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Optimized and validated RP-UPLC method for the study of forced degradation and determination of mycophenolate mofetil in tablet dosage forms

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ABSTRACT

A simple, sensitive, selective and fast reverse phase ultra performance liquid chromatographic (RP-UPLC) method is developed for the determination of mycophenolate mofetil (MMF) in tablet dosage forms and to study the degradation products of mycophenolate mofetil in forced degradation. The chromatographic separation is achieved on a Waters Acquity UPLC system equipped with auto sampler and PDA detector (2996). A volume of 6 µl of the standard or test is injected into a symmetry C18 (2.1 x 100mm, 1.7 µm, Make: BEH) column, the components are eluted by using mobile phase of potassium dihydrogen phosphate buffer of pH=4.0 and acetonitrile in the ratio 35:65 v/v at a flow rate of 0.4 ml /min. for a short runtime of 2.40 min. and the components are monitored at a detection wavelength of 216 nm. The system suitability parameters are found to be with in the limits of acceptance with retention time of 0.717min. The linearity limits, correlation coefficient, LOD and LOQ are found to be 2.5-15µg/ml, 0.9998, 0.0521 µg/ml and 0.171µg/ml respectively. Forced degradation studies are also performed for the drug sample by subjecting the drug to acid hydrolysis, base hydrolysis, oxidation, photolytic and thermal decomposition and found to be stable. The developed method is successfully applied for the analysis of pharmaceutical formulations and the mean percent of the recovery of the drug is found to be 99.2.

KEYWORDS

RP-UPLC,
Mycophenolate,
Validation,
Forced degradation,
Assay.

INTRODUCTION

Mycophenolate mofetil (MMF) (brand names CellCept, Myfortic) is an immunosuppressant and prodrug of mycophenolic acid, used extensively in transplant medicine. It is a reversible inhibitor of inosine monophosphate dehydrogenase[1] (IMPDH) in purine biosynthesis, more specifically guanine synthesis, which is necessary for the growth of T cells and B cells. MMF is also used in the treatment of autoimmune diseases, such as Behcet's disease, pemphigus vulgaris and systemic lupus erythematosus. The chemical name for mycophenolate mofetil is 2-morpholinoethyl (E)-6-(1, 3-dihydro-4-hydroxy-6-methoxy-7-methyl-3-oxo-5-isobenzofuranyl)-4-methyl-4-hexenoate. The empirical formula and molecular weight of the drug are $C_{23}H_{31}NO_7$ and 433.50 respectively. The chemical structure of Mycophenolate is presented in Figure 1.

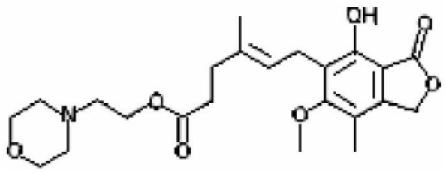

Figure 1 : Chemical structure of mycophenolate mofetil

An extensive literature surrey is carried out and found one UV-Spectrophotometric method[2] and a high-performance liquid-chromatography method[3] using photo diode array detector to estimate mycophenolate mofetil in bulk and pharmaceutical formulations. A few LC-MS/MS[4-10] methods and some HPLC[11-27] have been reported for the determination of Mycophenolate mofetil present in biological fluids or biological matrixes. Several authors have reported some HPLC methods for the study impurity profiling of mycophenolate mofetil[29], simultaneous determination of mycophenolate mofetil and its degradation product mycophenolic acid in dosage form[30-31] and stability of mycophenolate mofetil[32]

Very few reverse phase-HPLC methods[33-34] are also reported for the determination of the drug in dosage forms. But it is found that no UPLC method is reported to determine the quantity of Mycophenolate in pharmaceutical formulations. Simultaneous determination of mycophenolic acid and valproic acid in human plasma by HPLC[35] is reported based on derivatization by high-performance liquid chromatography with fluorescence detection. Two spectrophotometric methods are also present[36-37] to determine the drug in formulations. UPLC can be regarded as new invention for liquid chromatography. UPLC brings dramatic improvements in sensitivity, resolution and speed of analysis can be calculated. It has instrumentation that operates at high pressure than that used in HPLC & in this system uses fine particles(less than 2.5µm) & mobile phases at high linear velocities decreases the length of column, reduces solvent consumption and saves time. In the present work, this technology has been applied to the method development and method validation study of related substances determination of drug in pharmaceutical dosage forms. Therefore the authors are interested in developing a new RP-UPLC method for the assay of Mycophenolate in pharmaceutical formulations. The scope of the present investigation is to develop time saving, high sensitive and selective reverse phase ultra performance liquid chromatographic (RP-UPLC) method to determine the amount of mycophenolate mofetil (MMF) in pharmaceutical formulations and to study the degradation products of mycophenolate mofetil by forced degradation.

EXPERIMENTAL

Equipment

UPLC system is equipped with binary gradient pumps with auto sampler and auto injector (Model Acquity UPLC from Waters, USA) connected with a photo diode array detector (PDA) controlled with Empower software(Waters).

Materials and methods

Analytical grade potassium dihydrogen phosphate and ortho phosphoric acid are purchased from Qualigens Fine Chemicals Ltd., Mumbai. Acetonitrile of HPLC grade is obtained from E.Merck. (India) Ltd., Mumbai. Mycophenolate mofetil of highest purity (99.8%) is gifted by Dr.Reddy's Laboratories Ltd., Hyderabad. The commercially available mycophenolate mofetil tablets Cell Cept and Myfortic are purchased from the local market.

Preparation of buffer solution

Mobile phase is prepared by adding of 650ml acetonitrile (HPLC grade) to 350ml of 0.7% potassium dihydrogen phosphate buffer of pH 4.0 (the pH of the solution is adjusted to 4.0 by adding 1% ortho phosphoric acid solution), sonicated and filtered through 0.2µm PTFE filter (make: Millipore) filter before use.

Preparation of standard and sample solutions

Stock solution of the Mycophenolate mofetil is prepared by dissolving accurately weighed 10mg of mycophenolate standard in 7ml of mobile phase in a 10 ml volumetric flask, sonicated, made up to the mark with the mobile phase and filtered through 0.2µm PTFE filter (make: Millipore) filter. Further 1 ml of the above stock solution is accurately transferred into a 10ml volumetric flask and diluted up to the mark with diluents mixed well and again 0.75 ml of the above diluted solution is transferred into a 10ml volumetric flask and diluted up to the mark with diluents mixed well and

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filtered through 0.2µm PTFE filter. The concentration of the solution thus prepared is 7.5µg/ml. A series dilute solutions ranging from 2.5-15µg/ml of the drug are prepared in mobile phase Weight of five mycophenolate tablets is determined and calculated the average weight. An amount of the powder equivalent to 10mg of mycophenolate mofetil is accurately weighed and transferred into a 10 ml volumetric flask. About 7 ml of diluent is added, sonicated to dissolve, make volume up to the mark with diluents and filtered through 0.2µm PTFE filter. Further a solution of concentration 7.5µg/ml is prepared as above given in the standard.

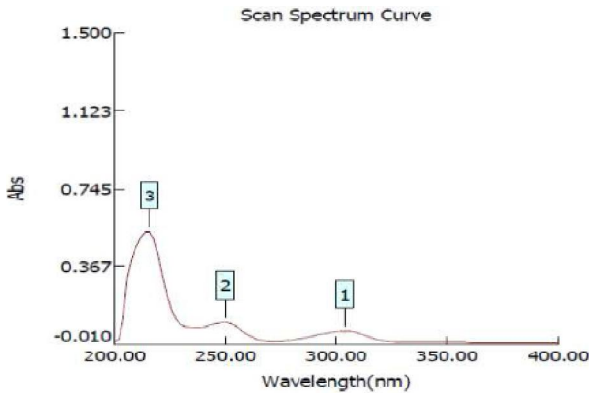

Figure 2 : UV Absorption spectrum of the mycophenolate mofetil

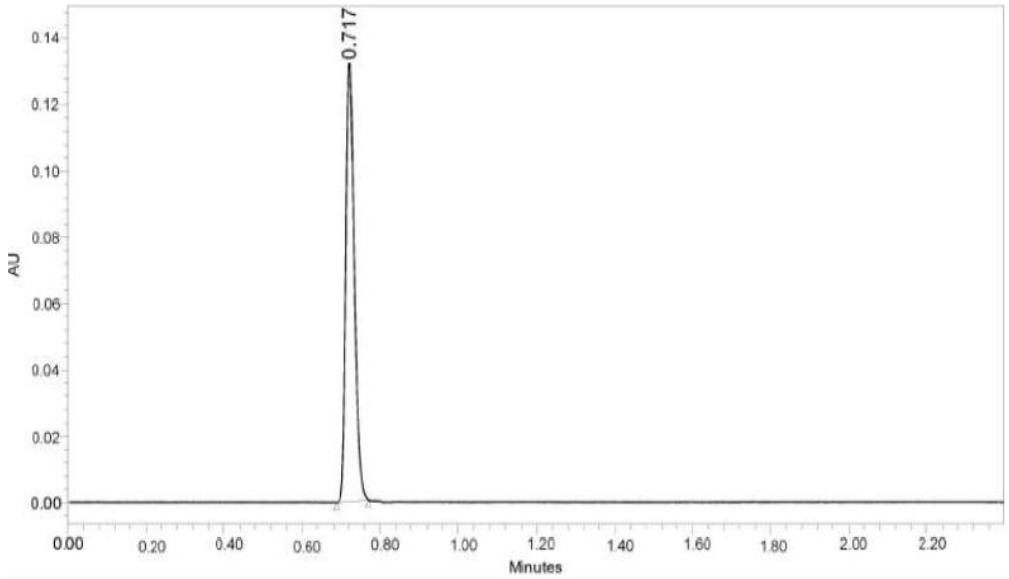

Figure 3 : A typical RP-HPLC chromatogram of mycophenolate standard mofetil

TABLE 1: Intra-day precision of the proposed RP-UPLC method

Injection ID	Area of the peak
Injection-1	762990
Injection-2	762890
Injection-3	762895
Injection-4	762895
Injection-5	762890
Average	762912
Standard Deviation	43.67
%RSD	0.005

TABLE 2 : Inter-day precision of the proposed RP-UPLC method

Injection ID	Area of the peak
Injection-1	762950
Injection-2	762910
Injection-3	762925
Injection-4	762965
Injection-5	762895
Average	762929
Standard Deviation	28.59
%RSD	0.003

TABLE 3 : Accuracy of the proposed method at three different spike levels

Spike Level	Area	Amount Adde (mg)	Amount Found (mg)	Percent of Recovery*	Mean % Recovery**
50%	380655	5.0	4.98	99.6	
100%	762892	10.0	99.98	99.9	99.2
150%	1124526	15.0	14.7	98.1	

***Average of three determinations at each spike level; ** Average of triplicate measurements at three spike levels**

Chromatographic conditions and optimization parameters

The mobile phase is allowed to pass through the column at a flow rate of 0.4 ml/min at ambient temperature for 30 min. to equilibrate the column; the response of the detector is recorded continuously at 216 nm. Different trails are performed by injecting different volumes working standard of different concentrations at different wavelengths using different composition of mobile phase until to get a reasonable retention time of the drug and valid system suitability parameters. Finally, the chromatogram of 6µl of 7.5µg/ml working standard at 216nm using a mobile phase of composition of 35:65 (v/v/) of acetonitrile and buffer for a run time of 2.4 min is found to be suitable for the assay analysis. The UV absorption spectrum of Mycophenolate mofetil and a standard chromatogram of the drug are presented in figure 2 and figure 3 respectively.

TABLE 4 : Linearity of the peak area with concentration of the drug

S.No.	Concentration (µg/ml)	Area
1	2.5	254297
2	5.0	508595
3	7.5	762892
4	10.0	1017190
5	12.5	1271488
6	15.0	1528785
Correlation Coefficient		0.9999
Slope		101848
Intercept		536

RESULTS AND DISCUSSION

System suitability

Standard solution (7.5µg/ml) of the mycophenolate is injected into the column and the chromatogram is recorded under the set of optimized chromatographic conditions. The USP plate count and tailing factor for the peak are calculated and found to be 10452 and 1.286 respectively which are within the limits specified (% RSD NMT 2.0%).

Intra-day and inter day precision

Precision of a method is generally expressed in terms of a statistical parameter %RSD. In the present investigation to find out intra day precision, the %RSD is calculated from the values of standard deviation and mean area of five replicate measurements of the working standard solution (7.5µg/ml) and found to be 0.005 which is within the limits specified (% RSD NMT 2.0%). Inter-day precision of the method is also determined by carrying out the same experiment on different days using same instrument and same column under similar chromatographic conditions. The %RSD value for the five replicate measurements is found to be 0.03 which is again within the limits specified (% RSD NMT 2.0%). The intra day and inter day precision results are presented in TABLE 1 and TABLE 2 respectively.

Accuracy

To demonstrate the accuracy a known amount of standard drug is added to the fixed amount of pre-analyzed tablet solution at three different spike levels (50%, 100% and 150% level of the working standard) in triplicate at each level. Percent recoveries are calculated by comparing the area before and after the addition of the standard drug and results are presented in TABLE 3. Satisfactory recoveries ranging from 98.1-99.6 indicate that the proposed method is accurate.

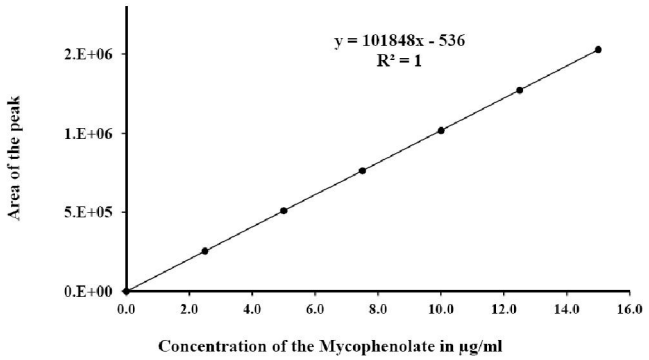

Figure 4 : Linearity of the drug concentration against the area of the peak

TABLE 5 : Study of robustness of the proposed method

Parameter	Sample ID	Value of parameter	USP Platecount	USP Tailing
Flow rate	1	0.3 ml/min.	11492	1.244
	2	0.4 ml/min.	11064	1.245
	3	0.5 ml/min.	10432	1.225
Composition of mobile phase	1	10% less	11456	1.224
	2	†Actual	11064	1.245
	3	10% more	11115	1.246

†Actual flow 0.7 ml/min and actual mobile phase composition 65:35Acetonitrile: Buffer have been considered from Assay standard

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TABLE 6 : A report on the study of degradation of mycophenolate in the presence of different laboratory conditions

Condition	Statistical Parameters #	Retention Time (min.)	Peak Area	USP Plate count	Tailing factor	Percent Of assay
0.1N HCl	Mean	0.713	705530.33	11322.00	1.242	96.84
	SD	0.0000	15.9478	2.6458	0.0010	0.1443
	%RSD	0.0000	0.0023	0.0234	0.0805	0.1491
0.1N NaOH	Mean	0.715	690341.66	11246.33	1.240	96.26
	SD	0.001	3.0550	16.2890	0.0030	0.0000
	%RSD	0.13986	0.0004	0.1448	0.2466	0.0000
$3\%H_2O_2$	Mean	0.712	614473.67	11129.00	1.220	92.93
	SD	0.0026	12.8582	5.5678	0.0231	0.0000
	%RSD	0.3706	0.0021	0.0500	1.8845	0.0000
Photo light	Mean	0.723	637234.00	11121.67	1.240	93.92
	SD	0.0020	18.5203	7.0238	0.0026	0.0058
	%RSD	0.2797	0.0029	0.0632	0.2129	0.0061
Thermal	Mean	0.711	652419.67	11121.33	1.240	94.59
	SD	0.0010	6.6583	7.2342	0.0021	0.0000
	%RSD	0.1404	0.0010	0.0650	0.1683	0.0000

#Statistical parameters are calculated for triplicate measurements of each condition

Linearity

The UPLC chromatogram data for determination of linearity is mentioned in TABLE 4. The linearity of the optimized method was determined for five concentrations and the correlation coefficient was found to be 0.99 for mycophenolate which is within the limits specified (NLT 0.99). It showed that the developed method followed Beer-Lambert's law within the range of 2.5-15.0µg/ml (Figure 4). The correlation coefficient, slope and intercept of the straight line are evaluated by linear regression analysis using linear least square method and these values are given in TABLE-4. The limit of detection (LOD) and limit of quantification (LOQ) are experimentally determined and are found to be 0.0521 µg/ml and 0.171µg/ml respectively.

Analysis of pharmaceutical formulations

Pharmaceutical formulations (tablet forms) are analyzed by the developed method. A sample solution of 7.5µg/ml is prepared and used for the assay analysis. The assay of the drug present in the each tablet is calculated by comparing the area of the peak of test with the standard. Atypical chromatogram of the sample is given in Figure 5.

Robustness

To determine the robustness of the developed method, experimental conditions such as flow rate, composition of the mobile phase, detection wavelength and pH of the buffer are deliberately altered, and system suitability parameters are checked. The normal flow rate of the mobile phase is 0.4 ml/min. To study the effect of flow rate, flow rate is changed by 0.1 units from 0.3 to

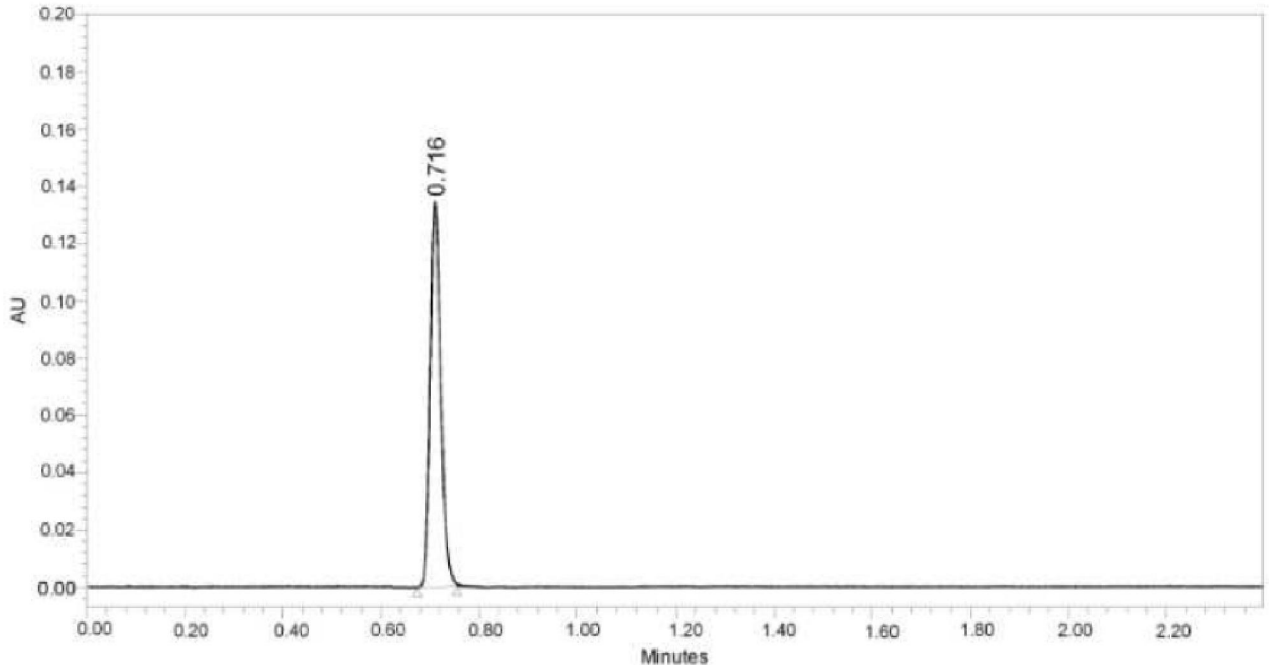

Figure 5 : A typical UP-HPLC chromatogram of mycophenolate sample

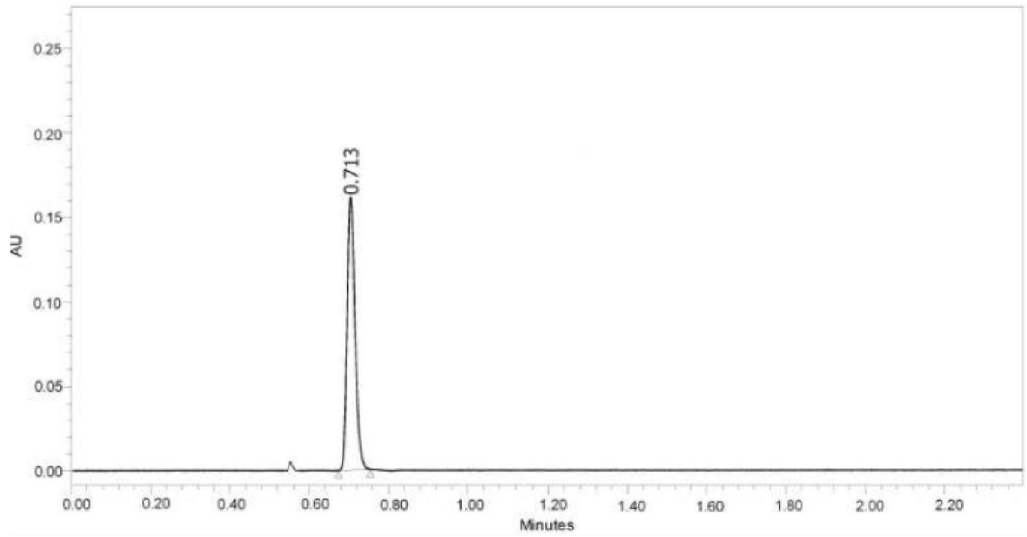

Figure 6 : A typical chromatogram of degradation of mycophnolate standard in 0.1N HCl

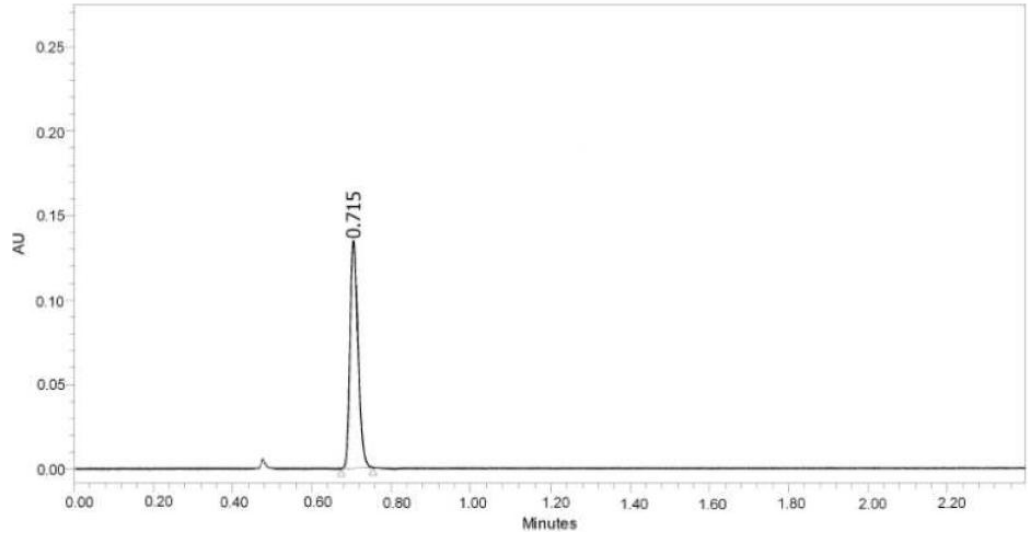

Figure 7 : A typical chromatogram of degradation of mycophnolate standard in 0.1N NaOH

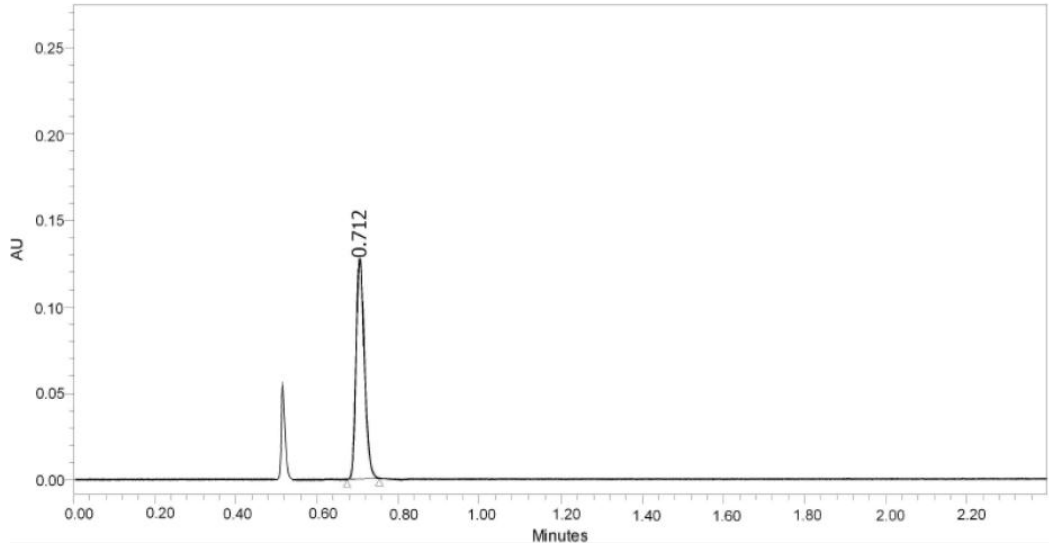

Figure 8 : A typical chromatogram of degradation of mycophnolate standard in 3% H_2O_2

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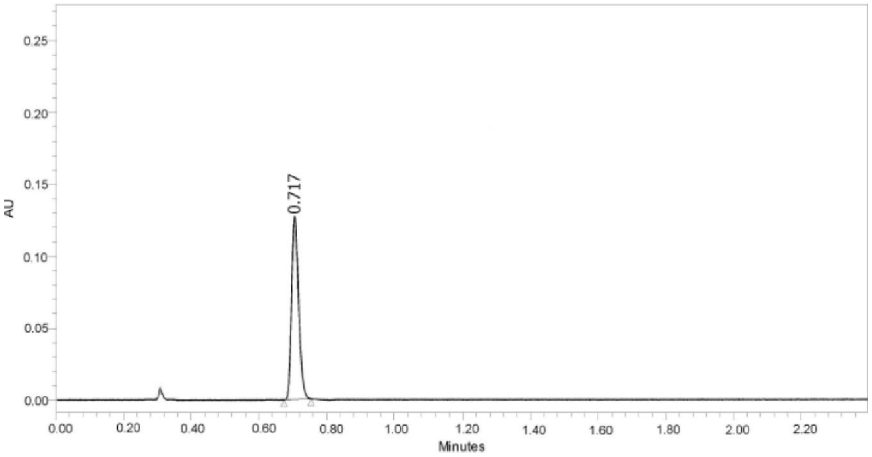

Figure 9 : A typical chromatogram of photolytic degradation of mycophnolate standard

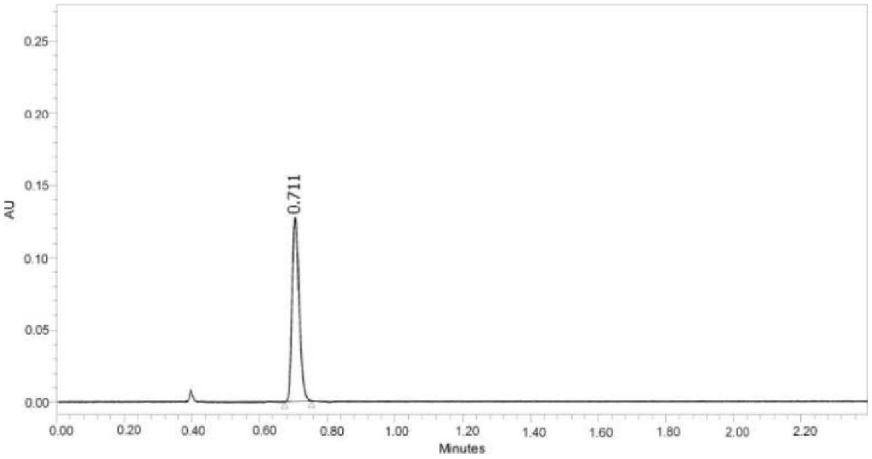

Figure 10 : A typical chromatogram of thermal degradation of mycophnolate standard

0.5 ml/min, the proportion of acetonitrile in the mobile phase is changed by ±3.5 %, and UV detection wavelength (216nm) is changed ± 3nm. To study the effect of pH variation in the mobile phase, pH is altered by ±0.2 units, i.e., to 4.2 and 3.8. Changes in chromatographic parameters, i.e., theoretical plates, tailing factor and % R.S.D. are evaluated for each change and are presented in TABLE 5.

Study of forced degradation

To study the percent of degradation of mycophenolate, it is subjected to forced degradation under different laboratory conditions such as 0.1N HCl, 0.1N NaOH, 3% H_2O_2, UV light and heat at 40°C. 1.0 ml of the stock solution is transferred accurately into three different 10ml volumetric flasks and made up to the mark with aqueous solutions of 0.1 N HCl, 0.1N NaOH and 3% hydrogen peroxide respectively. After 48 hours 0.75 ml of the above each solution is taken into 10 ml volumetric flask, neutralized and make up to the mark with mobile phase. 6 µl of each solution is injected into the system, obtaining the chromatograms and percent of degradation is evaluated by comparing the area of the peak of hydrolyzed compounds with area of standard compound. The typical chromatograms for the above study are presented in Figure 6-8. A second peak having considerable area is observed in Figure 7 at retention time 0.516 which may be due to peroxide. To study the degradation the mycophenolate in the presence of UV light and heat, the drug is kept in open petri dishes and exposed to UV light or kept in oven at 40°C; after 48 hours standard solution is prepared using the exposed drug by following the same procedure as mentioned above and 6 µl of each solution is injected into the system, chromatograms are recorded and the percent of degradation of the drug is evaluated under the same chromatographic conditions. The model chromatograms for these studies are given in Figure 9-10. The results of the degradation studies are presented in TABLE 6.

CONCLUSION

The developed RP-UPLC method is useful to determine the quantity of Mycophenolate even at low concentrations which indicates that the developed method is more sensitive than the previous methods. The retention time of the drug is greatly reduced to 0.717 min. this directly indicates that proposed method is time saving and less consumption of solvent as per the standard procedures indicates the cost reduction. The percent of recovery of the drug indicates that the proposed method is selective and hence the developed RP –UPLC method may be used as an analytical method for routine analysis bulk and formulations.

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