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Determination of total mycophenolic acid and its glucuronide metabolite using liquid chromatography with ultraviolet detection and unbound mycophenolic acid using tandem mass spectrometry

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Abstract

Two simple, sensitive and reproducible methods for determination of total mycophenolic acid (MPA) and its glucuronide metabolite (MPAG) as well as unbound MPA (fMPA) was developed by the use of HPLC-UV and LC–MS/MS methods, respectively. For the total MPA/MPAG method, the analytes were extracted using Isolute C_2 solid-phase extraction (SPE) cartridges and analyzed at 254 nm over a Zorbax Rx C_8 column (150 mm × 4.6 mm, 5 μm). The mobile phase was a gradient mixture of methanol and water (containing 0.1% (v/v) phosphoric acid). The total run time was 18 min and the extraction recovery was 77% for MPA and 84% for MPAG. The method was precise and accurate with a lower limit of quantification (LLOQ) of 0.5 mg/l for MPA and 5.0 mg/l for MPAG. For the fMPA method, plasma was subjected to ultrafiltration followed by SPE using C_{18} cartridges. Analytical column was the same as the HPLC-UV method and the mobile phase was a gradient composition of methanol:0.05% formic acid with a flow rate of 0.6 ml/min for the first 3 min and 0.7 ml for the last 4 min. The chromatographic method separated MPA from its metabolites MPAG and Acyl-MPAG. Mass transitions in negative ionization mode for MPA and the internal standard, indomethacin were *m/z*: 319 → 190.9 and *m/z*: 356 → 312.2, respectively. The assay was linear in the concentration range of 1–1000 μg/l for fMPA with a LLOQ of 1 μg/l and an accuracy of >95%. The two methods reported have an adequate degree of robustness and dynamic concentration range for the measurement of MPA, MPAG and fMPA for therapeutic drug monitoring purposes or pharmacokinetics investigations.

Keywords: MPA; MPAG; HPLC-UV; LC–MS/MS; Unbound concentration

1. Introduction

Mycophenolic acid (MPA) is a potent immunosuppressive agent commonly used following organ transplantation and for the treatment of autoimmune diseases [1]. To improve the oral absorption and bioavailability of MPA, it is administered as a morpholino ester prodrug, mycophenolate mofetil (MMF) that is rapidly and completely hydrolyzed in the gut. The oral absorption of MMF is fast and the peak plasma concentration of MPA is attained within one hour of oral administration with a mean relative bioavailability of 94% [2]. At clinically relevant concentrations, MPA is approximately 97% bound to plasma albumin [3]. Nowak and Shaw [3] have demonstrated that the unbound, rather than the total concen-

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tration of MPA is a predictor of MPA inhibitory effect on inosine monophosphate dehydrogenase, therefore, it is advisable to measure unbound or free concentration of MPA (fMPA), in addition to the total concentration for pharmacokinetic investigations.

Mycophenolic acid is metabolized in the body by the uridine diphosphate glucuronyl transferases to an inactive glucuronide conjugate MPAG, a pharmacologically active acyl glucuronide metabolite (AcMPAG) and a 7-*O*-glucoside metabolite [4]. Mycophenolic acid is primarily excreted renally as MPAG and to some extent in the bile [1]. Mycophenolic acid glucuronide is excreted in the gut and reabsorbed as MPA, a mechanism commonly known as enterohepatic recirculation resulting in the occurrence of a secondary plasma peak 6 h after oral administration [5].

As a guide to dosage adjustment, it is advisable to measure the concentration of MPA and MPAG in transplant recipients, however, this practice is not fully implemented in many centers [6,7]. An enzyme multiplied immunoassay technique, for the measurement of MPA was developed by Dade Behring, however, to our knowledge this method is only licensed for research use. Because of cross-reactivity with the AcMPAG, this method somewhat overestimates the MPA concentration and does not accurately measure the MPAG concentration [8]. High-performance liquid chromatography-based methods, either coupled with ultraviolet or mass spectrometry detections, therefore, remains the standard method for determination of MPA and its major metabolite MPAG. This manuscript describes the development and validation of a simple and rapid method for simultaneous determination of total MPA and MPAG using HPLC-UV. We also report a method to quantitate fMPA in plasma using LC–MS/MS. Both methods have been validated according to the Guidelines for Bioanalytical Method Validation published by the Food and Drug Administration (FDA) of the United States [9].

2. Experimental

2.1. Human plasma, chemicals and materials

For the preparation of in-house quality control and calibration standards, a pool of six plasma samples from healthy donors (Rhode Island Blood Center, Providence, RI) was used. In addition, subsequent to signing of an informed consent form, ethylenediaminetetraacetic acid anticoagulated plasma were obtained from 41 kidney transplant recipients attending the outpatient clinic at Rhode Island Hospital, Providence, Rhode Island.

Standard samples of MPA, MPAG and MPA internal standard (IS), a carboxy butoxy ether derivative of MPA (MPAC) were kindly donated by Roche Pharmaceuticals (Palo Alto, CA). The sample of MPAG was produced by the Analytical Services International Ltd, London, UK and was 98.7% pure with less than 0.1% MPA impurity. Phenolphthalein glucuronic acid (PGA) (Sigma, St. Louis, MO) was used as IS for MPAG determination.

Prior to use, all glass and plastic ware was treated with AquaSil™ Siliconizing Fluid (Pierce Rockford, IL). All solvents were HPLC grade and all reagents were analytical grade. HPLC quality deionized water was prepared using Milli Q50 (Millipore, Bedford, MA) water purification system. HPLC grade methanol was purchased from Pharmco Products Inc. (Brookefield, CT) and phosphoric acid (85%, v/v) ACS reagent was purchased from Sigma–Aldrich (St. Louis, MO).

2.2. Total MPA and MPAG

2.2.1. Stock solutions, calibrators and quality control standards

Stock solutions, containing 500 mg/l MPA and 2500 mg/l MPAG were prepared in absolute methanol and stored at −20 °C until use. Because MPAG is known to degrade to MPA upon long storage, the stock solution of MPAG was checked by HPLC-UV for the presence of MPA contamination before use. Aliquots of the stock MPA and MPAG solutions were diluted with drug free plasma to give seven combined calibration standards, containing 0.5, 1.5, 3.0, 6.0, 12.0, 25.0, 50.0 mg/l MPA and 5.0, 10.0, 25.0, 50.0, 125.0, 250.0, 500.0 mg/l MPAG, respectively. Three in-house quality control standards (QCs), representing the low, medium and high concentrations were prepared in drug-free plasma with a final concentration of 2.0, 10.0 and 30.0 mg/l MPA and 30.0, 100.0 and 300.0 mg/l MPAG. In addition, three reference standards, containing the same concentrations of MPA and MPAG as the QCs were prepared in 50:50% (v/v) methanol:water. The working solution of IS contained 20 mg/l MPAC and 200 mg/l PGA. All calibration, quality control and reference standards and the combined IS solutions were aliquoted and stored at −20 °C until use.

2.2.2. Sample preparation

Mycophenolic acid and MPAG were extracted from plasma matrix using Isolute C_2, 100 mg, 3 ml SPE cartridges (Argonaut Technologies Inc., Foster City, CA). Calibrators, QCs or patient plasma samples were thawed at 37 °C using a reciprocal shaking water bath. To 100 μl of the samples, 100 μl of combined internal standard solution and 2 ml of 5% (v/v) phosphoric acid were added and samples were vortex mixed. This was then loaded onto SPE cartridges mounted on a VisiPrep®DL SPE manifold (Supelco, Bellefonte, PA) previously primed with 3 ml of methanol and 3 ml of 5% (v/v) phosphoric acid and allowed to drain. The cartridges were then washed with 3 ml of 5% (v/v) phosphoric acid solution and 3 ml of 5% (v/v) methanol containing 1% (v/v) phosphoric acid solution in deionized water. The analytes were eluted with 750 μl of 50:50% (v/v) methanol:water containing 0.1% (v/v) phosphoric acid and 100 μl of this was injected onto the analytical column. All the stages

of the SPE procedure were carried out at atmospheric pressure.

2.2.3. HPLC-UV apparatus and conditions

The chromatographic separation was performed on a Hitachi D-7000 series instrument (San Jose, CA) consisting of an autosampler fitted with a 200 μl sample loop, a quaternary pump, a column oven and a variable wavelength UV detector set at 254 nm. Peak areas were integrated using the Hitachi System Manager (HSM) software. Mobile phase was filtered and degassed using 0.45 μm Nylon filters (Millipore, Bedford, MA) under vacuum. Chromatographic separation of individual analytes was achieved using a Zorbax Rx C_8, 150 mm × 4.6 mm, 5 μm particle size (Agilent Technologies, Palo Alto, CA) analytical column preceded by a Supelco 1/16″, peek, 2 μm frit, pre-column filter (Supelco, Bellefonte, PA) maintained at 35 °C. Elution of the analytes were carried out by switching mobile phase composition at 5, 9 and 14 min post injection corresponding to compositions of 48:52, 60:40 and 48:52% (v/v) methanol:water containing 0.1% (v/v) phosphoric acid, respectively. The flow rate was maintained at 1 ml/min over the 18 min run and all mobile phase changes occurred within 6 s. Calibration curves consisted of respective concentrations of MPA and MPAG plotted separately against MPA to MPAC and MPAG to PGA peak area ratios, respectively.

2.2.4. Assay validation

The specificity of the assay was evaluated by comparing the retention times of each of the analytes against the chromatogram of extracted plasma with no drugs added or containing the internal standards only. The peaks of each of the components were sought to be well resolved, and also, that there was no interference from endogenous or exogenous materials at the retention times of the analytes. Furthermore, to investigate possible chromatographic interference by drugs administered to transplant recipients including other immunosuppressive agents, plasma samples from 25 transplant recipients who were not receiving MPA were analyzed.

The chromatograms were checked for consistency in retention times and concentration dependent peak areas of the analytes. The sensitivity of the analytical procedure was expressed as the lower limit of quantification (LLOQ) or the lowest concentrations of total MPA or MPAG in plasma that can be quantitatively determined with suitable accuracy and precision and that after extraction gave a peak height to baseline noise ratio of at least 10:1, while the limit of detection (LOD) was taken as the minimum concentrations in plasma that after extraction gave a peak height to baseline noise ratio of at least 3:1. Assay linearity was assessed using an unweighted linear regression method between the LLOQ and the sample representing the upper limits of clinically relevant concentrations in plasma [9].

The recovery of the extraction procedure was assessed by comparing the peak areas of the extracted QCs with peak areas of reference standards prepared in 50:50% (v/v) methanol:water and injected directly onto the analytical column, and is expressed as a percentage area of the extracted QC relative to the directly injected reference standard. The extraction procedure was modified so that the recovery of the extraction procedure remains constant at the low, medium and high QC concentrations. The closeness of the validation results for QCs obtained by the method to the true value and the degree of agreement among the individual results for multiple analytical runs of the same sample were taken as the accuracy and precision, respectively. To evaluate freeze-thaw stability, aliquots of the QC plasma samples were subjected to freezing for 24 h at −20 °C and thawed unassisted at room temperature for three cycles.

2.3. Unbound MPA concentration (fMPA)

2.3.1. Materials

Indomethacin (Alfa Aesar, Ward Hill, MA) was used as the internal standard for determination of unbound MPA. Amicon Centrifree® ultrafiltration devices (Millipore, Bedford, MA) assembled with regenerated cellulose membranes with a molecular weight cut-off of 30 kD was used to generate plasma ultrafiltrate and the devices were centrifuged in a Marathon 22 KBR centrifuge with fixed angle rotor (Fisher Scientific, Hanover park, IL). The MPA from ultrafiltrates was then extracted using Sep-Pak® Vac C_{18}, 200.0 mg, 3.0 ml SPE cartridges (Waters Corporation, Milford, MA) and drying of the samples was performed using a centrifugational evaporator (SPD 1010 SpeedVac® system, ThermoSavant, Holbrook, NY).

2.3.2. Stock solutions, calibrators and quality control standards

Aliquots of the MPA stock solutions were diluted to give two sub-stocks, containing 0.5 and 50.0 mg/l MPA in absolute methanol and a working stock solution, containing 1.0 mg/l indomethacin is prepared in absolute methanol. The MPA sub-stocks were used to prepare eight calibrators in ultrafiltrate of drug free plasma, containing 1.0, 2.5, 5.0, 10.0, 50.0, 100.0, 500.0 and 1000.0 μg/l MPA. Three sets of QCs were prepared in absolute methanol, isotonic saline solution and ultrafiltrate of drug free plasma at MPA concentrations of 7.5, 400 and 750 mg/l and stored at −20 °C until use.

2.3.3. Sample preparation

Calibrators and QCs were directly subjected to SPE, while patients' samples were first subjected to ultrafiltration followed by SPE. Eight hundred microliters of patient plasma was transferred to an ultrafiltration device and centrifuged for 30 min at 3000 × *g*. To 200 μl of patient ultrafiltrate or other samples 100 μl of indomethacin working stock solution and 500 μl of 0.05 M hydrochloric acid were added and the samples were vortex mixed and centrifuged for 1 min at 850 × *g*. The mixtures were then loaded onto SPE cartridges previously primed with 2 ml of methanol and 2 ml of 90:10% (v/v) water:methanol. The cartridges were washed with 2 ml

of 90:10% (v/v) water:methanol, subjected to full vacuum for 15 min and the analytes were eluted with 1 ml of absolute methanol. The extracts were then dried in a centrifugational evaporator at 50 °C, reconstituted in 100 μl of absolute methanol and 20 μl of this was injected onto the analytical column.

2.3.4. LC–MS/MS apparatus and conditions

A turboion spray liquid chromatography tandem mass spectrometry technique was used. The LC–MS/MS system consists of a Perkin-Elmer 200 series micropump and autosampler (Perkin-Elmer, Norwalk, CT) attached to a PE Sciex API2000 series tandem mass spectrometer (Applied Biosystems, Toronto, Canada). High-purity nitrogen gas obtained from a 240 l Liquid Nitrogen Dewar (Medford, MA) was used as nebulizer (Gas 1), auxiliary (Gas 2), and collision gases.

Chromatographic separation was achieved using the analytical column described in Section 2.2.3. Elution of MPAG, AcMPAG, MPA and indomethacin from the chromatographic column was carried out with a gradient mobile phase composition consisting of 72:28% (v/v) methanol:0.05% formic acid for the first 3.5 min at a flow rate of 0.6 ml/min then switching to 85:15% (v/v) methanol:0.05% formic acid at a flow rate of 0.7 ml/min for the next 2.5 min and switching back to 72:28 %(v/v) methanol:0.05% formic acid composition for 1 min with a flow rate of 0.7 ml/min.

The LC–MS/MS compound parameters were adjusted to obtain optimum conditions for improving sensitivity and to detect and quantitate MPA and indomethacin in the multiple reactant monitoring (MRM) mode. Detection of the analytes was performed in negative ionization mode using the mass transitions of *m/z*: 319.0 → 190.8 for MPA, *m/z*: 355.9 → 312.2 for indomethacin and *m/z*: 495.0 → 319.2 for both MPAG and AcMPAG. Flow injection analysis was performed at a flow rate of 0.8 ml/min to obtain optimum source parameters for the analysis. The following compound parameters were used for MPA, and indomethacin, respectively: declustering potential, −30 and −20 V; focusing potential, −335 and −385 V; entrance potential, −9 and −8 V; collision cell entrance potential, −18 and −18 V; collision energy, −30 and −15 V and collision cell exit potential, −7 and −8 V. The optimum source parameters that gave the highest MPA intensity were: curtain gas, 20 psi; collision gas, 4 psi; ion spray voltage, −4000 V; temperature, 550 °C; ion source gas 1, 30 psi and ion source gas 2, 85 psi. Integration of the peaks was performed by manual baseline adjustment using the ANALYST SP version 1.2 software. All quantifications were performed using peak area ratios and calibration curves consisted of MPA to indomethacin concentration ratios plotted against MPA to indomethacin peak area ratios.

2.3.5. Assay validation

Chromatographic separation of MPAG and AcMPAG was ensured by injection of individual analytes and determining their respective retention times. The similarities of mass transitions selected for MPAG and AcMPAG was due to the fact that both analytes have same masses but differ only in the position of the glucuronide within the molecule and tend to have similar mass transition in the MRM mode. The chromatographic separation of MPAG and AcMPAG from MPA ensures that MPA generated from MPAG and AcMPAG at the ion source did not interfere and contribute to unbound MPA concentrations.

The LLOQ and LOD were determined as described in Section 2.2.4. The recovery of the extraction procedure was assessed by comparing the peak area rations of the extracted QCs prepared in isotonic saline and drug free ultrafiltrate with peak areas of QCs prepared in absolute methanol and injected directly onto the analytical column. Assay linearity was assessed in the concentration ranging from the LLOQ to the concentration covering the upper limits of clinically relevant concentrations of unbound MPA in plasma by preparing at least 10 calibration curves and determining the correlation coefficient of the curves. Accuracy and precision were determined by evaluating the closeness of the true concentration values of the QCs to the experimentally determined concentrations obtained using the calibration with $1/x^2$ weighting. Each of the validation parameters was determined six times and in duplicate injections.

2.3.6. Ion suppression test

Ion suppression is a common problem encountered in a LC–MS/MS analytical method and occurs due to the presence of matrix components that may prevent the ionization of the analyte. The test is performed by infusion of an analyte solution consisting of a mix of MPAG, AcMPAG, MPA and indomethacin (1 mg/l each) prepared in mobile phase at an infusion rate of 20 μl/min and simultaneously injecting ($n = 6$) samples that have been extracted from plasma ultrafiltrate. A dip in the baseline indicates that the eluent from HPLC column competes with ionization of the analyte of interest, a phenomenon that is commonly referred to as ion suppression. Ion suppression test was also performed for injection of deionized water ($n = 6$) and is referred to as a water dip [10]. When ion suppression is present in a method, the elution time of the analyte should preferably be different from the matrix dip or otherwise the sensitivity, as well as accuracy and precision of the method for detection of the compound of interest may be compromised.

3. Results

3.1. Total MPA and MPAG

Each of the four components, MPA, MPAG and their respective internal standards MPAC and PGA were well resolved and no interference was observed from plasma peaks at the elution times of these analytes. In addition, analysis of plasma samples from 25 transplant recipients receiving immunosuppressive agents other than MMF revealed no inter-

Table 1
Assay parameters for determination of total mycophenolic acid and mycophenolic acid glucuronide concentrations using HPLC-UV

Component	Concentration range (mg/l)	Retention time (min)[a]	LOD (mg/l)	LLOQ (mg/l)	Recovery[a]	Regression coefficient
Phenolphthalein glucuronic acid	N/A	3.90 ± 0.03	N/A	N/A	89.1 ± 2.2%	N/A
Mycophenolic acid glucuronide	5.0–500.0	4.68 ± 0.04	1.5	5.0	84.5 ± 3.7%	0.988–0.999
Mycophenolic acid	0.5–50.0	11.20 ± 0.08	0.15	0.5	78.0 ± 2.9%	0.979–0.998
Carboxy butoxy ether-mycophenolic acid	N/A	12.44 ± 0.10	N/A	N/A	83.2 ± 0.8%	N/A

[a] Mean ± S.D. of six replicates; LOD, limit of detection; LLOQ, lower limit of quantification.

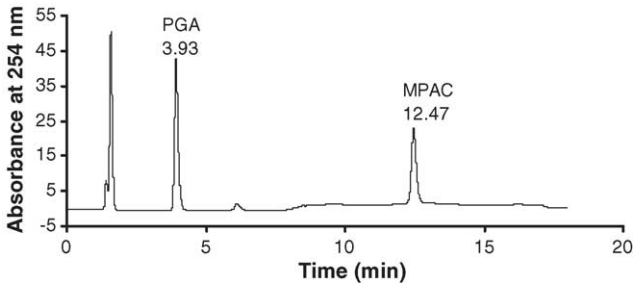

Fig. 1. Chromatogram of drug free plasma, containing internal standards, MPAC and PGA, extracted using the solid-phase extraction method described in Section 2.2.2.

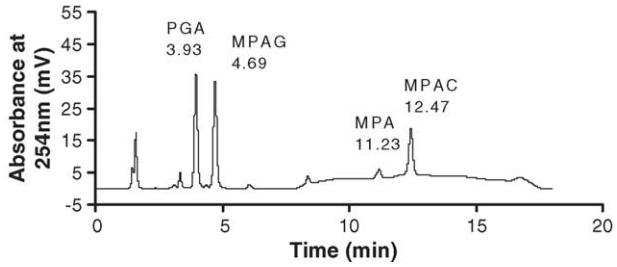

Fig. 2. Chromatogram of a plasma sample from a representative kidney transplant recipient; the concentration of MPA and MPAG was calculated to be 3.6 and 95.5 mg/l, respectively.

fering peaks from possible endogenous or exogenous compounds. The retention times of MPA and MPAG and their respective internal standards are given in Table 1. The solid-phase extraction procedure had an average recovery of 78.0% for MPA and 84.0% for MPAG, analyzed for each of the low, medium and high level QCs (Table 1). A chromatogram of drug free plasma extracted with the SPE method indicated no interference with plasma peaks (Fig. 1) and a typical chromatogram depicting MPA and MPAG extracted from plasma of a kidney transplant recipient is shown in Fig. 2. The LOD was found to be 0.15 mg/l for total MPA and 1.5 mg/l for MPAG. Similarly, the LLOQ was found to be 0.5 mg/l for total MPA and 5 mg/l for MPAG.

The assay was linear in the concentration range from 0.5 to 50.0 mg/l for MPA and 5.0 to 500.0 mg/l for MPAG with a mean regression coefficient (r^2) value of 0.9996, $y = 0.0539x + 0.0088$ for MPA and 0.9979, $y = 0.0135x + 0.0878$ for MPAG, of 10 replicated calibration curves performed in duplicates on different days. The method was accurate and precise with an inter- and intra-day inaccuracy of less than 12% (Table 2). The accuracy at LLOQ concentration for MPA (0.5 mg/l) was 106% and the inter-day CV was 12%, whereas, the accuracy for MPAG determinations at LLOQ (5.0 mg/l) was 116% with an inter-day CV of 1.5%. The concentrations of MPA and MPAG in the QCs remained unchanged after three cycles of freeze and thaw. The extracted samples were stable in the autosampler for about 40.0 h at room temperature.

3.2. Unbound MPA concentration

Mycophenolic acid and indomethacin were found to elute at 5.03 and 6.38 min, respectively, whereas, MPAG and AcMPAG eluted at 3.58 and 4.26 min, respectively. Fig. 3 depict chromatogram of a sample, containing MPAG, AcMPAG, MPA and indomethacin. As shown in Fig. 4a the ion suppression due to the matrix effect is found to occur at approximately 4.1 min, whereas, the water dip occurs at 2.25 and

Table 2
Imprecision and accuracy data for total mycophenolic acid (MPA) and mycophenolic acid glucuronide (MPAG) using HPLC-UV

Analyte	Actual concentration (mg/l)	Observed concentration[a] (mg/l)	Inter-day (%CV)[b]	Intra-day (%CV)[b]	Inter-day accuracy (%)	Intra-day accuracy
MPA						
QC_1	2	1.94 ± 0.25	12.7	13.8	96.8	93.5
QC_2	10	10.94 ± 0.45	4.2	2.9	109.4	107.4
QC_3	30	30.45 ± 0.67	2.2	2.1	101.5	102.7
MPAG						
QC_1	30	31.86 ± 1.52	4.8	3.0	106.2	108.5
QC_2	100	115.93 ± 3.03	2.6	3.2	115.9	111.0
QC_3	300	304.90 ± 5.25	1.7	1.4	101.6	102.4

[a] Mean ± S.D. of ten replicates.
[b] Coefficient of variation.

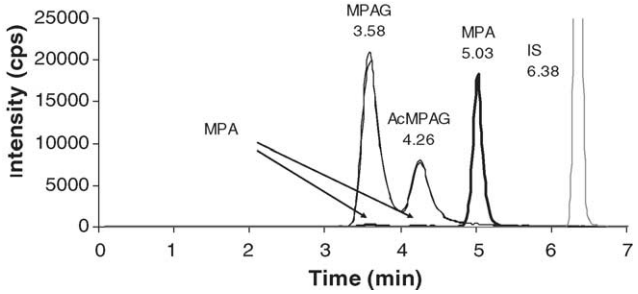

Fig. 3. Mass spectrometric chromatogram of an extracted sample after ultrafiltration, containing 1.0 mg/l each of MPAG, AcMPAG, MPA and indomethacin using Multiple Reactant Monitoring in negative ionization mode; transitions (MPA *m/z*: 319.0 → 190.9) (indomethacin *m/z*: 355.7 → 312.2), (MPAG *m/z*: 495.0 → 319.2) and (AcMPAG *m/z*: 495.0 → 319.1). Figure also shows small MPA peaks due to MPAG and AcMPAG breakdown to MPA, at ion source of MS.

6.18 min, respectively (Fig. 4b). The time of both ion suppressions differ from the retention time of MPA, and hence, can be concluded that ion suppression does not hinder the sensitivity of the mass spectrometer. The average recovery of the solid-phase extraction procedure was evaluated to be 92.7% for low, 95.1% for medium and 95.9% for high concentration of the QCs prepared in isotonic saline and drug free ultrafiltrate. The LLOQ was 1.0 μg/l and the method was linear in the concentration range from 1.0 to 1000 μg/l of MPA with the mean regression coefficient of 0.9988, $y = 0.0012x - 0.008$ of 10 replicated calibration curves performed in duplicates on different days.

The degradation of MPAG during drying at 50 °C after solid-phase extraction of the ultrafiltrates was studied for plasma samples that were spiked with MPAG only (200 mg/l). Samples that were extracted and dried were compared with samples that were extracted and injected directly onto the LC–MS/MS without drying. The non-dried samples did not show the presence of any traces of MPA (no peak was obtained at MPA retention time), whereas, samples that were dried showed about less than 0.06% degradation of MPAG to MPA. This degradation can be considered negligible and does not contribute significantly to the unbound concentration of MPA.

The method was accurate and precise with intra- and inter-day inaccuracy of less than 15% (Table 3). The concentrations of MPA in the QCs prepared in isotonic saline and drug free ultrafiltrate remained unchanged after three cycles of freeze and thaw. The extracted samples remained stable in the autosampler for about 48.0 h at room temperature.

3.3. MPA, MPAG and fMPA concentrations from clinical studies

Both methods have been successfully used to measure plasma concentrations of MPA in plasma from 41 adult renal transplant patients, at 12 h post MMF dose. The average time post transplant was 17 months and the average MMF dose was 500 mg/day. The mean ± S.D. of total MPA was 3.07 ± 2.42 mg/l with a minimum concentration of 0.58 mg/l and a maximum of 11.47 mg/l. The mean MPAG concentration was 84.95 ± 44.27 mg/l (range: 25.32–198.33 mg/l). The concentration of fMPA was found to be 12.08 ± 6.93 μg/l with a concentration range of 4.52–28.34 μg/l.

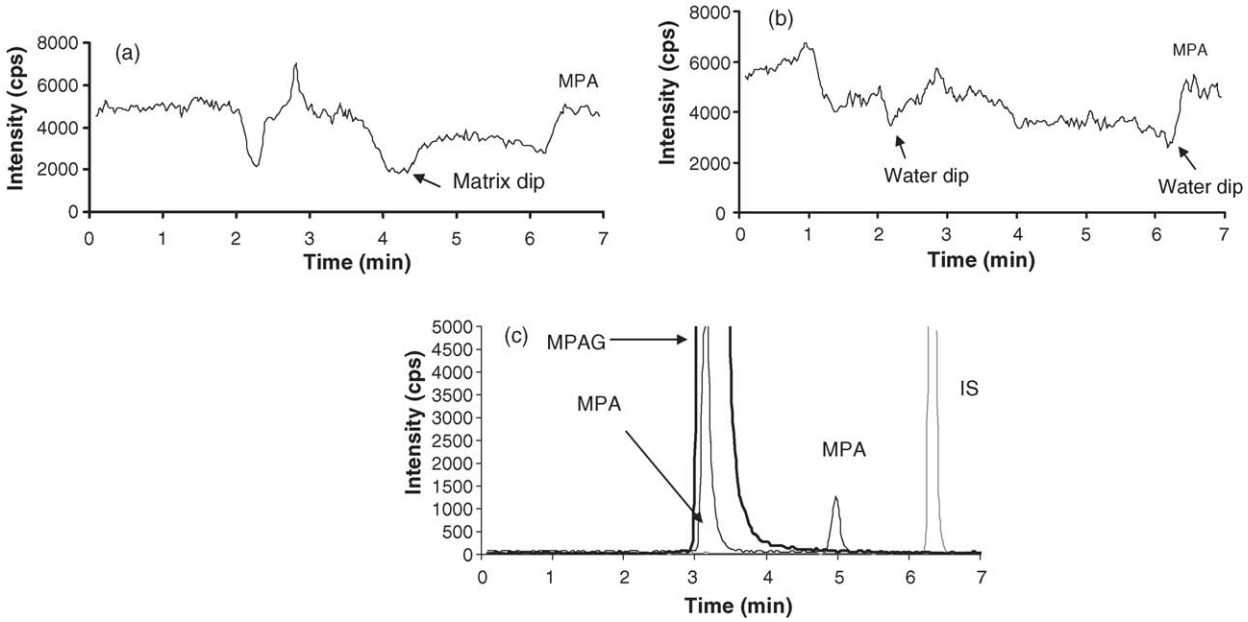

Fig. 4. (a) Chromatogram depicting the ion suppression test performed by the infusion of a 1 mg/l solution MPA in methanol with the simultaneous injection (20 μl) of a sample of drug free ultrafiltrate after subjecting to SPE. (b) Chromatogram depicting the ion suppression test performed by the infusion of a 1 mg/l solution of MPA in methanol with the simultaneous injection (20 μl) of deionized water. (c) Liquid chromatography–tandem mass spectrometry of unbound MPA (fMPA) in plasma sample from a representative kidney transplant recipient; the concentration of fMPA was determined to be 70 μg/l.

Table 3
Imprecision and accuracy of the unbound mycophenolic (fMPA) assay using quality control samples in absolute methanol, isotonic saline and drug free plasma ultrafiltrate

Sample	Actual concentration (μg/l)	Observed concentration (μg/l)[a]	Inter-day (%CV)[b]	Inter-day accuracy
Absolute methanol				
QC_1	7.5	7.3 ± 0.3	4.0	96.7
QC_2	400.0	378.3 ± 29.3	7.7	94.6
QC_3	750.0	779.3 ± 39.4	5.1	103.9
Isotonic saline				
QC_1	7.5	7.3 ± 0.2	2.2	97.6
QC_2	400.0	410.1 ± 24.8	6.1	102.5
QC_3	750.0	795.4 ± 84.4	10.6	106.1
Drug free ultrafiltrate				
QC_1	7.5	7.5 ± 0.3	4.0	99.3
QC_2	400.0	395.2 ± 42.0	10.6	98.8
QC_3	750.0	796.5 ± 18.2	2.3	106.2

[a] Mean ± S.D. of ten replicates.
[b] Coefficient of variation.

4. Discussion

We have described a simple and rapid HPLC-UV method for simultaneous determination of total MPA and MPAG in human plasma. A single continuous run was employed that allowed gradient elution of MPAG and its IS phenolphthalein glucuronic acid followed by MPA and its internal standard MPAC. We have also developed a highly sensitive and specific LC–MS/MS method for determination of unbound MPA in human plasma with a LLOQ of 1 μg/l. Furthermore, we have rigorously validated both methods using guidelines provided by the Food and Drug Administration of the United States [9].

To date several methods have been described for determination of MPA and MPAG using robotic extraction [11], ion pair reagent with UV [12] or fluorescence detection [13]. A number of HPLC methods were also developed that employed either MPAC [14] or PGA [15] as the internal standard for both MPA and MPAG. Considering the differences in the hydrophilicity of MPA and MPAG, using two internal standards, ensures comparable recovery between the analytes with their respective internal standard thereby providing higher accuracy and precision in determining the concentration of each analyte. In fact, in our assay when MPAC was used as the internal standard for determination of both MPA and MPAG, the concentration of MPAG for QC_1 and QC_2 standards were determined to be 27 and 21% higher, respectively, than the actual concentrations of these standards. In addition, our method utilizes a simple extraction procedure using SPE columns and does not involve any centrifugation, use of vacuum or drying of residues following extraction. The robustness of the method makes it easy for an operator to learn the technique quickly and to generate reproducible results. The method indeed is very economical with an approximate cost per sample of less than two US dollars for the supply and material. A single analytical column under the assay condition has lasted for the entire period of method validation and clinical study. In fact, this method is routinely used in three laboratories (The Analytical Services International, London UK; Department of Pharmacology, Papworth Hospital Cambridge UK and Pharmacokinetics Research Laboratory, College of Pharmacy, University of Rhode Island, USA).

The LLOQ of the HPLC-UV method is 0.5 mg/l for MPA and 5 mg/l for MPAG that is somewhat higher than some of the other published methods [10,16]. In this method we extract the analytes from 100 μl of plasma using SPE cartridges, elute the residues with 750 μl of solvent and inject 100 μl of this eluent directly onto the HPLC column. This dilution reduces the sensitivity of the assay by a factor of 7.5 but generates very clean extracts that increases the robustness and reliability of the assay and decreases the likelihood of column failure. Indeed, the on column limit of quantification for the method was 0.06 mg/l for MPA and 0.8 mg/l for MPAG. Furthermore, this LLOQ is adequate considering the plasma concentration range of 1–10 mg/l reported to occur for total MPA and 130–200 mg/l for total MPAG in normal adult renal transplant patients at least 3 weeks post transplantation [17]. The clinical suitability of the concentration range covered by this method could be demonstrated by our clinical study on trough plasma samples from 41 transplant recipients.

A number of methods for determination of fMPA in plasma using LC–MS/MS have been reported [10,16,18]. All these methods separate unbound drug using ultrafiltration followed by analysis of MPA concentration in the ultrafiltrate using LC–MS/MS, however, none of these methods have reported the possibility of occurrence of MPAG and AcMPAG in the ultrafiltrate. The presence of these two metabolites poses a major problem if they are not chromatographically separated from MPA because of the breakdown of both these metabolites at the ion source to MPA would contribute to the peak area of MPA [19].

In addition, we have used indomethacin instead of MPAC as the IS for determination of fMPA. The reason is early in the method development process we had observed that samples of MPAC were contaminated with MPA, and therefore, may interfere with the MPA measurements especially at low

concentrations. Injection of a 1 mg/l solution of MPAC using our LC–MS/MS method showed the presence of about 0.136 mg/l of MPA. In addition to this MPA peak at the usual retention time of MPA, another MPA peak was present that was due to the in source degradation of MPAC to MPA. MPAC a carboxy butoxy ether derivative of MPA is an impurity of mycophenolate mofetil. Considering that both MPAC and MMF are derivatives of MPA, it is conceivable to believe that MPAC may contain a small amount of MPA impurity. We do not anticipate any problems from the small amount of MPA impurity in the MPAC samples when HPLC with UV detection is used because of inherent low sensitivity of this technique. Moreover, the ion suppression test performed for both, the extracted matrix (drug free plasma ultrafiltrate) and directly injected deionized water sample has shown that suppression does not occur at the retention times of MPA hence will not affect the sensitivity of the fMPA determination. The separation achieved between MPAG, AcMPAG and MPA for the LC–MS/MS method can be utilized for the future prospects of quantifying MPAG and AcMPAG in plasma ultrafiltrate.

In conclusion, two robust and reproducible methods were developed. Both methods were validated according the guidelines published by the Food and Drug Administration of the United States. We currently use these methods to measure the concentration of total and unbound MPA and total MPAG for our pharmacokinetic studies. Further these methods are suitable for use in the therapeutic drug monitoring of MPA, MPAG and fMPA.

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