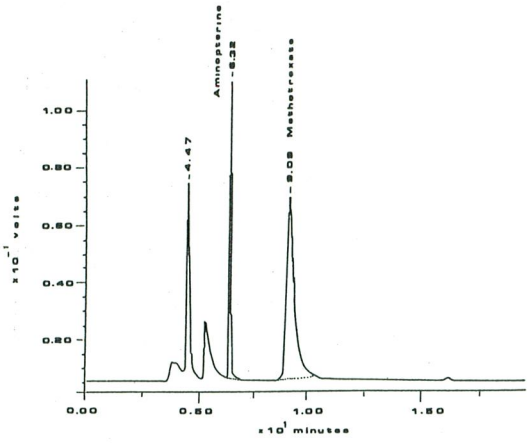

Fig - 2 : Chromatogram representing serum spiked with internal standard and MTX .

| MTX concentration (μg/ml) | n | X ± SD (μg/ml) | CV (%) |
|---|---|---|---|
| 0.1 | 10 | 0.104 ± 0.0063 | 6.05 |
| 7.5 | 10 | 7.79 ± 0.449 | 5.77 |

Table 1 : Reproducibility data for MTX . The recovery of MTX for this method was 97.9 %.

| Patients | Sex | Age (years) | n | 24 hours μmol/l | 36 hours μmol/l | 48 hours μmol/l | 72 hours μmol/l |
|---|---|---|---|---|---|---|---|
| 1 | m | 5 | 7 | 9.31 ± 2.31 | 0.35 ± 0.14 | 0 | 0.29 ± 0.29 |
| 2 | m | 6 | 4 | 3.66 ± 1.13 | 0.77 ± 0.31 | 0 | 0 |
| 3 | m | 10 | 2 | 11.33 ± 9.07 | 0.2 ± 0.07 | 0 | 0 |
| 4 | f | 12 | 5 | 9.69 ± 1.74 | 2.52 ± 1.67 | 0 | 0 |
| 5 | m | 10 | 3 | 11.27 ± 10.53 | 3.68 ± 2.96 | 0.08 ± 0.03 | 0 |
| 6 | m | 13 | 5 | 7.94 ± 3.48 | 0.64 ± 0.64 | 0 | 0 |
| 7 | m | 5.5 | 6 | 5.51 ± 1.18 | 0.19 ± 0.19 | 0 | 0.17 ± 0.17 |
| 8 | m | 3 | 6 | 5.32 ± 1.29 | 0.23 ± 0.19 | 0.06 ± 0.06 | 0.03 ± 0.03 |
| 9 | m | 5 | 5 | 13.61 ± 4.73 | 2.55 ± 1.51 | 0.42 ± 0.42 | 0.04 ± 0.04 |
| 10 | m | 5.5 | 3 | 18.29 ± 7.49 | 0.17 ± 0.11 | 0 | 0.02 ± 0.02 |

n : number of infusion.

Table 2 : Demographic data and serum MTX concentrations (mean ± Sx) of the patients with NHL.

## DISCUSSION

In view of the inherent risk of toxicity from high dose therapy, patient management requires an efficient analytical method for the determination of MTX in plasma or serum. HPLC is capable of quantifying MTX in patients on low dose therapy.

The major metabolite, 7-hydroxymethotrexate (7 - OHMTX ), may play a role in the action of MTX (14, 15). The immunological assays are limited by

their inability to measure metabolites and by possible interference (cross - reactivity) resulting from them. HPLC technique provides a method for measuring MTX and its metabolite (16).

The cytotoxic effects of MTX are due to the inhibition of dihydrofolate reductase and the subsequent lowering of reduced folate pools. The effects of MTX can be negated by administering reduced folates (i.e. leucovorin rescue). The administration of high dose MTX may be severely toxic if adequate leucovorin rescue is not given. For example; myelosupression, orointestinal mucositis, renal injury, or liver toxicity may occur. Results of a survey by Von Hoff et al. showed an approximately 6 % incidence of drug related deaths after high dose MTX and leucovorin rescue (17). It is generally agreed that a concentration in the plasma higher than 1μM at 48 hours after the beginning MTX treatment is associated with an increased risk of toxicity.

So, the serum levels of MTX in pediatric patients were calculated. At the end of the infusion, MTX levels were measured at the 24 th, 36th, 48th and 72th hours. This method was sensitive enough to measure even very small quantities of MTX ($10^{-10}$ - $10^{-11}$M ) present at the 72th hour. Sample preparation was rapid and simple, employing a few steps. This method also has the advantage of having a relatively short chromatography time and requiring a small sample volume, without loss of sensitivity. Excellent reproducibility and recovery were obtained for MTX. Reproducibility study was carried out at two concentrations (0.1 and 7.5 μg/ml) and the coefficients of variation were found to be 6.05 % and 5.77 %, respectively. The recovery for this method was found to be 97.9 %.

In conclusion, the proposed method offers an efficient extraction procedure and an accurate quantification of MTX in serum samples.

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