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Development and Validation of High Performance Liquid Chromatography Method for Quantitation of Methotrexate in Plasma

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Abstract

Purpose: Although Methotrexate (MTX) is a commonly used therapeutic agent in the treatment of cancer, its use in high doses leads to some toxic effects. Thus, we have aimed that to develop and validate sensitive, fast, inexpensive High Performance Liquid Chromatography-UV method for monitoring MTX concentration in plasma samples which is applicable for routine clinical analysis.

Methods: Plasma was deproteinized with acetone and the chromatographic separation was performed on C18 column (250 x 4.6 mmx 5µm) using mobile phases composed of 0.05 M sodium phosphate buffer/ tetrahydrofuran (95:5) (pH=4.85) (mobile phase A) and 0.05 M sodium phosphate buffer/tetrahydrofuran (75:25) (pH=4.0) (mobile phase B) at a flow rate of 0.6 mL/min. Ultraviolet detection was done at 313 nm and at ambient temperature.

Results: Retention time for MTX was 7.78 minutes. The linearity is evaluated by a calibration curve in the concentration range of 1.0-50.0 µmol/L and presented a correlation coefficient of 0.9999. Precision of method within a day was 0.67-4.02 % and between days was 1.16-5.19%. The limits of detection and quantification achieved 0.1 and 0.9 µmol/L, respectively.

Conclusion: The fast and precise method enables to analyze large number of samples by using less mobile phase that makes it to be cost-effective. Also, this method is suitable for quantitation of MTX after infusion of high doses of this drug and has good accuracy, precision and quantitation limit.

Introduction

Methotrexate (MTX) is an analog of folic acid and acts as a dihydrofolate reductase inhibitor. It is one of the most widely used drugs in the treatment of acute lymphoblastic leukemia and trophoblastic neoplasm as well as in the treatment of psoriasis and active Rheumatoid Arthritis (RA) that is unresponsive to nonsteroidal anti-inflammatory agents due to its chemotherapeutic, anti-inflammatory and immunosuppressive effects. Two different mechanisms of action have been identified for these effects; 1) MTX competitively inhibits the dihydrofolate reductase by blocking folate binding, leads to reduction in the synthesis of active tetrahydrofolate which results in inhibition of the synthesis of purine and pyrimidine bases effectively limiting DNA and RNA synthesis and cancer cell growth. 2) MTX has an impact on several pathways resulting in inhibition of T cell activation and suppression of T cell expression of intercellular adhesion molecules, inhibition of methyl transferase activity and increased CD95 sensitivity leading to apoptosis in active T cells [1]. Although originally developed as an anti-neoplastic agent, low dose MTX (5-25 mg/week), has been demonstrated to be highly effective to treat immune-mediated disorders such as RA, psoriatic arthritis, inflammatory myopathies, prophylaxis against graft versus host disease and other inflammatory conditions [2]. However, toxicity, which usually encountered in the first year of treatment, may prevent many patients to obtain benefit from it. It has been reported that mild toxicity occurs in about 60% of patients, and 7-30% of patients discontinue MTX therapy within the first year of treatment due to toxicity [3,4]. Toxic findings may vary from simple gastrointestinal problems to hepatotoxicity. Pulmonary toxicity may also be seen with both high- and low-dose treatment and may present with acute or chronic symptoms. Myelosuppression is another major dose-limiting side effect of high-dose MTX, but is infrequent in patients receiving low-dose therapy.

Due to all these reasons, monitoring MTX levels is important to assure appropriate levels are maintained during therapy or treatment. Several analytical methods based on different binding assays like fluorescence polarization immunoassay [5] radioimmunoassay [6] and enzyme immunoassay [7] have been developed over time for monitoring the plasma levels of MTX. At the present time, automated binding assays are being widely used in clinical practice, however there are studies reporting that there may be unreliable results due to potential antibody cross reactivity

between MTX and its metabolites [8,9]. Consequently, numerous chromatographic methods have been developed using different conditions for sample preparation, analyte extraction, separation and detection of MTX in biological samples. Despite the prominence of tandem mass spectrometry with higher sensitivity among these methods, MS facilities are not always available in hospital laboratories.

Because of the need for a sensitive, fast and inexpensive method broadly applicable to clinical routines for monitoring of MTX, we aimed to develop and validate a High Performance Liquid Chromatography (HPLC) method with UV detection for the quantitation of MTX in plasma samples.

Material and Methods

Chemicals and Reagents

Chemicals and solvents used for this study such as sodium phosphate, tetrahydrofuran and MTX standard were of high quality and HPLC grade (Sigma-Aldrich, St. Louis, USA).

Instruments and Liquid Chromatographic Conditions

After selecting an efficient extraction method for the analyte, HPLC-UV method was developed and several parameters validated using an HP 1200 series HPLC system interfaced with a HP 1200 series UV detector (Agilent Technologies, Santa Clara, CA, USA).

The chromatographic separation was performed on C18 column (250 x 4.6 mm i.d., 5µm particle size), purchased from Waters, USA with the mobile phases composed of 0.05 M sodium phosphate buffer/ tetrahydrofuran (pH 4.85) (95:5%) and 0.05 M sodium phosphate buffer/tetrahydrofuran (pH 4.00) (75:25%) respectively at a flow rate of 0.6 mL/min. The wavelength of the detection was at 313 nm. The injection volume was 10 µL and the analysis time was 10 min per sample.

Preparation of Stock Solutions and Calibration Samples

Stock solution of MTX was prepared in distilled water at a concentration of 130 µmol/L. Subsequently, working solutions were prepared by diluting stock solution to concentrations of 1, 2.5, 5.0, 10.0, 12.5, 25.0 and 50.0 µmol/L by spiking of blank plasma. For each validation and assay run, the calibration curve standards were prepared freshly, protected from light and stored at 4°C.

Extraction Procedure

We added 250 µL of acetone to 250 µL of plasma. The contents were mixed in a vortex mixer for 15s. The phases were separated by centrifugation at 1900 rpm for 5 minutes and the supernatant was transferred to a conical tube and dried under nitrogen. The residue was dissolved in 300 µL of distilled water and 10 µL of sample solution was injected into HPLC system.

Results and Discussion

Method Development

In method development study, a combination of 0.05M sodium phosphate buffer and 5% tetrahydrofuran were initially selected as mobile phase according to G Biberoglu et.al. [10] However, we have modified the mobile phase since mobile phase peaks have suppressed MTX peaks and peak shape of MTX was not sharp enough. Therefore we have tried to use different organic phases like acetonitrile, methanol i.e. with various concentrations but different concentrations of tetrahydrofuran in sodium phosphate buffer were selected as mobile phases because of good resolution and symmetric peak shapes for MTX with a short run time. According to the chemical properties of MTX, appropriate amount of formic acid added into mobile phase for pH adjustment. It was found that a mixture of 0.05M sodium phosphate buffer + 5% tetrahydrofuran (pH=4.85) and 0.05M sodium phosphate buffer + 25% tetrahydrofuran (pH=4.00) were finally exerted as the mobile phase.

In the first stage, several kinds of columns were compared, such as ACE Phenyl column (4.6x150mmx2µM), ACE C18 column (4.6x100mmx2µM), and ACE C8 column (4.6x5mmx2.1µM). For ACE Phenyl column, retention time of MTX was too long and for ACE C18 column the shapes of the peaks were not sharp enough. In ACE C8 column, we have not controlled stability of system pressure. Finally, Waters C18 analytical column (4.6x250 mm, 5µM) was used with a flow rate of 0.6 mL/min to obtain good peak shapes and permit a run time of 10 min.

Since plasma has complex nature, to prevent potential interferences a pre-treatment procedure is necessary to remove protein in plasma before HPLC analysis. With this purpose, we used acetone for protein precipitation before spiking of MTX solution to the calibration sample. By doing that we increased sample throughput and observed high resolution.

Method Validation

Method Validation study was conducted in accordance with the standards established by the Clinical and Laboratory Standards Institute (CLSI), The validation experiments and results are described below.

Linearity

MTX working solutions were prepared by diluting the stock solution (130 µmol/L) to concentrations range of 1.0-50.0 µmol/L by spiking of blank plasma. Each of these standard solutions was injected three times into the HPLC-column and the peak area was calculated using Agilent ChemStation software. Calibration curve was prepared by plotting peak area (y) versus MTX concentrations (µmol/L) (x) for the method (Figure 1). The regression line (r2> 0.99) demonstrates the excellent relationship between peak area and MTX concentration in the method, over a concentration range of 1.0-50.0 µmol/L.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

Detection limits for MTX are shown in (Table 1).

Precision and Accuracy

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample, while the accuracy of an analytical method describes the closeness of the test results obtained by the method to the normal value of the analyte. The intra-day accuracy and precision of the assay were determined by analyzing 5 replicates containing MTX at two different concentration levels i.e., 5.0 and 50.0 µmol/L. (Table 2) presents the intra- and inter-day

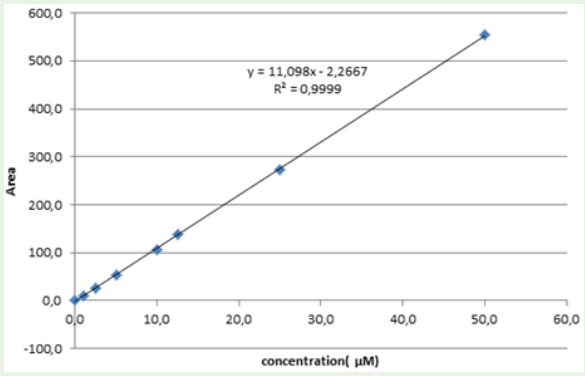

Figure 1: Calibration curve of Methotrexate (peak area versus concentration).

Table 1: Summary of assay parameters for calibrators.

Exact concentration (µmol/L)	1.0 (LLOQ)	2.5	5	10	12.5	25	50.0 (ULOQ)
Mean	1.06	2.45	5.04	9.76	12.53	24.92	50.2
SD	0.05	0.05	0.05	0.04	0.08	0.41	0.34
CV%	4.72	0.82	0.99	0.41	0.64	1.65	0.68
Bias%	6	-2	0.8	-2.4	4.42	-0.32	0.4

% CV calculated as (SD/mean) ×100

% Bias calculated as 100×(mean-exact concentration)/exact concentration

Table 2: Summary of intra-day and inter-day assay parameters for samples.

	Exact concentration (µmol/L)	5	50
	Mean	4.83	49.81
	SD	0.19	0.34
Intra-day	**CV%**	4.02	0.67
	Bias%	-3.4	-0.38
	Mean	4.78	49.7
Inter-day	**SD**	0.28	0.58
	CV%	5.19	1.16
	Bias%	-4.4	-0.6

%CV calculated as (SD/mean)×100,

%bias calculated as 100×(mean-exact concentration)/ exact concentration

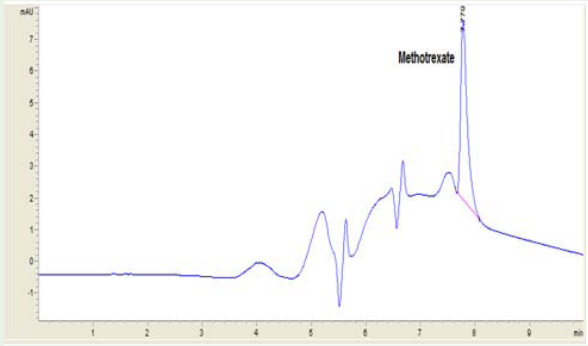

Figure 2: Chromatogram of methotrexate for 5.0 µmol/L. Retention time was 7.78 minutes.

Figure 3: Chromatogram of methotrexate for 50.0 µmol/L. Retention time was 7.78 minutes.

accuracy and precision for each of the samples and (Figures 2 & 3) illustrate chromatograms of them. Our intra- and inter-day accuracy and precision (CV %) acceptance criterion for each sample was ≤20%.

Conclusion

A sensitive, specific and validated gradient HPLC-UV method for quantitative determination of MTX in plasma is described. This simple, rapid, accurate and reproducible method utilizes a single step direct extraction without involvement of expensive solid phase cartridges. The chromatogram yields a well-resolved peak for MTX with good intra-and inter-day precision. This simple HPLC-UV method can be conveniently used as a routine clinical application in conventional hospitals and research laboratories.

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