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Research Article

A NEW RAPID AND SIMPLE ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF ESTIMATION THE MYCOPHENOLATE IN DOSAGE FORM BY UPLC TECHNIQUE

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ABSTRACT

A new rapid, reproducible and selective reverse phase UPLC method has been developed for the estimation of Mycophenolate in dosage form.It was resolved by using a mobile phase of Potassium dihydrogen phosphate: acetonitrile in the ratio 35:65 v/v at a flow rate of 0.2 ml/min. on UPLC system using UV - Visible detector at the wavelength of 228 nm. The column used was C18 (4.6 x 100mm, 3.5 mm, Make: XBridge).The linearity range was found to be 10-50 µg/ml. Quantification was achieved with UV detections at 216 nm based on peak area and absorbence. As per USP requirements system suitability studies were carried out and freshly prepared standard solutions are Mycophenolate The proposed new method is found to be economic, sensitive, precise, rapid and reproducible.

Key words:, Mycophenolate,UPLC, new method development, validation.

INTRODUCTION

Mycophenolate mofetil is the 2-morpholinoethyl ester of mycophenolic acid (MPA), an immunosuppressive agent and it is a inosine monophosphate dehydrogenase (IMPDH) inhibitor[1].The chemical name for mycophenolate mofetil (MMF) is 2-morpholinoethyl (E)-6-(1,3-dihydro-4-hydroxy-6-methoxy-7-methyl-3-oxo-5-isobenzofuranyl)-4-methyl-4-hexenoate[2]. It has an empirical formula of C23H31NO7, a molecular weight of 433.50, and the following chemical structure of Mycophenolate[3] is given in Fig.1.

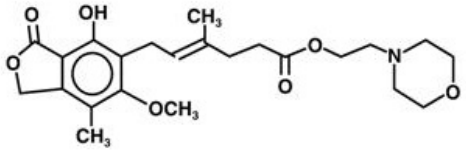


Fig.1: Chemical structure of Mycophenolate.

MPA is a potent, selective, uncompetitive, and reversible inhibitor of inosine monophosphate dehydrogenase (IMPDH), and without incorporation into DNA it inhibits the de novo pathway of guanosine nucleotide synthesis[4,5]. Because T- and B-lymphocytes are critically dependent for their proliferation on de novo synthesis of purines, whereas other cell types can utilize salvage pathways, MPA has potent cytostatic effects on lymphocytes. MPA inhibits proliferative responses of T- and B-lymphocytes to both mitogenic and allospecific stimulation. Addition of guanosine or deoxyguanosine reverses the cytostatic effects of MPA on lymphocytes5. MPA also suppresses antibody formation by B-lymphocytes. MPA prevents the glycosylation of lymphocyte and monocyte glycoproteins that are involved in intercellular adhesion to endothelial cells and may inhibit recruitment of leukocytes into sites of inflammation and graft rejection[6,7].

MATERIALS AND METHODS

Reagents and Standard – Mycophenolate tablets:

a. Water UPLC Grade.
b. Mycophenolate Working Standard.
c. Methanol UPLC Grade.
d. Ortho phosphoric acid.

Chromatographic Parameters

Equipment: Ultra performance liquid chromatography equipped with Auto Sampler and DAD or UV detector.

Column: Symmetry C18 (4.6 x 100mm, 3.5 mm, Make: XBridge) or equivalent
Flow rate: 0.2mL per min
Wavelength: 216 nm
Injection volume: 20 ml
Column oven : Ambient
Run time : 1.2 min

Preparation of Phosphate buffer: Weigh 7.0 grams of Potassium di hydrogen phosphate into a 1000ml beaker, dissolve and diluted to 1000ml with UPLC water. Adjusted the pH to 4.0 with ortho phosphoric acid

Preparation of mobile phase Mix a mixture of above buffer 350mL (35%) and 650 mL of Acetonitrile UPLC (65%) and degas in ultrasonic water bath for 5 minutes.Filter through 0.45 µ filter under vacuum filtration.

Diluent Preparation: Mobile phase as diluent

Preparation of the Mycophenolate Standard & Sample Solution

5.1 Standard Solution Preparation

Accurately weigh and transfer 10mg of Mycophenolate Working standard into a 10 mL volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.3 ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent. Mix well and filter through 0.45µm filter.

Sample Solution Preparation

Weigh 5 Mycophenolate Tablets and calculate the average weight. Accurately weigh and transfer the sample equivalent to 10 mg of Mycophenolate into a 10 mL volumetric flask. Add about 7 mL of diluent and sonicate to dissolve it completely and make volume up to the mark with diluent[8,9,10]. Mix well and filter through 0.45µm filter.Further pipette 0.3 ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent. Mix well and filter through 0.45µm filter.

Procedure

Inject 20 mL of the standard, sample into the chromatographic system and measure the area for the Mycophenolate peak and calculate the %Assay by using the formulae.

System Suitability

Tailing factor for the peak due to Mycophenolate in Standard solution should not be more Than 2.0.Theoretical plates for the Mycophenolate peak in Standard solution should not less than 2000

Calculation

Assay % =

$$\frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \frac{Avg.\ Wt}{Label\ Claim} \times 100$$

Where:

AT = Peak Area of Mycophenolate obtained with test preparation
AS = Peak Area of Mycophenolate obtained with standard preparation
WS = Weight of working standard taken in mg
WT = Weight of sample taken in mg
DS = Dilution of Standard solution
DT = Dilution of sample solution
P = Percentage purity of working standard

RESULTS

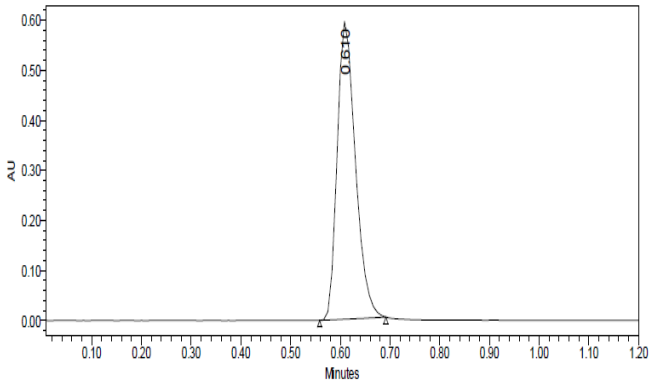
System Suitability Results

1). Tailing factor Obtained from the standard injection is 1.3
2). Theoretical Plates Obtained from the standard injection is 8287.2

Assay Results

Weight of 5 tablets: 4.265 grams
Average Weight : 0.853 grams

$$\frac{1501193}{1524093} \times \frac{10}{10} \times \frac{0.3}{0.3} \times \frac{10}{10} \times \frac{10}{100} \times \frac{99.8}{180} \times \frac{853}{1} \times 100 = 98.4\%$$



Experimental and method validation

Precision

Preparation of stock solution

Accurately weigh and transfer 10 mg of Mycophenolate Working standard into a 10 mL volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Preparation of 30 µg/ml solution

Further pipette 0.3 ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent. Mix well and filter through 0.45µm filter

Procedure

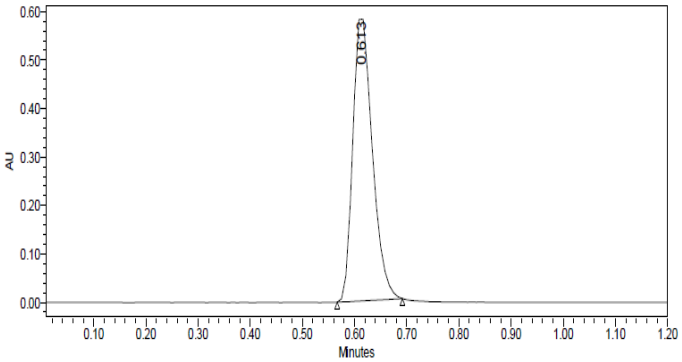
The standard solution was injected for Six times and measured the area for all six injections in UPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

The results are summarized

Injection	Area
Injection-1	1524180
Injection-2	1511154
Injection-3	1512869
Injection-4	1510828
Injection-5	1519370
Injection-6	1512168
Average	1515094.8
Standard Deviation	5441.9
%RSD	0.4

Acceptance Criteria

The % RSD for the area of six standard injections results should not be more than 2%.



Intermediate Precision/Ruggedness:

To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different day by using different make column of same dimensions[11,12].

Preparation of stock solution

Accurately weigh and transfer 10 mg of Mycophenolate Working standard into a 10 mL volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and

make volume up to the mark with the same solvent. (Stock solution)

Preparation of 30 µg/ml solution

Further pipette 0.3 ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent. Mix well and filter through 0.45µm filter

Procedure

The standard solution was injected for six times and measured the area for all six injections in UPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

The results are summarized in

Injection	Area
Injection-1	1527765
Injection-2	1524242
Injection-3	1527152
Injection-4	1519157
Injection-5	1519175
Injection-6	1525370
Average	1523810.0
Standard Deviation	3809.7
%RSD	0.3

Acceptance Criteria

The % RSD for the area of six standard injections results should not be more than 2%.

Accuracy

Preparation of stock solution

Accurately weigh and transfer 10 mg of Mycophenolate Working standard into a 10 mLvolumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Preparation of 30 µg/ml solution

Further pipette 0.3 ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent. Mix well and filter through 0.45µm filter.

Preparation Sample solutions

For preparation of 50% solution (With respect to target Assay concentration)

Accurately weigh and transfer 5.0 mg of Mycophenolate API sample into a 10 mL volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.3ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent. Mix well and filter through 0.45µm filter.

For preparation of 100% solution (With respect to target Assay concentration)

Accurately weigh and transfer 10.0mg of Mycophenolate API sample into a 10 mL volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)Further pipette 0.3 ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent. Mix well and filter through 0.45µm filter.

For preparation of 150% solution (With respect to target Assay concentration)

Accurately weigh and transfer 14.8mg of Mycophenolate API sample into a 10 mL volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)Further pipette 0.3 ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent. Mix well and filter through 0.45µm filter.

Procedure

Inject the standard solution, Accuracy -50%, Accuracy -100% and Accuracy -150% solutions.

Calculate the Amount found and Amount added for Mycophenolate and calculate the individual recovery and mean recovery values.

The results are summarized

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	773817.1	5.1	5.0	102.0%	
100%	1514099.3	9.98	10.0	99.8%	100.3%
150%	2227360.7	14.6	14.8	99.1%	

Acceptance Criteria

The % Recovery for each level should be between 98.0 to 102.0%.

LINEARITY

Preparation of stock solution

Accurately weigh and transfer 10mg of Mycophenolate API sample into a 10 mL volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Preparation of Level – I (10µg/ml)

0.1ml of stock solution has taken in 10 ml of volumetric flask dilute up to the mark with diluent.

Preparation of Level – II (20µg/ml)

0.2ml of stock solution taken in 10 ml of volumetric flask dilute up to the mark with diluent.

Preparation of Level – III (30µg/ml)

0.3ml of stock solution taken in 10 ml of volumetric flask dilute up to the mark with diluent.

Preparation of Level – IV (40µg/ml)

0.4ml of stock solution taken in 10 ml of volumetric flask dilute up to the mark with diluent.

Preparation of Level – V (50µg/ml)

0.5ml of stock solution taken in 10 ml of volumetric flask dilute up to the mark with diluent.

Procedure

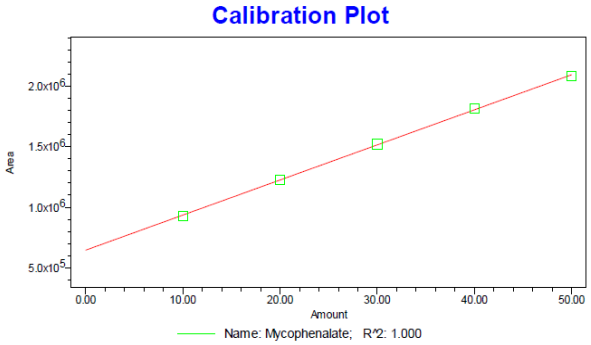
Inject each level into the chromatographic system and measure the peak area. Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient[13,14].

Linearity Results

S.No	Linearity Level	Concentration	Area
1	I	10µg/ml	928526
2	II	20µg/ml	1227166
3	III	30µg/ml	1522364
4	IV	40µg/ml	1817614
5	V	50µg/ml	2083661
Correlation Coefficient			0.999

Acceptance Criteria

Correlation coefficient should be not less than 0.999.



LIMIT OF DETECTION

Preparation of 30µg/ml solution

Accurately weigh and transfer 10mg of Mycophenolate Working standard into a 10 mL

Volumetric flasks add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.3 ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent. Mix well and filter through 0.45µm filter

Preparation of 0.008% solution At Specification level (0.002µg/ml solution)

Pipette 1mL of 10µg/ml solution into a 10 ml of volumetric flask and dilute up to the mark with diluent.Further pipette 0.02mL of above diluted solution into a 10 ml of volumetric flask and dilute up to the mark with diluent.

Calculation of S/N Ratio

Average Baseline Noise obtained from Blank

Signal Obtained from LOD solution (0.008% of target assay concentration) : 152µV

S/N = 152/51= 2.98

Acceptance Criteria

S/N Ratio value shall be 3 for LOD solution.

10.6 LIMIT OF QUANTIFICATION

Preparation of 30µg/ml solution

Accurately weigh and transfer 10mg of Mycophenolate Working standard into a 10 mL

Volumetric flasks add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution) Further pipette 0.3 ml of the above stock solution into a 10ml volumetric flask and dilute upto the mark with diluent. Mix well and filter through 0.45µm filter

Preparation of 0.02% solution At Specification level (0.008µg/ml solution):

Pipette 1mL of 10µg/ml solution into a 10 ml of volumetric flask and dilute up to the mark with diluent.

Further pipette 0.02mL of above diluted solution into a 10 ml of volumetric flask and dilute up to the mark with diluent.

Calculation of S/N Ratio

Average Baseline Noise obtained from Blank

Signal Obtained from LOD solution (0.02% of target assay concentration)

S/N = 507/51 = 9.94

Acceptance Criteria

S/N Ratio value shall be 10 for LOQ solution.

ROBUSTNESS

As part of the Robustness, deliberate change in the Flow rate, Mobile Phase composition, Temperature Variation was made to evaluate the impact on the method.

a). the flow rate was varied at 0.1 to0.3ml/min. Standard solution 30 µg/ml was prepared and analysed using the varied flow rates along with method flow rate.

The results are summarized On evaluation of the above results, it can be concluded that the variation in flow rate do not affect the method significantly. Hence it indicates that the method is robust even by change in the flow rate ±10%.

S.No	Flow Rate (ml/min)	System Suitability Results USP Plate Count	USP Tailing
1	0.1	8263.6	1.2
2	0.2	8287.2	1.3
3	0.3	8190.4	1.2

*** Results for actual flow (0.2 ml/min) have been considered from Assay standard.**

b). The Organic composition in the Mobile phase was varied from75% to 55%.

Standard solution 30 µg/ml was prepared and analysed using the varied Mobile phase composition along with the actual mobile phase composition in the method. The results are summarized

On evaluation of the above results, it can be concluded that the variation in 10% Organic composition in the mobile phase do not affect the method significantly. Hence it indicates that the method is robust even by change in the Mobile phase ±10%.

: 51µV

S.No	Change in Organic Composition in the Mobile Phase	System Suitability Results USP Plate Count	USP Tailing
1	10% less	8250.2	1.3
2	*Actual	8287.2	1.3
3	10% more	8174.9	1.2

*** Results for actual Mobile phase composition (65:35Acetonitrile: Buffer) have been considered From Assay standard**

RESULT AND DISCUSSION

A New simple, precision and accuracy UPLC method was developed the estimation of Mycophenolate analysis , consisting of an Acetonitrile: buffer system (65: 35 % v/v). The chromatographic condition was set at a low rate of 0.2 ml/min with the UV detector at 230 nm. The above method was optimized with a view to develop an assay method for Mycophenolate.Several mobile phase compositions were tried to resolve the peaks of Mycophenolate. The optimum mobile phase containing Acetonitrile: KH2PO4 buf fer (65: 35 % v/v) was selected because it was found ideal to resolve the analytic peaks of the drugs. Quantification was achieved with UV detections at 216 nm based on peak area and absorbence. As per USP requirements system suitability studies were carried out and freshly prepared standard solutions are Mycophenolate . Various parameters obtained with 20 µl of injection volume are summarized in the table given below.Validation and system suitability parameters

Table 5: : 51µV

S.NO	PARAMETERS	LIMIT	OBSERVATION
1	System suitability (%RSD of tailing factor)	suitable	1.3
2.	Specificity	No interferences	Specific
3	Precision: A)System Precision B).Method precision	RSD NMT 2.0% RSD NMT 2.0%	0.4 0.3
4	Linearity	Correlation coefficient NLT 0.999	1.000
5	Accuracy	%Recovery range98-102 %	100.3
6	Robustness	RSD NMT 2%	Robustted
7	LOD	S:N Ratio should be more than 3:1	2.98
8	LOQ	S:N ratio should be more than 10:1	9.94

The system is suitable for tailing factor, theoretical plate, resolution. The data obtained from the precision experiments. The R.S.D. value for precision was indication that the method was efficiently precise.Percentage recovery was calculated from 80% to 120% by

injecting to UPLC. The excellent recovery was made at each added concentration. There is allowable variation in flow rate, wave length which indicates that method is robust enough.The LOD for Mycophenolate were found to be 2.98 µg/ml The LOQ for Mycophenolate were found to be 9.94µg/ml The chromatogram of sample showed a single peak at the retention time of Mycophenolate indicating that there is no interference of the changing the persons for injecting the sample to the instrument.

CONCLUSION

The reliability and suitability of the method could be seen from recovery studies. Further there is no interference due to excipients . System suitability parameters were calculated which includes efficiency, resolution and tailing factor. Precision of the methods were studied by making repeated injections of the samples and system precision values were determined[14, 15, and 16]. The method was validated for linearity, accuracy, precision, robustness. The method is new simple, specific & easy to perform and requires short to analyse the samples. Low limit of Quantification and limit of detection makes this method suitable for Quality control. This new method enables Simultaneous determination of because of good separation and Resolution of the Chromatographic Peaks[17].The method was found to be accurate, precise and robusted. Hence it was concluded that the UPLC method developed was very much suit for routine analysis.Mycophenolate in tablet formulations and future planings use this method for estimation of Mycophenolate in clinical trials.

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