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DEVELOPMENT AND VALIDATION OF RP- HPLC METHOD FOR THE DETERMINATION OF METHOTREXATE IN BULK AND PHARMACEUTICAL TABLET DOSAGE FORM

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ABSTRACT

A new RP-HPLC method was developed and validated for determination of Methotrexate in bulk and tablet dosage form. The estimation was carried out on Enable C18 (250 mm ×4.6 mm, 5 µm) column using Distilled water: Acetonitrile in the ratio of 80:20 (v/v) at pH 3 with Formic acid as mobile phase. The flow rate was 1.0 ml/min and the effluent was monitored by UV detector at 211 nm. The retention time was 3.28 min and linearity was observed in the concentration range of 8-60µg/ml. The percentage recovery was in good agreement with the labelled amount in the pharmaceutical formulations and the method was simple, precise and accurate for the determination of Methotrexate in bulk and pharmaceutical formulations.

KEYWORDS: Methotrexate, HPLC, Validation.

INTRODUCTION

Methotrexate is described chemically L-Glutamic acid, N-{4-[[(2, 4-Diamino-6-pteridinyl) methyl] methylamine] benzoyl}-, Folex: Methotrexate; Mexate. It is a class of anticancer drug. It is abbreviated MTX and as amethopterin is antimetabolite and antifolate drug.[1-3] The drug is official in Indian pharmacopoeia[4], USP[5] and BP.[6] Literature survey reveals that there are few UV Spectroscopic methods[7-11] and one HPLC.[12] Method is reported for the determination of methotrexate in plasma and urine of humans, rats and dogs. The target of this study is to develop new, simple and fast analytical methods by HPLC to quantify Methotrexate in bulk and its tablet dosage forms together with its latter validation study. This validation study is defined as the laboratory studies by which it is established that the performance characteristics of the method meet requirements for the intended analytical application.

This work describes the validation parameters stated by the International Conference on Harmonization[ICH] guidelines includes specificity, linearity, range, accuracy, precision, robustness, LOD, LOQ.

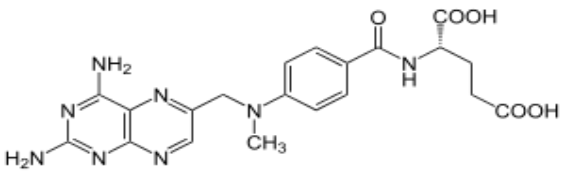

Figure 01 Structure of Methotrexate

MATERIALS AND METHODS

INSTRUMENT

The equipment used for the method was Agilent Technology, Singapore 1120 Compac LC. The Column selected for the method was Cogent C18, 250mm x 4.6mm, 5µ. The flow rate was monitored at 1.0 mL/min. The temperature of the column oven was 25ºC± 2º C. Shimazdu Ax200 balance was used. Calibrated glassware's were used for the study. Modern Industrial Corporation ultrasonicator.

Reagents and chemicals

The chemicals were used for the process double Distilled Water [HPLC grade] Acetonitrile [HPLC grade] and Formic Acid [AR grade] all these chemicals were from Merck.

METHOD DEVELOPMENT

Preparation of mobile phase

The mobile phase was consists of Distilled water: Acetonitrile in the ratio of 80:20 (v/v). The prepared mobile phase was degassing in ultrasonic water bath for 5 minutes and it was filtered through 0.45 µ filter under vacuum filtration.

Preparation of standard stock solution

Take approximately 10 mg of standard Methotrexate sample into a 100 ml of volumetric flask. Add 70 ml of acetonitrile and allow to sonicate for 15 minutes, then volume make upto 100 ml with distilled water.

Chromatographic conditions

The optimum compositions of the mobile phase containing Distilled water: Acetonitrile 80:20 (v/v) was selected because the peak pattern and retention time were satisfactory. The flow rate was set to 1 ml/min and UV detection was carried out at 211 nm for the analysis of Methotrexate. The mobile phase and samples were degassed by replacing in ultrasonicator for 30min and filtered through 0.22µ membrane filter paper respectively. All determinations were performed at ambient column temperature. The chromatogram of standard Methotrexate is shown in figure 02.

Preparation of calibration curves of Methotrexate

Appropriate aliquots of the standard stock solution of Methotrexate were pipetted out and transferred to a serious of 10 volumetric flasks. The volume was made up to the mark with mobile phase to obtained working standard solution of Methotrexate of concentrations 20µg/ml. Triplicate dilutions of each solutions, 20µl injections of each concentration of the drug were injected into the HPLC system three times separately and chromatographed under the conditions as desired above. Evaluation of the drug was performed with the UV detector set at 211 nm and the peak areas were recorded.

RESULTS AND DISCUSSION

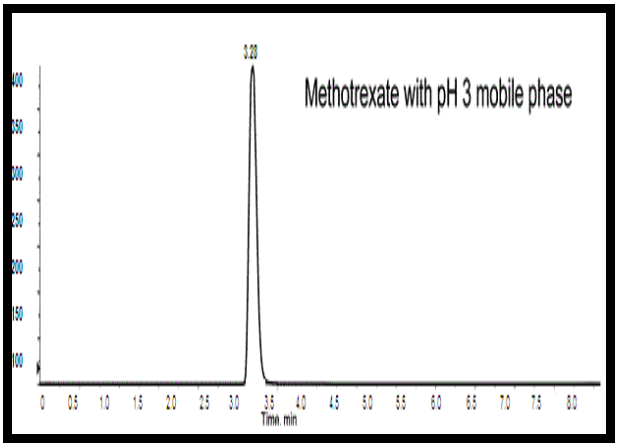

Figure 02 HPLC chromatogram of standard Methotrexate.

Calibration curves of Methotrexate

The standard calibration table and graph are shown in Table 01 and figure 03 respectively.

Table 01: Calibration Table for Methotrexate.

Sr. No.	Conc. of Methotrexate (µg/ml)	Area under curve (AUC)*
1	8	1510021
2	10	19589846
3	20	28686723.39
4	30	38284845
5	40	47254318
6	50	58744982
7	60	69477868

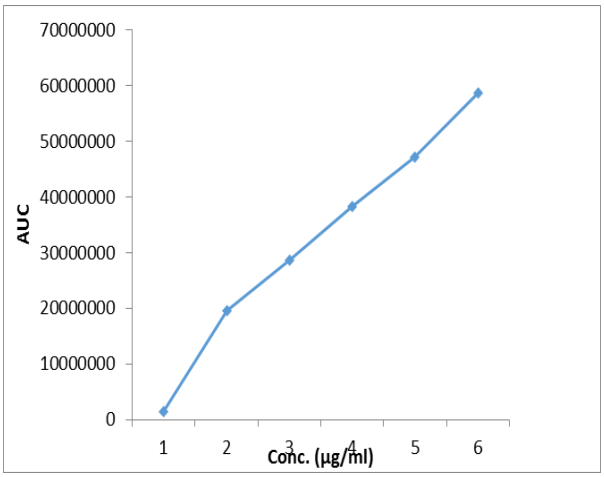

Figure 03: Standard calibration curve of Methotrexate.

Linearity

The calibration curve was found to be linear and in adherence to Beer's law over the concentration range of 8-60µg/ml. The linearity was validated by the value of the correlation coefficient (r^2±S.D. =0.9847±0.000198).The results of the linearity studies given in table 02.

Table 02: Linear regression data for calibration curves of Methotrexate.

Drug	Linearity Range (µmg/ml)	Slope ±S.D.*	y-intercept ±S.D.*	Regression Coefficient (r^2±S.D.)*	LOD (µg/ml)	LOQ (µg/ml)
METHOTREXATE	8-60	143381	94672	0.9847 ±0.000198	0.0000279 x10^{-05}	0.0000847 x10^{-05}

*n=6

Analysis of the Tablet formulation (Assay of Tablet)

A single peak at a retention time of 3.28 min was observed in the chromatogram of the drug samples extracted from tablet, indicating that there is no interference of the excipients presents in tablet formulation. The mean % drug content was found to be 102.489% with a mean % R.S.D. of 0.3969.The results of the analysis of tablet formulations T_1 is given in table 03 respectively with statistical validation data are given in table 04.

Tablet T_1: Methotrexatein
Table 03: Analysis of the Tablet formulation (T_1).

Table 04: Statistical Validation of Tablet Analysis.

Tablet	% Mean	S.D.*	% R.S.D.*	S.E.*
T_1	100.79	0.4778	0.3969	0.1951

*n=6, Tablet T_1

Recovery studies (Accuracy)
The recovery studies were carried out at 80, 100 and 120% of the test concentration as per ICH guidelines. The results of the recovery studies and the statistical validation are given in Table 05and 06 respectively.

Table 05: Recovery Studies.

Tablet Sample	Level of recovery (%)	Amount present (mg/tab)	Amount of Std. added (mg)	Total amount recovered (mg)	% Recovery
T_1	80	2.5	8	10.47	99.76
		2.5	8	10.48	99.89
		2.5	8	10.48	99.862
	100	2.5	10	12.47	99.83
		2.5	10	12.498	100
		2.5	10	12.489	99.95
	120	2.5	12	14.46	99.74
		2.5	12	14.41	99.413
		20	12	14.38	99.238

*n=3 at each level of recovery.

Table 06: Statistical Validation of Recovery Study.

Sr. no.	Tablet Sample	Type of Recovery (%)	(%) Mean*	S.D.*	C.O.V.*	S.E.*
1	T_1	80	99.83	0.272	4.7	0.065421
2		100	99.92	0.309	3.2	0.1408
3		120	99.46	0.646	3.3	0.1067

*n= at each level of recovery

Precision:
The repeatability of sample application and measurement area of peak area were expressed in terms of % R.S.D. and found to be less than 2%. The mean intra -day and inter- day precision was found to be 0.3154 and o.5257 respectively and given in Table 07.

Table 07: Precision of the method.

Precision	% Mean*	S.D.*	% R.S.D.	S.E.*
Intra-day	98.21	0.3098	0.3154	0.0178
Inter –day	99.43	0.5228	0.5257	0.03018

*n=6

Robustness of the method.
To evaluate the robustness of the method, each parameter selected was varied at three levels (-1, 0, 1). The results presented in table 08 Indicated that the selected factors (retention time t_R and tailing factor t) were unaffected by small variations in the selected method parameters.

Table08: Robustness evaluation of the HPLC method (n=3*)

Changes Factor	Level	Chromatographic t_R	t
Flow Rate (ml/min)			
0.9	-1	3.08	0.76
1.0	0	3.28	0.72
1.1	1	3.18	0.81
Mean ± S.D. (n=3)		3.18±1.847	0.76 ± 0.0450

% of acetonitrile in the mobile phase (v/v)			
78:22	-1	3.20	0.61
80:20	0	3.28	0.74
82:18	1	3.10	0.69
Mean ± S.D. (n=3)			3.19±0.3686
0.68±0.0655			
Temperature (^{0}c)			
23	-1	1.913	0.62
25	0	1.914	0.65
27	1	1.913	0.60
Mean ± S.D. (n=3)		1.913 ± 0.0005	0.62 ±0.025
PH			
2.9	-1	1.921	0.64
3	0	1.943	0.65
3.1	1	1.976	0.67
Mean± S.D. (n=3)			1.946 ±0.0276
0.065±0.015			

Where t_R= Retention time and t=Tailing factor

SUMMARY AND CONLUSION

Literature survey reveals that there are few methods like separation and identification of Methotrexate by using HPLC and HPLC-EIMS in human plasma. Keeping this point in consideration, an attempt was made to develop a simple, fast accurate and precise alternative HPLC method, with a mobile phase consisting of Distilled water: Acetonitrile (80:20 v/v). The chromatographic condition was set at a flow rate of 1ml/min with the UV detector at 211nm.

Review of literature indicates that the developed method for analysis of Methotrexate is simple, accurate, precise and economic as compared to the reported methods as the developed methods uses diode array detector. The retention time for Methotrexate was found to be 3.28 min. Hence it can be conclude that the developed HPLC method can be employed successfully for the estimation of Methotrexate in both bulk and Tablet formulation.

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