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Determination of mycophenolic acid in human plasma by high-performance liquid chromatography

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Abstract

The development, validation and evaluation of high-performance liquid chromatography (HPLC) method for quantifying mycophenolic acid in human plasma is described. The method involved protein precipitation using acetonitrile, after addition of terazosin as an internal standard. Separation was achieved with a reversed-phase C_{18} column (250 mm × 4.6 mm) employing UV detection at 215 nm. The mobile phase consisted of 0.02 M potassium dihydrogenphosphate solution adjusted to pH 6.9 with 2 M potassium hydroxide solution–acetonitrile (80:20 (v/v)) at a flow rate of 1.5 ml/min. The total run time was 21.0 min. The assay was linear from 0.2 to 25 μg/ml with goodness of fit (r^2) greater than 0.99 observed with three precision and accuracy batches during validation. The observed mean recoveries were 89.3 and 98.0% for drug and internal standard, respectively. The applicability of this method to pharmacokinetic studies was established after successful application during a 34-subject bioavailability study. The method was found to be precise, accurate and specific during the study.

Keywords: Validation; Mycophenolic acid; Terazosin

1. Introduction

Mycophenolate mofetil is the morpholinoethyl ester pro-drug of mycophenolic acid (Fig. 1a). It is rapidly hydrolyzed to mycophenolic acid, the active form of this immunosuppressant agent, after absorption. Mycophenolic acid exerts its immunosuppressive activity through noncompetitive and reversible inhibition of inosine monophosphate dehydrogenase, thus decreasing guanine nucleotide levels, with a subsequent reduction in lymphocyte proliferation. Inosine monophosphate dehydrogenase differentially affects lymphocytes that are dependent on two enzymes for the de novo synthesis of purine nucleotides [1].

An high-performance liquid chromatography (HPLC) method is available for measuring mycophenolic acid with a sensitivity of 0.4 ng/ml [1]. Also an HPLC method using fluorescence detector for measuring mycophenolic acid in human plasma in range from 0.2 to 20.0 μg/ml is available [2]. Few more methods are also available for the determination of mycophenolic acid in human plasma using liquid–liquid extraction or solid-phase extraction techniques for sample preparation with the limit of quantification upto 0.1 μg/ml [3–7].

This article describes a simple, sensitive and specific method for the determination of mycophenolic acid in human plasma using UV detection. The preparation of the samples was based on simple protein precipitation instead of liquid–liquid extraction or solid-phase extraction. This assay allowed determination of 0.2 μg/ml of mycophenolic acid using 250 μl plasma sample.

The calibration curve standards and quality control samples for mycophenolic acid were prepared in bulk and stored in a freezer below −60 °C. The above mentioned samples were used during method validation to establish precision and accuracy as well as stability.

2. Experimental

2.1. Chemicals and reagents

Mycophenolic acid and terazosin hydrochloride (Fig. 1b) working standards were procured from Biocon India, India, and Astron Research, India. respectively. Acetonitrile

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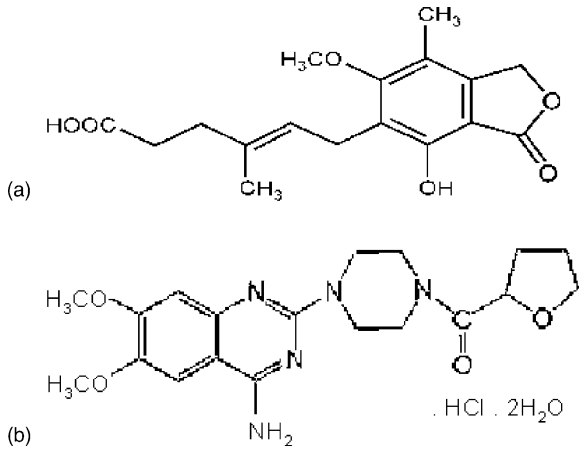

Fig. 1. Structure of (a) mycophenolic acid and (b) terazosin hydrochloride.

(HPLC grade) and methanol (HPLC grade) were purchased from Spectrochem, India, and potassium dihydrogenphosphate (GR grade) and potassium hydroxide (GR grade) were purchased from Merck, India. Laboratory-prepared water (Milli-Q) was used. Plasma was procured from Prathama Blood Centre, India.

2.2. Equipment

HPLC, Shimadzu 10 A *VP* Series System was supplied by Shimadzu, Japan; the Milli-Q water (category no. QGARD00R1) system was supplied by Millipore India, India; the Cyclo mixer was supplied by Remi equipments, India; and the centrifuge (Megafuge 2.0 R) was supplied by Heraeus Instruments, Germany.

2.3. Preparation of calibration curve standards

A stock solution of mycophenolic acid of 2500 μg/ml was prepared using methanol as diluent (stable for 14 days at 2–8 °C.). Calibration standards were prepared by mixing appropriate volumes of the serial dilutions and control plasma to achieve eight different concentrations from 0.2 to 25.0 μg/ml of mycophenolic acid. The standards were then stored in a freezer below −60 °C.

2.4. Preparation of quality control samples

Quality control samples were prepared by mixing appropriate volumes of the serial dilutions prepared from the stock solution prepared for calibration curve standards and control plasma to achieve four different concentrations at LOQ (limit of quantification), low, medium and high levels each for mycophenolic acid. The QC (quality control) samples were then stored in a freezer below −60 °C.

2.5. Sample preparation for analysis

The frozen samples were thawed in water at room temperature. The thawed samples were vortexed to ensure complete mixing of contents. Plasma (0.25 ml) was transferred into pre-labeled tubes and 25 μl of IS dilution (about 500 μg/ml of terazosin) was added to each tube and this solution was vortexed. After adding 1.0 ml of acetonitrile, it was vortexed for 30 s to ensure uniform mixing. The tubes were centrifuged at 1891 × *g* for 5 min at 10 °C. Evaporated supernatants at about 40 °C under nitrogen gas stream and reconstituted the residue with 200 μl of reconstitution solution (Milli-Q water–acetonitrile, 80:20). The reconstituted samples were transferred into HPLC autosampler vials for analysis. All the procedures from thawing to placement in the autosampler were performed at room temperature.

2.6. Chromatographic conditions

The samples were chromatographed on a Kromasil C_{18} 5 μm (250 mm × 4.6 mm) column supplied by Flexit, India, at 30 °C. The mobile phase consisted of a mixture of acetonitrile–0.02 M potassium dihydrogenphosphate buffer, pH adjusted to 6.9 with 2 M potassium hydroxide solution (20:80). The autosampler was set at 4 °C and the injection volume was 15 μl. The pump flow was 1.5 ml/min and the absorbance detector was set at 215 nm.

2.7. Linearity, precision and accuracy

Linearity, precision and accuracy were established for mycophenolic acid during the course of the validation by analysing three precision and accuracy batches consisting 8-point calibration curve and eight sets of each LOQ, low, medium and high quality control samples. Eight different calibration curve standards (range from 0.2 to 25.0 μg/ml) of mycophenolic acid along with eight sets of QC samples (each of LOQ, low, medium and high) were retrieved from the deep freezer and processed as per the procedure mentioned in Section 2.5.

2.8. Stability

2.8.1. Freeze thaw

Eight high and low quality control samples each, which were prepared and stored as mentioned in Section 2.4 were retrieved from the deep freezer after they had been frozen completely after storage (about 24 h) and thawed at room temperature. After complete thawing of the samples the same were put back into the freezer. The above step was repeated after freezing the samples for 12–24 h for another two more times. Thus, all the samples went through three freeze thaw cycles.

All the stability samples (after three freeze thaw cycles) were processed as per Section 2.5 with freshly thawed QCs (eight each high and low). The means of responses were compared of all the QC samples (that had gone through three freeze thaw cycles) with those of freshly thawed QC samples for stability.

Table 1
Curve parameter summary and back-calculated calibration curve concentrations for mycophenolic acid in human plasma

	Concentration (μg/ml)							
	0.207	0.414	1.254	3.742	12.474	17.569	22.524	25.027
Mean	0.2110	0.4003	1.2380	3.6737	12.1763	17.9210	22.7887	25.9457
S.D.	0.00100	0.00473	0.01253	0.05601	0.24974	0.26700	0.31107	0.18604
R.S.D. (%)	0.5	1.2	1.0	1.5	2.1	1.5	1.4	0.7
Accuracy (%)	101.9	96.7	98.7	98.2	97.6	102.0	101.2	103.7

2.8.2. Bench top

Eight samples each of high and low QCs were taken out from deep freezer, thawed and kept at room temperature for 6.0 h. After 6.0 h, another set of eight high and eight low QC samples were taken out from deep freezer and thawed. Both the sets of low and high QC samples were processed as per Section 2.5 and the means of responses of stability samples with those freshly thawed QC samples were compared for stability.

2.8.3. Autosampler

After completion of the analysis of precision and accuracy batch, all the QC samples (low, medium and high) were stored in autosampler at 4 °C and re-injected after 43 h as stability samples along with another set of freshly processed QCs (eight each of high, medium and low). The means of responses of stability samples with those of freshly processed QC samples were compared for stability.

2.8.4. Long term

The QC samples (low and high) prepared and stored below −60 °C as mentioned in Section 2.4 were stored for 31 days. Few days prior to the analysis, fresh QC samples were prepared (as per Section 2.4) from freshly prepared drug stock and used as comparison samples. All the stability samples (eight high and eight low) and comparison samples (eight high and eight low) were processed as per Section 2.5. The mean of responses was compared of all the QC sample (stored below −60 °C for 31 days) with those of freshly prepared QC samples for stability.

Table 2
Between-batch precision and accuracy of mycophenolic acid

	Concentration (μg/ml)			
	LOQ QC (0.210)	LQC (0.601)	MQC (12.013)	HQC (20.022)
Mean	0.2236	0.6228	12.1083	21.2762
S.D.	0.01214	0.02090	0.22246	0.46433
R.S.D. (%)	5.4	3.4	1.8	2.2
Accuracy (%)	106.5	103.6	100.8	106.3

2.9. Collection and storage of plasma samples

The blood samples were collected in vacutainers containing EDTA as the anticoagulant. The samples were then centrifuged (1891 × *g*, 10 °C, 15 min) and separated plasma samples were stored in polypropylene tubes below −60 °C.

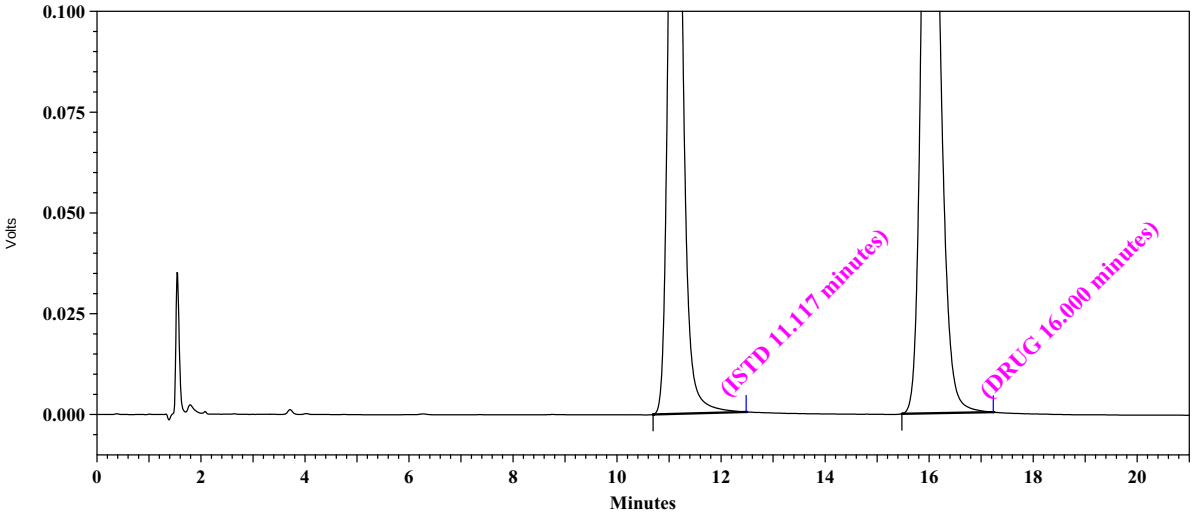

Fig. 2. Representative chromatogram of aqueous standard (18.070 μg/ml).

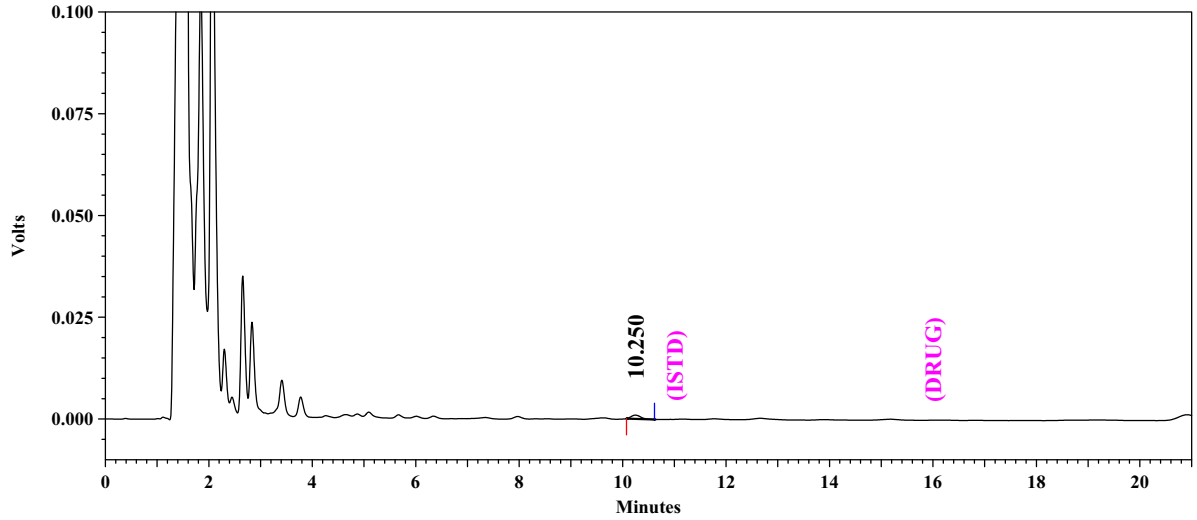

Fig. 3. Representative chromatogram of standard blank.

3. Results and discussion

3.1. Linearity

A linear equation was judged to produce the best fit for the concentration/response relationship. The regression type was 1/concentration^2 and peak area ratio for an 8-point calibration curve was found to be linear from 0.207 to 25.027 μg/ml. The goodness of fit were consistently greater than 0.99 during the course of validation (Table 1).

3.2. Precision and accuracy

The precision of the assay was measured by the percent coefficient of variation over the concentration range of LOQ, low, medium and high quality control samples, respectively of mycophenolic acid during the course of the validation. The accuracy of the assay was defined as the absolute value of the ratio of the back-calculated mean values of the quality control samples to their respective nominal values, expressed as percentages (Table 2).

Representative chromatograms of aqueous standard, standard blank, high QC and LOQ QC are shown in Figs. 2–5.

3.3. Stability

The concentration values obtained during the stability experiments, i.e. autosampler stability after 43.0 h at 4 °C, freeze thaw stability after three cycles, bench top stability after 6.0 h at room temperature and long-term stability after 31 days below −60 °C, suggest that there was no significant loss of mycophenolic acid concentrations (Table 3).

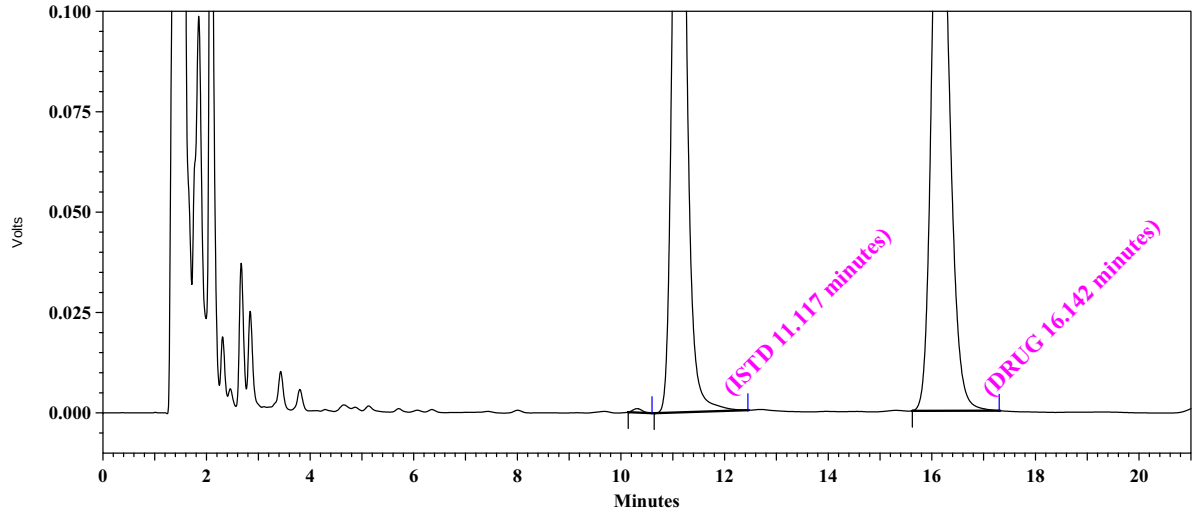

Fig. 4. Representative chromatogram of high QC.

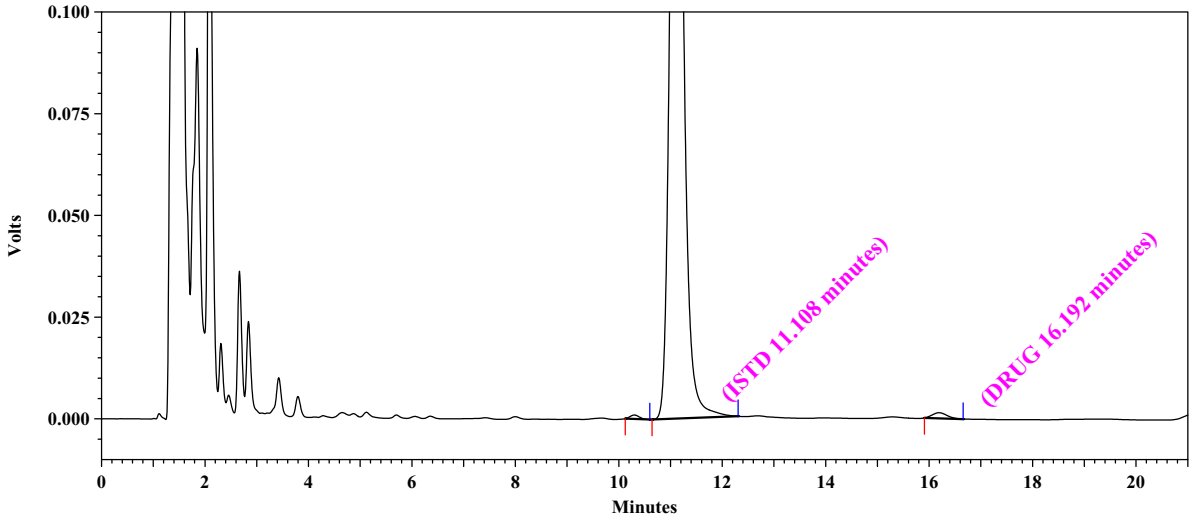

Fig. 5. Representative chromatogram of LOQ QC.

3.4. Specificity

Ten lots of human plasma, commercially procured, were chromatographically screened for interfering substances and did not show significant interference at the retention time of mycophenolic acid and IS (internal standard).

3.5. Recovery

3.5.1. Mycophenolic acid

The areas of low, medium and high quality control samples were compared against the area of respective aqueous quality control samples. The mean recoveries for mycophenolic acid for the low, medium and high QCs were 86.54, 89.98 and 91.39%, respectively.

3.5.2. Internal standard

The internal standard peak areas of low, medium and high quality control samples were compared to the internal standard peak areas of respective aqueous quality control samples. Mean recovery for terazosin for the low, medium and high QCs was 98.01%.

3.6. Carryover

No significant carryover was observed when standard blanks were injected immediately following high standards.

3.7. Application

The applicability of this method to pharmacokinetic studies was established after successful application during a 34-subject bioavailability study. Tablets containing mycophenolate mofetil 500 mg were dosed to healthy, adult, male, human subject under fasting conditions. Precision of calibration curve standards and quality control samples was obtained, 0.9–1.7% and 1.7–3.7%, respectively. Accuracy of calibration curve standards and quality control samples was obtained as 96.6–103.1% and 102.0–106.6%, respectively.

4. Conclusion

The method described here for assay of mycophenolic acid in plasma was found to be specific during

Table 3
Result table of stability data obtained during validation

Parameters	Results (%)	
	Drug	IS
Short-term stock solution (after 6.0 h at room temperature)	101.3	97.4
Long-term stock solution (within 2 to 8 °C)	97.6 (after 14 days)	104.5 (after 16 days)
Autosampler/wet extract (low, medium and high) (after 43 h at 4 °C)	102.4, 100.1 and 101.4	NA
Dry extract (low and medium) (after 73.0 h below −20 °C)	104.2 and 104.5	NA
Freeze thaw(low and high) (3 cycles)	98.3 and 99.7	NA
Bench top (low and high) (after 6.0 h at room temperature)	99.7 and 100.9	NA
Long-term stability of drug in plasma (low and high) (after 31 days below −60 °C)	93.9 and 102.9	NA

a bioavailability study. This demonstrated the suitability of the analytical method for use in bioavailability studies.

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