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Short communication

Liquid chromatographic determination of mycophenolic acid and its metabolites in human kidney transplant plasma: Pharmacokinetic application

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Abstract

Background and objective: Difference in the hydrophilic properties of mycophenolic acid metabolites makes it technically difficult to simultaneously determine their plasma levels in one analytical run. Therapeutic drug monitoring (TDM) for MPA ensures adequate MPA exposure levels to both prevent rejection and avoid related toxicity. One measure limitation for TDM for MPA is the availability of simple, rapid and reproducible method for determination of MPA derivatives.
Method: Herein we report a single method to measure MPA and its metabolites using a gradient elution system in less than 10 min. We further tested applicability of our method in both stable and unstable renal transplant recipients with a wide range of levels.
Results: Intra- and inter-day imprecision were less than 8% and 10%, respectively. Accuracy of the estimated concentrations ranges from 90% to 108%.
Conclusion: Collectively these data show that the new method is reasonably accurate and precise for the simultaneous determination of MPA and its metabolites in human plasma.

Keywords: Pharmacokinetics; Mycophenolic acid; Therapeutic drug monitoring; Transplantation; HPLC assay; Validation

1. Introduction

Mycophenolic acid (MPA) is a potent, selective, reversible and uncompetitive inhibitor of inosine monophosphate dehydrogenase and thus inhibits the *de novo* pathway of guanine nucleotide synthesis, limiting lymphocyte proliferation [1]. MPA derivatives became widely accepted as an efficacious adjunctive immunosuppressant in transplantation following studies showing beneficial effects on rejection in cyclosporine (CsA)-based [2] and tacrolimus-based regimens [3,4].

MPA is extensively metabolized by UDP-glucuronsyltransferase enzymes (UGTs) in the liver, gut and kidney to its inactive metabolite, phenyl mycophenolic acid glucuronide (MPAG), Fig. 1. This metabolite exists in plasma in up to 100-fold greater concentration than MPA. MPAG is extensively bound to serum albumin, from which it can displace MPA, and is excreted in both urine and bile [1]. Excretion of MPAG into bile allows enteric organisms with glucuronidase activity to cleave MPAG back to MPA which can undergo enterohepatic recirculation. Accordingly, two MPA peaks in plasma are usually seen: one following the first absorption and occurs 0.5–1 h after oral administration and a second and smaller peak represents absorption from the distal bowel following enterohepatic recirculation 4–6 h after oral intake of the parent drugs Cellcept [5]. A second and less abundant metabolite is the acyl glucuronide (AcMPAG), Fig. 1. Unlike MPAG, AcMPAG it is pharmacologically active and cross-reacts with the antibody used in the immunoassays used for MPA quantification.

Recently we reviewed current literature to support the notion that therapeutic drug monitoring (TDM) of MPA and its metabolites improves monitoring of transplant patients on calcineurin inhibitor minimization or withdrawal protocols [6].

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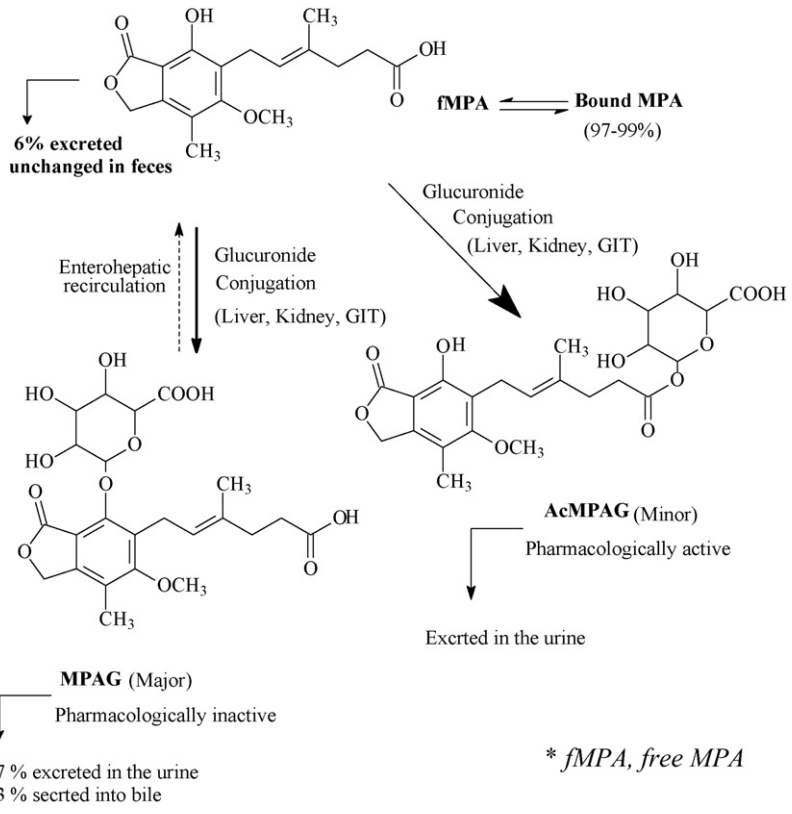

Fig. 1. Metabolic biotransformation of MPA and its metabolites. Adapted with permission from [6].

Establishment of easy-to-use, sensitive and reproducible analytical method for the simultaneous determination of MPA and its metabolites is a basic requirement to achieve this goal. Among the numerous reported methods, high-performance liquid chromatographic (HPLC)-based analyses are the standard methods for the determination of MPA and its metabolites. Differences in hydrophilic properties of MPA (relatively non-polar, $\log P = 3.88 \pm 0.38$ for uncharged form) and its glucuronide metabolites (relatively polar, $\log P = 0.49 \pm 0.52$ for uncharged form) makes it difficult to simultaneously determine these components in one analytical run. Accordingly, the previously published HPLC methods used either gradient elution system [7–11] or different stationary phases [12].

Although several methods have been developed for the quantification of MPA and MPAG in human plasma, very few methods have been developed for the simultaneous determination of MPA, MPAG and AcMPAG [7,10,13]. These methods suffer from either laborious or expensive sample preparation and extraction or longer run time that is not suitable for routine drug monitoring.

Herein, we describe a new, simple, rapid, and reproducible method for the simultaneous determination of MPA and its glucuronide metabolites which offers a wide scope of applications. Thereafter we applied the new method for the determination of pharmacokinetic parameters of these compounds in a randomly selected eight renal transplant patients.

2. Experimental

2.1. Chemicals and reagents

MPA was purchased from Toronto Research Chemicals (North York, Ont., Canada). Phenolphthalein glucuronide (PG) was purchased from Sigma–Aldrich Canada Ltd. (Oakville, Ont., Canada). MPAG and AcMPAG were kindly donated by Roche Pharmaceuticals (Palo Alto, CA). HPLC grade acetonitrile and methanol were purchased from EMD chemicals (Darmstadt, Germany). A Milli-Q Synthesis (Millipore, Bedford, MA) water purification system provided purified deionized water. All other chemicals used were analytical grade.

2.2. Plasma samples

Drug-free plasma samples from healthy donors ($n = 8$) were pooled and used for constructing calibration curves and quality control (QC) samples. In addition as part of a clinical pharmacokinetic study approved by the institutional review board at the University of Saskatchewan, serial blood samples were collected from kidney transplant recipients at specific time intervals over a period of 12 h in EDTA anticoagulant tubes after oral administration of 1 or 1.5 g mycophenolate mofetil (MMF). Blood samples were centrifuged at $15{,}000 \times g$ for 10 min, and plasma was stored at $-20\,^{\circ}C$ until time of analysis.

2.3. Preparation of standard solutions and calibration standards

MPA, MPAG and AcMPAG were dissolved in methanol to prepare stock solutions (1.0 mg/mL). The stock solutions were stored at −20 °C. Calibration standards were prepared by spiking drug-free plasma samples with aliquots of the stock solutions to obtain final concentrations in the range of 0.5–40 μg/mL for MPA, 10–280 μg/mL for MPAG, and 0.5–20 μg/mL for AcMPAG. Three quality control (QC) samples were prepared by spiking blank plasma samples with MPA combined with its metabolites for method validation studies. Internal standard solution was prepared by dissolving PG in methanol to obtain 600 μg/mL.

2.4. Sample preparation

Aliquots (200 μL) of plasma, spiked plasma standards, or quality control samples were mixed with 100 μL internal standard and 200 μL of 0.1 M phosphoric acid in acetonitrile in an eppendorf tube. The tubes were mixed using vortex device for 30 s and then centrifuged at 5000 × *g* for 5 min. Aliquots of 30 μL of the clear supernatant were injected into the HPLC for analysis.

2.5. Apparatus and HPLC conditions

The HPLC system consisted of Waters model 2695 Alliance separation module, model 2996 photodiode array detector and Empower data module (Millipore-Waters, Milford, MA, USA). Absorbance was monitored at 215 nm (λ_{max} for MPA). Chromatographic separation was carried out on Zorbax Eclipse XDB C_{18} column (150 mm × 4.6 mm i.d., 5 μm particle size). The column was kept at 25 °C. The analytes were eluted under gradient conditions using a mobile phase composed of methanol and 0.15% phosphoric acid.

2.6. Method development and validation

Differences in polarity between MPA and its glucuronide metabolites precluded their simultaneous separation with an isocratic elution. Accordingly, a gradient elution system of methanol and 0.15% phosphoric acid was used. The mobile phase composition remained at 48:52, respectively for the first 7 min. For the last 3 min of the run, the % methanol was increased to 60% to elute the relatively non-polar drug, MPA. We have used a detection wavelength of 215 nm based upon the spectra obtained by scanning the chromatograms using photodiode array detector. This low wavelength was associated with interference from endogenous compounds at lower flow rates. Using a flow rate of 1.15 mL/min during the first 3 min significantly reduced such interference. For the last 7 min of the run, flow rate remained at 1.0 mL/min.

Method validation procedures were performed according to FDA guidelines (www.fda.gov/cder/guidance/cmc3.pdf) to evaluate the suitability of the method for the quantitative determination of MPA and its metabolites in human plasma. Specificity was tested by analysis of eight-independent drug-free human plasma samples supplemented only with the internal standard to ensure the absence of endogenous compounds with the same retention times as the analytes of interest.

The linearity of the method was evaluated by processing a six-point calibration curves range from 0.5 to 40 μg/mL (MPA), 10–280 μg/mL (MPAG) and 0.5–20 μg/mL (AcMPAG) on five different days. The peak height ratios between each analyte and the internal standard were plotted against the analyte's nominal concentration. A linear least-squares regression analysis was conducted to determine slope, intercept and coefficient of determination (r^2) to demonstrate linearity of the method.

The accuracy and precision of the proposed method were determined by analysis of the QC samples. The intra-day accuracy and precision were assessed from the results of six replicate analyses of QC samples on a single assay day. The inter-day accuracy and precision were determined from the same QC samples analyzed on six consecutive days. Precision is expressed as % relative standard deviation (R.S.D.), while accuracy (%) is expressed as [(calculated amount/predicted amount) × 100].

The low limit of detection (LLOD) was defined as the lowest detectable concentration, taking into consideration a signal-to-noise (S/N) ratio of 3 [14]. Low limit of quantification (LLOQ) was determined as the lowest concentration at which the precision, expressed as % R.S.D., is less than 20% and S/N ratio of 10 [14].

The recovery of MPA and its metabolites was determined by comparison of peak heights obtained from injection of 30 μL aliquots of standards prepared in methanol with samples containing the same amount after spiking in blank human plasma and processed as indicated above. Recovery was calculated using the following equation:

$$\text{recovery}\,(\%) = \frac{\text{peak height from spiked plasma}}{\text{peak height from methanol solution}} \times 100$$

2.7. Pharmacokinetic (PK) analysis

PK parameters including area under concentration–time curve (AUC), maximum plasma concentration (C_{max}), time to reach C_{max} (T_{max}), apparent volume of distribution at steady state (V_d/F), oral clearance (Cl/*F*), and mean residence time (MRT) were estimated using a non-compartmental analysis using WinNonLin 5.1 (Pharsight Corporation, Mountain View, CA, USA).

3. Results and discussion

Recent years have witnessed more attention toward the clinical importance of TDM for MPA, the active metabolite of the pro-drug MMF [6,15]. Monitoring of the MPA and its metabolites, MPAG and AcMPAG, may also indicate the degree of immunosuppressive activity and adverse effects of the drug [16,17]. Accordingly, several methods have been developed for the quantitation of MPA in human plasma at a high degree of accuracy and sensitivity. MPAG is extensively bound to serum albumin, from which it can displace MPA and increases

its free (unbound) fraction (fMPA) [1]. Significant increase in fMPA is observed during kidney impairment where MPAG is accumulated [18]. Also, MPAG undergoes enterohepatic recirculation and re-enters the systemic circulation as MPA, Fig. 1 [5]. Although it is considered a less abundant metabolite, AcMPAG is believed to have immunosuppressive activity [16,17] and its concentration correlates with the gastrointestinal adverse effect of MMF [16]. Accordingly, availability of a simple and accurate analytical method for the simultaneous determination of MPA and its metabolites is important to facilitate the TDM and pharmacokinetic studies of MAP.

Several studies have failed to separate MPA and its metabolites in one chromatographic run by isocratic elution (data not shown) due to difference in polarity. Similar to other published methods, we used a gradient elution system for the simultaneous separation and quantification of MPA and its glucuronide metabolites [7,10,13]. However, in comparison with these methods, we achieved good resolution with acceptable accuracy and precision in less than 10 min. Using gradient elution, the retention times were 4.6, 6.3, 7.7, and 8.6 min for internal standard (PG), MPAG, AcMPAG, and MPA, respectively. Fig. 2 shows chromatograms of blank plasma samples spiked with the internal standard, MPAG, AcMPAG, and MPA at concentrations close to the claimed LLOQ for each analyte (Fig. 2a), blank plasma samples supplemented only with the internal standard (Fig. 2b), and plasma sample of a kidney transplant patient 4 h after oral administration of mycophenolate mofetil (MMF) (Fig. 2c). No interfering peaks were observed at the retention times of MPA, MPAG, AcMPAG or the internal standard.

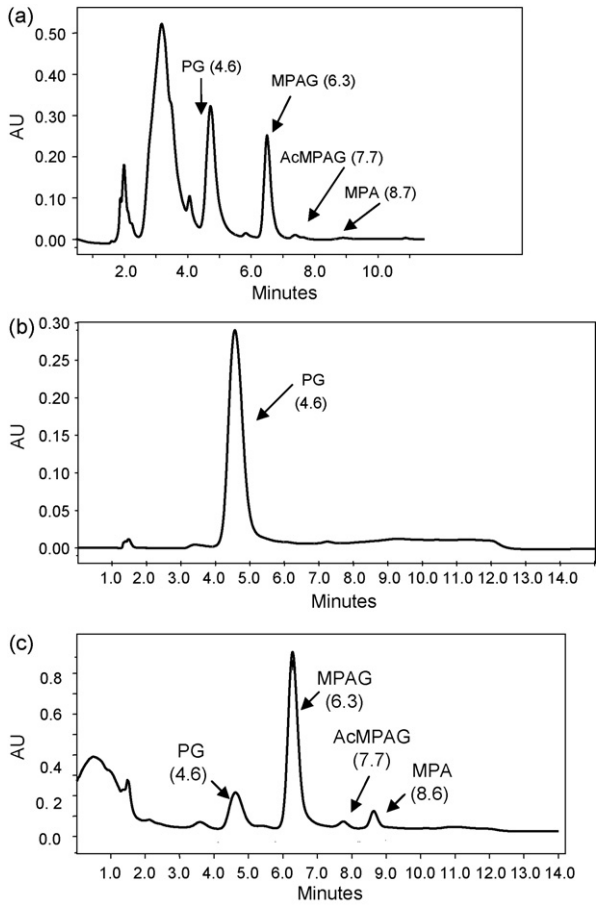

Fig. 2. Typical HPLC chromatograms of MPAG, AcMPAG and MPA in human plasma samples. (a) Blank plasma sample spiked with internal standard, MPAG (20 μg/mL), AcMPAG (0.75 μg/mL), and MPA (0.75 μg/mL). (b) Blank plasma sample spiked only with the internal standard (120 μg/mL). (c) Plasma sample from a kidney transplant patient, 4 h after MMF administration. The results show concentrations of 86.5, 2.5, and 5.5 μg/mL for MPAG, AcMPAG and MPA, respectively.

3.1. Method validation

3.1.1. Extraction efficiency and selectivity

The extraction recovery of MPA and its metabolites was measured using three levels of concentration in human plasma, while the recovery of the internal standard was determined only at one concentration level (Table 1). Triplicates of these samples were extracted and analyzed as described in the method section. Despite the simple sample preparation method, recovery of all analytes from spiked plasma samples were in acceptable range (83–97%), Table 1. Compared with other methods utilizing protein precipitation [19–21], our method shows better or similar results in addition it includes the simultaneous determination of MPA and its metabolites.

All studied plasma samples were obtained from kidney transplant patients who are taking many other drugs.

Table 1
Extraction recovery of MPA, its metabolites and the internal standard from spiked human plasma ($n = 3$)

	Nominal concentration (μg/mL)	Estimated[a] concentration (μg/mL)	Recovery (%)	CV (%)
MPAG	20	19.5 ± 0.8	97.5	6.9
	100	94.6 ± 3.22	94.6	3.2
	200	192.7 ± 4.5	96.5	2.3
AcMPAG	1	0.93 ± 0.04	93	4.3
	10	9.3 ± 0.37	93	4
	20	19.13 ± 0.51	95.6	2.7
MPA	1	0.84 ± 0.05	83.7	6.1
	15	13.16 ± 0.76	87.7	5.8
	30	25.9 ± 2.2	86.3	4.9
PG	120	110 ± 5	91.7	4.6

[a] Expressed as mean ± S.D.

Table 2
Intra-day ($n = 6$) and inter-day (six consecutive days) accuracy and precision data for the quantitation of MPA and its metabolites in human plasma by the proposed HPLC method

	Nominal concentration (μg/mL)	Accuracy (%)		Imprecision (%)	
		Intra-day	Inter-day	Intra-day	Inter-day
MPAG					
QC1	20	98 ± 4.1	96 ± 5.5	2.5	3.6
QC2	100	98.2 ± 3.5	95 ± 4.6	1.25	2.7
QC3	200	101 ± 4.3	98 ± 5	3.4	5
AcMPAG					
QC1	1	103.5 ± 5.2	100.5 ± 7	5.4	8.1
QC2	10	101 ± 3.2	99.5 ± 6.5	2.4	5.4
QC3	20	100.5 ± 2.0	98.7 ± 4.2	3.1	6.7
MPA					
QC1	1	97.5 ± 3	99 ± 4.8	7.9	9
QC2	15	100 ± 4.0	101.5 ± 7.2	5.2	7.2
QC3	30	100.5 ± 2.5	102 ± 4.8	4.6	6.9

These co-administered drugs include; immunosuppressive agents (corticosteroids, cyclosporine A, sirolimus, tacrolimus), antibiotics (amikacin, amoxicillin, vancomycin), analgesics (acetaminophen, salicylates), and other cardiovascular and psychotic drugs. Analysis of plasma samples from these patients did not show any significant interference from the co-administered medication at the retention time of MPA or its metabolites (Fig. 2a and c). In addition, the interference from endogenous compounds was evaluated by separate analysis of plasma samples from 20 normal volunteers. Again, under the assay conditions, no interfering peaks were observed at the retention times of the analytes of interest (Fig. 2b).

3.1.2. Linearity

The calibration curves of MPA and its metabolites in human plasma were linear over concentration ranges of 0.5–40 μg/mL MPA, 10–280 μg/mL MPAG, and 0.5–20 μg/mL AcMPAG. The determination coefficient (r^2) was greater than 0.995 in all calibration curves ($n = 6$).

3.1.3. Limit of detection and quantitation

Determination of low limit of detection (LLOD) and quantitation (LLOQ) was determined based upon signal-to-noise (S/N) ratio of 3 and 10, respectively [14]. Spiked human plasma samples were assayed for all analytes in decreasing concentration. Chromatograms from the assay of blank plasma samples were used for the establishment of the background noise. LLOD of MPA, MPAG and AcMPAG were 0.15, 2.5, and 0.15 μg/mL, respectively. Although more sensitive methods have been reported, such methods were either mass spectrometry-based [8,22–24], or involve time-consuming sample preparation procedures [11,25,26]. LLOQ of MPA, MPAG and AcMPAG were 0.5, 10, and 0.5 μg/mL, respectively. The obtained LLOQ in our method were sufficient for our preliminary pharmacokinetic study in human plasma in renal transplant patients. These limits of detection and quantitation are, in general, comparable to the published HPLC–UV methods that enable simultaneous determination of MPA and its glucuronide metabolites [7,10,13].

3.1.4. Accuracy and precision

To determine inter- and intra-day accuracy and precision of the assay, replicate set ($n = 6$) of three concentrations (low, medium and high) of each analyte in human plasma were analyzed. Accuracy and precision were calculated as described in the materials and methods section and results are summarized in Table 2. During the course of method validation, intra- and inter-day precision were less than 8% and 10%, respectively. The accuracy of the estimated concentrations ranged from 90% to 108% (Table 2). These data show that this new method is both accurate and precise for the determination of MPA and its metabolites in human plasma.

3.2. Application of the method

The method was successfully applied for monitoring of plasma levels of MPA and its metabolites in eight kidney transplant patients. Fig. 3 represents concentration–time profile for MPA, MPAG, and AcMPAG in plasma samples collected

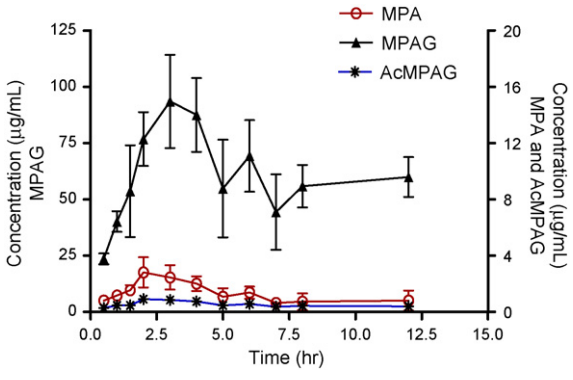

Fig. 3. Plasma concentration–time curves for MPA, MPAG and AcMPAG from kidney transplant recipients ($n = 6$) over a period of 12 h after administration of 1000 mg MMF twice daily.

Table 3
Pharmacokinetic profile of MPA and its metabolites in kidney transplant patients ($n = 8$)

	MPA		MPAG		AcMPAG	
	Average	±S.D.	Average	±S.D.	Average	±S.D.
T_{max} (min)	105.0	57.8	205.7	114.1	187.5	74.8
C_{max} (μg/mL)	25.6	22.1	97.2	53.6	5.8	2.1
$AUC_{0–12}$ (mg h/L)	101.2	86.2	608.1	374.2	31.8	14.0
V_d/F (L)	48.4	32.0	11.9	12.0	133.6	73.3
Cl/F (L/h)	15.4	15.3	2.0	2.1	18.9	8.4
MRT (h)	3.6	1.2	4.6	1.1	4.4	0.9

$AUC_{0–12}$: area under the concentration–time curve from 0 to 12 h; C_{max}: maximum plasma concentration; T_{max}: time to maximum plasma concentration; V_d/F: apparent volume of distribution; Cl/F: oral clearance; MRT: mean residence time.

over a period of 12 h after administration of MMF. Only samples from patients taking 1000 mg MMF twice daily were used to construct this figure. Actual sampling times were used for all analysis. Plasma concentration–time profiles of MPA show evidence of enetrohepatic recirculation characterized by a secondary peak at approximately 6 h in most of the tested patients.

These patients were randomly selected during the month of December 2006. Plasma samples in two fresh transplant recipients were tested at day 3 while on 1.5 g of Cellcept twice daily; two kidney transplant recipients requiring admission to hospital because of Norwalk virus induced moderate and severe gastroenteritis associated with pre-renal failure; one patient with Tacrolimus induced renal dysfunction (biopsy proven) and one patient with MMF-induced leucopenia and two other stable recipients. We randomly selected these patients to test the applicability of our method under different clinical conditions. A total of 40 patients were tested with this method. It should be noted, however, that the primary aim of this paper is to present the pharmacokinetic profiles of MPA and its glucuronide metabolites in different renal patients as shown in Table 3.

The eight patients were selected to demonstrate the marked inter-individual variability in pharmacokinetics of MPA and its metabolites in different clinical sittings, which calls for the importance of therapeutic drug monitoring of MPA and its active metabolites [6,15,27] in both stable and unstable patients. The observed inter-patient variability may result in MPA exposure levels that falls outside the defined target range even after administration of a standard MMF dose [15,28]. Such fluctuation in MPA concentrations in patients following administration of the same MMF dose can result in MPA levels that may be toxic or sub-therapeutic.

4. Conclusion

We describe a simple and accurate method for the simultaneous determination of MPA and it major metabolites, MPAG and AcMPAG in less than 10 min. The method was used to determine PK parameters of MPA and its metabolites in eight kidney transplant patients. The PK results show large inter-individual variability. This suggests that MMF dose individualization can be further improved by regular TDM, particularly whenever MPA level is expected to change.

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