

Optimization and validation of an analytical method for the estimation of methotrexate in rabbit plasma

Hafiz Rashid Hussain¹, Sajid Bashir^{1*}, Daulat Haleem Khan², Irum Shahzadi³,
Sheikh Abdur Rashid⁴, Muhammad Hashim Khan⁵, Zaineb Abdullah²,
Asif Mahmood⁶ and Muhammad Sarfraz¹

¹College of Pharmacy, University of Sargodha, Sargodha, Pakistan

²Department of Pharmacy, Lahore College of Pharmaceutical Sciences, Lahore, Pakistan

³Department of Biotechnology, COMSATS University Islamabad, Abbottabad campus, Abbottabad, Pakistan

⁴Faculty of Pharmacy, Gomal University, Dera Ismail Khan, Pakistan

⁵Department of Pharmacy, Gomal center of biotechnology and biochemistry, University of Agriculture Dera Ismail Khan, Pakistan

⁶Department of Pharmacy, University of Lahore, Lahore, Pakistan

Abstract: Methotrexate (MTX) is an anticancer drug used for the treatment of various cancers and autoimmune diseases. In this study, High Performance Liquid Chromatography (HPLC) method was developed and validated for the estimation of MTX in rabbit plasma with high estimation rate and recovery. Various validation parameters like, sensitivity, sample recovery, accuracy and precision analysis were studied. The pre-saturated reversed C18 end capped HPLC column was used to separate MTX present in rabbit plasma. A solvent mixture of 100mM phosphate buffer pH 7.4 and acetonitrile (92:8 percent v/v) was employed as the mobile phase. Analysis was carried out at λ max 303 nm and retention time of MTX was found 5.32 min. During the method development and validation ICHQ2 (R1) guidelines were strictly followed. Developed method was found excellent in terms of recovery of MTX from plasma samples (98.6%). It is obvious from the current study that the developed HPLC method can be utilized to analyze the level of MTX in patients. Furthermore, the cost of the developed method for the determination of MTX would be very low as compared to the previously reported methods.

Keywords: Methotrexate, HPLC method development, estimation of MTX in plasma.

INTRODUCTION

Methotrexate (MTX) is an anti-folate agent and is well recognized for the treatment of various categories of cancers and autoimmune diseases. It has been known as a folic acid analogue and is often utilized for the treatment of a variety of malignant and non-malignant illnesses (Cronstein and Aune, 2020). Initially developed as a chemotherapeutic agent, MTX is now the most important disease-modifying antirheumatic drug (DMARD) in the management of different types of arthritis like rheumatoid arthritis, juvenile idiopathic arthritis, and psoriasis.

Chemically, MTX is referred as (2S)-2-[(4-{[2,4-diamino-7,8-dihydropteridin-6yl] methyl} (methyl amino) phenyl) form-amido] pen-tanedioic acid (Choi *et al.*, 2018). Chemical structure (fig. 1a) of MTX (Patel *et al.*, 2018) is very similar to that of folic acid (fig. 1b) (Eftekhari *et al.*, 2020). MTX consists of a pteridine-diamine core and a p-aminobenzoyl portion, linked to a glutamic acid part containing two highly ionizable carboxylic acid groups. MTX is classified as a BCS class IV drug having poor solubility in water (0.01mg/mL at 20°C) and permeability (Giri *et al.*, 2021). MTX solubility in other organic solvents including chloroform is also very low

*Corresponding author: e-mail: rashid.hussain@uos.edu.pk

(Demirbolat *et al.*, 2021). However, its solubility is pH-dependent, requiring neutral or basic solutions for its solubility. The presence of an asymmetric carbon in the structure results in S and R stereoisomers. S-MTX and R-MTX are considered as an active form and an impurity, respectively (Guichard *et al.*, 2017).

The quantification of MTX within physiological samples has been carried out using a wide range of analytical approaches, mostly based on various binding assays. Analytical techniques used in the past were labor-intensive and required the use of radioisotopes (Myers *et al.*, 1975; Steven, 2017)(Agata, 2019). However, other methods have proven to be inaccurate and specific because of possible cross-reactivity between MTX and its metabolites' antibodies (Estève *et al.*, 2006). Different conditions for sample preparation, analyte extraction, separation and detection of MTX in biological samples have resulted in several chromatographic methods being developed. Some of the more sensitive methods, such as fluorescence and tandem mass spectrometry, have been used to find MTX. Because of the continued use of MTX and the fact that MS facilities are not always available as standard equipment in hospital laboratories. Furthermore, handling of mobile phase and flow rate adjustments were also found problematic during the handling of LC-MS

(Karami *et al.*, 2019). Hence, a sensitive, fast, and low-cost method for therapeutic drug monitoring (TDM) of MTX is still required.

The aim of the current study is to develop a simple, rapid, highly specific and sensitive HPLC method for quantifying MTX in serum samples using high-performance liquid chromatography (HPLC). In the present study, HPLC method was developed and validated in accordance to the ICHQ2(R1) guidelines for the estimation of MTX in animal (Rabbit) model. Method developed was also employed to elucidate various validation parameters such as, linearity, selectivity, robustness, limit of detection (LOD), limit of quantification (LOQ), accuracy and precision. Furthermore, estimation of MTX in plasma samples was conducted to determine the reliability of developed method for the MTX separation from the biological fluids. Sample recovery (%) was also measured to see the amount of sample detected by the developed method.

MATERIALS AND METHODS

All materials and reagents used were of analytical grade. Potassium dihydrogen phosphate, ortho-phosphoric acid and Sodium hydroxide, HPLC grade methanol and Acetonitrile (ACN) were purchased from Sigma-Aldrich Co., St Louis, MO, USA. MTX was purchased from Vanz Pharma, Hubei, China. Experimental subjects (Albino rabbits) were procured from animal house of University of Sargodha, Sargodha, Pakistan.

HPLC method Development

Animals

Twenty-four healthy adult albino rabbits (acquired from Animal House Facility, University of Sargodha, Pakistan) were used as experimental subjects to perform the study. Albino rabbits (weight range of 2.5 kg \pm 0.1) were included in this study while, pregnant female rabbits were excluded before assigning into control or experimental group. Subjects were provided with *ad libitum* of water and standard rodent chow along with standardized conditions in a transient room at 25 \pm 2 $^{\circ}$ C while keeping relative humidity at 65 \pm 5%, and light/dark cycle ratio at 12h (Cho *et al.*, 2021). Subjects were divided into A & B groups (12 subjects in each group) for the comparison of variation in plasma levels after administration of MTX. They were kept in especially designed wooden boxes during dosing and specimen collection. Each animal was administered with 20 mg MTX oral suspension for the determination of MTX in the plasma of experimental subjects.

Chromatographic instrumentation and conditions

The method development was performed on HPLC Agilent 1200 series (M/s Agilent, Germany), installed with the ATVP quaternary pumps (G1311A), thermostat

columns, autosamplers (G1329A) and UV-visible Detector. The pre-saturated reversed C18 end capped HPLC column (Agilent Eclipse \times DB, 5 μ m, 4.6 \times 150 mm, M/s Agilent, USA) was used to separate the chromatograph. A solvent mixture of 100mM phosphate buffer pH 7.4 and acetonitrile (92:8 percent v/v) was employed as the mobile phase. Analysis was carried out at λ max 303 nm and results were analyzed by Chemstation software of Agilent 1200 series provided by M/s Agilent, Germany.

Sample Preparation procedure

Various organic solvents including methanol, ACN, perchloric acid, silver nitrate trichloroacetic acid, and aqueous phase combinations of organic solvents are widely utilized for protein precipitation present in biological samples (Li *et al.*, 2015). In the present study, equal amounts of ACN and plasma samples were mixed vortexed for 5 min and then centrifuged at 1000 rpm for 10 min to cause protein precipitation during sample preparation. Supernatant was then passed from the 0.45 μ m pore size syringe filters before injecting into the HPLC column.

Preparation of standard solution of MTX

MTX stock solution (100mg/mL) was prepared in 100mM phosphate buffer solution pH 7.4. Various dilutions (20-1000ng/mL) were prepared from MTX stock solution and passed through membrane filters (0.45 μ m) before injecting into the chromatographic column.

Analytical method validation

Analytical method validation is a critical requirement during the development of a robust, reliable and reproducible analytical method. By employing ICHQ2(R1) guidelines, the optimized HPLC technique was validated for linearity, accuracy, precision, detection limit (DL), and quantification limit (QL) (Kumar *et al.*, 2007).

Linearity

Samples (20 μ L) of each concentration of MTX dilutions (20ng/ml to 1000ng/ml) were injected in HPLC column to determine the linearity range of the developed HPLC method. Results obtained for peak area were further analyzed by Least Square Regression analysis against each concentration. In addition, residual analysis was also performed between observed responses and predicted values at a confidence interval of 95% by employing an MS-Excel 2019 spreadsheet (M/s Microsoft Inc., Washington, USA) (Beg *et al.*, 2016).

Accuracy

To estimate the accuracy of the developed method, MTX recovery (%) from plasma sample were analyzed with MTX dilutions (20ng/mL, 300ng/mL, and 1000ng/mL). Furthermore, (SEM) and relative standard deviation (% RSD) were also measured (De Abreu *et al.*, 2015).

Precision

Precision is a significant parameter to analyze the method reliability developed in similar instrumental conditions over time. Measurements of MTX dilutions (LQC, 20ng/mL; MQC, 300ng/mL, and HQC, 1000ng/mL) were conducted intra-days (repeated observation on the same day) and inter-days (repeated observations on the next day) to elucidate the recovery (%) of MTX. The percentage of RSD, SEM, and the standard deviation (SD) value were also measured in accuracy studies.

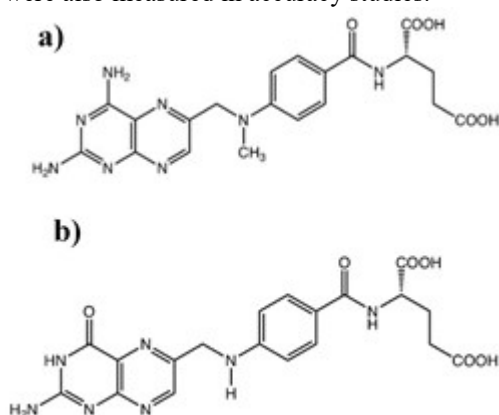


Fig. 1: Chemical Structure of a) MTX and b) Folic acid

Quantification limit (QL) and detection limit (DL)

Sensitivity of developed HPLC method was assessed by QL and DL, depending on the values of SD and slope of the calibration curve by the equation given below (Karami et al., 2019).

$$DL = 3.3 \times \frac{\sigma}{s} \quad QL = 10 \times \frac{\sigma}{s}$$

Where "σ" is the standard deviation and "s" is the slope.

The experimental procedure utilized in the current study was reviewed and authorized by Institutional Biosafety and Ethical Review Committee, vide notification number SU/ORIC/79.

STATISTICAL ANALYSIS

For the statistical data analysis, GraphPad Prism version, 5.01 was employed. Results were presented as mean standard deviation (SD). Obtained results were further processed statistically by employing one-way ANOVA. If the calculated $*p \leq 0.05$ it showed significant difference between two groups. Similarly, $**p \leq 0.01$ for very significant and $***p \leq 0.001$ for highly significant difference.

RESULTS

Development and validation of HPLC method for the estimation of MTX in rabbit plasma

For the optimization of HPLC method to separate MTX from plasma samples, different mobile phase combinations were also investigated either by varying the

percentage of organic and aqueous components or by employing various buffer systems with different gradients with methanol, acetonitrile, or distilled water. Different buffers such as Na Acetate, Acetate, sodium phosphate buffer, and phosphate buffer solution were also investigated to achieve an optimum separation of MTX (Begas et al., 2014; Li et al., 2015; Michail and Moneeb, 2011; Uchiyama et al., 2012). Results revealed that phosphate buffer pH 7.4 was found appropriate to obtain the symmetry within peaks. Furthermore, a mobile phase having potassium dihydrogen phosphate (100mM), sodium hydroxide buffer pH 7.4 and acetonitrile (92:8 % v/v) produced adequate peak symmetry. Other optimized chromatographic conditions are enlisted in table 1.

Analytical method validation

Selectivity

By adjusting above mentioned conditions, chromatograms of different samples such as blank plasma (fig. 2), blank MTX (fig. 3), and spiked MTX in plasma (fig. 4) were recorded. It has been observed that there was a good separation of MTX in the plasma with no interference between the two peaks. Retention time of plasma and MTX in spiked plasma was found to be 1.98 min and 5.32 min, respectively.

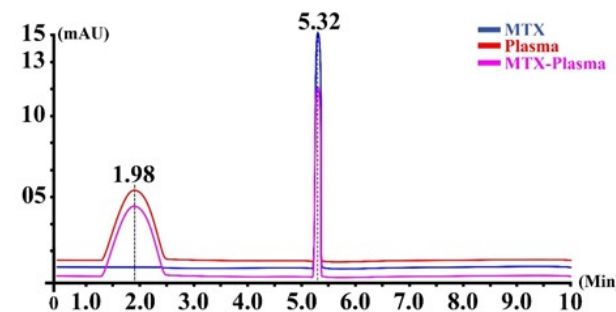


Fig. 2: Chromatogram of MTX, Plasma sample and Plasma spiked with MTX under optimized chromatographic Conditions.

Standard Curve and Linearity

Standard curve was developed by plotting MTX plasma concentrations against peak area of the chromatograms. Standard curve was made on three consecutive days to avoid any type of variation and characterized for linearity. Different concentrations of MTX (20ng/mL to 1000 ng/mL) were prepared and analyzed on each day. Various parameters were checked during the construction of calibration curves (table 2).

Value of regression coefficient (R^2) was found to be 0.999, which indicate an acceptable linearity for the estimation of different concentration of MTX in plasma.

Accuracy & Precision

Standard curve has also been analyzed for accuracy and precision by performing HPLC under optimized

Table 1: Optimized Chromatographic conditions

Chromatographic Parameters	Optimized Conditions
Flow rate	0.8 mL / min
Wave length	303 nm
Injection volume	20 μ L
Retention Time	5.32 min
Run Time	10 min
Temperature	25 ⁰ C
Pressure	110-126 bar
Mobile Phase	PBS pH 7. 4: ACN (92: 8 % V/V)
pH	pH of the buffer is 7.4
HPLC column	C18
HPLC detector	UV

Table 2: Standard Curve Analysis for Linearity Measurements

Run Number	Equation Form Y=mx+ b		Correlation Coefficient (r ²)
	Slope (m)	Intercept (b)	
Day 1	0.07	44.31	0.999
Day 2	0.04	23.68	0.998
Day 3	0.03	17.78	0.999
Mean \pm SD	0.047 \pm 0.002	28.59 \pm 3.58	0.999 \pm 0.0003

conditions. For this purpose, high, medium and low concentrations of MTX spiked plasma were studied for intra-day and inter-day variations (table 3). Furthermore, statistical difference was not found between inter-day and intra-day samples when ANOVA was applied on the results.

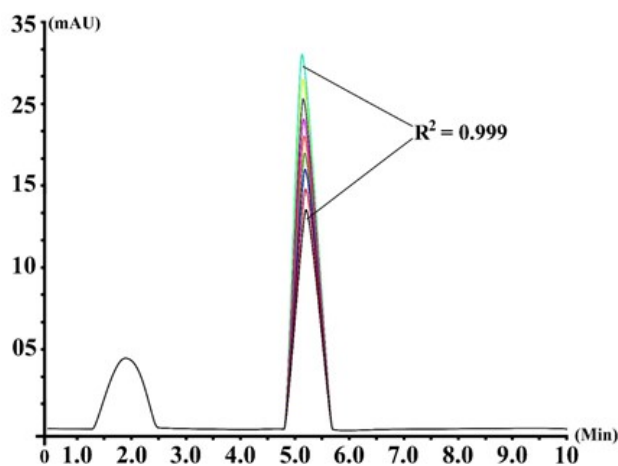


Fig. 3: Standard Curve and linearity data obtained under optimized HPLC conditions after spiking plasma samples with different concentrations of MTX.

Sensitivity and the measurement of Quantification limit (QL) and detection limit (DL)

Sensitivity of developed HPLC method was investigated for the MTX detection in plasma samples. Calculated lower limit of detection (LLOD) value was found to be 10ng/mL. However, amount of quantified MTX in plasma

was 20ng/mL which was subjected to the calibration curve analysis for measured precision and accuracy.

Recovery of MTX from Plasma Samples

Optimization of the technique for the extraction of MTX from plasma sample is a significant factor as it presents a source of error. ACN was added in equal quantities with plasma samples and MTX recovery (%) was analyzed by optimized HPLC conditions. MTX recovery (%) from the samples was found in different QC levels. Low, medium and high recovery rates were 96.8%, 97.2% and 98.4%, respectively (table 4). Hence, ACN was found to be an optimum solvent for the MTX extraction from the plasma samples.

DISCUSSION

The results of linearity analysis are concordant with previously reported study where fluorometric estimation of MTX in human plasma by liquid chromatography declared the regression coefficient (R²=0.999) (Uchiyama *et al.*, 2012).

During accuracy and precision analysis calculated values of % RSD and % RE were found to be within the limits of acceptability (\pm 15 %), presenting the ruggedness of the established HPLC method for the identification of MTX in plasma samples (Jain *et al.*, 2019).

Limit of quantification is also compared with a previous study, LLOD and lower limit of quantification (LLOQ) reported by Karami *et al.*, for MTX in plasma sample

Table 3: Intra-day and Inter-day accuracy & precision data

Conc. of MTX in plasma	Observed Conc. Of MTX by the purposed Method					
	Intra day			Inter day		
	Mean (n=12)	%RSD	%RE	Mean (n=12)	%RSD	%RE
20	19.90	0.96	0.51	19.94	1.60	0.30
300	297.71	0.70	0.77	296.05	1.01	1.31
1000	994.9	0.43	0.53	991.59	0.69	0.84

Table 4: Recovery (%) of MTX from plasma samples

Level	Concentration (ng/ml)		% Recovery of pure drug	Mean SD RSD (%)
	Amount added	Amount recovered		
LQC	20	9.92± 0.08	96.8%	0.81
MQC	300	29.42± 0.09	97.2%	0.31
UQC	1000	69.02± 0.51	98.4%	0.74

were 4ng/ml and 5ng/mL, respectively, showing an enhanced sensitivity of their developed method (Karami et al., 2019).

Uchiyama et al., (2012) employed trichloric acid (5%) with ACN (5%) to remove plasma proteins and found efficient MTX recovery (81.2% to 81.5%) in the plasma samples (Uchiyama et al., 2012). This indicates the highly efficient recovery obtained during current study in contrast to the reported method.

CONCLUSION

During this study, HPLC method was developed and validated for an efficient detection of MTX in the plasma of rabbits. MTX recovery (%) from the plasma sample was found to be 98.6% while, the detection limit was found very low (20ng/mL). Furthermore, the retention time of the MTX was found 5.32min in the HPLC chromatograph. Therefore, the developed HPLC method may be used for the separation of MTX from plasma samples with high throughput, high accuracy, and recovery. In addition, developed HPLC method may be utilized for the estimation of MTX in the plasma of patients receiving the MTX therapy to treat various chronic diseases and hence, can provide appropriate tool in the Therapeutic drug monitoring.

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