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Simultaneous Estimation by RP-HPLC Method for the Immunosuppressant Drug Combination: Mycophenolate Mofetil, Tacrolimus with Prednisolone

Pardeep Kr. Sharma[1], Vijay Mishra[1], Surajpal Verma[1]* and Amit Bhatia[2]

[1] School of Pharmaceutical Sciences, Lovely Professional University, Phagwara 144411, Punjab, India
[2] Department of Pharmaceutical Sciences and Technology,
Maharaja Ranjit Singh Punjab Technical University, Bathinda 151001, Punjab, India.

ABSTRACT

In current scenario, treatment of any disease depends upon two major factors i.e. patient compliance and effective dosage regimen. The effective dose delivered by a dosage form to a patient depends on various parameters, which can be assessed by an effective and economic analytical method. In the present study a precise analytical method for estimating the combination of immunosuppressant drugs mycophenolate mofetil (MMF), tacrolimus (TAC) and prednisolone through RP-HPLC was developed. The mobile phase contained a mixture of acetonitrile and 0.35% triethylamine (pH 4.2) with orthophosphoric acid (70:30). As per ICH guidelines the optimized RP-HPLC method was validated with respect to linearity, limit of detection (LOD), limit of quantitation (LOQ), accuracy, precision, repeatability, robustness, ruggedness. The accuracy of the method was determined in terms of % recovery of the standard. The obtained test results were compared with that of the standard drug. The results of the recovery study were found to be within the acceptance criteria (96.93- 103.99%), which indicated a good degree of sensitivity of the developed method in detection of analytes in a sample.

Keywords: Dosage regimen, mycophenolate mofetil, prednisolone, RP-HPLC, ICH, tacrolimus

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E-mail addresses:
parryds2@gmail.com (Pardeep Kr. Sharma)
vijaymishra2@gmail.com (Vijay Mishra)
surajpal.15834a@gmail.com (Surajpal Verma)
drbhatiaamit@gmail.com (Amit Bhatia)
* Corresponding author

INTRODUCTION

Immunosuppression causes decrease in the immunity of the body and its ability to fight with various infections. Immunosuppressant drugs generally weaken the immune system so that it cannot differentiate the transplanted organ from the rest of the body, resulting

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in a decrease in the rejection rate. Some of these drugs are used to treat autoimmune disorders. In current scenario, under the combination therapy patient receives more than one therapy during the treatment. Several individual pills, which may contain a particular drug or the multiple drugs, are given to the patient during the treatment. The multiple drugs incorporated in a single dosage form generally improve patient compliance, which involves how correctly a patient follows dosage regimen. In industrial point of view, it is easy to formulate and analyze the single drug formulation. But, as the number of drugs increases, the complexity of the formulation increases. It generates the necessity of the development of reliable and rapid analytical method for routine analysis of the drugs in combination.

Mycophenolate Mofetil (MMF) (Figure 1), chemically 2-morpholinoethyl (E)-6-(1,3-dihydro-4-hydroxy-6-methoxy-7-methyl-3-oxo-5-isobenzofuranyl)-4-methyl-4-hexenoate is a potent, non-competitive, specific and reversible inhibitor of inosine monophosphate dehydrogenase (IMPDH) (Tripodi et al., 2001). IMPDH is an important enzyme in B- and T-cells for the synthesis of guanosine nucleotides. MMF is an ester prodrug of mycophenolic acid (MPA) and is converted to MPA by hepatic esterase (Fujiyama et al., 2010). MPA shows five-fold potency as an inhibitor of type II isoform of IMPDH, resulting in more strong inhibition of cell growth and multiplication of lymphocytes (Allison & Eugui, 2000). MMF inhibits the production of antibodies and the proliferation of lymphocytes (Birnbaum et al., 2009; Häntzschel et al., 2008; Iaccarino et al., 2007). MMF generally blocks the early events of proliferation and DNA synthesis. But, it does not inhibit the initial events like the production of interleukins (IL-1 and IL-2) during the activation of human peripheral blood mononuclear cells (HPBMC) (Sepe et al., 2008; Tjeertes et al., 2007). Since MMF is an ester prodrug of MPA, hence MPA may be present as a synthetic impurity in MMF (Tang et al., 2005). On 3rd May 1995, United States Food and Drug Administration (USFDA) approved MMF as an immunosuppressant used in kidney transplantation in combination with corticosteroids (Kim, Rostas, & Gabardi, 2013).

Tacrolimus (TAC) (Figure 2), an immunomodulator (FK506), was isolated from the fungus *Streptomyces tsukubaensis* in 1984. TAC, chemically (1R, 9S, 12S, 13R, 14S, 17R, 18E, 21S, 23S, 24R, 25S, 27R)-1, 14-dihydroxy-12-{(E)-2[(1R,3R,4R)-4-hydroxy-3-methoxycyclohexyl]-1-methylethenyl}-23,25-dimethoxy 13, 19, 21, 27-tetramethyl-17-prop-2-en-1-yl-11, 28-dioxa-4-azatricyclo [22.3.1.04,9] octacos-18-ene-2, 3, 10, 16-tetrone, is T-lymphocyte-specific macrolide calcineurin inhibitor, which inhibits the transcription of IL-2 and other cytokines (Homey et al., 1998) via T-cell activation through tumor necrosis factor-α (TNF-α), IL-1ß and IL-6 (Kawai & Yamamoto, 2005; Kondo et al., 2004). In late 80's TAC is used to prevent the rejection of solid organ post transplantation (Starzl et al., 1989). But after USFDA approval in year 2000, TAC ointment was used for many skin diseases like lupus dermopathy (Lampropoulos et al., 2004), atopic dermatitis psoriasis (Yamamoto & Nishioka, 2003), localized scleroderma (Mancuso & Berdondini,

2003), chronic actinic dermatitis (Evans, Palmer, & Hawk, 2004), pyoderma gangrenosum (Petering et al., 2001), Behçet's disease (Sakane et al., 1995), lichen planus (Lener et al., 2001), rheumatoid ulcers (Schuppe et al., 2000) and steroid rosacea (Goldman, 2001). The efficacy of TAC was found to be much better as compared to the corticosteroids due to less or no dermal side effects and systemic absorption (Jan, 2003). Some common adverse effects during the treatment of skin diseases are itching or erythema, burning sensations, which diminish as treatment progress (Soter et al., 2001).

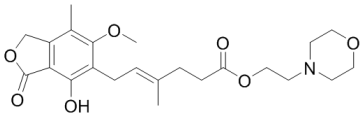

Figure 1. Chemical structure of MMF

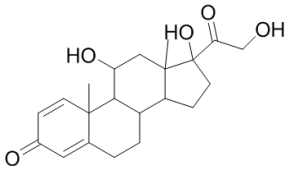

Figure 3. Chemical structure of prednisolone

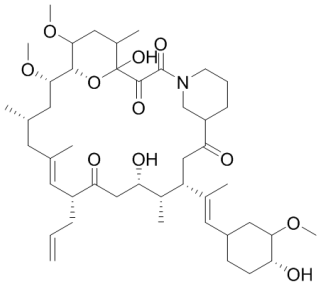

Figure 2. Chemical structure of TAC

Prednisolone (PRED) (Figure 3), chemically 11,17-dihydroxy-17-(2-hydroxyacetyl)-10,13-dimethyl-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydrocyclo penta[a] phenanthren-3-one is a synthetic corticosteroid, and always remains at the forefront of anti-inflammatory and immunosuppressive therapies (Ashok et al., 2011; Morrison, 2013). The exact mechanism of immunosuppressant activity of PRED is not known, however in vitro experiments demonstrated that PRED inhibited platelet aggregation by repressing the cellular adhesion molecule (CAM1) (Hirsch et al., 2012; Liverani et al, 2012; Wehling-Henricks, Lee, & Tidball, 2004).

Various analytical techniques like spectrophotometry (Singh & Nath, 2011), spectroscopy (such as NMR) (Touzani, 2011), chromatography (such as TLC or preparative TLC, HPTLC, gas chromatography, HPLC and HPLC coupled with other techniques like MS/MS-MS) (Danafar & Hamidi, 2015; Difrancesco et al., 2007; Kawanishi et al., 2015; Douma et al., 2016; Rissling et al., 2016; Sobiak et al., 2016; Tron et al., 2016; Wang et al., 2017) are available for the detection and quantification of drugs/compounds present in a sample. No official RP-HPLC method is available for the assay of MMF, TAC and prednisolone in single formulation (Benech et al., 2007; Chozas et al., 2012; Gonzalez-Ramirez et al., 2014; Kirresh et al., 2017; Parant et al., 2017; Rivera et al., 2017; Tölgyesi et al., 2017; Tummala et al., 2013; Vosough & Tehrani, 2018; Wiesen et al., 2012). So, there

is a need for method development for the assay of MMF, TAC, and PRED in combination (Snyder, Kirkland, & Glajch, 2012).

MATERIALS AND METHODS

In present work, several attempts have been made for the simultaneous estimation of MMF, TAC and PRED and its pharmaceutical dosage forms. A number of trials have been made concerning the mobile phase, and in addition UV detector's wavelength to develop an appropriate and quick technique for the study of all the three drugs, at the same time.

Materials, Reagents, and Chemicals

Drugs Mycophenolate Mofetil, Tacrolimus and Prednisolone were received as gift samples from Biocon Ltd., Bangalore, India and Jackson Laboratories Pvt Ltd., Amritsar, Punjab, India, respectively. Acetonitrile and other HPLC grade solvents and chemicals were purchased from Thermo Fisher Scientific, Vadodara, Gujarat, India. Orthophosphoric acid and Triethylamine of analytical grade were obtained from Merck, Mumbai, India. For the entire HPLC method, in-house produced double-distilled water was used.

The HPLC (Shimadzu, Kyoto, Japan) instrument equipped with two LC-10 ATVP pumps, SPD-10AVP UV-vis detector, injector with a 20 μL loop and Kinetex Polar, C18, 5 µm, 4.6 × 250 mm column was used for the experimental analysis. The results were acquired and processed using Shimadzu LC-solution version 6.42 software. A mixture of acetonitrile and 0.35% Triethylamine pH 4.2 with orthophosphoric acid (70:30) was used as mobile phase. Injection volume (20 µL) was injected into the column using a syringe and the linear gradient flow rate was set at 1.2 mL/min. The drugs were detected at 254 nm for Prednisolone and Mycophenolate and 210 nm for Tacrolimus.

Preparation of Standard Stock Solution

10 mg of each drug was accurately weighed and transferred into 10 mL volumetric flask containing 5 mL of acetonitrile and sonicated for 10 min then the volume was made up to 10 mL with acetonitrile.

Preparation of Sample Solution

Sample solutions of different concentrations ranging from 10-100 µg/mL were prepared from stock solution by diluting with acetonitrile.

Method Validation

As per ICH guidelines the optimized RP-HPLC method was validated with respect to Linearity, Limit of Detection (LOD), Limit of Quantitation (LOQ), Accuracy, Precision, Repeatability, Robustness, and Ruggedness.

RESULTS AND DISCUSSION

HPLC Chromatogram of Individual Drug and Mixture Sample

On HPLC analysis, chromatograms of individual drugs and in combination were optimized in terms of their retention time as shown in Figure 4.

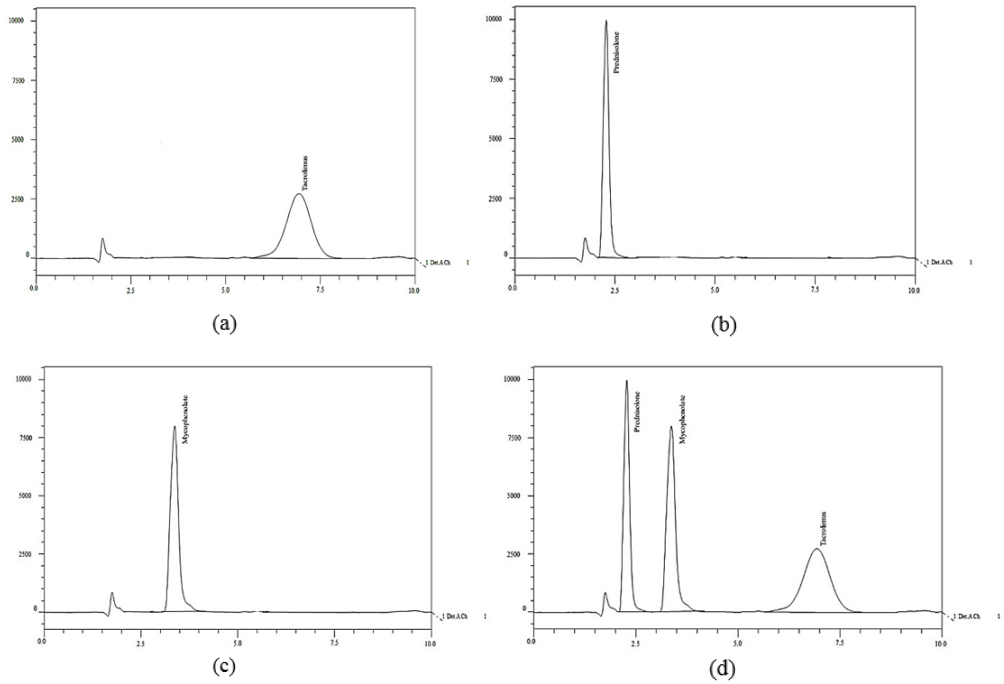

Figure 4. HPLC chromatogram of individual drugs (a) TAC, (b) PRED, (c) MMF and (d) drugs in combination

Linearity

For linearity, different concentrations ranging from 10-100 µg/mL of MMF, TAC and PRED were prepared. All the dilutions were filtered through 0.22 µm nylon filter and injected. Each concentration was used in triplicate. A calibration curve was plotted and r^2 was determined (Figure 5). All the drugs shows the linearity in the concentration ranging from 10-100 µg/mL (Table 1).

Accuracy and Precision

The accuracy of the method was determined in terms of percent recuperation of standard. Recuperation studies were carried out by extending the standard drug solution at the level of lower, medium and higher concentration of each drug in the pre-analyzed sample (Table 2). Results were found to be within the acceptance criteria (96.93-103.99%) representing

Table 1

Linearity observation of Prednisolone, Mycophenolate, and Tacrolimus (n=3)

Concentration (µg/mL)	PRED		MMF		TAC	
	Area	SD	Area	SD	Area	SD
10	305579.3	4048.347	192027.3	2066.726	112687.3	407.6547
20	634971	6180.709	370155.7	7401.618	239156.3	535.3264
30	1003729	2957.649	612807.7	10224.46	421809.3	4883.271
40	1329168	21710.31	768419.7	9407.889	524716	5618.779
50	1626347	16692.96	942059.7	4499.839	654758	10412.89
60	1976215	30299.37	1134766	14594.69	795546.7	2262.619
70	2321734	8966.311	1290143	11643.82	921787	1761.694
80	2651374	17168.08	1497025	20125.89	1042911	2222.671
90	2943142	366.5601	1668172	15772.36	1170387	4833.766
100	3224405	510.6959	1844333	2933.77	1286536	3677.219

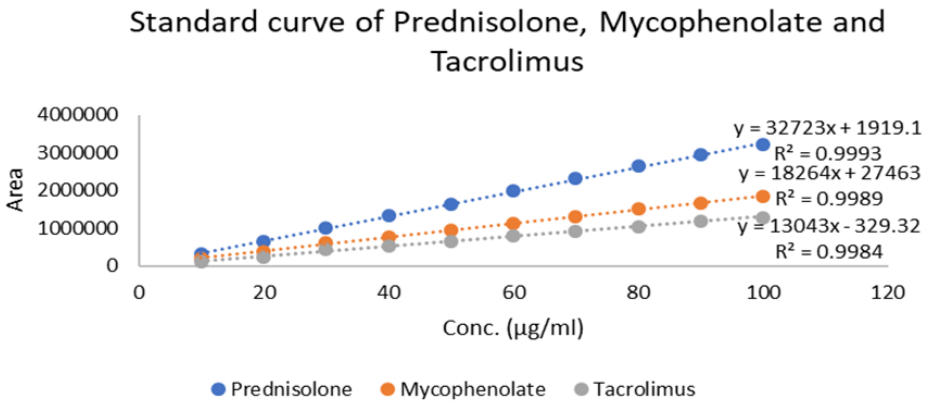

Figure 5. A standard curve of Prednisolone, Mycophenolate and Tacrolimus by RP-HPLC

a good degree of sensitivity of the method towards detection of analytes in a sample.

The intra-day and inter-day variation for determination of all the three drugs were carried out with 3 concentrations levels (i.e. low, medium and high) in the same day and 3 consecutive days where repeatability was determined with a lower concentration and injected six times and relative standard deviation (%RSD) was calculated.

Repeatability

The repeatability is established only when an observer is carrying the same experiment multiple times over a short period of time at the same place, on the same instrument, under

Table 2

Data Accuracy

conc. (µg/mL)	Intraday precision			Inter-day-1 precision			Inter-day-2 precision		
	PRED	MMF	TAC	PRED	MMF	TAC	PRED	MMF	TAC
50	1593277	814082	644690	1612348	783409	654833	1631418	746736	664976
50	1591086	814278	646370	1590942	778282	647281	1590797	740286	648192
50	1591739	811789	647013	1591951	779398	649052	1590162	737007	651091
50	1590479	809657	642863	1590849	779049	644703	1591218	736441	646543
50	1590472	807816	647326	1599090	769750	650780	1607707	731683	654234
50	1590916	805905	644609	1590694	776861	645968	1590472	747816	647326
Mean	1591328	810588	645479	1595979	777792	648769	1600296	739995	652060
SD	970.341	3102.36	1569.57	7889.46	4112.8	3353.88	15249.9	5735.01	6328.48
%RSD	0.06098	0.38273	0.24316	0.49433	0.52878	0.51696	0.95294	0.77501	0.97054

same conditions. The repeatability for the determination of MMF and TAC was estimated three times around the same day and for three continuous days. The percent RSD was calculated for each situation for all three drugs (Table 3). Repeatability was analyzed in six replicates for lowest concentration level. Intraday and inter-day studies were made in triplicate for each concentration level. In all the cases the %RSD was less than 2.

Table 3

Precision results showing repeatability

conc. (µg/mL)	Intraday precision			Inter-day-1 precision			Inter-day-2 precision		
	PRED	MMF	TAC	PRED	MMF	TAC	PRED	MMF	TAC
50	1593277	814082	644690	1612348	783409	654833	1631418	746736	664976
50	1591086	814278	646370	1590942	778282	647281	1590797	740286	648192
50	1591739	811789	647013	1591951	779398	649052	1590162	737007	651091
50	1590479	809657	642863	1590849	779049	644703	1591218	736441	646543
50	1590472	807816	647326	1599090	769750	650780	1607707	731683	654234
50	1590916	805905	644609	1590694	776861	645968	1590472	747816	647326
Mean	1591328	810588	645479	1595979	777792	648769	1600296	739995	652060
SD	970.341	3102.36	1569.57	7889.46	4112.8	3353.88	15249.9	5735.01	6328.48
%RSD	0.06098	0.38273	0.24316	0.49433	0.52878	0.51696	0.95294	0.77501	0.97054

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD and LOQ of developed method were accomplished according to ICH guidelines. A few methodologies for deciding the LOD and LOQ are conceivable, contingent upon the strategy i.e. a non-instrumental or instrumental. Among them, the following method was employed-

LOD= 3.3σ/S and LOQ= 10σ/S

where, σ = the standard error of response and S = the slope of the calibration curve. Results are represented in Table 4.

Table 4

LOD and LOQ

Sr. No.	Sample	LOD (µg/mL)	LOQ (µg/mL)
1.	Mycophenolate	0.210193	0.636949
2.	Prednisolone	0.442067	1.339597
3.	Tacrolimus	0.038667	0.117171

The LOD and LOQ were calculated on the basis of standard deviation of the response and the slope (s) of the calibration curve at approximate levels of LOD and LOQ. The obtained results were found to be within the limit.

Robustness and Ruggedness

These terms refer to the capability of an analytical method to remain unchanged by deliberately changing the method parameters like change in flow rate, and change in wavelength. The concept of remaining unchanged by deliberately varying the method parameters has two possible elucidations such as- (a) no change of the identified measure of the analyte in a specific test disregarding the variation in the method parameter or (b) no change is observed in the critical performance characteristics disregarding the variation in the method parameter.

For the calculation of robustness, the sample with lowest concentration was analyzed by deliberately changing the flow rate about ±15%, i.e. 1 and 1.4 mL/min and changing the wavelength by ±5 nm, i.e. 245 and 255 nm.

The robustness was studied by analyzing the sample containing lower concentration with deliberate variation in the method parameters. Robustness of the method was studied by a change in wavelength or change in flow rate. The change in the responses of drugs was noted in terms of %RSD (Table 5 and Table 6).

The ruggedness was studied by analyzing the same samples of three drugs by changing the analyst. The change in the responses of drugs was noted in terms of %RSD. Results are represented in Table 7.

Table 5

Change in wavelength

Concentration (µg/mL)	249 nm and 205 nm			254 nm and 210 nm			259 nm and 215 nm		
	Area			Area			Area		
	PRED	MMF	TAC	PRED	MMF	TAC	PRED	MMF	TAC
50	1776659	907237	933558	1573979	712879	679282	1371098	518520	425005
50	1775023	904786	935429	1571931	712900	679780	1369639	517013	424131
50	1774938	902279	935611	1572804	709292	682122	1370269	516305	426633
mean	1775540	904767	934866	1572905	711690	680395	1370335	517279	425256
SD	970.014	2479.05	1136.41	839.117	1695.9	1238.21	731.758	1131.26	1269.79
%RSD	0.05463	0.274	0.12156	0.05335	0.23829	0.18198	0.0534	0.21869	0.2986

The %RSD should not be more than 2. The %RSD obtained for a change of flow rate and wavelength was found to be below 2, which was within the acceptance criteria and indicated that the method was robust.

Table 6

Change in flow rate

Flow rate	1.0 mL/min			1.2 mL/min			1.4 mL/min		
Concentration (µg/mL)	Area			Area			Area		
	PRED	MMF	TAC	PRED	MMF	TAC	PRED	MMF	TAC
50	1602980	796699	646079	1590471	788571	651924	1577962	779842	643768
50	1603518	796200	648935	1591569	788276	645042	1579619	778152	641149
50	1604539	793512	651320	1591989	785249	643826	1579439	776986	635332
mean	1603679	795470	648778	1591343	787365	646931	1579007	778327	640083
SD	791.872	1714.22	2624.02	639.994	1501.31	3565.55	909.173	1435.99	4317.85
%RSD	0.04938	0.2155	0.40446	0.04022	0.19068	0.55115	0.05758	0.1845	0.67458

Table 7

Ruggedness data

Concentration (µg/mL)	Analyst 1			Analyst 2		
	Area			Area		
	PRED	MMF	TAC	PRED	MMF	TAC
50	1593277	814082	644690	1590797	740286	648192
50	1591086	814278	646370	1593832	739687	640289
50	1591739	811789	647013	1592418	738565	638463
50	1590479	809657	642863	1595121	738156	642563

Table 7 *(Continue)*

Concentration (µg/mL)	Analyst 1			Analyst 2		
	Area			Area		
	PRED	MMF	TAC	PRED	MMF	TAC
50	1590472	807816	647326	1592162	737007	651091
50	1590916	805905	644609	1591218	736441	646543
Mean	1591328	810588	645479	1592591	738357	644524
SD	970.341	3102.36	1569.57	1486.52	1358.13	4456.69
%RSD	0.06098	0.38273	0.24316	0.09334	0.18394	0.69147

The %RSD obtained was found to be below 2, which was within the acceptance criteria. So, the method was found to be rugged.

Specificity

Specificity of the HPLC method was demonstrated by the separation of the analytes from other potential components such as impurities, degradants or excipients. A volume of 20 µL of individual ingredients and excipients solution was injected and the chromatograms were recorded**.**

The test results obtained were compared with the results of those obtained for the standard drug. It was shown that potential components except drug were not interfering with the developed method. Results are represented in Table 8.

Table 8

Specificity data

Concentration (µg/mL)	Area		
	PRED	MMF	TAC
50	1536476	726475	622028
50	1536951	726409	621097
50	1534978	726462	611111
50	1539824	726385	621563
50	1533361	725767	618875
50	1538822	725034	621366
Mean	1536735.333	726088.6667	619340
SD	2178.766904	531.4836676	3814.259736
%RSD	0.141778929	0.073198177	0.615858775

CONCLUSION

The analytical strategy depicted in the present study has great precision, accuracy, linearity and is found appropriate for the simultaneous estimation of immunosuppressant drugs like MMF and TAC. As the technique was effectively validated as per ICH guidelines, it can be promptly utilized as a part of value control laboratories for the standard pharmaceutical investigation. Additionally, this straightforward and quick technique can streamline execution in developing new formulations containing immunosuppressant drugs like MMF, TAC and PRED.

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