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RESEARCH ARTICLE

Rapid Reverse-phase High-performance Liquid Chromatography Estimation of Methotrexate in Bulk, Pharmaceutical Preparation and in Spiked Plasma Samples

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ABSTRACT

A simple, sensitive, and selective reverse-phase high-performance liquid chromatography (RP-HPLC) method with ultraviolet (UV) detection for the estimation of methotrexate in pharmaceutical formulation and in spiked plasma developed and validated in the present work. Chromatographic separation of drug is performed with a 250 mm × 4.6 mm, 5 µm diameter particles RP C-18 column and the mobile phase consisted of a mixture of methanol and water (80:20, v/v), containing 0.1% HPLC-grade glacial acetic acid for the adjustment of pH to 4.5. Isocratic elution at a flow rate of 1 ml/min with UV detection at 256 nm at ambient temperature is used in this method. The proposed RP-HPLC method is successfully applied for the determination of methotrexate in pharmaceutical preparation and spiked plasma samples. The validation studies are carried out and it's fulfilling ICH requirements. The method is found to be specific, linear, precise (including both intra- and inter-day precision), accurate, and robust. This proposed method may represent a valuable aid in the laboratory monitoring of the toxicity of anticancer chemotherapy.

Keywords: Estimation, methotrexate, reverse-phase high-performance liquid chromatography method, spiked plasma samples, toxicity monitoring

INTRODUCTION

Methotrexate (MTX) [Figure 1] is the most common and widely used anticancer drug which is official in both the USP[1] and the BP.[2] MTX is a drug included into the antineoplastic and antirheumatic therapeutic categories. It belongs to antifolates, which employed frontline for the chemotherapy of leukemia, solid tumor, and other choriocarcinomas.[3-7] Many analytical methods have been reported for the analysis of MTX in pharmaceutical formulations and in biological fluids using liquid chromatography (LC),[8-14] capillary zone electrophoresis,[15,16] spectrophotometric,[17,18] and voltammetric techniques.[19-21] These methods suffer from long-time analysis, pre-step derivatization or not satisfactory purity estimation in pharmaceutical formulations. Till this date, no simple reverse-phase high-performance LC (RP-HPLC) method has been reported for the estimation of MTX in pharmaceutical preparation and in spiked plasma samples. In general, anticancer drugs are highly toxic with a narrow margin of safety. Therefore, patients should be carefully supervised since therapeutic response is unlikely to occur without some evidence of toxicity. This establishes the need to develop a method that permits determination MTX in plasma of cancer patients. The aim of the present study was to develop and validate a simple RP-HPLC method with ultraviolet (UV) detection for the direct analysis of MTX in pharmaceutical formulations such as tablets and injections and in spiked plasma samples. The proposed method is quite fast and effective for the determination of MTX in plasma sample.

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MATERIAL AND METHOD

Experimental instrumentation

The chromatographic system consisted of an L-7110 solvent delivery system (Merck, Hitachi), L-7170 UV-Visible detector (Merck, Hitachi), and Rheodyne injector valve bracket fitted with a 20 µl sample loop. HPLC separations were performed on a stainless-steel LichroCART C-18 analytical column (250 mm × 4.6 mm) packed with 5 µm diameter particles, LichroCART, HPLC guard cartridge system, and a Winchrom software on an IBM-compatible PC connected to a printer.

Materials and reagents

Methotrexate tablets (Neotrexate Tablets, Emil Pharmaceutical, Tarapur, India) and injection (Alltrex Inj, VHB) were purchased from the market. All reagents were of HPLC analytical grade, namely: Methanol and acetic acid (Merck) while HPLC-grade water from Qualigens, Mumbai (India). The reference standard of MTX was received as gift samples from Dabur Research Foundation, Hyderabad, India.

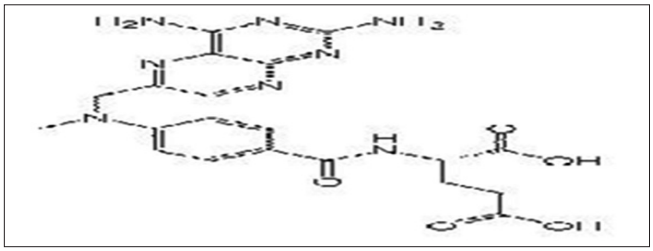

Figure 1: Chemical structure of the methotrexate

Table 1: System suitability parameters of MTX

Compound	*t*	*n*	*R*	*T*
MTX	2.5	2562	0.95	0.58

t: Retention time (min), *n*: Number of theoretical plates, *R*: Retention factor, *T*: Tailing factor. MTX: Methotrexate

Chromatographic conditions

The mobile phase consisted of mixture of methanol and water (80:20, v/v) containing acetic acid (0.1% v/v) to maintain the pH to 4.5. Prior usage, the mobile phase was degassed and filtered by passing through a 0.45 µm pore size membrane filter (Millipore, Milford, MA, USA). The elution was carried out under isocratic condition at a flow rate of 1.0 ml/min, with UV detection at 256 nm at ambient temperature. The system suitability parameters of MTX are summarized in Table 1.

Standard solutions and calibration graphs

Reference standard of MTX (10.0 mg) was transferred to 100 ml volumetric flask and dissolved in 0.01 N NaOH. The flask was shaken for 5 min and the volume was made up to the mark with same solvent to obtain standard stock solution of MTX (100 µg/ml). This stock solution was further diluted with appropriate quantity of mobile phase to obtain working standard solutions of suitable concentrations (corresponding to the linearity range stated in Table 2). Triplicate 20-µl injections were made for each concentration and chromatographed under the above-mentioned conditions. The peak area of each concentration was plotted against the corresponding concentration to obtain the calibration graph.

Analysis of standard laboratory samples

Three standard laboratory samples of MTX (2, 4, and 8 µg/ml) were prepared in triplicate. About 20 µl injections were made for each concentration and are chromatographed under the above-mentioned conditions. The peak area of each concentration was extrapolated in calibration curve to obtain the corresponding concentration [Table 3].

Table 2: Linear regression and statistical parameters of MTX by proposed HPLC method

Linearity range* (µg/ml)	Regression data*			S^{da}	S^{db}	LOD (µg/ml)	LOQ (µg/ml)
	a	b	c				
0.5–16	3947	82465	0.9975	1026	2326	0.25	0.40

*Denotes average of three determinations. [a]Intercept, [b]slope, [c]correlation coefficient, [d]standard deviation of intercept. MTX: Methotrexate, HPLC: High-performance liquid chromatography

Table 3: Result of precision and accuracy in standard laboratory samples of MTX by developed method

Sample concentration (µg/ml)	% Recovery±SD[a]	RSD[b] (%)	Er[c] (%)
2	99.6±0.22	0.52	0.4
4	99.2±0.69	0.61	0.8
8	99.5±0.33	0.72	0.5

[a]Mean±standard deviation of three determinations, [b]percentage relative standard deviation, [c]percentage relative error. MTX: Methotrexate

Table 4: Result of the analysis of commercial formulation of MTX by proposed method

Commercial MTX tablet	Amount	Amount present*	Percentage of drug found	SD[a]	RSD[b]
Neotrexate tablets	2.5 mg/tab	2.49 mg/tab	99.98	0.362	0.135
Emil pharmaceutical Alltrex Inj, VHB	50 mg/2 ml	49.97 mg/2 ml	99.94	0.295	0.117

*Average of three determinations. [a]Standard deviation, [b]relative standard deviation. MTX: Methotrexate

Table 5: Assay result of analysis of commercial formulation of MTX in spiked plasma samples by proposed method

Spiked concentration (µg/ml)	% Recovery±SD[a]	RSD[b] (%)	Er[c] (%)
2	99.1±0.22	0.22	0.9
4	99.0±0.69	0.41	1.0
8	99.2±0.31	0.52	0.8

[a]Mean±standard deviation of three determinations, [b]percentage relative standard deviation, [c]percentage relative error. MTX: Methotrexate

Analysis of commercial pharmaceutical formulation

Twenty tablets were weighed and powdered; the quantity of the powder equivalent to 10 mg of MTX was weighed accurately and transferred into a 100-ml volumetric flask and the volume made up to 100 ml with 0.01 N NaOH. This solution was sonicated for 10 min, filtered; the filtrate containing extracted MTX was diluted appropriately with mobile phase and chromatographed exactly as under the assay of MTX as presented in Table 4.

Analysis of spiked plasma samples was used which thawed at room temperature before use. About 0.5 ml aliquots of plasma were transferred into centrifuge tubes. The plasma sample in each tube was spiked with a suitable amount of standard MTX solution as presented in Table 5. For protein precipitation, 4.5 ml methanol was mixed with each sample and centrifuged for 10 min at 1000 rpm. After decantation, the solutions were injected into HPLC and chromatographed under above-mentioned conditions.

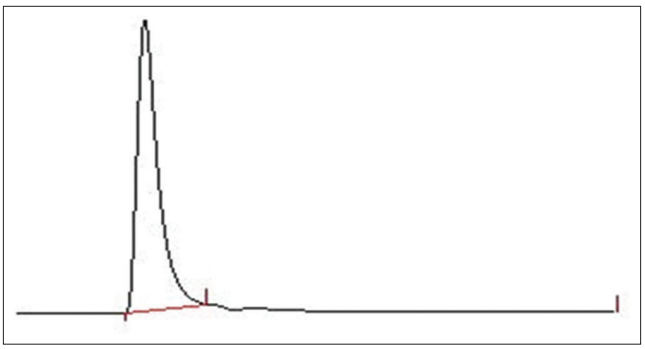

Figure 2: Chromatogram of 20-µl injection of pure methotrexate

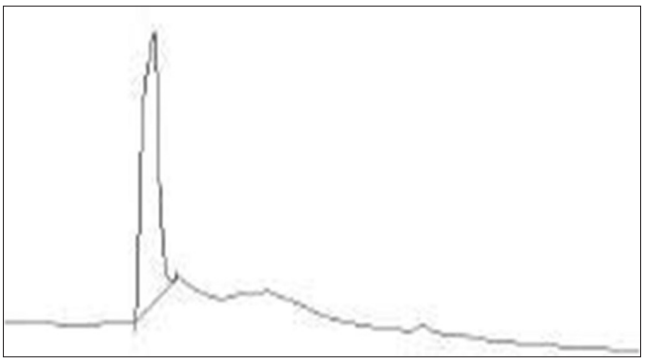

Figure 3: A chromatogram of a 20-µl injection of plasma sample spiked with methotrexate

RESULTS AND DISCUSSION

The typical peak and response obtained from the proposed analytical method on the newer HPLC system as shown in Figure 2. The typical chromatogram of a plasma sample spiked with MTX shown in Figure 3. The absorption spectrum of MTX in aqueous acid exhibits three maxima in the UV region at 256, 304, and 373 nm, but maximum absorbance of MTX was found at

256 nm. Therefore, the wavelength of 256 nm was selected for the estimation of MTX. Estimation of MTX was achieved by LichroCART RP C18 column and methanol:water:acetic acid pH 4.5, (80:20:0.1 v/v) as mobile phase, at a flow rate of 1.2 ml/min. These experimental conditions allowed the accurate determination of MTX with peak at retention time of 2.5 min.

Optimization of chromatographic conditions

To optimize the HPLC assay conditions, the effects of the percentage of methanol as well as the effect of pH of the mobile phase were studied.

Effect of methanol percentage in the mobile phase

The results showed that a satisfactory peak was obtained with a mobile phase consisting of 80% methanol for MTX. Different percentage of methanol in the mobile phase was studied as a function of retention time as well as peak symmetry for MTX. It was observed that 80% of methanol provided optimum resolution with the most symmetric and well-defined peak. At lower methanol concentration, the peak showed excessive tailing and increased retention times. Increasing methanol concentration led to improper peak of MTX.

Effect of pH

The effect of the pH of the aqueous component of the mobile phase was studied using aqueous phases at various pH values between 3.0 and 7.0 (adjusted using acetic acid/sodium hydroxide). These solutions were used with 80% of methanol as the mobile phase. The pH showed marked effect on the retention and symmetry of MTX, where a pH 4.5 was selected as it provided optimum resolution that was similar to that achieved at higher pH values but with the added advantage of increased speed and compound being eluted out within only 2.5 min.

Statistical analysis of results

Concentration ranges and calibration graphs

Under the above described experimental condition, linear relationship was observed by plotting graph between drug concentrations against peak area. The corresponding concentration ranges are listed in Table 2. The slopes, intercepts, and correlation coefficients obtained by the linear least 5. Squares regression treatments of the results are also given in the same table. The high values of the correlation coefficients (r = 0.999) with negligible intercepts indicated good linearity of the calibration graphs. Standard deviations of intercept (Sa) and slope (Sb) were also calculated.

Detection and quantitation limits

Limit of detection (LOD) and limit of quantitation (LOQ) were calculated from the linear regression equation. The LOD and LOQ were found to be 0.25 and 0.45 μg/ml, respectively, which are presented in Table 2.

Precision and accuracy

To assess the precision, as percentage relative standard deviation; and the accuracy, as percentage relative error, of the proposed HPLC method; triplicate determinations were carried out onto the samples of different proportions. The data represented in Tables 3 and 5 show good accuracy and precision of the proposed analytical procedure.

Analysis of pharmaceutical formulation

Appropriate dilutions of pharmaceutical formulation of MTX including tablet and injection were made and analyzed by the proposed HPLC method. The obtained results are listed in Table 4. The accuracy and precision were found satisfactory to be with labeled claim.

Analysis of spiked plasma samples

The proposed HPLC method was applied for the determination of MTX in plasma of female BALB/c mice spiked with drug. Methanol was used for protein precipitation before sample preparation in proposed method. Specificity of the method was assessed after carrying out the chromatographic procedure on blank plasma samples (after protein precipitation) and since no interfering peaks were detected at retention

time of the analyte, it was concluded that no endogenous substance from plasma interfered with the assay. To assess precision of method, three determinations for each concentration were examined and standard deviation was calculated. The results obtained are listed in Table 5 that shows that the proposed method is quite effective and reproducible for the routine determination of MTX in plasma sample.

CONCLUSION

The proposed HPLC method can be readily applied for the determination of MTX in pharmaceutical formulation and in plasma samples. The proposed analytical procedure is simple and economic in terms of both, cost and time. The method is highly specific and there is no interference from any of the additive present in the sample. The method is quite selective, sensitive, and suitable for routine blood drug monitoring of MTX that may represent a valuable aid in the laboratory monitoring of the toxicity of anticancer therapy.

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