ORIGINAL ARTICLE

Determination of mycophenolic acid in human plasma by ultra performance liquid chromatography tandem mass spectrometry

Vivek Upadhyaya, Vikas Trivedi, Gaurang Shah, Manish Yadav, Pranav S. Shrivastava

Chemistry Department, Kadi Sarva Vishwavidyalaya, Gandhinagar 382015, India
Department of Chemistry, School of Sciences, Gujarat University, Navrangpura, Ahmedabad 380009, India

Received 28 February 2013; accepted 3 June 2013
Available online 12 June 2013

KEYWORDS
Mycophenolic acid; Mycophenolic acid-d3; UPLC–MS/MS; In-source conversion; Incurred sample reanalysis

Abstract A simple, sensitive and high throughputs ultra performance liquid chromatography tandem mass spectrometry method has been developed for the determination of mycophenolic acid in human plasma. The method involved simple protein precipitation of MPA along with its deuterated analog as an internal standard (IS) from 50 μL of human plasma. The chromatographic analysis was done on Acquity UPLC C18 (100 mm × 2.1 mm, 1.7 μm) column under isocratic conditions using acetonitrile and 10 mM ammonium formate, pH 3.00 (75:25, v/v) as the mobile phase. A triple quadrupole mass spectrometer operating in the positive ionization mode was used for quantitation. In-source conversion of mycophenolic glucuronide metabolite to the parent drug was selectively controlled by suitable optimization of cone voltage, cone gas flow and desolvation temperature. The method was validated over a wide concentration range of 15–15000 ng/mL. The mean extraction recovery for the analyte and IS was 49.5%. Matrix effect expressed as matrix factors ranged from 0.97 to 1.02. The method was successfully applied to support a bioequivalence study of 500 mg mycophenolate mofetil tablet in 72 healthy subjects.

1. Introduction

Mycophenolic acid (MPA) is the active metabolite of ester prodrug mycophenolate mofetil (MPM) and is widely used as an immunsuppressant drug to prevent the rejection of organ transplantation and in the treatment of autoimmune disease [1,2]. To improve its oral bioavailability MPA is administered as MPM, which is completely absorbed and rapidly hydrolyzed by esterases to MPA. It is a selective, reversible and non-competitive inhibitor of inosine...
Monophosphate dehydrogenase (IMPDH), the rate limiting enzyme in the *de novo* synthesis of guanosine nucleotides [3]. MPA is primarily metabolized by uridine diphosphate glucuronosyl enzyme in the liver, kidney and intestine to phenolic mycophenolic acid glucuronide (MPAG) metabolite which is recovered in urine. MPAG is pharmacologically inactive and can hydrolyze back to MPA by β-glucuronidase during enterohepatic recirculation [4,5]. This leads to a second concomitant peak in the plasma concentration-time profile between 6–12 h after administration and might contribute to the gastrointestinal toxicity [6]. Additionally, MPA is further metabolized to two minor metabolites namely acyl glucuronide (AcMPAG) and phenolic glucoside of MPA. MPA is highly bound to plasma proteins, mainly to human serum albumin (97–99%) [7]. Clinical pharmacokinetics of MPA reveal significant inter and intra subject variation in plasma concentration [8], which can be associated with different factors such as renal allograft function, hepatic function, MPA unbound fraction, enterohepatic recirculation, and concomitant immunosuppressant therapy [9]. Hence therapeutic drug monitoring for MPA is necessary to optimize outcomes, especially in patients with high rejection risk [10]. Moreover, due to low therapeutic levels it is essential to develop sensitive, rugged and rapid bioanalytical methods for its determination in biological fluids to minimize the risk of drug accumulation, and for optimization of therapy to reduce the frequency of adverse effects.

Several methods have been reported for the analysis of MPA and its metabolites in different biological fluids by electrophoresis [11–14], immunoassay technique [15–17], high performance liquid chromatography using diode array [18], fluorescence [19–21], UV [22–29], and mass spectrometry detection [30–41]. Relatively few UPLC–MS/MS based methods are available in literature for rapid analysis of MPA in biological samples [42,43]. Kuhn et al. [42] have developed a rapid method to determine MPA and MPAG with a limit of quantitation (LOQ) of 50 ng/mL and 2300 ng/mL respectively in serum and plasma. A comparable method with LOQ of 100 ng/mL for MPA was used for a pharmacokinetic study in kidney transplant recipients [43]. A summary of salient features of liquid chromatographic methods with mass spectrometry detection for determination of total MPA concentration in human plasma is presented in Table 1.

Ultra performance liquid chromatography (UPLC) is an ideal tool for rapid separation of complex mixtures in both isocratic and gradient modes. Improved separation efficiency and a decrease in the analysis time can be realized by reducing the particle size of the column packing material. The advantage of UPLC over conventional HPLC is the ability to increase the speed without sacrificing efficiency [44]. In the present work, a simple, sensitive, selective and rapid UPLC–MS/MS method has been developed

**Table 1**: Comparative summary of liquid chromatographic methods with mass detection developed for determination of total MPA in plasma.

| Sr. no. | Detection technique | Human plasma volume (µL) | Extraction procedure; internal standard | Recovery (%) | Linear range (ng/mL) | Run time (min) | Application | Ref. |
|---------|---------------------|--------------------------|------------------------------------------|--------------|----------------------|----------------|-------------|------|
| 1*      | LC–MS/MS 100        | PP with perchloric acid and sodium tungstate; carboxy butoxy ether of MPA | 91–110 | 100–50000 | 4.0 | – | Pharmacokinetic study with 1.5 g MPM in 52 healthy volunteers | [31] |
| 2*      | LC–MS/MS 100        | SPE on Phenomenex Strata-X cartridges; indomethacin | 82–92 | 50–50000 | 7.0 | – | Pharmacokinetic study with MPM in healthy volunteers | [33] |
| 3*      | LC–MS/MS 100        | PP with acetonitrile and formic acid; carboxy butoxy ether of MPA | 98.5–101.7 | 50–30000 | 3.0 | – | Pharmacokinetic study with MPM in healthy volunteers and patients | [35] |
| 4*      | LC–MS/MS 100        | LLE with diethyl ether at pH 4.0; PPA | 43.3–60.0 | 50–40000 | 2.0 | – | Method comparison with commercially available EMIT | [36] |
| 5*      | LC–MS/MS 10         | Ultrafiltration followed by online SPE; mycophenolic acid-d3 | 89.3–99.1 | 100–100000 | 100–400000 | – | Analysis in dried blood/plasma spots | [37] |
| 6       | LC–MS/MS 50         | SPE on Waters MAX Oasis plates; MPA cyclopropane analog | 81.3–84.7 | 19.95–19955 | – | – | Bioequivalence study with 500 mg MPM in 103 healthy subjects | [38] |
| 7       | LC–MS/MS 250        | LLE; pioglitazone | – | 75.43–24965 | 4.0 | – | Bioequivalence study with 500 mg MPM in 117 healthy subjects | [39] |
| 8*      | LC–MS/MS 20         | PP with acetonitrile; [13C, 2H3]–MPA | 82.0 | 80–20000 | 2.0 | – | Pharmacokinetic study with 500 mg MPM twice daily in a renal pediatric patient | [40] |
| 9*      | UPLC–MS/MS 10       | PP with acetonitrile; PPA | 78.1–129.7 | 50–100000 | 2.0 | – | Analysis of 121 heart transplantation patients who used MPM as part of multiple-drug regime | [42] |
| 10*     | UPLC–MS/MS 50       | PP with acetonitrile; mycophenolic acid-d3 | – | 100–20000 | 2.0 | – | Analysis of clinical samples collected from 15 *de novo* kidney transplant recipients | [43] |
| 11      | UPLC–MS/MS 50       | PP with acetonitrile; mycophenolic acid-d3 | 96.8–101.1 | 15–15000 | 2.0 | – | Bioequivalence study with 500 mg MPM in 72 healthy subjects | PM |

*Along with metabolites; MPA: mycophenolic acid; MPM: mycophenolate mofetil; PP: protein precipitation; SPE: solid phase extraction; LLE: liquid-liquid extraction; PPA: N-phthaloyl-L-phenylalanine; EMIT: enzyme multiplied immunoassay technique; PM: present method.
and fully validated for reliable measurement of MPA in human plasma samples. Potential interference due to in-source conversion of its principal metabolite MPAG to MPA was suitably controlled through optimized mass parameters. The method requires only 50 μL human plasma for sample processing and demonstrates excellent performance in terms of ruggedness and efficiency (2.0 min). It was successfully applied to support a pivotal bioequivalence study in 72 healthy subjects.

2. Experimental

2.1. Chemicals and materials

Reference standards of mycophenolic acid (MPA, 98%), mycophenolic acid-d3 [MPA-d3 as an internal standard (IS), 98%], and phenyl glucuronide of mycophenolic acid (MPAG, 98%) were obtained from Toronto Research Chemicals (Toronto, Canada). HPLC grade acetonitrile was procured from Merck (Darmstadt, Germany). Formic acid and ammonium formate were purchased from Spectrochem Pvt. Ltd. (Mumbai, India), and Sigma-Aldrich (St. Louis, MO, USA) respectively. Puriﬁed water was obtained from a Milli-Q water puriﬁcation system from Millipore (Bangalore, India). Blank human plasma was obtained from in-house clinical department and was stored at −20 °C until use.

2.2. Liquid chromatographic and mass spectrometric conditions

A Waters Acquity UPLC system (MA, USA) consisting of binary solvent manager, sample manager and column manager was used for setting the reverse-phase liquid chromatographic conditions. The analysis of MPA and IS was performed on Waters Acquity UPLC BEH C18 (100 mm × 2.1 mm, 1.7 μm) column maintained at 40 °C. The mobile phase consisted of acetonitrile and 10 mM ammonium formate, pH 3.00 adjusted with formic acid (75:25, v/v). The flow rate of the mobile phase was kept at 0.4 mL/min. The sample manager temperature was maintained at 5 °C.

Ionization and detection of MPA and IS was carried out on a Quattro Premier XE™ mass spectrometer from Waters – Micro Mass Technologies (MA, USA), equipped with turbo ion spray interface and operated in positive ion mode. Quantitation was performed using multiple reaction monitoring (MRM) mode to monitor precursor → product ion transitions for MPA m/z 321.1 → 207.0 and IS m/z 324.1 → 210.1. The source dependent parameters maintained for MPA and IS were desolvation gas: 700 L/h; capillary voltage: 3.5 kV; ion source temperature: 120 °C; desolvation gas flow: 600 L/h. The optimum values for compound dependent parameters like cone voltage and collision energy were 40 V and 207.0 and IS respectively. Purified water was obtained from a Milli-Q water puriﬁcation system from Millipore (Bangalore, India). Blank human plasma was obtained from in-house clinical department and was stored at −20 °C until use.

2.2. Liquid chromatographic and mass spectrometric conditions

A Waters Acquity UPLC system (MA, USA) consisting of binary solvent manager, sample manager and column manager was used for setting the reverse-phase liquid chromatographic conditions. The analysis of MPA and IS was performed on Waters Acquity UPLC BEH C18 (100 mm × 2.1 mm, 1.7 μm) column maintained at 40 °C. The mobile phase consisted of acetonitrile and 10 mM ammonium formate, pH 3.00 adjusted with formic acid (75:25, v/v). The flow rate of the mobile phase was kept at 0.4 mL/min. The sample manager temperature was maintained at 5 °C.

Ionization and detection of MPA and IS was carried out on a Quattro Premier XE™ mass spectrometer from Waters – Micro Mass Technologies (MA, USA), equipped with turbo ion spray interface and operated in positive ion mode. Quantitation was performed using multiple reaction monitoring (MRM) mode to monitor precursor → product ion transitions for MPA m/z 321.1 → 207.0 and IS m/z 324.1 → 210.1. The source dependent parameters maintained for MPA and IS were desolvation gas: 700 L/h; capillary voltage: 3.5 kV; ion source temperature: 120 °C; desolvation temperature: 300 °C; entrance potential: 1.0 V; and cone gas flow: 120 L/h. The optimum values for compound dependent parameters like cone voltage and collision energy were 40 V and 14 eV respectively for MPA and IS. Quadrupole 1 and 3 were maintained at unit mass resolution and the dwell time was set at 200 ms for both the drugs. Data collection, peak integration, and calculations were performed using Mass Lynx software version 4.1.

2.3. Calibrators and quality control samples

The standard stock solution of MPA (1000 μg/mL) was prepared by dissolving accurately weighted amount in methanol. Calibration standards (CSs) and quality control (QC) samples were prepared by spiking blank plasma with stock solution. CSs were made at 15.0, 30.0, 75.0, 150, 300, 750, 1500, 7500, 12000, and 15000 ng/mL concentrations respectively, while QC samples were prepared at 13000 ng/mL (HQC, high quality control), 6500/455 ng/mL (MQC-1/2, middle quality control-1/2) and 40.0 ng/mL (LQC, low quality control). Stock solution (1.0 mg/mL) of IS was prepared by dissolving 25.0 mg in 25.0 mL of methanol. Its working solution (1500 ng/mL) was prepared by appropriate dilution of the stock solution in acetonitrile. Standard stock and working solutions used were stored at 5 °C, while CSs and QC samples in plasma were kept at −70 °C until use.

2.4. Sample preparation

Prior to analysis, all frozen subject samples, calibration standards and quality control samples were thawed and allowed to equilibrate at room temperature for 30 min. To an aliquot of 50 μL of spiked plasma/subject samples, 250 μL of IS was added and vortexed for about 2 min. The samples were centrifuged at 1431 g for 10 min at 10 °C. Subsequently, 100 μL of supernatant was transferred into pre-labeled tubes containing 100 μL of 10 mM ammonium formate solution. After brief vortex, 5 μL was used for injection into the chromatographic system.

2.5. Method validation

System suitability experiment was performed by injecting six consecutive injections using aqueous standard mixture of MPA (6500 ng/mL) and IS (1500 ng/mL) at the start of each batch. System performance was checked by injecting one extracted lower limit of quantitation (LLOQ) sample with IS at the beginning of each analytical batch and before re-injecting any batch during method validation. Autosampler carryover was evaluated by sequentially injecting extracted blank plasma → upper limit of quantitation (ULOQ) sample → two extracted blank plasma samples → LLOQ sample → extracted blank plasma at the start and end of each batch.

Selectivity of the method was assessed for potential matrix interferences in ten batches (6 normal lots of K2EDTA, 2 haemolysed, and 2 lipemic) of blank human plasma by extraction and inspection of the resulting chromatograms for interfering peaks. Selectivity in the presence of MPAG was also evaluated to check its impact on quantitation of MPA.

Five calibration lines containing ten non-zero concentrations were used to determine linearity. A quadratic, 1/x², least-squares regression algorithm was used to plot the peak area ratio (analyte/IS) from multiple reaction monitoring versus concentration. The linear equations were then used to calculate the predicted concentrations in all samples within the analytical runs. The correlation coefficient for each calibration curve must be ≥0.99 for both the analytes. The lowest standard on the calibration line was accepted as the LLOQ, if the analyte response was at least ten times more than that of extracted blank plasma. Rejection reproducibility for extracted samples was also checked by re-injection of an entire analytical run after storage at 5 °C.

Intra-batch accuracy and precision were evaluated by replicate analysis of plasma samples on the same day. The analytical run consisted of a calibration curve and six replicates of LLOQ, LQC, MQC-1/2 and HQC samples. The inter-batch accuracy and precision were assessed by analysis of five precision and accuracy batches on three consecutive validation days. The precision (% CV) at each concentration level from the nominal concentration
should not be greater than 15%. Similarly, the mean accuracy should be within 85–115%, except for the LLOQ where it can be within 80–120% of the nominal concentration [45].

Ion suppression/enhancement effects on the MRM LC–MS/MS sensitivity were evaluated by post column analyte infusion experiment as described previously [46]. Briefly, a standard solution containing MPA (at MQC-1 level) was infused post column into the mobile phase at 10 μL/min employing infusion pump. Aliquots of 5 μL of extracted control blank plasma sample were then injected into the column by the autosampler and chromatograms were acquired for the analyte and IS.

Extraction recovery of MPA and IS from human plasma was evaluated in six replicates by comparing the mean peak area responses of pre-extraction fortified samples to those of post-extraction fortified samples representing 100% recovery. Matrix effect, expressed as matrix factors (MFs) was assessed by comparing the mean area response of post-extraction fortified samples with mean area of solutions prepared in mobile phase solutions (neat standards) at HQC, MQC-1/2 and LQC levels. To evaluate the relative matrix effect in six plasma sources, pre-extraction fortified samples were prepared in triplicate at LQC and HQC concentration and assessed for accuracy (%) and precision (% CV). In order to meet acceptance criteria for MF, the % CV must be ≤15% and for relative matrix effect the accuracy should not be more than ±15% and % CV must be ≤15%. Further, at least 90% of the lots at each QC level should be within the aforementioned criteria.

Stock solutions of MPA and IS were checked for short term stability at room temperature and long term stability at 5 °C. Stability results in plasma were evaluated by measuring the area ratio response (MPA/IS) of stability samples against freshly prepared comparison standards with identical concentration. The solutions were considered stable if the deviation from nominal value was within ±10.0%. Autosampler (wet extract at 5 °C), bench top (at room temperature), freeze–thaw (at −20 °C and −70 °C) and long term stability (at −20 °C and −70 °C) was performed at LQC and HQC levels using six replicates. The stability samples were quantified against freshly prepared quality control samples. Stability data were acceptable if the % CV of the replicate determinations did not exceed 15.0% and the mean accuracy value was within ±15.0% of the nominal value.

Method ruggedness was verified using two precision and accuracy batches. The first batch was analyzed on two different columns of the same make but different batch number, while the second batch was analyzed by two different analysts who were not part of method validation. The ability to dilute samples which could be above the upper limit of the calibration range was validated by analyzing six replicate samples containing 27000 ng/mL of MPA after two-/ten-fold dilution respectively. The precision and accuracy for dilution reliability was determined by comparing the samples against freshly prepared calibration curve standards.

2.6. Application of the method and incurred sample reanalysis (ISR)

The aim of the study was to determine the bioequivalence of 500 mg MPM test (Generic Company, India) with a reference (CellCept®, Roche Laboratories Inc. New Jersey, USA) tablet formulation. The design was randomized, open label, balanced, two-treatment, two-period, two-sequence, crossover study in 72 healthy adult Indian subjects under fasting. The primary target variables of the study were $C_{\text{max}}$, $AUC_{0–\text{inf}}$, and $AUC_{0–\text{inf}}$, which were analyzed using the confidence interval approach. The secondary end points of the study included $T_{\text{max}}$, $K_{\text{el}}$ and $t_{1/2}$. The study was conducted as per the International Conference on Harmonization, E6 Good Clinical Practice guidelines [47]. After an overnight fasting, the subjects were orally administered a single dose of test and reference formulations with 240 mL of water. Blood samples were withdrawn at pre-dose time 0.00, 0.17, 0.25, 0.33, 0.42, 0.50, 0.58, 0.67, 0.83, 1.00, 1.17, 1.33, 1.50, 1.67, 1.83, 2.00, 2.17, 2.33, 2.50, 2.67, 2.83, 3.00, 3.25, 3.50, 3.75, 4.40, 4.33, 4.67, 5.00, 5.50, 5.0, 6.0, 7.0, 8.0, 10.0, 12.0, 24.0 and 36.0 h and collected in labeled K$_3$EDTA-vacuettes. After blood collection, plasma separation was done by centrifugation at 1431g for 10 min at 4 °C. The plasma samples were separated and divided into two aliquots and stored in two different pre-labeled radioimmunoassay (RIA) vials at −20 °C until analysis.

The statistical analysis for pharmacokinetic parameters of MPA included descriptive statistics, analysis of variance and two one-sided tests for bioequivalence using SAS® software version 9.2 (SAS Institute Inc., Cary, NC, USA). To determine whether the test and reference formulations were pharmacokinetically equivalent, $C_{\text{max}}$, $AUC_{0–\text{inf}}$, and $AUC_{0–\text{inf}}$ and their ratios (test/reference) using log transformed data were assessed. The drug formulations were considered pharmacokinetically equivalent if the difference between the compared parameters was statistically non-significant ($P\geq0.05$) and the 90% confidence intervals (CI) for these parameters were within 80–125%.

Fig. 1 Product ion mass spectra of (A) MPA (m/z 321.0 → 207.0) and (B) internal standard, IS (m/z 324.1 → 210.1) in 100–420 amu scan range.
Fig. 2  MRM ion-chromatograms of (A) mobile phase, (B) aqueous standard of MPA (15000 ng/mL), (C) aqueous standard of MPAG (15000 ng/mL), (D) MPA (15000 ng/mL) spiked with MPAG (15000 ng/mL) before fine tuning, (E) MPA (15000 ng/mL) spiked with MPAG (15000 ng/mL) after fine tuning of mass parameters and (F) MPA and MPAG in subject sample at 2.0 h after administration of 500 mg MPM. Optimized mass parameters before and after fine tuning were cone voltage: 45/40 V, desolvation temperature: 400/300 °C and cone gas flow: 150/120 L/h respectively.
An ISR for assay reproducibility was also done by reanalysis of 317 samples, which were near the $C_{max}$ and the elimination phase in the pharmacokinetic profile of the drug. The results were compared with initial pharmacokinetic study using the same procedure. As per the acceptance criterion at least two-thirds of the original and repeat results should be within 20% of each other [48].

3. Results and discussion

3.1. Method development

Due to in-source conversion of MPAG metabolite to MPA it is essential to separate both the analytes chromatographically. Back conversion of this unstable metabolite to MPA must be considered during sample preparation and subsequent analysis especially for subject samples. In the present work, UPLC-MS/MS analysis was done in the positive ionization mode to attain high sensitivity and a good linearity in regression curves. The Q1 MS full scan spectra for MPA and IS predominantly contained protonated precursor $[M+H]^+$ ions at $m/z$ 321.0 and 324.1 respectively. The most abundant and consistent product ions in Q3 MS spectra for MPA and IS were found at $m/z$ 207.0 and 210.1 respectively at 14 eV collision energy (Fig. 1). Unlike previous report [36], maximum sensitivity for quantitation purpose was achieved using the protonated molecular ions instead of MPA ammonium ion adducts $[MPA+NH_4]^+$ having $m/z$ 338. In another report, in-source fragmentation of MPAG was detected by measuring $[MPA+H]^+$ and not with $[MPA+NH_4]^+$ as the precursor ion [42]. The MPA peak resulting from in-source fragmentation of MPAG was seen well before the original MPA peak when $[MPA+H]^+$ was measured from a patient sample under treatment of MPM. MPAG undergoes in-source fragmentation to MPA via loss of the glucuronic acid moiety which can lead to overestimation of MPA concentration and could be a major problem when both co-elute [41]. Thus it was imperative to minimize the interference of MPAG for accurate quantitation of MPA, as $[MPA+H]^+$ ions were studied in the present work. Initially chromatographic trails were taken to ensure an acceptable separation between MPA and MPAG and to avoid any false concentration during subject sample analysis (Fig. 2A–F). In-source fragmentation of MPAG to MPA was observed in our study with aqueous standard of MPAG (15000 ng/mL) and also when MPA aqueous sample (at ULOQ) spiked with MPAG (15000 ng/mL) was injected (Fig. 2C and D). In the MRM window of MPA an additional peak was observed at 1.11 min.

Fig. 3 Fine tuning of mass parameters (A) cone voltage, (B) desolvation temperature and (C) cone gas flow to minimize in-source conversion of MPAG to MPA.
which was well resolved from MPA peak and did not contribute to its peak area. Furthermore, to reduce in-source fragmentation, cone voltage, desolvation temperature and cone gas flow were fine tuned without compromising MPA response. Significant reduction in conversion was possible by maintaining the desolvation temperature at 300 °C, cone voltage 40 V and cone gas 120 L/h (Fig. 2E). Noticeably there was no significant conversion during subject sample analysis after optimization of mass conditions (Fig. 2F). The effects of ion source temperature, cone voltage and cone gas flow on MPAG in-source conversion are presented in Fig. 3. A dwell time of 200 ms for MPA and IS was adequate and no cross talk was observed between their MRMs.

Different combinations of methanol/acetonitrile with acidic buffers (formic acid/acetic acid-ammonium formate/ammonium acetate) in different volume ratios were tested. Better separation, higher sensitivity, efficiency and symmetric peak shapes were obtained with acetonitrile: 10 mM ammonium formate, pH 3.0

![Representative MRM ion-chromatograms of (A) double blank blood (without MPA and IS), (B) blank plasma with IS, (C) MPA at LLOQ and IS, and (D) subject sample at 2.0 h after administration of 500 mg dose of MPM.](image)
As the mobile phase. Under these conditions, retention times of 1.21, 1.57 and 1.56 min were achieved for MPAG, MPA and IS respectively with a run time of 2.0 min. The reproducibility of retention times for MPA, expressed as % CV was \(\leq 0.34\%\) for 100 injections on the same column. Unlike the previous methods that have employed a general internal standard \[33,36,39,42\], a deuterated analog was used in the present work. MPA-d3 adequately compensated for any variability during extraction and UPLC–MS/MS analysis. Sample preparation by protein precipitation afforded clean chromatograms with no interfering peaks as evident from representative MRM ion chromatograms of extracted blank plasma (without MPA and IS), blank plasma fortified with IS, MPA at LLOQ and an actual plasma sample at 2.0 h (Fig. 4). The mean extraction recovery across QC levels for MPA and IS was 98.4% and 96.6% respectively (Table 2). The post column infusion experiment confirmed the absence of signal suppression or enhancement at the retention time of MPA and IS (Fig. 5). Noticeably an ion-suppression/enhancement was observed at 0.4–0.6 min; however, it did not interfere in the quantitation of the analyte.

| Table 2 | Extraction recovery and matrix factors for mycophenolic acid and mycophenolic acid-d3. |
|---|---|---|---|---|
| Chemical | QC level | Area response | Extraction recovery (B/A) (%) | Matrix factor (A/C) |
| | | A | B | C |
| Mycophenolic acid | HQC | 473124 | 478372 | 464086 | 101.1 | 1.02 |
| | MQC-1 | 228384 | 223792 | 232486 | 97.9 | 0.98 |
| | MQC-2 | 16092 | 15574 | 16175 | 96.8 | 0.99 |
| | LQC | 1395 | 1362 | 1428 | 97.6 | 0.97 |
| Mycophenolic acid-d3 | HQC | 222575 | 217678 | 220371 | 97.8 | 1.01 |
| | MQC-1 | 218966 | 213054 | 214673 | 97.3 | 1.02 |
| | MQC-2 | 209917 | 201101 | 207859 | 95.8 | 1.01 |
| | LQC | 214792 | 205341 | 219175 | 95.6 | 0.98 |

A: Mean area response of six replicates prepared by spiking in extracted blank plasma.
B: Mean area response of six replicates prepared by spiking before extraction.
C: Mean area response of six replicates prepared by spiking in mobile phase (neat samples).

![Fig. 5](image_url)

Injection of extracted blank human plasma during post column infusion of (A) MPA and (B) IS at MQC-1 level.

| Table 3 | Relative matrix effect in six different lots of human plasma for mycophenolic acid at HQC and LQC levels. |
|---|---|---|---|---|
| Plasma lots | Observed concentration (ng/mL) | | |
| | LQC (40.0 ng/mL)* | HQC (13000 ng/mL)* |
| | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| 1 | 38.4 | 40.4 | 39.2 | 12667 | 12775 | 12636 | 12727 | 12829 | 12697 |
| 2 | 39.9 | 39.2 | 39.1 | 12587 | 12701 | 12573 | 12668 | 12879 | 12665 |
| 3 | 39.6 | 39.8 | 40.5 | 12593 | 12353 | 12967 | 12593 | 12353 | 12967 |
| 4 | 40.1 | 40.9 | 40.3 | 11692 | 11985 | 11943 | 11692 | 11985 | 11943 |
| 5 | 38.6 | 37.7 | 38.7 | 11943 | 12552 | mean | 39.2 | 12552 | mean |
| 6 | 37.6 | 37.9 | 37.5 | 12775 | 12879 | 12665 | 12668 | 12879 | 12665 |
| Mean (ng/mL) | 39.2 | 12775 | 12665 | 12552 | 12775 | 12665 |
| Standard deviation | 0.95 | 273.6 | 2.18 | 2.18 |
| Coefficient of variation (%) | 2.42 | 0.95 | 2.42 | 2.18 |
| Accuracy (%) | 12967 | 12665 | 12665 | 96.6 |

*Three replicates for the same concentration.

(75:25, v/v) as the mobile phase. Under these conditions, retention times of 1.21, 1.57 and 1.56 min were achieved for MPAG, MPA and IS respectively with a run time of 2.0 min. The reproducibility of retention times for MPA, expressed as % CV was \(\leq 0.34\%\) for 100 injections on the same column. Unlike the previous methods that have employed a general internal standard \[33,36,39,42\], a deuterated analog was used in the present work. MPA-d3 adequately compensated for any variability during extraction and UPLC–MS/MS analysis. Sample preparation by protein precipitation afforded clean chromatograms with no interfering peaks as evident from representative MRM ion chromatograms of extracted blank plasma (without MPA and IS), blank plasma fortified with IS, MPA at LLOQ and an actual plasma sample at 2.0 h (Fig. 4). The mean extraction recovery across QC levels for MPA and IS was 98.4% and 96.6% respectively (Table 2). The post column infusion experiment confirmed the absence of signal suppression or enhancement at the retention time of MPA and IS (Fig. 5). Noticeably an ion-suppression/enhancement was observed at 0.4–0.6 min; however, it did not interfere in the quantitation of the analyte.
Presence of unmonitored, co-eluting compounds from the matrix can directly impact the accuracy, precision, ruggedness and overall reliability of a validated method. Thus, it is recommended that evaluation of matrix factors can help to assess the matrix effect. In the present work, the matrix factor ranged from 0.97 to 1.02 for MPA and IS (Table 2). The effect of different plasma sources on analyte quantitation, expressed as relative matrix effect was established in four normal K3EDTA, 1 haemolysed and 1 lipemic plasma sources. The % CV values ranged from 2.18 to 2.42 and the accuracy was within 96.6–97.9% (Table 3), which is well within the acceptance criteria.

3.2. UPLC–MS/MS assay results

The precision (% CV) of system suitability test was observed in the range of 0.05–0.11% for the retention time and 1.63–2.53% for the area response for MPA and IS. The signal to noise ratio for system performance was ≥70 for analyte and IS. Carryover evaluation showed minimal carryover (<2.64% of LLOQ area) in extracted blank plasma after succeeding injection of highest calibration standard at the retention time of analyte and IS.

Five MPA calibration curves were linear over the concentration range of 15–15000 ng/mL with a correlation coefficient \( r^2 \geq 0.9995 \). The mean linear equation obtained for MPA was \( y = (0.99838 \pm 0.00074)x + (0.00836 \pm 0.00002) \). The accuracy and precision (% CV) for the calibration curve standards ranged from 98.8% to 102.8% and 1.67% to 6.53%. The LLOQ (S/N ≥70) and limit of detection (LOD, S/N ≥10) were 15 ng/mL and 2.5 ng/mL respectively.

The intra-batch and inter-batch precision and accuracy for MPA were established from validation runs performed at five QC levels.
samples, while the remaining 29 samples showed 5% change for 155 samples, 5 adverse event during the course of the study. Furthermore, there was no bioequivalence of the test sample with the reference product in terms of rate and extent of absorption. Furthermore, there was no bioequivalence of the test sample with the reference product in mean for test/reference was within 80–100.5%.

Stock solutions of MPA and IS for short term at room temperature and long term at 5±3 °C were stable at room temperature up to 8 h and for minimum period of 40 days respectively. MPA in control human plasma (bench top) at room temperature was stable for at least 9 h at 25 °C and for minimum of five freeze–thaw cycles. Wet extract stability of the extracted quality control samples was determined up to 36 h without significant loss of MPA. Long term stability of the spiked quality control samples was unaffected up to minimum 93 days. The detailed results for stability experiments are presented in Table 5.

The precision (% CV) and accuracy for method ruggedness with different columns ranged from 2.9% to 3.7% and 94.5% to 97.4% respectively across five quality control levels. For the experiment with different analysts, the results for precision and accuracy were within 1.7–3.2% and 96.1–100.6% respectively at these levels. The precision (% CV) for dilution reliability of 1/2 and 1/10th dilution was between 2.3% and 3.3% respectively, while the accuracy results were within 95.8–96.8%, which is within the acceptance limit of 15% for precision (% CV) and 85–115% for accuracy.

3.3. Application to bioequivalence study and ISR results

The developed method was used to estimate MPA concentration in human samples after administration of 500 mg oral dose of MPM. Fig. 6 shows the mean plasma concentration vs. time profile of MPA in healthy subjects. The method was sensitive enough to monitor MPA concentration up to 36.0 h. The mean pharmacokinetic parameters and log transformed geometric least squares mean values for \( \text{AUC}_{0-36}, \text{AUC}_{0-\text{inf}} \) and \( C_{\text{max}} \) under fasting conditions are summarized in Table 6. The results obtained can be compared with a similar study with identical MPM dose strength in healthy Indian subjects [39]. The mean \( C_{\text{max}} \), \( \text{AUC}_{0-36} \) and \( \text{AUC}_{0-\text{inf}} \) values were a bit lower in the present work, albeit with much less variation. However, \( t_{1/2} \) and \( K_{\text{el}} \) values were comparable with that study. The 90% confidence interval of individual ratio geometric mean for test/reference was within 80–125%, which corroborates bioequivalence of the test sample with the reference product in terms of rate and extent of absorption. Furthermore, there was no adverse event during the course of the study.

ISR results showed % change for assay reproducibility within 5% for 155 samples, 5–10% for 73 samples, 10–15% for 60 samples, while the remaining 29 samples showed % change between 15% and 20% (Fig. 7). This authenticates the reproducibility of the proposed method.

3.4. Comparison with reported procedures

The proposed method is more sensitive compared to all other methods developed for estimation of total MPA concentration in plasma and rapid compared to several HPLC [18–29] and LC–MS/MS [31,33,35,36,39] methods in biological samples. The present method employed only 50 µL plasma volume for processing, which is low compared to several methods [31,33,35,36,39] and identical with few others [38,43]. Only three other methods employ volumes less than 50 µL [37,40,42]. Additionally, the on-column loading of MPA is 16 ng per injection at ULOQ level, which is much less compared to previous reports [31,33,35,36,38,39,43]. Further, assay reproducibility is comprehensively demonstrated by incurred sample reanalysis, which is not reported in methods employing mass spectrometric detection [30–43].

4. Conclusions

The UPLC–MS/MS method for the quantitation of MPA in human plasma was developed and fully validated as per the USFDA guidelines. A total of approximately 9500 samples were analyzed during a period of 12 days, which included calibration, QC and

![Fig. 7 % Change for assay reproducibility results with 317 incurred study samples.](image)

| Parameter          | Test (ng/mL) | Reference (ng/mL) | Ratio (test/reference) (%) | 90% confidence interval (Lower–Upper) | Intra-subject variation (% CV) |
|--------------------|--------------|-------------------|----------------------------|--------------------------------------|-------------------------------|
| \( C_{\text{max}} \) | 11271±4298   | 10752±3599        | 103.4                      | 98.3–108.8                           | 5.25                          |
| \( \text{AUC}_{0-36} \) (b ng/mL) | 23590±4921   | 23910±5086        | 99.3                       | 95.7–102.9                           | 5.17                          |
| \( \text{AUC}_{0-\text{inf}} \) (b ng/mL) | 26248±6363   | 26532±7175        | 99.7                       | 94.9–104.7                           | 6.34                          |
| \( T_{\text{max}} \) (h) | 1.81±0.73    | 1.84±0.79         | —                          | —                                    | —                             |
| \( t_{1/2} \) (h) | 11.76±5.84   | 10.97±5.47        | —                          | —                                    | —                             |
| \( K_{\text{el}} \) (1/h) | 0.072±0.025  | 0.078±0.023       | —                          | —                                    | —                             |
subject samples and the precision and accuracy were well within the acceptable limits. The advantages of this method include high sensitivity, small sample volume for processing, and short chromatographic run time. The efficiency of protein precipitation and a chromatographic run time of 2.0 min per sample make it an attractive procedure in high-throughput clinical analysis of MPA. Based on dilution reliability results it is possible to extend the ULOQ to 27000 ng/mL. In addition, assay reproducibility is effectively proved by reanalysis of 317 subject samples.

Acknowledgments

The authors are thankful to scientists, Mr. Pankaj Sarkar, Mr. Sujal Shah and the management of Cadila Pharmaceuticals Ltd., India for providing infrastructure facility to carry out this work.

References

[1] M. Krejci, M. Doubek, T. Buchler, et al., Mycophenolate mofetil for the treatment of acute and chronic steroid-refractory graft-versus-host disease, Ann. Hematol. 84 (2005) 681–685.

[2] R.E. Bullingham, A.J. Nicholls, B.R. Kamm, Clinical pharmacokinetics of mycophenolate mofetil, Clin. Pharmacokinet. 34 (1998) 429–455.

[3] J.T. Ransom, Mechanism of action of mycophenolate mofetil, Ther. Drug Monit. 17 (1995) 681–684.

[4] P.I. Mackenzie, Identification of uridine diphosphate glucuronosyltransferases involved in the metabolism and clearance of mycophenolic acid, Ther. Drug Monit. 22 (2000) 10–13.

[5] L.M. Shaw, T. Pawinski, M. Korecka, et al., Monitoring of mycophenolic acid in clinical transplantation, Ther. Drug Monit. 24 (2002) 68–73.

[6] H.W. Sollinger, Mycophenolates in transplantation, Clin. Transplant. 18 (2004) 485–492.

[7] I. Nowak, L.M. Shaw, Mycophenolic acid binding to human serum albumin: characterization and relation to pharmacodynamics, Clin. Chem. 41 (1995) 1011–1017.

[8] G. Filler, J. Bendrick-Pearl, U. Christians, Pharmacokinetics of mycophenolate mofetil and sirolimus in children, Ther. Drug Monit. 30 (2008) 138–142.

[9] L. Zeng, C.E. Nath, P.J. Shaw, et al., HPLC-UV assay for monitoring total and unbound mycophenolic acid concentrations in children, Biomed. Chromatogr. 23 (2009) 92–100.

[10] T. van Gelder, Y.L. Meur, L.M. Shaw, et al., Therapeutic drug monitoring of mycophenolate mofetil in transplantation, Ther. Drug Monit. 28 (2006) 145–154.

[11] C. Farulucci, M. Anzini, M. Rovini, et al., Development of a CE method for the determination of mycophenolic acid in human plasma: a comparison with HPLC, Electrophoresis 28 (2007) 3908–3914.

[12] K. Ohyama, N. Kinoshita, N. Kishikawa, et al., A simple and rapid CZE method for the analysis of mycophenolic acid and its phenol glucuronide metabolite in human serum, Electrophoresis 29 (2008) 3658–3664.

[13] K. Ohyama, N. Kishikawa, H. Nakagawa, et al., Simultaneous determination of mycophenolic acid and its acyl and phenol glucuronide metabolites in human serum by capillary zone electrophoresis, J. Pharm. Biomed. Anal. 47 (2008) 201–206.

[14] Z.K. Shihabi, Enhanced detection in capillary electrophoresis: Example determination of serum mycophenolic acid, Electrophoresis 30 (2009) 1516–1521.

[15] K. Boer, S. Brehmer-Streck, T. Deufel, et al., Automated monitoring of C2 and C0 blood levels of mycophenolic acid and cyclosporine on the Abbott Architect c8000. Clin. Biochem. 40 (2007) 1163–1167.

[16] B. Blanchet, F. Taief, F. Conti, et al., Comparison of a new enzymatic assay with a high-performance liquid chromatography/ultraviolet detection method for therapeutic drug monitoring of mycophenolic acid in adult liver transplant recipients, Liver Transpl. 14 (2008) 1745–1751.

[17] N. Rebollo, M.V. Calvo, A. Martin-Saurez, et al., Modification of the EIT immunoassay for the measurement of unbound mycophenolic acid in plasma, Clin. Biochem. 44 (2011) 260–263.

[18] M. Daurel-Receveur, K. Titier, S. Picard, et al., Fully automated analytical method for mycophenolic acid quantification in human plasma using on-line solid phase extraction and high performance liquid chromatography with diode array detection, Ther. Drug Monit. 28 (2006) 505–511.

[19] H. Hosotsubo, S. Takahara, Y. Kokado, et al., Rapid and simple determination of mycophenolic acid in human plasma by ion-pair RP-LC with fluorescence detection, J. Pharm. Biomed. Anal. 24 (2001) 555–560.

[20] J. Shen, Z. Jiao, Y. Yu, et al., Quantification of total and free mycophenolic acid in human plasma by liquid chromatography with fluorescence detection, J. Chromatogr. B 817 (2005) 207–213.

[21] Y. Zhong, Z. Jiao, Y. Yu, Simultaneous determination of mycophenolic acid and valproic acid based on derivatization by high-performance liquid chromatography with fluorescence detection, Biomed. Chromatogr. 20 (2006) 319–326.

[22] C.G. Patel, F. Akhlaghi, High-performance liquid chromatography method for the determination of mycophenolic acid and its acyl and phenol glucuronide metabolites in human plasma, Ther. Drug Monit. 28 (2006) 116–122.

[23] G. Bahrami, B. Mohammadi, An isocratic high performance liquid chromatographic method for quantification of mycophenolic acid and its glucuronide metabolite in human serum using liquid-liquid extraction: Application to human pharmacokinetic studies, Clin. Chim. Acta 370 (2006) 185–190.

[24] F.A. Elbarty, A.S. Shoker, Liquid chromatographic determination of mycophenolic acid and its metabolites in human kidney transplant plasma: Pharmacokinetic application, J. Chromatogr. B 859 (2007) 276–281.

[25] B. Chen, W. Zhang, Z. Yu, et al., Determination of mycophenolic acid (MPA) and its acyl and phenol glucuronide metabolites simultaneously in human plasma by a simplified HPLC method, Anal. Lett. 40 (2007) 2465–2475.

[26] F.A. Elbarty, A.S. Shoker, Simple high performance liquid chromatographic assay for mycophenolic acid in renal transplant patients, J. Pharm. Biomed. Anal. 43 (2007) 788–792.

[27] Y. Mino, T. Naito, T. Matsushita, et al., Simultaneous determination of mycophenolic acid and its glucuronides in human plasma using isotonic pair high-performance liquid chromatography, J. Pharm. Biomed. Anal. 46 (2008) 603–608.

[28] F.T. Musumba, V.D. Fazio, R. Vanbinst, et al., A fast ultra-performance liquid chromatography method for simultaneous quantification of mycophenolic acid and its phenol- and acyl-glucuronides simultaneously in human plasma by a simplified HPLC method, J. Chromatogr. A 1216 (2009) 177–185.

[29] Q. Zhang, Y. Tao, Y. Zhu, et al., Bioequivalence and pharmacokinetic comparison of two mycophenolate mofetil formulations in healthy Chinese male volunteers: An open-label, randomized-sequence, single-dose, two-way crossover study, Clin. Ther. 32 (2010) 171–178.

[30] C. Willis, P.J. Taylor, P. Salm, et al., Quantification of free mycophenolic acid by high-performance liquid chromatography–atmospheric pressure chemical ionization tandem mass spectrometry, J. Chromatogr. B 748 (2000) 151–156.

[31] G. Brandhorst, F. Streit, S. Goetze, et al., Quantification by liquid chromatography tandem mass spectrometry of mycophenolic acid and its phenol and acyl glucuronide metabolites, Clin. Chem. 52 (2006) 1962–1964.

[32] A.E. Mendonza, R.Y. Gohh, F. Akhlaghi. Analysis of mycophenolic acid in saliva using liquid chromatography tandem mass spectrometry, Ther. Drug Monit. 28 (2006) 402–406.

[33] M.O. Benoit-Biancambano, P. Caron, E. Levesque, et al., Sensitive high-performance liquid chromatography–tandem mass spectrometry method for quantitative analysis of mycophenolic acid and its
glucuronide metabolites in human plasma and urine, J. Chromatogr. B 858 (2007) 159–167.
[34] H. Benech, S. Hascoet, V. Furlan, et al., Development and validation of an LC/MS/MS assay for mycophenolic acid in human peripheral blood mononuclear cells, J. Chromatogr. B 853 (2007) 168–174.
[35] M.J. Figurski, M. Korecka, L. Fields, et al., High-performance liquid chromatography-mass spectrometry/mass spectrometry method for simultaneous quantification of total or free fraction of mycophenolic acid and its glucuronide metabolites, Ther. Drug Monit. 31 (2009) 717–726.
[36] J. Kuhn, C. Prante, K. Kleesiek, et al., Measurement of mycophenolic acid and its glucuronide using a novel rapid liquid chromatography–electrospray ionization tandem mass spectrometry assay, Clin. Biochem. 42 (2009) 83–90.
[37] K. Heinig, F. Bucheli, R. Hartenbach, et al., Determination of mycophenolic acid and its phenyl glucuronide in human plasma, ultrafiltrate, blood, DBS and dried plasma spots, Bioanalysis 2 (2010) 1423–1435.
[38] S. Almeida, A. Filipe, R. Neves, et al., Mycophenolate mofetil 500-mg tablet under fasting conditions: single-dose, randomized-sequence, open-label, four-way replicate crossover, bioequivalence study in healthy subjects, Clin. Ther. 32 (2010) 556–574.
[39] S. Patel, V. Chauhan, J. Mandal, et al., Single-dose, two-way crossover, bioequivalence study of mycophenolate mofetil 500 mg tablet under fasting conditions in healthy male subjects, Clin. Ther. 33 (2011) 378–390.
[40] M.H.J. Wiesen, F. Farowski, M. Feldkötter, et al., Liquid chromatography–tandem mass spectrometry method for the quantification of mycophenolic acid and its phenolic glucuronide in saliva and plasma using a standardized saliva collection device, J. Chromatogr. A 1241 (2012) 52–59.
[41] A. Buchwald, K. Winkel, T. Epting, Validation of an LC–MS/MS method to determine five immunosuppressants with deuterated internal standards including MPA, BMC Clin. Pharmacol. 12 (2012) 1–11.
[42] J. Kuhn, C. Göttig, K. Kleesiek, 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 80 (2010) 1894–1898.
[43] X. Delavenne, L. Juthier, B. Pons, et al., UPLC–MS/MS method for quantification of mycophenolic acid and metabolites in human plasma: Application to pharmacokinetic study, Clin. Chim. Acta 412 (2011) 59–65.
[44] N. Wu, A.M. Clausen, Fundamental and practical aspects of ultra high pressure liquid chromatography for fast separations, J. Sep. Sci. 30 (2007) 1167–1182.
[45] Guidance for Industry, Bioanalytical Method Validation, US Department of Health and Human Services, Food and Drug Administration Centre for Drug Evaluation and Research (CDER), Centre for Veterinary Medicine (CVM), May 2001.
[46] A. Gupta, S. Guttikar, P.S. Shrivastav, et al., Simultaneous quantification of produg oseltamivir and its metabolite oseltamivir carboxylate in human plasma by LC–MS/MS to support a bioequivalence study, J. Pharma. Anal. 3 (2013) 149–160.
[47] Guidance for Industry: ICH E6 Good Clinical Practice, U.S. Department of Health and Human Services, Food and Drug Administration Centre for Drug Evaluation and Research (CDER), Centre for Biologics Evaluation and Research (CBER), April 1996.
[48] M. Yadav, P.S. Shrivastav, Incurred sample reanalysis (ISR): a decisive tool in bioanalytical research, Bioanalysis 3 (2011) 1007–1024.