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QbD Approach Method Development and Validation for the Simultaneous Estimation of Methotrexate and Folic acid by UV Spectrophometric and RP-HPLC in Bulk and Tablet Dosage Forms

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Abstracts:

An accurate, precise and reproducible UV Spectrophometric and RP-HPLC method was developed for the simultaneous quantitative determination of Methotrexate and Folic acid in tablet dosage forms with the help of QbD approaches. Agilent (S.K.) Gradient System UV Detector and C_{18} column with 100mm x 4.6 mm i.d and 5µm particle size Methanol: ph Buffer (75:25v/v) (pH 3.3 0.1% OPA with TEA) was used as the mobile phase for the method. The detection wavelength was 249 nm and flow rate was 1.0 ml/min. In the developed method, the retention time of Methotrexate and Folic acid were found to be 5.25 and 7.35min. The LOD and LOQ of Methotrexate were found to be 0.1272 µg/ml and 0.3875, Folic acid were found to be 0.028 µg/ml and 0.087 µg/ml, respectively. The proposed method is The developed method was validated according to the ICH guidelines. The linearity, precision, range, robustness was within the limits as specified by the ICH guidelines. Hence the method was found to be simple, accurate, precise, economic and reproducible. The developed method was validated according to the ICH guidelines. In this methods linearity, precision, range, robustness were observed. The method was found to be simple, accurate, precise, economic and reproducible. So the proposed methods can be used for the routine quality control analysis of Methotrexate and Folic acid in bulk drug as well as in formulations.

Keywords: QBD, RP-HPLC, UV, Methotrexate, Folic acid, development, validation,

INTRODUCTION

The ultimate goal of chemotherapy is a cure, suppression of every neoplastic cell require a true treatment. If treatment is not achievable, then the goal becomes control of the disease to extend survival and maintain the best quality of life. This allows the individual to maintain a normal existence with the cancer thus being treated as a chronic disease. In either case neoplastic cell burden is initially reduced, either by surgery or by radiation followed by chemo therapy immunotherapy or a combination of these treatment modalities. In advanced stages of cancer, the likelihood of controlling the cancer is far from reality and the goal is palliation. This mean that chemotherapeutic drugs may be used to relieve symptoms caused by the cancer and improve the quality of life, even though the drugs may not lengthen life. Treatment of cancer include log kill, pharmacologic sanctuaries, combinations of drugs – cytotoxic agents with qualitatively different toxicities, and with different molecular sites and mechanisms of action, are usually combined at full doses. This results in higher response rates, due to additives and potentiated cytotoxic effects, and non- overlapping host toxicities, advantages of drug combinations, treatment protocol. Some problems associate with chemotherapy like resistance, multidrug resistance, toxicity followed by common adverse effect, minimizing adverse effects, treatment include tumor.

Methotrexate is an antimetabolites which structurally related to normal compounds that exist within the cell. And interfere with purine /pyrimidine nucleotide precursors available by inhibit their synthesis, their maximum cytotoxic effect are in s-phase. The vitamin folic acid plays a central role in a variety of metabolic reactions involving the transfer of one carbon units and is essential for cell replication. Methotrexate is structurally related to folic acid and acts as an antagonist of that vitamin by inhibiting dihydrofolate reductase. Folic acid is obtained from dietary sources or from that produced by intestinal flora. It undergoes reduction to the tetrahydrofolate form via a reaction catalyzedby intracellular nicotinamide-adenine dinucleotide phosphate-dependent. Methotrexate enters the cell by active-transport processes that normally mediate the entry of N^5-Methyl-FH_4. Literature gave brief information of method development on bulk of methotrexate and folic acid followed validate that method as per ICH guideline on spectrophotometry and HPLC method over a QbD approach. Specific method are reported for the analysis and determination of MTX & FA in bulk and dosage form. The reported method is complex and time consuming hence there was a need for developing a validated method for estimation of MTX & FA in pharmaceutical dosage form [1-2].

Methotrexate (MTX), 4-amino-N10-methylpteroylglutamic acid (Figure 1)., is an antifolate drug, developed as the first targeted anticancer agent in 1940 [3]. It is administered at a high dose to treat several types of cancers, such as acute human leukemia, breast cancer, osteogenic sarcoma, head and neck carcinomas, prostate and bladder cancers [4-7]. It is also administered at a low dose as a remedy for a variety of autoimmune and inflammatory diseases, such as rheumatoid arthritis (RA), psoriasis, sarcoidosis, and systemic lupus erythematosus [8].

Folic acid chemically known as (2S)-2-[(4-{[(2,4-Diaminopteridin6yl) methyl] (methyl) amino} benzoyl) amino]pentanedioic acid (2S) 2 [[4[(2 Amino 4 oxo 1Hpteridin 6 yl) methyl amino] benzoyl] amino]

pentanedioic acid (Figure 2). Molecular formula $C_{19}H_{19}N_7O_6$ [9-10]

Literature review reveals that, Methotrexate also reported in combination with other drugs Similarly, Folic Acid is reported for spectrophotometric, RP-HPLC and simultaneous estimation with other combinations [11-17]. Since no spectrophotometric method is reported for simultaneous estimation of Methotrexate and Folic acid in combination therefore, the present work, a successful attempt has been made to estimate both these drugs simultaneously by to simple UV spectrophotometric and RP-HPLC methods development. The present study aimed to develop a simple, sensitive, short retention time and accurate UV spectrophotometric and RP-HPLC method for the simultaneous determination of both Methotrexate and Folic acid together in pure and tablet dosage forms with high sensitivity, selectivity that can be used for the routine analysis of production samples. Validation of the developed method done in accordance with ICH guidelines. [13]

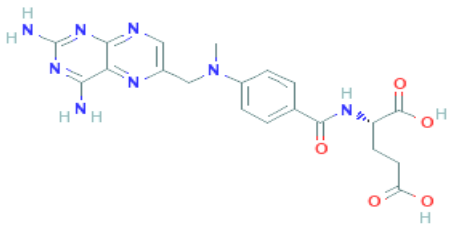

Figure 1: Structure of Methotrexate

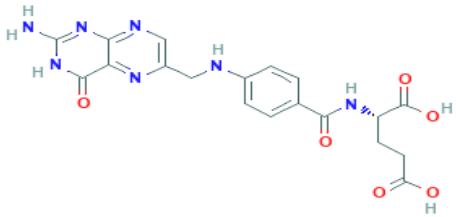

Figure 2: Structure of Folic acid

MATERIALS AND METHODS

Materials and Reagents

The analysis of the drug was carried out on Agilent (S.K.) gradient system UV detector. Equipped with reverse phase (Agilent) C_{18} column (4.6mm x 100mm; 2.5µm), a SP930d pump, a 20µl injection loop and UV 730d (dad) absorbance detector and running Chemstation software.

Methotrexate and Folic acid were procured from R.S.I.T.C Jalgaon. Orthophopsphoric acid (OPA) (Avantor Performance material India Ltd. Thane, Maharashtra) and methanol, acetonitrile, (HPLC grade Merck Specialties Pvt. Ltd. Shiv Sager Estate 'A' Worli, Mumbai.), water, 0.45 µm filter (Millipore, Bangalore). A combination of Methotrexate 7.5mg and 1mg Folic acid in tablet formulation was procured from Intra Lab. India Pvt. Ltd. (Truxofol 7.5 : 1mg brand).

Chromatographic Conditions

Column C_{18} (100 mm× 4.6 mm); particle size packing 2.5 µm ; detection wavelength of 249 nm; flow rate 1.0 ml/min; temperature ambient; sample size 20 µl; mobile phase Methanol : water (75 : 25) (pH 3.3 0.1% OPA with TEA); run time of 15 mins.

Preparation of standard stock solution

Folic acid standard stock solution (Stock I)

An accurately weighed quantity, 10 mg of Folic acid (FA) was dissolved in methanol in a 10 ml volumetric flask and volume made up to 10 ml to produce a solution of 1000 µg/ml.

Methotrexate standard stock solution (Stock II)

An accurately weighed quantity, 75 mg of Methotrexate (MTX)was dissolved in methanol in 10 ml volumetric flask and volume made up to 10 ml to produce a solution of 7500 µg/ml **(**Figure 3).

Preparation of Stock Standard Combination Solution (Stock III) [FA + MTX]

Accurately weight and transfer 10 mg Folic acid and Methotrexate 75 mg working standard into 10 ml volumetric flask as about diluent methanol completely and make volume up to the mark with the same solvent to get 1000 &7500 µg/ml standard (stock solution) and 15 min sonicate to dissolve it and remove the unwanted gas, further an aliquots portion of Folic acid and Methotrexate stock solution in ratio of 1:7.5 were mixed in volumetric flask in 10 ml and volume was adjusted up to mark with mobile phase from the resulting solution 0.1ml was transferred to 10 ml volumetric flask and the volume was made up to the mark with MEOH :Water (0.1% OPA), prepared in (75 ml MEOH : 25 ml Water (0.1% OPA) solvent .Result as shown as (Figure 4);

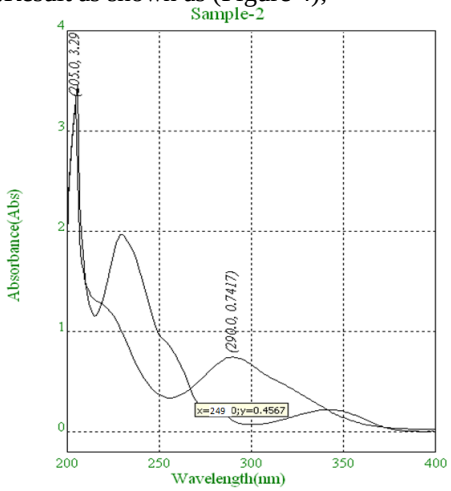

Figure 4: Typical UV spectrum of methotrexate and folic acid

Instrumentation:

The study was processed on Agilent 1100 series instrument. Chemstation software using c18 column, length 100 mm, internal diameter 4.6 µm. Particle size DAD detector.

Methotrexate (BIOTREXATE; EMTEXATE; NEOTREXATE), It is a 4-amino-4-deoxy-10-methylpteroyl-L-glutamic acid. It is yellow to orange-brown, crystalline powder. It is practically insoluble in water. It dissolves in dilute solutions of alkali hydroxides and carbonates. The solution are sterilized by filtration. It

is stored in well- closed, light-resistant containers. Methotrexate is mainly used in the management of acute lymphoblastic leukemia and for the treatment of chorio carcinoma. It has been used as an immunosuppressant. It is given by mouth, or by injection as methotrexate sodium. Folic acid having structural units corresponding to pteridine, p-amino benzoic acid, L-glutamic acid. Chemical name of folic acid is 4-(2-amino-4-hydroxypteridin-6-yl) methyl amino benzoyl-L-glutamic acid. Folic acid is free or combined with several L-glutamic acid moieties in peptide linkage, in liver, yeast, leafy green vegetables, and certain other natural products. It may be prepared synthetically. It is yellow to yellowish orange, odorless crystalline powder, practically insoluble in cold water, soluble in dilute sodium hydroxide solution. Fitting the experimental data to a suitable mathematical model followed by exhaustive data analysis was carried out by using JMP software. Polynomial equations in the form of prediction expression were prepared considering the coefficients of significant model terms with probability value less than 0.05 according to Analysis of Variance (ANOVA). Model suitability was evaluated through lack of fit and correlation coefficient values. Two-dimensional contour (2-D) and three-dimensional response surface (3-D) profiler were analysed to establish the relationship between CMVs and CAA. The optimum method conditions were optimized by numerical and graphical mode within the robust design space. (Figure 5 and 6).

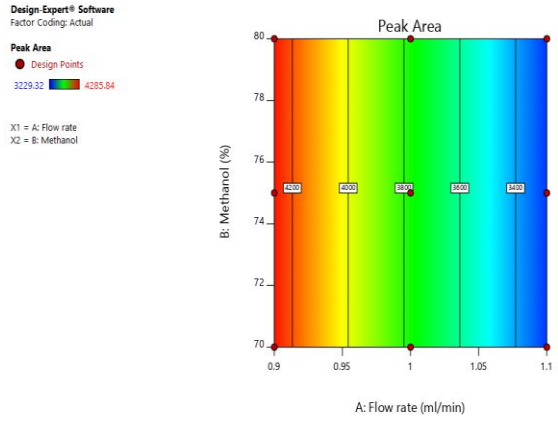

Figure Error! No text of specified style in document.**5: Design space obtained for optimized experimental condition**

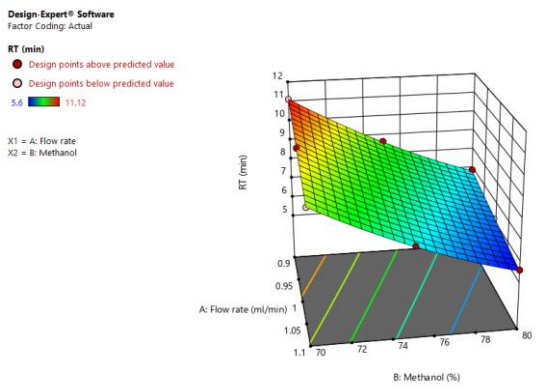

Figure 6: 3D structure for response at flow rate and composition

Assay preparation of marketed formulation

Determination of assay method followed by weighing a 20 tablet of marketed brand contain MTX and FA. Calculate the total weight into average weight of tablet for measure the equivalent with methotrexate 7.5mg and folic acid 1 mg crush the all 20 weighed tablet into fine powder with help of mortar and pestle, take out 165 mg powder which equivalent with MTX and FA. Dilute in 10 ml MEOH. To ensure complete extraction it was sonicated for 15 min. 0.3 ml of supernatant was then diluted up to 10 ml with mobile phase. The resulting solution was injected in HPLC and drug peak area was noted (Figure 7 and Table 1).

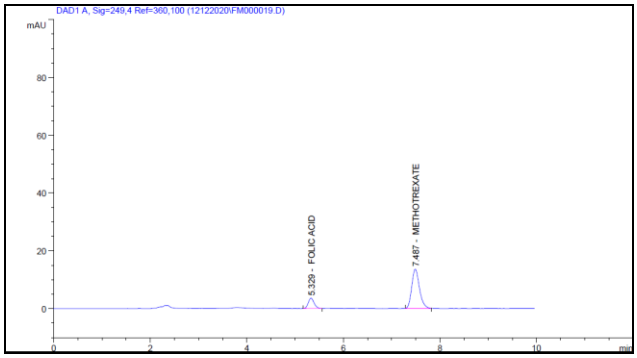

Figure 7: Chromatograph of analyzed marketed dosage form

Table 1: Analysis of marketed formulation

Assay	Drug	Amt. Found	%Label Claim	SD	%RSD
Rp-HPLC Method	FA	15.038	101.33	0.04	0.16
	MTX	50.14	99.40	0.04	0.16
UV Method	FA	24.85	100.88	0.35	0.37
	MTX	20.73	100.13	0.64	0.61

Method validation:

The proposed methods were validated in accordance to ICHQ2 (R1) guideline for precision, accuracy, linearity, robustness, limit of detection and limit of quantification [18-21].

RESULTS

Linearity and Range

The mobile phase was allowed to equilibrate with the stationary phase until OPA by baseline was obtained. From the freshly prepared standard stock solution, pipette out 75 mg MTX and 10 mg FA in 10 ml of volumetric flask and diluted with the mobile phase. From it 0.05, 0.1, 0.15, 0.2 and 0.25 of solution were pipette out in 10 ml volumetric flask and volume were made up to 10 ml with mobile phase to get final concentration 5, 10, 15, 20 and 25 µg/ml of Folic acid and 37.5, 75, 112.5, 150 and 187.5 µg/ml of Methotrexate. The respective linear equation for Methotrexate wasy = 1.413x - 0.2545 and Folic acid equation y = 2.1861x - 0.4417where x is the concentration and y is area of peak. The correlation coefficient was 0.999 and 0.999. The calibration curve of Methotrexate and Folic acid is depicted in (Figure 8 and 9).

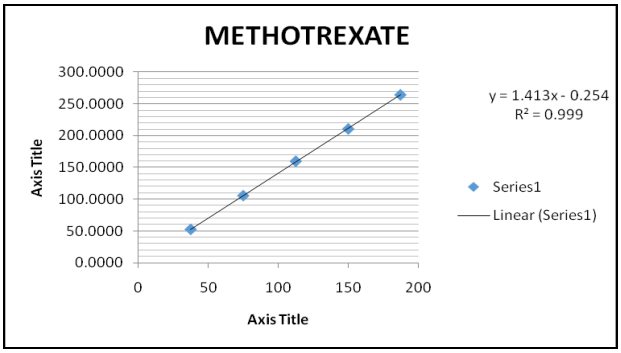

Figure 8: Calibration curve for methotrexate.

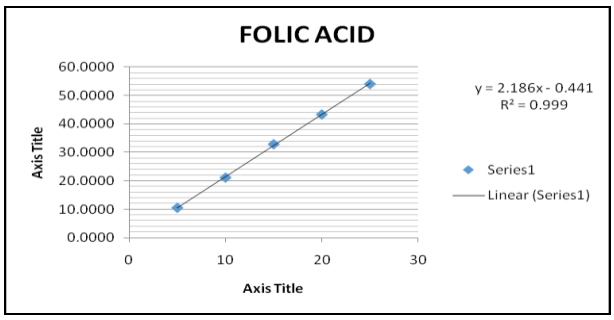

Figure 9: Calibration curve for folic acid

Accuracy

Recovery study done to validate the accuracy of the developed method. To pre-analyzed tablet solution, a definite concentration of the standard drug (80%, 100% and 120%) was added, and then its recovery was analyzed Table 5. The accuracy of UV spectroscopic method was ascertained by recovery studies performed at different levels of concentrations (80%, 100%, and 120%). The % recovery was found to be within 98-101%. Statistical validation of recovery studies shown in (Table 2 and 3).

Precision

Precision was studied to find out intra and inter-day variations in the test method of MTX and FA. Intra-day precision was determined by analyzing three concentrations in three replicate measurements of within the linearity range of drugs on three different times in the same day. Inter-day precision was conducted during routine operation of the system over a period of 3 consecutive days. Intraday and Inter day Precision studies on UV method for MTX and FA, which shows the high precision % amount in between 98% to 101% indicates to analytical method that concluded (Table 4).

Table 2: Recovery data of methotrexate and folic acid by HPLC method

Method	Drug	Level %	Amt. taken µg/ml	Amt. added µg/ml	Absorbance mean ± S.D	Amount recovery mean ± S.D	% recovery mean ± S.D
RP-HPLC Method	FA	80%	10	8	18.02±0.021	8.02±0.021	100.333±0.26
		100%	10	10	19.98±0.007	20.58±0.007	99.80±0.007
		120%	10	12	21.96±0.022	20.58±0.022	99.67±0.18
	MTX	80%	75	60	135.29±0.69	60.29±0.69	100.48±1.14
		100%	75	75	150.33±0.15	20.58±0.15	100.44±0.20
		120%	75	90	164.42±0.59	20.58±0.59	99.36±0.66
UV Method	FA	80%	1	0.8	1.81±0.01	0.81±0.01	101.25±0.88
		100%	1	1.0	2.01±0.01	1.01±0.01	101.00±1.41
		120%	1	1.2	2.22±0.01	1.22±0.01	101.25±1.18
	MTX	80%	7.5	6	13.36±0.04	5.86±0.03	97.65±0.49
		100%	7.5	7.5	15.17±0.02	7.63±0.04	102.28±0.25
		120%	7.5	9.0	16.78±0.04	9.28±0.04	102.84±0.09

Table 3: Recovery data of methotrexate and folic acid by UV method

Method	Level of Recovery (%)	Drug	% RSD	Standard Deviation*	Mean % Recovery
Rp-HPLC Method	80%	FA	0.26	0.26	100.33
		MTX	1.14	1.14	100.48
	100%	FA	0.07	0.07	99.80
		MTX	0.20	0.20	100.44
	120%	FA	0.18	0.18	99.67
		MTX	0.66	0.66	99.36
UV Method	80%	FA	0.87	0.88	101.25
		MTX	0.50	0.49	97.65
	100%	FA	1.40	1.41	101.00
		MTX	0.25	0.25	102.28
	120%	FA	1.16	1.18	101.25
		MTX	0.09	0.09	102.84

Table 4: Intra day and Inter day precision of methotrexate and folic acid

Method	Drug	Conc. (µg/ml)	Interday Precision		Intraday Precision	
			Mean± SD	%Amt Found	Mean± SD	%Amt Found
RP-HPLC Method	FA	10	9.89±0.96	98.93	10.00±0.65	100.00
		15	15.37±0.11	102.51	15.45±0.28	102.05
		20	19.76±0.15	98.80	19.87±0.14	99.38
	MTX	75	105.55±0.9	99.82	105.41±0.65	99.69
		112.5	161.51±0.8	102.39	161.02±0.73	101.44
		150	209.37±0.17	98.84	209.37±0.17	98.84

UV Method	FA			MTX		
	1	0.13±0.001	98.50	15	0.41±0.001	100.95
	2	0.15±0.003	98.83	22.5	0.61±0.001	101.02
	3	0.17±0.001	101.63	30	0.81±0.001	101.83

Limit of detection and quantification

LOD is the lowest amount of analyte in a sample that can be detected but not necessarily quantify under the stated experimental conditions. LOQ is the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under stated experimental conditions. The LOD and LOQ of MTX were found to be 0.023 µg/ml and 0.71 µg/ml, FA were found to be 0.033 µg/ml and 0.01 µg/ml, respectively.

DISCUSSION

The proposed methods for simultaneous estimation of MTX and FA in tablet dosage forms were found to be simple, accurate, economical, and rapid. The method was validated as per the ICH Q2 (R1) guidelines. Standard calibration yielded a correlation coefficient (r2) of 0.999 for both MTX and FA at all the selected wavelengths. The values of % RSD are within the prescribed limit of 2%, showing high precision of methods, and recovery was close to 100% for both drugs. Results of the analysis of pharmaceutical formulations reveal that the proposed method is suitable for their simultaneous determination with virtual interference of any additive present in pharmaceutical formulations. Hence, the above methods can be applied successfully for simultaneous estimation of MTX and FA in formulations.

CONCLUSION

The developed UV spectrophotometric method in that linearity, precision, range, and robustness were found to be more accurate, precise, and reproducible. The methods were found to be simple and time-saving. All proposed methods could be applied for routine analysis in quality control laboratories.

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Conflict of Interest

Authors have no conflicts of interest to declare.

Abbreviation used:

HPLC: High performance liquid chromatography; **UV:** Ultraviolet; **ICH:** International Conference on Harmonization; **LOQ:** Limit of quantitation; **LOD:** Limit of detection; **RSD:** Relative standard deviation; **RT:** Retention time; **OPA:** Orthophosphoric acid; **MTX:** Methotrexate; **FA:** Folic acid; **FDA:** Food and Drug Administration; **SD:** Standard deviation.

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