British Journal of Pharmaceutical Research
3(3): 363-373, 2013

SCIENCEDOMAIN *international*
www.sciencedomain.org

Development and Validation of New RP-UPLC Method for the Quantitative Analysis of Mycophenolate in Tablet Dosage Form

P. Raja Abhilash[1*], K. Vasanth Kumar[2] and V. Sateesh Kumar[2]

[1]Department of Pharmaceutical Analysis and Quality Assurance, S.V.S. School of Pharmacy, Bheemaram, Hanamkonda, India.
[2]Department of Pharmaceutics, Jangaon Institute of Pharmaceutical Sciences, Jangaon, India.

Authors' contributions

This work was carried out in collaboration between all authors. Author PRA designed the method, wrote the first draft of manuscript and evaluated validation parameters. Author KVK has reviewed the various literatures. Author PRA has prepared the necessary sample and standard solutions. Author VSK has performed the statistical analysis and managed the analyses of the study. All authors read and approved the final manuscript.

Research Article

Received 6th October 2012
Accepted 23rd January 2013
Published 12th April 2013

ABSTRACT

Aim: To develop and validate a novel and rapid reverse phase ultra-performance liquid chromatographic (RP-UPLC) method for the estimation of mycophenolate in tablet formulations.
Place and Duration of Study: Department of pharmaceutical analysis, S. R. College of Pharmacy ananthasagar, between February 2012 and June 2012.
Methodology: Chromatographic separation was achieved on a Symmetry C_{18} (4.6x100mm, 3.5µm, Make: XBridge) column using an isocratic method with mobile phase composed of Potassium di-hydrogen phosphate: Acetonitrile in the ratio 35:65 v/v). The flow rate was 0.2 ml/min, temperature of the column was maintained at ambient and detection was made at 228 nm. The run time was as short as 1.2 min. The developed method was validated according to the International Conference on Harmonization (ICH) guidelines with respect to linearity, accuracy, precision, specificity and robustness.
Results: The developed method was linear for mycophenolate from 10-50µg/ml and the linear regression obtained was > 0.999. Precision, evaluated by intra- and inter-day

**Corresponding author: Email: abhilashmpharm@gmail.com;*

assays had relative standard deviation (R.S.D) values within 1.5%. Recovery data were in the range 99.1-102.0% with R.S.D. values < 1.5%.
Conclusion: The method is precise, accurate, linear, robust and fast. The short retention time of 0.613 min allows the analysis of a large number of samples in a short period of time and, therefore, should be cost-effective for routine analysis in the pharmaceutical industry.

Keywords: Mycophenolate; UPLC; new method development; validation.

1. INTRODUCTION

Mycophenolate mofetil is an immunosuppressive agent. 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[1]. It has an empirical formula of $C_{23}H_{31}NO_7$, a molecular weight of 433.50g, and the following chemical structure of Mycophenolate mofetil is given in Fig.1.

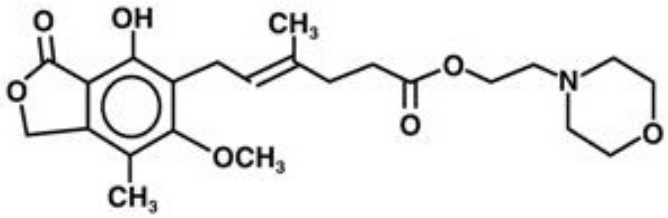

Fig. 1. Chemical structure of mycophenolate mofetil

MPA, which is released after hydrolysis of the morpholino ethylester linkage, is a potent, selective, uncompetitive, and reversible inhibitor of inosine monophosphate dehydrogenase (IMPDH) inhibits the de novo pathway of guanosine nucleotide synthesis [2]. 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 lymphocytes. 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.

Ultra performance liquid chromatography systems take advantage of technological pace in particle chemistry performance, system optimization, detector design and data processing. When taken together, these achievements have created an improvement in chromatographic performance. This new category of analytical separation science retains the practicality and principles of HPLC while increasing the overall interrelated attributes of speed, sensitivity and resolution. Today's pharmaceutical industries are looking for new ways to cut cost and shorten time for development of drugs while at the same time improving the quality of their products and analytical laboratories are not exception in this trend. Speed allows a greater number of analyses to be performed in a shorter amount of time thereby increasing sample throughput and lab productivity. These are the benefits of faster analysis and hence the ultra

performance liquid chromatography. A typical assay was transferred and optimized for UPLC system to achieve both higher sample analysis throughput and better assay sensitivity. Analysis of operation cost and sample throughput found UPLC cost advantageous over HPLC. In the present work, this technology has been applied to the method development and validation study of related substance and assay determination of Mycophenolate mofetil.

Though the literature provides some of the references on the estimation of mycophenolate mofetil tablets by HPLC method. Various authors have proposed a method for determination of mycophenolate mofetil in human and rat plasma by HPLC [3-7]**.** Simple determination of mycophenolic acid in human serum by column-switching high-performance liquid chromatography [8,9] and Determination of mycophenolic acid in human plasma by ion-pair RP-LC with fluorescence detection[10] was reported. Few literatures have revealed various methods to determine mycophenolate mofetil in human plasma and urine by LC-MS [11-16]. A simultaneous determination of mycophenolic acid and valproic acid in human plasma by HPLC [17] was reported. However there were no reports available on the estimation of mycophenolate mofetil by UPLC method. The reported HPLC methods are more time consuming, complex mobile phase mixtures, use high flow rate of analysis, lack of sensitivity and peak symmetry. It is, therefore, felt necessary to develop a new rapid method for the determination of mycophenolate mofetil by UPLC method. Hence a reproducible RP UPLC method was developed for the quantitative determination of mycophenolate mofetil tablets by using Symmetry C_{18} (4.6 x 100mm, 3.5 µm, Make: XBridge) UPLC column. The proposed method was validated as per the guidelines suggested by ICH [18].

2. EXPERIMENTAL DETAILS

2.1 Materials and Reagents

Mycophenolate mofetil Working Standard was procured from Teena laboratories, Hyderabad, India. Commercially available Mycophenolate mofetil purchased from local pharmacy. Methanol, acetonitrile HPLC Grade and Ortho phosphoric acid AR grade were obtained from Merck chemicals, Mumbai. Water was prepared by using Millipore Milli Q Plus water purification system.

2.2 Chromatographic Conditions

Chromatography separation was performed on Waters Aquity UPLC with photodiode array detector. The output signal was monitored and processed using empowers software. The chromatographic column used Symmetry C_{18} (4.6 x 100mm, 3.5 µm, Make: XBridge). The mobile phase of Potassium di-hydrogen phosphate: acetonitrile in the ratio 35:65 v/v at a flow rate of 0.2 ml/min. The detection was monitored at the Wavelength of 228 nm. The injection volume was 3.0 µL and the chromatographic runtime of 1.2 min was used.

2.3 Preparation of Solutions

2.3.1 Preparation of phosphate buffer

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

2.3.2 Preparation of mobile phase

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

2.4 Preparation of the Mycophenolate Mofetil Standard & Sample Solution

2.4.1 Standard solution preparation

Accurately transferred 10mg of Mycophenolate mofetil Working standard into a 10 mL volumetric flask and about 7 mL of Diluent added then sonicated to dissolve it completely and the volume was made up to the mark with the same solvent (Stock solution). Further pipetted 0.3 ml of the above stock solution into a 10ml volumetric flask and diluted up to the mark with diluent. Mix well and filter through 0.45µm filter.

2.4.2 Sample solution preparation

Accurately transferred the sample equivalent to 10 mg of Mycophenolate mofetil into a 10mL volumetric flask. About 7mL of diluent added and sonicated to dissolve it completely and the volume is made up to the mark with diluent. Mixed well and filtered through 0.45µm filter. Further pipetted 0.3 ml of the above stock solution into a 10ml volumetric flask and diluted up to the mark with diluent. Mixed well and filtered through 0.45µm filter.

2.5 Method Validation

2.5.1 Precision

The precision of the method was evaluated by carrying out six independent asses of test sample against a qualified reference standard and the %RSD of assay was calculated (% RSD should not be more than 2%).

2.5.2 Intermediate precision/ruggedness

2.5.2.1 Intra-day precision

Intraday precision was evaluated on same day by carrying out six independent assays of mycophenolate mofetil (50,100, 150% i.e. 5.0, 10.0, 15.0 µg/ml.) test samples against qualified reference standard. The percentage of RSD of six assay values was calculated.

2.5.2.2 Intermediate precision (inter-day)

The inter day precision was evaluated on different days by different analyst from the same laboratory and by using different column of same brand. This was performed by assaying the six samples of mycophenolate mofetil against qualified reference standard. The percentage of RSD of six assay values was calculated. The %RSD for the area of six replicate injections was found to be within the specified limits (% RSD should not be more than 2%).

2.5.3 Accuracy

Recovery of the assay method for mycophenolate mofetil was established by three determinations of test sample using tablets at 50%, 100% and 150% of analyte concentration. Each solution was injected thrice (n=3) into UPLC system and the average peak area was calculated from which Percentage recoveries were calculated. (% Recovery should be between 98.0 to 102.0%).

2.5.4 Linearity

Test solutions were prepared from stock solution at 5 concentration levels (0.008, 0.010, 0.012, 0.014, and 0.016µg/ml). Each solution was injected thrice (n=3) into UPLC system and the peak area vs. concentration data treated by least square linear regression analysis. (Correlation coefficient should be not less than 0.999).

2.5.5 Limit of detection (LOD) limit of quantification (LOQ)

LOD and LOQ for the were determined at signal to noise ratios of 3:1 and 10:1, respectively by injecting series of dilute solutions with known concentrations.

2.5.6 Robustness

To prove the reliability of the analytical method during normal usage, some small but deliberate changes were made in the analytical method (e.g., flow rate, column temperature, and mobile phase composition). Changes in the chromatographic parameters (i.e., theoretical plates and the tailing factor) were evaluated for the studies.

2.5.7 Solution stability and mobile phase stability

Stability of mycophenolate mofetil solution was studied by injecting the sample into the chromatographic system at different time interval. The peak area was recorded in the time intervals of 0, 12, and 24 hrs. Freshly prepared solution was injected at the same time intervals for mobile phase stability (0, 12, and 24 hours).

3. RESULTS

3.1 Method Development

Different chromatographic conditions were experimented to achieve better efficiency of the chromatographic system. Parameters such as mobile phase composition, wavelength of detection, column, column temperature, pH of mobile phase, and diluents were optimized. Several proportions of buffer, and solvents (water, methanol and acetonitrile) were evaluated in order to obtain suitable composition of the mobile phase. Choice of retention time, tailing, theoretical plates, and run time were the major tasks while developing the method. Acquity BEH C18, 50mm × 2.1 mm, 1.7 µm column used for the elution, but the peak eluted before 1.5 minutes with a tailing factor of 2. Experiment with Phenyl 100mm × 2.1 mm, 2 µm column ended with inconsistent retention time and peak fronting. Buffers like sodium dihydrogen orthophosphate, dipotassium hydrogenorthophosphate, and disodium hydrogen orthophosphate did not yield desired results. Use of ion pair reagents also did not yield the expected peak.

At 35: 65 ratio of the mobile phase, a perfect peak was eluted. Thus the mobile phase ratio was fixed at 35:65 (buffer: solvent) in an isocratic mobile phase flow rate. The typical chromatogram obtained for Mycophenolate mofetil from final UPLC conditions are depicted in Fig. 2.

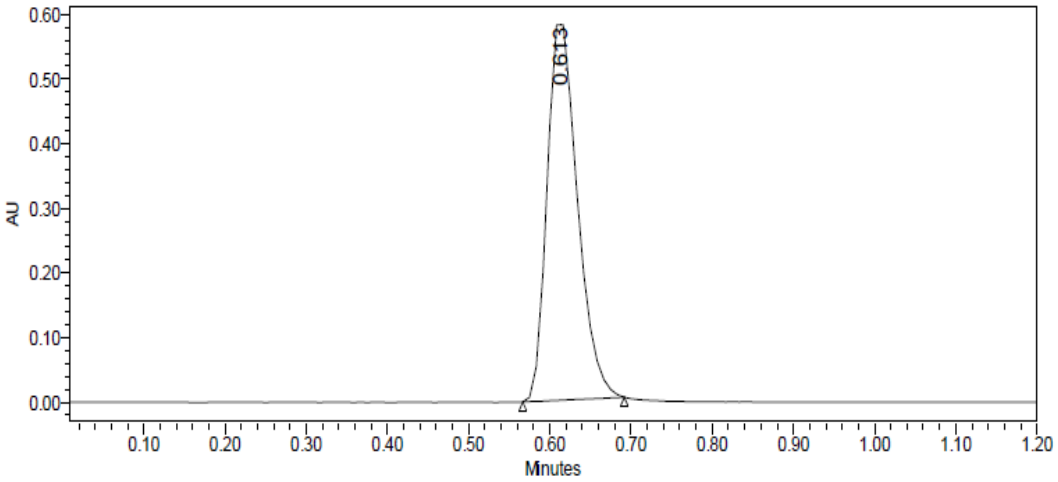

Fig. 2. Typical chromatogram of Mycophenolate mofetil by proposed method

3.2 Method Validation

Based on International Conference on Harmonization (ICH) guidelines, the method is validated with regard to system suitability, linearity, accuracy, precision, LOD, LOQ , robustness and sensitivity as follows.

3.2.1 System suitability

The system suitability results for the proposed UPLC method areTailing factor Obtained from the standard injection is 1.3. Theoretical Plates Obtained from the standard injection is 8287.2. The results proved that the optimized UPLC method fulfils these requirements within the USP accepted limits indicated in the 'Experimental' section.

3.2.2 Precision

The % R.S.D. of Mycophenolate mofetil assay during the method precision was found to be 0.4%, indicating good precision of the method. The results are summarized in Table 1.

3.2.3 Intermediate Precision

The % R.S.D. of Mycophenolate mofetil assay during the intermediate precision was found to be 0.3%, the value is well within the generally acceptable limits. The results are summarized in Table 2.

Table 1. Results of precision

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%

Table 2. Results of intermediate precision

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%

3.2.4 Limits of detection (LOD) and quantification (LOQ)

LOD and LOQ for Mycophenolate mofetil were 0.002 and 0.008 µg/ml, respectively. Since the LOQ and LOD values of Mycophenolate mofetil are achieved at a very low level, this method can be suitable for cleaning validation in the pharmaceutical industry.

3.2.5 Accuracy

Percent recovery of Mycophenolate mofetil samples ranged from 98.01% to 102.0% and the mean recovery is 99.84%, showing the good accuracy of the method. The result is shown in Table 3.

Table 3. Results of accuracy

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	773817.1	5.1	5.0	98.03%	99.84%
100%	1514099.3	9.98	10.0	100.2%	
150%	2227360.7	14.6	14.8	101.3%	

3.2.6 Linearity

The linearity of the calibration plot for the method was obtained over the calibration ranges tested, i.e., 0.008-0.016 µg/ml for three times, and the correlation coefficient obtained was 0.999, thus indicating excellent correlation between peak areas and concentrations of the analyte**.** The results were showed in Chart 1.

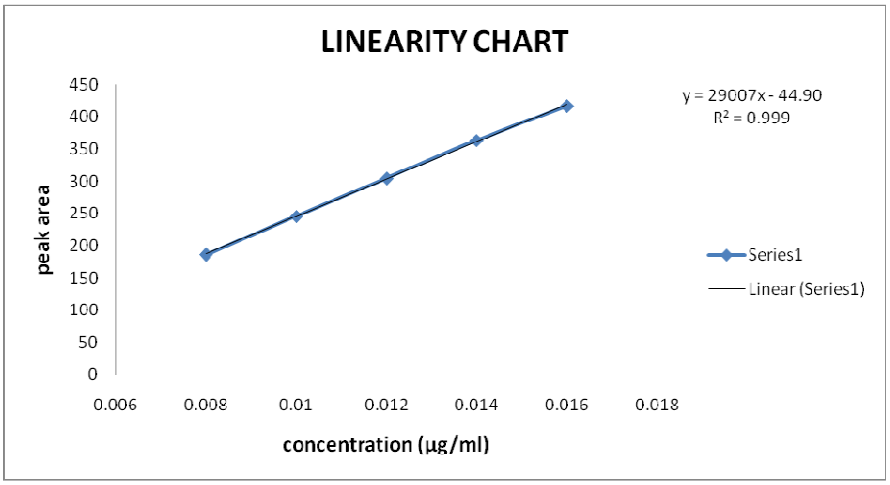

Chart-1- Linearity chart

3.2.7 Robustness

In all the deliberately varied chromatographic conditions in the concentration range for the evaluation of robustness is 10 -50 µg/ml, (n=3). It can be concluded that the variation in flow rate and the variation in 10% Organic composition do not affect the method significantly. Hence it indicates that the method is robust even by change in the flow rate ±10% and change in the Mobile phase ±10%. The results are summarized in Table 4.

Table 4. Results of robustness

Chromatographic changes	USP plate count	USP tailing
Flow rate(ml/min)		
0.1	8263.6	1.2
0.2*	8287.2	1.3
0.3	8190.4	1.2
Change in organic composition in the mobile phase		
10% less	8250.2	1.3
65:35 (Acetonitrile: Buffer)*	8287.2	1.3
10% more	8174.9	1.2
UV wavelength(nm)		
226	8154.5	1.2
228*	8214.3	1.3
230	8194.6	1.3

** optimized parameters*

3.2.8 Sensitivity

The LOQ concentration was found to be 0.008 µg/mL, The LOD concentration was found to be 0.002 µg/ mL, at an injection volume of 1 µL. The lower LOQ values with good precision indicate the better sensitivity of the UPLC method.

3.2.9 Application of the developed method to commercial mycophenolate mofetil tablets

When the developed method was used to analyze a commercial brand of Mycophenolate mofetil tablet formulation, the mean recovery of six replicates was 99.69% with % R.S.D. of 0.52. The % recovery value indicates non-interference from the excipients present in the dosage form.

4. DISCUSSION

4.1 Method Development and Optimization

The main aim of the developed method was to achieve separation and quantification of Mycophenolate mofetil using an isocratic mobile phase with UPLC system. Developing a UPLC method was to reduce the run time of the method and solvent consumption for routine analysis such as assay, dissolution and content uniformity during quality assurance. Detection of Mycophenolate mofetil was adequate at 228 nm. The initial trial was conducted using HPLC and chromatographic separation was obtained on a Waters symmetry C_{18} column (150 x 4.6mm, particle size 5µm). Mycophenolate mofetil is an acid labile compound and to avoid any degradation, a mobile phase with basic pH was selected. The mobile phase was optimized in the ratio of Potassium di-hydrogen phosphate: acetonitrile in the ratio 35:65 v/v at a flow rate of 0.2 ml/min. While developing the UPLC method, basic chromatographic conditions such as the column, solvents and UV detection employed in the HPLC method were taken into account. In selecting the UPLC column, its stability at the higher pH was taken into consideration to preserve the long life of the column. Most commercial C_{18} columns are not stable at high pH on the longer run, thus shortening their life span. Symmetry C_{18} (4.6 x 100mm, 3.5 µm, Make: XBridge) column was found to be more suitable and stable at this pH. The peak was sharp and acceptable. The flow rate also is scaled down from 1.0 to 0.2 ml/min. When these operating condition were applied to the developed method, a satisfactory peak was achieved for Mycophenolate mofetil, which eluted at around 0.612 min giving a total run time of1.2 min.

5. CONCLUSION

The new, isocratic RP-UPLC method proved to be simple, linear, precise, accurate, robust, rugged and rapid. The developed method was capable of giving faster elution, maintaining good separation more than that achieved with conventional HPLC. The short retention time of 0.612 min allows the analysis of a large number of samples in a short period of time and is therefore more cost-effective for routine analysis in the pharmaceutical industries. It is suitable for rapid and accurate quality control of Mycophenolate mofetil in tablet formulations.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Available from: www.chemblink.com.
2. Franklin T J and Cook J M.(1969). The inhibition of nucleic acid synthesis by mycophenolic acid, Biochem. J. 133(3):515-524.
3. Barzoki MA, Rouini M, Gholami K, Lessan-Pezeshki M, Rezaee S. Determination of mycophenolic acid in human plasma by high-performance liquid chromatography, DARU. 2005;13(3):120-126.
4. Srivatsan V, Dasgupta A K, Kale P, Verma R, Joshi P, Soni D, Patel M, Soni G, Patel J, Modi H. Determination of mycophenolic acid in human plasma by high-performance liquid chromatography, Journal of Chromatography. A. 2004;1031(1-2):259-264.
5. Na-Bangchang K, Supasyndh O, Supaporn T, Banmairuroi V, Karbwang J. Simple and sensitive high-performance liquid chromatographic method for the determination of mycophenolic acid in plasma, Journal of Chromatography. 2000;B738(1):169-173.
6. Tisna I, Kaloostian M, Lee R, Tarnowski T, Wong B. High-performance liquid chromatographic method for the determination of mycophenolate mofetil in human plasma, Journal of Chromatography. 1996;B681(2):347-353.
7. Sugioka N, Odani H, Ohta T, Kishimoto H , Yasumura T, Takada K. Determination of a new immunosuppressant, mycophenolate mofetil, and its active metabolite, mycophenolic acid, in rat and human body fluids by high-performance liquid chromatography, Journal of Chromatography. 1994;B654(2):249-256.
8. Teshima D, Kitagawa N, Otsubo K, Makino K, Itoh Y, Oishi R. Simple determination of mycophenolic acid in human serum by column-switching high-performance liquid chromatography, Journal of Chromatography. 2002;B780(1): 21-26.
9. Renner U D, Thiede C, Bornhauser M, Ehninger G and Hans-Michael Thiede. Determination of mycophenolic acid and mycophenolate mofetil by high-performance liquid chromatography using postcolumnderivatization, Analytical Chemistry. 2001;73(1):41-46.
10. Hosotsubo H, Takahara S, Kokado Y, Permpongkosol S, Wang J D, Tanaka T, Matsumiya K, Kitamura M, Okuyama A, Sugimoto H. Rapid and simple determination of mycophenolic acid in human plasma by ion-pair RP-LC with fluorescence detection, Journal of Pharmaceutical and Biomedical Analysis. 2001;24(2):555-560.
11. Kuhn J, Gotting C, Kleesiek K. Measurement of mycophenolic acid and its glucuronide using a novel rapid liquid chromatography–electrospray ionization tandem mass spectrometry assay, Clinical Biochemistry. 2009;42(1-2):83-90.

12. Kuhn J, Prante C, Kleesiek K, Gotting C. Sample cleanup-free determination of mycophenolic acid and its glucuronide in serum and plasma using the novel technology of ultra-performance liquid chromatography–electrospray ionization tandem mass spectrometry, Talanta. 2010;80(5):1894-1898.
13. Shen B, Li S, Zhang Y, Yuan X, Fan Y, Liu Z, Hu Q, Yu C. Determination of total, free and saliva mycophenolic acid with a LC–MS/MS method: Application to pharmacokinetic study in healthy volunteers and renal transplant patients, Journal of Pharmaceutical and Biomedical Analysis. 2009;50(3):515-521.
14. Marie-Odile, Benoit-Biancamano, Caron P, Levesque E, Delage R. Sensitive high-performance liquid chromatography–tandem mass spectrometry method for quantitative analysis of mycophenolic acid and its glucuronide metabolites in human plasma and urine, Journal of Chromatography. 2007;B858(1-2):159-167.
15. Benech H, Hascoet S, Furlan V, Pruvost A, Durrbach A. Development and validation of an LC/MS/MS assay for mycophenolic acid in human peripheral blood mononuclear cells, Journal of Chromatography. 2007;B853(1-2):168-174.
16. Platzer M, Jahn K, Wohlrab J, Neubert R H. Quantification of mycophenolate mofetil in human skin extracts using high-performance liquid chromatography–electrospray mass spectrometry, Journal of Chromatography. 2001;B755(1):355-359.
17. Zhong Y, Jiao Z, Yunqiu Y. Simultaneous determination of mucophenolic acid and valproic acid based on derivatization by high-performance liquid chromatography with fluorescence detection, Biomedical Chromatography. 2006;20(4):319-326.
18. International Conference on Harmonization, ICH Q2 (R1) Guideline on Validation of Analytical Procedures: Text and Methodology (2005) Yokohama, Japan.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history.php?iid=211&id=14&aid=1233