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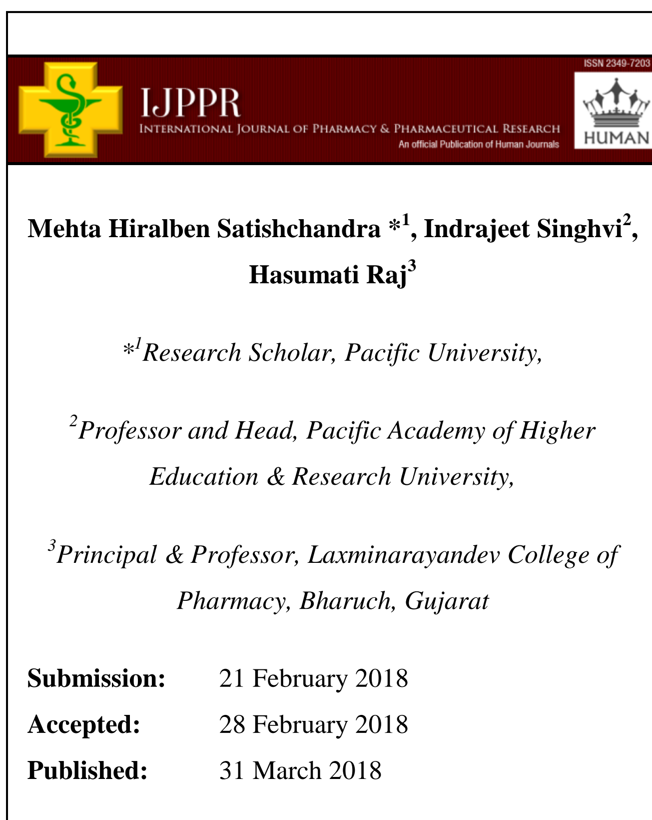
Forced Degradation Studies and Development and Validation of Stability-Indicating RP-HPLC Chromatographic Method for Mycophenolate Mofetil Assay and Related Substances



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ABSTRACT

Mycophenolate mofetil (MYCO) is 2-morpholin-4-ylethyl (E)-6-(4-hydroxy-6-methoxy-7-methyl-3-oxo-1H-2-benzofuran-5-yl)-4-methylhex-4-enoate, it's antineoplastic antibiotic derived from various Penicillium fungal species. In this study, degradation behavior of Mycophenolate mofetil was studied by subjecting the drug to various ICH stress conditions. A new, simple, sensitive and accurate stability-indicating method was established for quantitative determination of Mycophenolate mofetil in the presence of related compounds. An expectable separation was achieved with ODS C_{18} column with flow rate 1.0 ml/min. UV Detection wavelength at 250nm was used for estimation of Mycophenolate mofetil over a concentration range of 10 – 300 µg/ml with the mean recovery of 99.86 – 101.54 %. Methods can analysis Mycophenolate mofetil related compound LOQ limit up to 0.002 µg/ml. A method can well resolve all degraded product as compare to Mycophenolate mofetil. Developed method can routinely use for the estimation of Mycophenolate Mofetil related compounds from the dosage form and for stability sample.

INTRODUCTION

Stability testing and forced degradation studies play a very crucial role during drug development. Stability is fundamental to all product characteristics, and the term "stability indicating assay" has been used to describe "a procedure which affords specific determination of a drug substance in the presence of its degradation products". The prime goal of studying the stability of a drug is to determine the shelf life of the drug. Identification of the degradation products, the establishment of degradation pathways, determination of intrinsic stability of the drug molecules, and validation of the analytical procedure are some of the goals achieved by stress testing.

The various conditions specified for forced degradation studies include thermal, acidic, alkaline, and neutral hydrolysis conditions and oxidative and light stress.

Mycophenolate mofetil MYCO) is, 2-morpholin-4-ylethyl (E)-6-(4-hydroxy-6-methoxy-7-methyl-3-oxo-1H-2-benzofuran-5-yl)-4-methylhex-4-enoate, occurs as white or almost white crystalline powder (1).

Mycophenolic acid is an active metabolite of the prodrug Mycophenolate mofetil. Mycophenolic acid inhibits *inosine monophosphate dehydrogenase* (IMPDH), preventing the formation of guanosine monophosphate and synthesis of lymphocyte DNA that results in inhibition of lymphocyte proliferation, antibody production, cellular adhesion, and migration of T and B-lymphocytes. Mycophenolic acid also has antibacterial, antifungal, and antiviral activities. (NCI04) (2).

Several methods have been described for the determination of Mycophenolate mofetil in pharmaceutical preparations including HPTLC, HPLC and NMR had been used for the determination of mycophenolate mofetil. (1), (3), (4), (12), (13) the proposed method was validated as per ICH guidelines According to International Conference on Harmonization (ICH).

In the present study, during Alkali degradation, it was observed potential degradation behavior of MYCO to form Impurity-I, while during Oxidative degradation significant degradation product has been observed as Impurity-II. The structure of possible related compounds/degradants is identified/characterized by the various characterization techniques such as UV, IR, and NMR & Mass and chromatographically by HPLC spiking studies.

Also, developed methods are precise, accurate, specific and sensitive stability indicating methods for estimation of MYCO in presence of its degradation products.

MATERIALS AND METHODS

Instrumentations:

Instruments:

A Shimadzu HPLC, Model: LC-10ATvp (Shimadzu) with the rheodyne injector, UV-Visible detector, Model: SPD-10 AVP (Shimadzu) and class VP software.

HPLC Column, C_{18} (size-250 x 4.60 mm, I.D-5 μ) (Phenomenex).

Nylon filters 0.45 μm.

PH meter (Thermo Electro Corporation).

The drug was weighed on the balance, Model ALC 210.4 (Acculab).

Sonicator used was Ultra Sonicator (Fast Clean Ultrasonic Cleaner).

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Reagents and Materials

Mycophenolate Mofetil (MYCO) was kindly supplied as gift samples by Torrent Research Center, Ahmadabad, India. HPLC grade Acetonitrile and sodium acetate was purchased from S.D. Fine Chemicals Ltd. (Mumbai).

The water for HPLC was prepared by triple glass distillation and filtered through a nylon 0.45 μm – 47 mm membrane filter (Gelman Laboratory, Mumbai, India).

Sodium hydroxide, hydrochloric acid, and 30 % Hydrogen peroxide was purchased from Qualigens Fine Chemicals (Glaxo Ltd.).

AR grade Acetonitrile was purchase form ACS chemicals (Ahmedabad).

Impurity-Impurity-II and I are in-house isolated by degradation process of Mycophenolate Mofetil.

Chromatographic conditions

HPLC method. – The mobile phase has been used for separation consisting of Acetonitrile: sodium acetate buffer (40:60 v/v, pH 5.4, with phosphoric acid) using a phenomenax C_{18} column with flow rate 1.0 ml/min. The elution was monitored by peak area at 230 nm, and the injection volume was 20 µL.

Stress studies

Acidic conditions:- For acidic hydrolysis, Acid degradation study was performed by treating the sample with 5ml of 2 N hydrochloric acids, kept at about 60°C for 4 hours and mixture was neutralized by adding 5 ml of 2N NaOH analyzed as per method (Standard Solution preparation procedure).

Alkaline conditions: - Alkaline degradation studies were performed by keeping the drug content in 5 ml of 2 N NaOH, kept at about 60°C for 4 hours and the mixture was neutralized by adding 5 ml of 2N HCl and analyzed as per method (Standard Solution preparation procedure).

Oxidation: - Oxidative degradation study was performed by treating the sample with 30 %w/v Hydrogen peroxide, kept at about 60°C for 14 hours and analyzed as per method.

Photodegradation studies: - Photodegradation studies were carried out by exposing the drug powder drug in a photostability chamber for 10 days. The powder was spread as a thin layer in a Petri plate. The samples of both solution and powder were kept in parallel in darkness for the same period.

Thermal stress studies:- The bulk drug, in a thin layer in a Petri plate, and drug solution (1000 µg/ ml) were exposed to thermal stress conditions in a hot air oven at 100ºC for 24 hours.

Preparation of standard stock solutions

MYCO (100 mg) was weighed accurately and transferred to the 100 ml volumetric flask. It was dissolved in 50 ml acetonitrile properly and diluted up to mark with acetonitrile to obtain the final concentration of 1000 µg/ml. 10 µg/ml solution was prepared for the related compound.

Preparation of related compounds stock solutions

The separate stock solution of related compounds Impurity I and II of 10 µg/ml were prepared by dissolving 10 mg of each of related substance in 100 ml of acetonitrile. Further diluted 5 ml of resulted solution to 50 ml of Acetonitrile.

System Suitability Test

System suitability test of the chromatographic system was performed before each validation run using five replicate injections of a standard solution. Theoretical plates and tailing factor were determined.

Method validation

Calibration curve– From the stock solution of MYCO (1000 µg/ml) and MYCO related compound (10 µg/ml), appropriate aliquots selected to prepared final concentrations of 10 to 300 µg/ml of MYCO and 0.1 to 3 µg/ml of MYCO related compound solution. All these solutions were injected into HPLC column and the peak area of each solution was measured at the selected wavelength. The figure shows the resolution of MYCO and its related substance.

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Accuracy (percentage Recovery)

To ensure the accuracy of the method, recovery studies were performed by standard addition method at 80 %, 100 % and 120 % levels of drug concentrations, to the pre-analyzed samples and they were re-analyzed.

The accuracy of the method for all the related substances was determined by analyzing MYCO sample solutions spiked with all the related substances at three different concentration levels of 0.1 µg/ml, 1 µg/ml and 3 µg/ml and the sample concentration of 1000 µg/ml each in triplicate.

Precision

Repeatability

Repeatability was performed by analyzing six separate MYCO solutions of concentration 1000µg/mL that were prepared by spiking the related substances to give 1 µg/mL of each of Impurity I and II. The % R.S.D for each related substance was evaluated.

Intermediate Precision

The intermediate precision of the method for MYCO and related substances was determined on three separate sample solutions prepared by spiking the related substances by two different analysts on two different days. The mean values of results for each day and for each analyst were compared.

Robustness

The robustness of the method was checked by repeatedly injecting (n = 5) standard solutions of 100 µg/ml in two C_{18} column one was made by phenomenex and one by hypersil for the HPLC method.

Limit of Detection and Limit of Quantification

The limit of detection (LOD) and the limit of quantification (LOQ) of the drug were calculated using the following equations as per International Conference on Harmonization (ICH) guideline[17].

$$LOD = 3.3 \times (X/S)$$

$$LOQ = 10 \times (X/S)$$

Where X = the standard deviation of the response and S = the standard deviation of y-intercept of regression lines.

Isolation of MYCO related compounds

Drug substance was kept under Alkaline medium for 24 hrs and impurity formation compound was filtered and isolated to check the retention time of degradation product formation. Formed product retention time is matching with the degradation product observed

during alkali degradation. Further Impurity was purified by preparative TLC method. Alkali degradation impurity was detonated as Impurity – II.

Drug substance was kept with 30% H_2O_2 in the alkaline medium for 24 hrs and impurity formation compound was filtered and isolated to check the retention time of degradation product formation. The retention time of product formed is matching with the degradation product observed during peroxide degradation. Further Impurity was purified by preparative TLC method. Oxidative degradation impurity was detonated as Impurity –II.

Analysis of MYCO related compound in MYCO tablet formulation

The full content was transferred into a 10 ml volumetric flask containing 5 ml ACN, sonicated for 15 min and further diluted to 10 ml with ACN. The resulting solution was sonicated for 10 min and the supernatant was filtered through Whatman filter paper no.41. 20 µl of this solution was injected into HPLC column for two times and peak area was measured at 230 nm and an average was considered for HPLC method.

The amount of MYCO in sample solution was determined by fitting the responses into the regression equation of HPLC.

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RESULT AND DISCUSSION

HPLC Method

To optimize the HPLC parameters, several mobile phase compositions were tried. A satisfactory separation and good peak symmetry for MYCO was obtained with a mobile phase consisting of Acetonitrile: sodium acetate buffer (40:60 v/v, pH 5.4, with phosphoric acid). The elution was monitored by peak area at 230 nm, and the injection volume was 20 µL using phenomena C_{18} columns with flow rate 1.0 ml/min. The detection wavelength was 230 nm. A complete resolution of the peaks with clear baseline separation was obtained (Figure 1).

Three related substances were detected and well resolved by the method. The retention data for MYCO and related substances is indicated in Table 1.

Validation of the Proposed Method

Linearity: – Linear correlation was obtained between peak areas and concentrations of MYCO in the range of 10 – 300 µg/ml and 0.1 to 3 µg/ml for MYCO related compounds. The linearity of the calibration curves was validated by the high value of correlation coefficients of regression (Table 2). Figure 2 shows that MYCO can well separate from its all type of degradation products so developed HPLC method is specific and selective for MYCO.

Accuracy: – The recovery experiments were carried out by the standard addition method. The recoveries obtained were ranged between 99.23 – 101.76 %. The values of % assay range 99-102% indicated there is no any interference form excipient present in the formulation.

Precision was expressed in terms of % R.S.D. All values for precision were within recommended limits.

The % RSD values for precision and LOD and LOQ were reported in Table 2.

CONCLUSION

In this study, it was possible to develop a selective and validated stability-indicating HPLC assay method for Mycophenolate mofetil on a C_{18} column, which could separate the drug and its degradation products formed under a variety of stress conditions. MYCO was found to be sensitive to the alkali and oxidative condition, whereas it was comparatively stable in Acid, thermal and photolytic condition. TLC used to isolated and separate MYCO form its degradation products. Based on NMR and Mass data three Impurities were isolated and characterized as Impurity I, and Impurity II (2-(morpholin-4-yl)ethyl (4*E*)-6-(4,6-dihydroxy-7-methyl-3-oxo-1,3-dihydroisobenzofuran-5-yl)-4-methylhex-4-enoate, known as impurity A).

The results of the analysis of pharmaceutical dosage forms by the proposed methods are highly reproducible and reliable and are in good agreement with the label claim of the drug. The method can be used for relates substance analysis of MYCO in pharmaceutical preparation and it is hoped that this report on stability-indicating method and degradation of MYCO would be helpful for the multiple generic manufacturers of the drug around the globe by saving them for unnecessary repetition of the same studies.

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Table 1: System suitability parameters of MYCO.

Sr. No	System suitability parameters	Retention time (minutes)	RRT	Theoretical Plate	Tailing Factor
1	MYCO	6.852	**1.0**	5682	1.02
2	Impurity I	10.278	**1.5**	4785	1.05
3	Impurity II	2.740	**0.4**	9965	1.06

Table 2: Summary of Validation parameters by HPLC with UV detection

Sr. No	Parameters	MYCO	Impurity I	Impurity II
2	Linearity range	10-300µg/ml	0.1 – 3 µg/ml	
4	The correlation coefficient (r^2)	0.9999	0.9999	0.9999
5	Intercept	136522	54244	65825
6	Slope	3688	574	5424
8	Precision			
	Intra day Average % RSD (n = 5)	0.35	0.87	0.65
	Inter day Average % RSD (n = 5)	1.54	0.95	1.32
	Reproducibility of measurements %RSD	0.31	0.24	0.22
	% Recovery	99.32-101.54	99.99-100.16	99.96-101.10
9	Limit of detection (µg/ml)	0.013	0.0015	0.0017
10	Limit of quantification (µg/ml)	0.0562	0.0034	0.0042

%RSD calculated from five replication of readings

Table 3: Accuracy data of MYCO by HPLC with UV detection

Initial conc. (µg/ml)(A)	The quantity of std. Added (µg/ml)(B)	Total Amount (A + B)	Peak Area	
			Total quantity Found*± S.D.	%Recovery ± S.D
100	150	250	249.23 ± 0.35	99.32 ± 0.12
100	300	400	399.85 ± 0.28	99.95 ± 0.35
100	450	550	501.82 ± 0.15	101.22 ± 0.11

***Average of five readings**

Table 4: Accuracy data of MYCO related substance by HPLC with UV detection

Amount Added	Impurity I		Impurity II	
	Total quantity Found*	%Recovery ± S.D	Total quantity Found*	%Recovery ± S.D
0.1	0.099	99.00±0.96	0.100	100.05± 0.22
1	1.02	101.01±0.75	1.01	101.32±0.06
3	2.98	99.98±0.88	3.01	100.23±0.65

***Average of five readings**

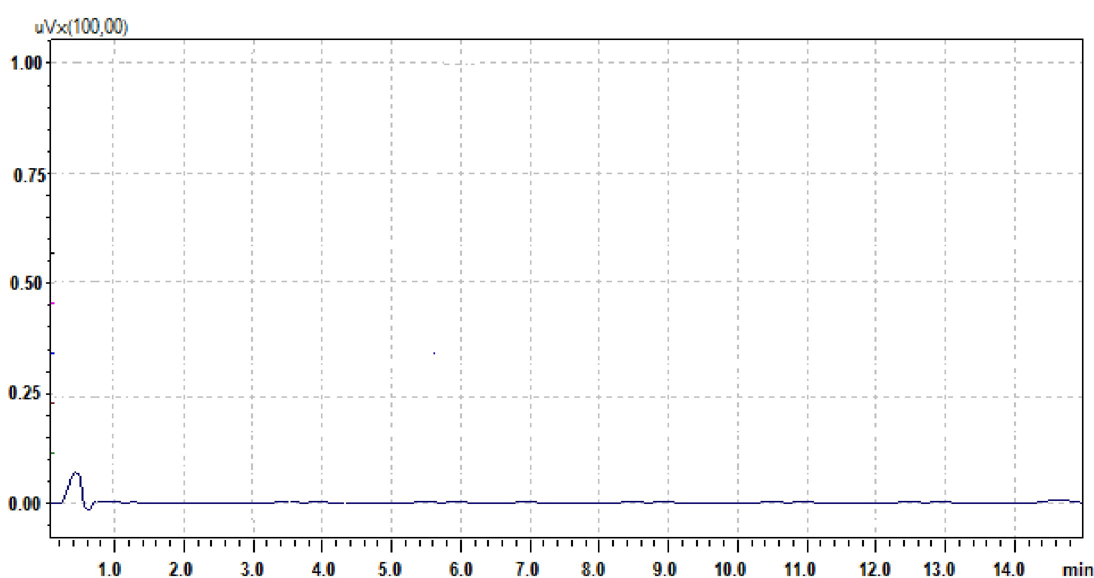

Figure 1: Blank solution by HPLC with UV detection

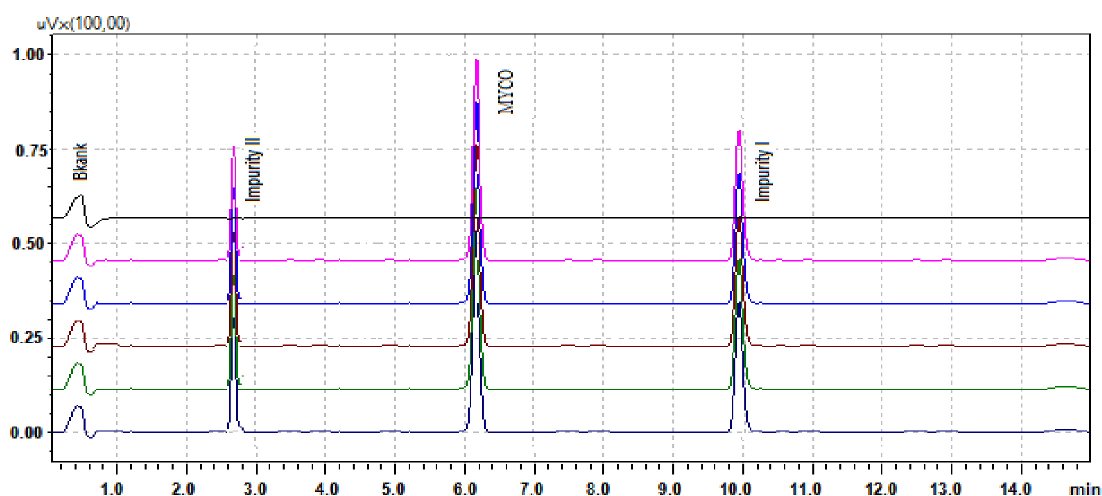

Figure 2. The linearity of Mycophenolate Mofetil and related substances.