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Development of RP- HPLC Method for Simultaneous Estimation of Mycophenolate Mofetil and Tacrolimus

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- ✓ UV-Visible.

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Abstract

This study was to develop a simple, fast, accurate and precise reverse phase high performance liquid chromatography (RP-HPLC) method for simultaneous estimation of mycophenolate mofetil and tacrolimus in unit dosage forms. In this RP-HPLC method we use the Linear gradient elution using a Kinetex Polar, C18, 5 µm, 4.6 × 250 mm column and mobile phase was acetonitrile, phosphate buffer, methanol and flow rate was 1.2 ml/min. The elution was detected and quantified at 250 nm using UV-Visible detector. The standard curves of Mycophenolate mofetiland Tacrolimus was following the linear relationship (r2 > 0.99) within the analytical range of 2-7 µg/ml and 500-5000 µg/ml. The mentioned method depicted in this paper has good accuracy, precision, linearity, robustness and was suitable for simultaneous estimation of mycophenolate mofetil and tacrolimus.

1. Introduction

Mycophenolate mofetil (Fig. 1) (MMF) "2-morpholinoethyl (E)-6-(1,3-dihydro-4-hydroxy-6- methoxy-7-methyl-3-oxo-5-isobenzofuranyl)-4-methyl-4-hexenoate"[1] is a selective, uncompetitive, potent and reversible inhibitor of inosine monophosphate dehydrogenase (IMPDH). IMPDH is an important enzyme for the synthesis of guanosine nucleotides [2]. MMF isanester prodrug of mycophenolic acid (MPA) and is converted by hepaticesterase to MPA [3]. During the initial studies, MPAis found to have antibacterial, antiviral, antifungal, antitumor, and immunosuppressive properties [4-7]. After getting approval from the United States Food and Drug Administration (FDA) in 1995 MMF is used for the prevention of renal, cardiac, hepatic, pancreatic allograft rejection, psoriasis, Lung transplant, Lupus glomerulonephritis, systemic sclerosis [8-12]. MMF inhibit the production of antibodies and the proliferation of lymphocytes [13-15]. "MMF did not inhibit early events in the activation of human peripheral blood mononuclear cells, such as the production of interleukin-1 (IL-1) and interleukin-2 (IL-2), but did block the coupling of these events to DNA synthesis and proliferation"[16,17]. MMF is a non-official ester of mycophenolic acid (Fig. 2) (MPA) so MPA present as asynthetic impurity in MMF [18]. MPA is five times more potent inhibitor of type II isoform of IMPDH. So, more strongly inhibition of cell growth and multiplication of lymphocytes[19].

Tacrolimus (TAC) (Fig. 3) is a macrolide immunomodulator (FK506), isolated in 1984 from the fungus *Streptomyces tsukubaensis*. TAC is T lymphocyte specific calcineurin inhibitor inhibits the transcription of interleukin (IL)-2 and other cytokines [20] through T-cell activation through tumor necrosis factor-a, IL-1ß and IL-6 [21,22]. In late 80's TAC is used to prevent the rejection rate after solid organ transplation [23]. But in 2000 after approval from US FDA TAC ointments was used for many skin diseases like lupus dermopathy[24], atopic dermatitis psoriasis [25], localized scleroderma [26], chronic actinic dermatitis [27], pyoderma gangrenosum[28], Behçet's disease [29], lichen planus [30], rheumatoid ulcers [31] and steroid rosacea[32],atopic dermatitis[33], periodontitis [34]. The efficacy of TAC is sometimes much better than corticosteroids due to less or no side effects on skin and uptake to the blood systemic absorption [35]. Some common adverse effects during treatment in skin diseases are itching or erythema, burning sensations and decreases as treatment progress [36].

There are various analytical techniques available for the detection and quantification of compounds present in the samples, like spectrophotometery[37], NMR[38], TLC or preparative TLC, HPTLC, Gas Chromatography, HPLC[39], etc. No official HPLC methods were found for the assay of MMF and TAC in combined formulations[40-45]. So, there is a need for method development for the assay of MMF in combined formulations (dosage forms)[46].

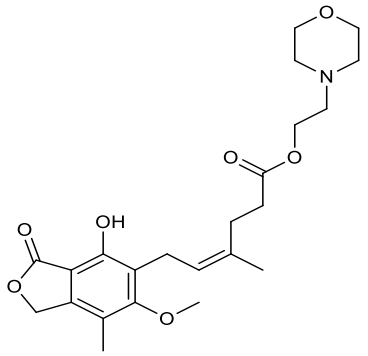

Figure 2: *Chemical structure of Mycophenolate mofetil (MMF)*

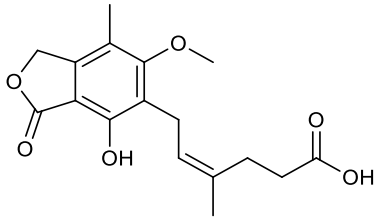

Figure 1: *Chemical structure of mycophenolic acid (MPA)*

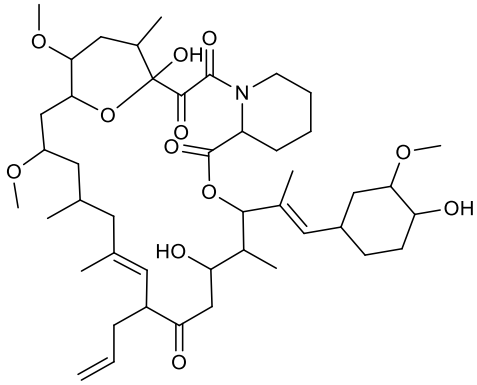

Figure 3: *Chemical structure of Tacrolimus (TAC)*

2. Materials and Methods

2.1 *Chemicals*

MMF and Tacrolimus were the gift samples from Biocon Ltd., (Bangalore, India). HPLC grade solvents, Acetonitrile, and other chemicals were purchased from Thermo Fisher Scientific (Vadodara, Gujarat, India). For the entire HPLC method, in house produced double-distilled water was used. Analytical grade Orthophosphoric acid, Triethylamine, and Potassium dihydrogen orthophosphate were obtained from Merck (Worli, Mumbai, India).

HPLC conditions the RP-HPLC (LC-2010, Shimadzu, Japan) with a variable wavelength UV-Visible detector set at 250 nm. For data acquisition and analysis, the LC-solution software was used. The HPLC column used for analysis was Kinetex Polar, C18, 5 µm, 4.6 × 250 mm. Column temperature was set at 35°C. The mobile phase was a mixture of A:B:C (25:60:15)v/v [A: Phosphate buffer pH 2.9 (2.488 gm of potassium dihydrogen orthophosphate was dissolved in 1000 ml of distilled water and 1 ml of triethylamine was added. pH was set upto 2.9 with orthophosphoric acid, B: ACN and C: Methanol]. Injection volume was 20 µl which was injected into the column using asyringe and the linear gradient flow rate was set at 1.2 ml/min. MMF and TACwere detected by UV absorption at 250 nm.

2.2 *Preparation of standard solutions*

The primary stock of MMF was prepared by dissolving 5 mg of drug was dissolved in 10 ml of diluent (mobile phase) to obtain a solution of 500 µg/ml.

The primary stock of Tacrolimus was prepared by dissolving 50 mg of drug was dissolved in 10 ml of diluent (mobile phase) to obtain a solution of 5000 µg/ml.

The working standards were prepared by serial dilution with diluent(mobile phase) to obtain MMF concentrations of 2-7 µg/ml and Tacrolimus 500-5000 µg/ml.

2.3 *Method Validation*

The optimized RP-HPLC method was validated with respect to Robustness, Linearity Range, Accuracy, Precision, Limit of Detection, Limit of Quantitation according to ICH guidelines.

3. Results and Discussion

3.1 *HPLC Chromatogram of Mixture Sample*

On HPLC analysis of amixture of standards, chromatogram was optimized in which retention time of drugs as shown in Table 1 and Figure 4.

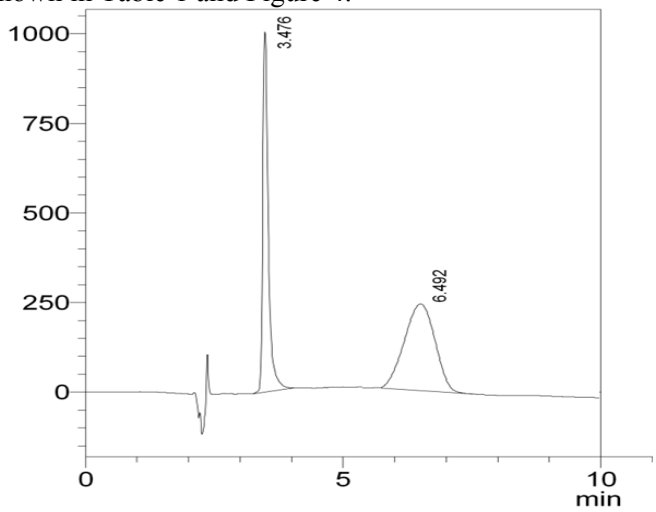

Figure 4: HPLC chromatogram of Mycophenolate Mofetil and Tacrolimus mixture

Table 1: Retention time of drugs (Mycophenolate Mofetil and Tacrolimus)

S.No.	Name of drug	Retention time (min.)
1.	Mycophenolate	3.476
2.	Tacrolimus	6.492

3.2 *Linearity*

The linearity of ananalytical method is its ability to elicit test results that are directly proportional to the concentration of ananalyte in thesample within a given range. The range of theanalytical method is the interval between the upper and lower levels of analyte that have been demonstrated to be determined with a suitable level of precision, accuracy, and linearity". Selected linearity range for MMF 2-7 µg/ml (Table 2 and Figure 5) and TAC 500-5000 µg/ml (Table 2 and Figure 6). All the dilutions were filtered through 0.22 µ filter and injected.

Table 2: Linearity Data

Mycophenolate Mofetil				Tacrolimus			
Concentration (µg/ml)	Area	Mean	Std. dev	Concentration (µg/ml)	Area	Mean	Std. dev
2	24620	25019.67	934.0002	500	150719	151946.3	1633.206
	26087				153800		
	24352				151320		
3	41324	40918.67	655.8486	1000	228691	226036	2437.966
	40162				223898		
	41270				225519		
4	59115	59938.33	717.5015	2000	307172	304898.7	1994.923
	60270				304084		
	60430				303440		
5	72703	72526.33	180.0926	3000	381813	383950.7	3337.922
	72343				387797		
	72533				382242		
6	96352	96362	135.2775	4000	483757	482531.3	3946.91
	96502				478117		
	96232				485720		
7	123017	124465	3292.182	5000	556968	548545.7	7293.958
	128233				544327		
	122145				544342		

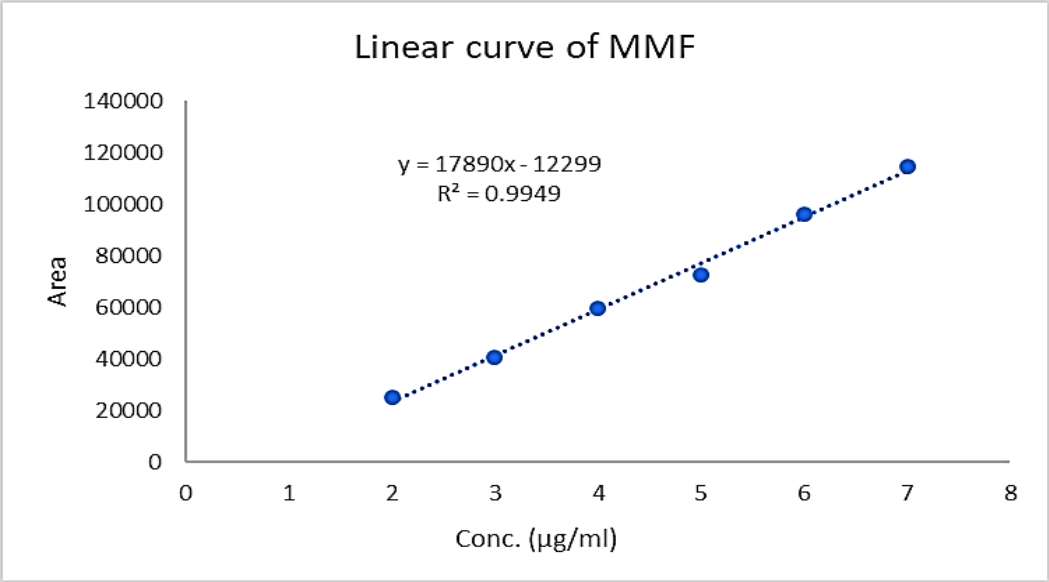

Figure 5: linear curve of Mycophenolate Mofetil (MMF)

A linear curve was obtained in the range of 2-7 µg/ml with an equation of y= 17890x - 12299 and R^2=0.994.

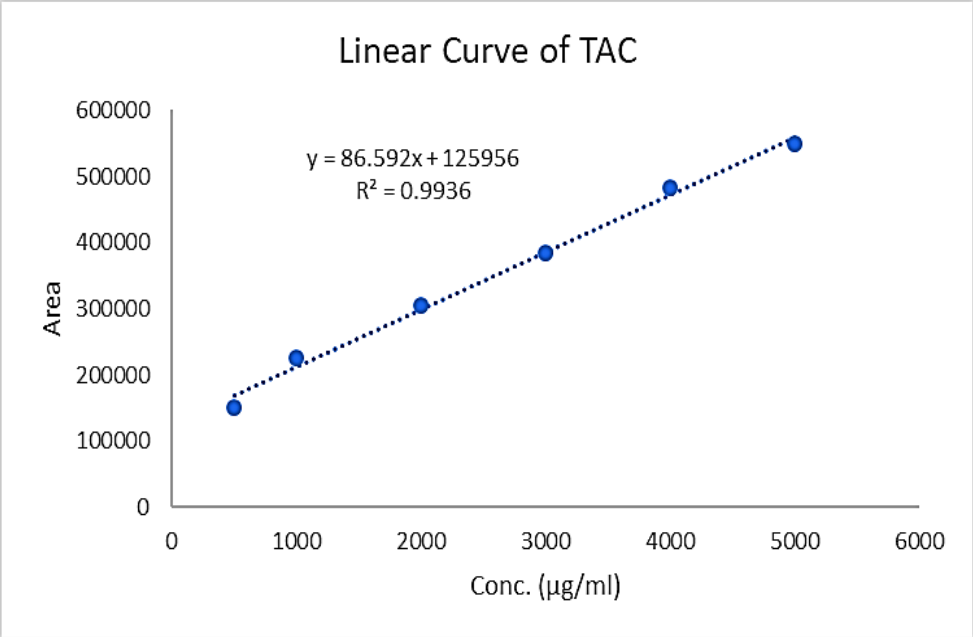

Figure 6: Linear curve of Tacrolimus

A linear curve was obtained in the range of 500-5000 µg/ml with an equation of y= 86.592x + 125956 and R^2=0.993.

3.3 *Limits of Detection (LOD) and Limits of Quantitation (LOQ)*

LOD and LOQ depend on the method's sensitivity. LOD is thelowest concentration detected and LOQ is the minimum sample concentration that can be measured. As per ICH guidelines, there are three different methods to calculate LOD and LOQ. A) visual evaluation method B) Signal to noise ratio method C) Slope method. Among them here employed method was :

LOD= 3.3σ/S	LOQ= 10σ/S

Where, σ = the standard deviation of response

S = the slope of thecalibration curve; and data were shown in Table 3.

Table 3: LOD and LOQ analysis of Mycophenolate and Tacrolimus.

S.No.	Drug Name	LOD	LOQ
1.	Mycophenolate Mofetil	11.4163 (µg/ml)	32.56454 (µg/ml)
2.	Tacrolimus	15.8764 (µg/ml)	46.0800 (µg/ml)

The results obtained were within the limit.

3.4 *Accuracy*

Determination of % recovery of standard compound. In this method, the calculation of % recovery was carried out by adding standard drug solution at the level of lower medium and ahigher concentration of each drug in thepre-analyzed sample. The recovery data of Mycophenolate and Tacrolimus in Table 4 and Table 5. Results were within the acceptance criteria 99.0% to 119%, indicating a good degree of sensitivity. In this method, the known concentration of standard drug was added to the assay sample.

Table 4: Recovery data of Mycophenolate Mofetil and Tacrolimus

Conc. Level	MMF Conc. (µg/ml)	Amount Recovered	Mean	Std. dev	% RSD	TAC Conc. (µg/ml)	Amount Recovered	Mean	Std. dev	% RSD
Lower	25	108.85	108.24	2.13	1.97	1000	101.49	101.67	0.86	0.85
		110.01					102.61			
		105.88					100.91			
Medium	50	119.03	117.54	1.43	1.22	2500	119.16	118.23	1.75	1.48
		117.39					119.33			
		116.18					116.22			
Higher	75	102.8	100.77	1.76	1.75	5000	111.76	114.29	2.19	1.91
		99.75					115.54			
		99.75					115.56			

The results indicate that the recoveries are well within the acceptance range of 99% – 119%, therefore, amethod is accurate and it can be used for the estimation of all the three drugs.

3.5 *Precision and Repeatability*

The intra-day and inter-day variation for determination of all the three drugs were carried out with concentrations over 3 levels on the same day (Table 5) and three consecutive days (Table 6) where repeatability was determined with alower concentration and injected six times and % RSD was calculated.

Table 5: Repeatability data

MMF Conc. (µg/ml)	Area	Mean	Std. dev	%RSD	TAC Conc.(µg/ml)	Area	Mean	Std. dev	%RSD
25	1046545	1045223	15232.09	1.46	1000	2229817	2270220	35591.47	1.57
	1029373					2283904			
	1059751					2296938			
	1045012					2219936			
	1041766					2231462			
	1044616					2244333			

3.6 *Robustness*

The robustness was carried out by taking the sample of lower concentration with deliberately changing the method parameters. The change in the responses of drugs was noted in terms of %RSD. Robustness of the method was studied by

a) Change in flow rate (Table 7)

b) Change in wavelength (Table 8).

Table 6: Intraday and Inter day study data

Intraday study									
MMF Conc. (µg/ml)	Area	Mean	Std. dev	%RSD	TAC Conc. (µg/ml)	Area	Mean	Std. dev	% RSD
25	260326	260815	426.98	0.16	1000	761961	767879	5125.37	0.67
	261005					770887			
	261114					770789			
50	622979	624395.7	1226.91	0.19	2500	808721	818834	8764.24	1.07
	625114					824218			
	625094					823563			
75	1005960	1005123	1288.12	0.13	5000	1682393	1670329	20915.09	1.25
	1003640					1646178			
	1005770					1682415			
Inter day study (Day-2)									
25	269070	267876.3	1760.87	0.66	1000	704714	707821.7	5442.5	0.77
	265854					714106			
	268705					704645			
50	746240	505162	3284.93	0.65	2500	713869	708160.8	4543.51	0.64
	740618					705968			
	740485					705663			
75	1297367	1298907	2366.33	0.18	5000	1325508	1324654	1262.92	0.09
	1301632					1323203			
	1297723					1325250			
Inter day study (Day-3)									
25	336749	335261.7	1293.83	0.39	1000	649997	636085	12057.51	1.89
	334396					629604			
	334640					628654			
50	771408	764830	5716.82	0.75	2500	554245	553878.3	447.28	0.08
	761062					553380			
	762020					554010			
75	1365779	1371250	9784.52	0.71	5000	1148445	1139757	14085.86	1.24
	1382546					1123505			
	1365424					1147321			

This developed method was found to be precise due to low values of the %RSD.

Table 7: Robustness data of Mycophenolate Mofetil and Tacrolimus with deliberate changes in flow rate

Flow rate (ml/min)	MMF Conc. (µg/ml)	Area	Mean	Std. dev	%RSD	TAC Conc. (µg/ml)	Area	Mean	Std. dev	%RSD
1	25	341748	342629	3764.63	1.09	1000	939429	941948	5442.81	0.58
		339383					948194			
		346756					938221			
1.2		329300	333502.7	3649.62	1.09		676264	680848	3969.93	0.58
		335874					683116			
		335334					683164			
1.4		288731	284818	3538.79	1.24		536046	544077	6977.36	1.28
		283881					548650			
		281842					547535			

Table 8: Robustness data of Mycophenolate Mofetil and Tacrolimus at different wavelengths

λ (nm)	MMF Conc. (µg/ml)	Area	Mean	Std. dev	%RSD	TAC Conc. (µg/ml)	Area	Mean	Std. dev	%RSD
245	25	288606	290481	1623.93	0.56	1000	932853	930667	1915.22	0.21
		291439					929864			
		291398					929284			
250		270848	273721.7	2535.78	0.93		705667	714534	8405.18	1.18
		275645					722385			
		274672					715550			
255		245762	245078.3	1670.92	0.68		550099	555936.3	8212.35	1.48
		243174					565327			
		246299					552383			

The acceptance criteria for %RSD should not be more than 2. The %RSD obtained for thechange in wavelength and change of flow rate was found to be less than 2. Hence the method was robust.

3.7 *Ruggedness*

The ruggedness was studied by analyzing the same samples of three drugs by changing analyst discussed in Table 9. The change in the responses of drugs was noted in terms of % RSD.

Table 9: Ruggedness data

Standard Name	Conc. (µg/ml)	Analyst-I				Analyst-II			
		Area	Mean	Std. dev	%RSD	Area	Mean	Std. dev	%RSD
MMF	25	1041598	1041475.83	1169.80	0.11	1067918	1070509.5	2476.95	0.23
		1042138				1070120			
		1041579				1072425			
		1041580				1072551			
		1042694				1072805			
		1039266				1067238			
TAC	1000	2267405	2242358.83	24637.24	1.09	2272586	2287140.67	14577.61	0.64
		2265621				2277023			
		2234424				2302026			
		2234425				2296356			
		2201497				2302287			
		2250781				2272566			

The acceptance criteria for %RSD should not be more than 2. The %RSD obtained for achange of analyst was found to be less than 2. Hence the method was rugged.

Conclusion

The analytical method described in this paper has good accuracy, precision, linearity and is suitable for simultaneous estimation of mycophenolate mofetil and tacrolimus. As the method was successfully validated based on ICH guidelines, it can be readily used in quality control laboratories for the routine pharmaceutical analysis. Also, this simple and rapid method can simplify performance in developing new formulations.

Conflict of Interest-There is no conflict of interest in this study.

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