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Simple reversed-phase ion-pair liquid chromatography assay for the simultaneous determination of mycophenolic acid and its glucuronide metabolite in human plasma and urine

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Abstract

A simple and reproducible reversed-phase ion-pair high-performance liquid chromatographic (HPLC) method using isocratic elution with UV absorbance detection is presented for the simultaneous quantitation of mycophenolic acid (MPA) and MPA-glucuronide (MPAG) in human plasma and urine. The sample preparation procedures involved simple protein precipitation for plasma and 10-fold dilution for urine. Each analytical run was completed within 15 min, with MPAG and MPA being eluted at 3.8 and 11.4 min, respectively. The optimized method showed good performance in terms of specificity, linearity, detection and quantitation limits, precision and accuracy. This assay was demonstrated to be applicable for clinical pharmacokinetic studies.

Keywords: Mycophenolic acid; Mycophenolic acid glucuronide

1. Introduction

Mycophenolate mofetil (MMF, Fig. 1) is an immunosuppressant that is approved by the US Food and Drug Administration (FDA) for the prophylaxis of organ rejection in patients receiving allogeneic renal transplants (May 1995), allogeneic cardiac transplants (February 1998) and allogeneic hepatic transplants (July 2000). MMF is used as the antimetabolite drug in place of azathioprine, in combination with a calcineurin inhibitor (cyclosporine or tacrolimus) or sirolimus (rapamycin) and corticosteroids, for the prevention of rejection in the above-mentioned transplant populations. It has been demonstrated in three randomised, double-blind, multicentre clinical trials that MMF, administered in combination with cyclosporine and corticosteroids, reduces the incidence of acute allograft rejection in renal transplantation [1–3].

MMF, the ester prodrug of mycophenolic acid (MPA, Fig. 1), is rapidly absorbed after oral administration and is rapidly hydrolysed to MPA, the active immunosuppressive entity, by esterases present in the gut wall, liver, and possibly lung and peripheral tissues [4]. MPA is a potent, selective, uncompetitive, reversible inhibitor of inosine monophosphate dehydrogenase (IMPDH) and thus, exerts potent cytostatic effects on both T and B proliferating lymphocytes by inhibiting the de novo pathway of guanosine nucleotide synthesis [5–7]. MPA is extensively metabolised by UDP-glucuronosyltransferases in the liver, gastrointestinal tract and possibly kidney [4,8], mainly into the inactive metabolite MPA-glucuronide (MPAG, Fig. 1) that is primarily excreted by the kidneys, and which also undergoes enterohepatic circulation to be converted back to MPA [4,6].

In clinical practice, the dose of MMF prescribed currently is mainly based on data from clinical trials carried out in the Western population. The recommended dosage of MMF for prophylaxis of allograft rejection is 2 to 3 g per day, given in two divided doses [1–3]. In the Chinese renal transplant population, studies have suggested that MMF given at 1.5 g per day is comparable in efficacy to that given at 2 g per day [9,10], and there is reduced occurrence of adverse effects (leukopenia, gastrointestinal side effects or cytomegalovirus infection) in patients treated with 1.5 g per day MMF as compared to those treated with 2 g per day MMF [9]. A

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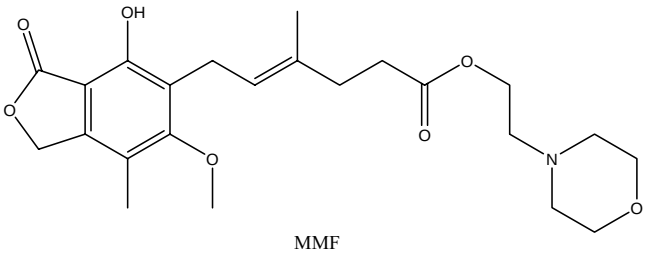
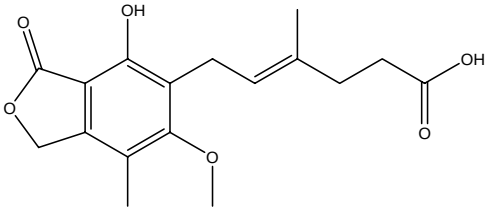
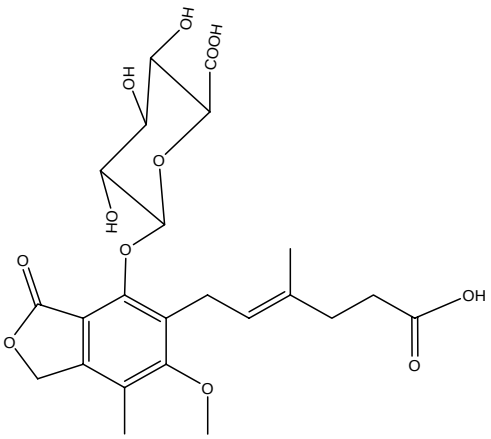


MMF



MPA



MPAG

Fig. 1. Chemical structures of mycophenolate mofetil (MMF), mycophenolic acid (MPA) and mycophenolic acid glucuronide (MPAG).

local trial conducted in Singapore General Hospital has also suggested the need for dosage reduction in the Asian population to minimise the adverse effect of leukopenia, and the dose of MMF in terms of mg/kg body weight was found to be higher in patients with leukopenia [11]. Due to the considerable intra- and inter-patient variability in the response to MMF [8,12], assay techniques for the simultaneous determination of MPA and MPAG are highly desirable as a means to understand the impact of pharmacokinetics of MPA and MPAG, in specific patient populations, on clinical outcome in terms of efficacy and tolerability.

To date, several reversed-phase high-performance liquid chromatographic (HPLC) methods have been reported for the quantitative determination of both MPA and MPAG in plasma [13–26], and only three of these published methods reported the quantitation of both analytes in urine as well [18,20,24]. Some of the earlier methods involved the indirect quantitation of MPAG by measuring MPA before and after hydrolysis of MPAG to MPA using β-glucuronidase [13–16]. HPLC methods for the direct quantitation of MPAG without enzymatic hydrolysis were also developed. However, due to the marked difference in polarity of MPA and MPAG, some of the published methods involved the use of two different sets of chromatographic conditions for analyzing MPA and MPAG on separate runs [17–20] or the use of gradient elution method for simultaneous analysis of both analytes [21]. Due to the complexity and inefficiency of these methods, other isocratic elution HPLC methods were developed for the simultaneous analysis of both MPA and MPAG, either without [22–24] or with [25,26] the use of an ion-pairing reagent.

For ionic compounds like MPA and MPAG, HPLC separation is normally performed by reversed-phase, ion-pair or ion-exchange chromatography [27]. Normally, reversed-phase chromatography is the method of choice but if it proves inadequate, the other two methods would then be considered [27]. The reversed-phase HPLC methods without ion-pairing reagent were first tried out but due to substantial endogenous plasma interferences to the MPAG peak, ion-pair chromatographic method was hence used in this study. This paper reports on the development and validation of a simple reversed-phase isocratic ion-pair HPLC method for the simultaneous analysis of both MPA and MPAG in both plasma and urine using direct UV absorbance detection. The applicability of this present simultaneous assay for clinical pharmacokinetic studies was also demonstrated. The simple sample preparation procedure and the use of isocratic elution in the chromatographic analysis contribute to the efficiency, and hence, the desirability of this assay for clinical pharmacokinetic studies and drug monitoring.

2. Experimental

2.1. Chemicals and reagents

MPA was obtained from Fluka Chemie (Buchs, Switzerland). MPAG was a generous gift from Roche Bioscience (Palo Alto, CA, USA). The ion-pair reagent, tetrabutylammonium hydrogen sulfate (TBA-HS), was purchased from Fluka Chemie (Buchs, Switzerland). Potassium hydroxide was from Merck (Darmstadt, Germany) and potassium dihydrogen phosphate was from Nacalai Tesque (Kyoto, Japan). HPLC-grade acetonitrile was purchased from Mallinkrodt Baker (Paris, KY, USA). Pooled blank human plasma was obtained from the blood bank of the National University Hospital, Singapore. Blank human urine was from a single healthy individual after an overnight fast. Milli-Q (18 MΩ) water, used throughout the study, was generated by a Milli-Q RG Millipore Water Purification System (Millipore, SA, Molsheim, France).

2.2. Instrumentation

Chromatographic analyses were carried out on a Shimadzu integrated HPLC system LC-2010A liquid

chromatograph (Shimadzu Corporation, Kyoto, Japan) equipped with a UV detector, autosampler and column oven. Instrument control, data collection and processing were performed using Shimadzu Class-VP software version 6.10. All chromatographic separations in this study were performed using an XTerra™ RP_{18} analytical column (150 mm × 4.6 mm i.d., particle size 5 μm) (Waters Corporation, Milford, MA, USA), connected with an XTerra™ RP_{18} guard column (20 mm × 3.9 mm i.d., particle size 5 μm) (Waters) and a 0.5 μm stainless steel frit pre-column filter (Upchurch Scientific, Oak Harbor, WA, USA).

2.3. Chromatographic conditions

The running buffer solution used for this reversed-phase ion-pair HPLC assay composed of 40 mM TBA-HS in 20 mM potassium dihydrogen phosphate that was prepared and adjusted to pH 5.50 (EcoMet pH meter, Istek, Seoul, Korea) with 2 M potassium hydroxide. This solution was then filtered through a 0.2 μm nylon filter membrane (Lida Manufacturing Corporation, Kenosha, WI, USA) and degassed using ultrasonic bath before use. The mobile phase for this simultaneous assay of MPA and MPAG consisted of the above running buffer solution—acetonitrile (73:27, v/v), mixed on-line and delivered isocratically at a flow rate of 1.0 ml/min. The temperature of the column oven was maintained at 24 °C. Each sample was injected onto the column at a constant volume of 20 μl for an analysis run of 15 min. The UV detection wavelength was set at 304 nm and 215 nm for the chromatographic analyses of plasma and urine samples, respectively.

2.4. Stock and working standard solutions

Standard stock solutions of MPA (1000 mg/l) and MPAG (5000 mg/l) were prepared in methanol and stored at −20 °C. These stock solutions were diluted to prepare working solutions in methanol–water (8:2, v/v) to yield concentrations from 5 to 500 mg/l for MPA, and from 20 to 4000 mg/l for MPAG.

2.5. Sample preparation

2.5.1. Calibration standards of plasma samples

Aliquots of 100 μl of blank human plasma were spiked with 10 μl each of the MPA (5, 10, 50, 100, 250 and 400 mg/l) and MPAG (100, 250, 500, 2000, 3000 and 4000 mg/l) working solutions to yield spiked plasma concentrations corresponding to 0.5, 1, 5, 10, 25 or 40 mg/l for MPA, and 10, 25, 50, 200, 300 or 400 mg/l for MPAG, respectively. These calibration standard samples were vortex-mixed for 1 min and allowed to equilibrate at room temperature for 10–15 min. Subsequently, protein precipitation was carried out by adding 100 μl of acetonitrile to each of the above samples, followed by vigorous vortex-mixing for 2 min. The mixtures were then left to stand at room temperature for 10–15 min, after which they were centrifuged at 8000 rpm for 20 min at 4 °C using an Avanti™ J-25 Centrifuge (Beckman Instruments, CA, USA). Aliquots of 20 μl of the clear supernatant were then injected onto the HPLC system for analysis.

2.5.2. Calibration standards of urine samples

Aliquots of 25 μl of blank human urine were spiked with 25 μl each of the MPA (5, 10, 25, 50, 100, 250 and 500 mg/l) and MPAG (20, 50, 100, 250, 500, 2000 and 4000 mg/l) working solutions to yield spiked urine concentrations corresponding to 5, 10, 25, 50, 100, 250 and 500 mg/l for MPA, and 20, 50, 100, 250, 500, 2000 and 4000 mg/l for MPAG, respectively. These calibration standard samples were vortex-mixed for 1 min and allowed to equilibrate at room temperature for 10–15 min. The samples were then diluted to 250 μl with water and vortex-mixed for 2 min. Aliquots of 20 μl of the diluted solutions were injected onto the HPLC system for analysis.

2.5.3. Specificity

Prior to assay validation, plasma from transplant patients receiving other medications had to be screened to ensure the absence of interfering peaks in the assay. Hence, blood samples from stable renal transplant patients, in Singapore General Hospital, receiving immunosuppressive therapy with and without MMF were used for screening. The blood samples were collected in EDTA Vacutainer tubes and centrifuged at 3000 rpm for 10 min at 25 °C using a Universal 32R centrifuge (Hettich AG, Bach, Switzerland) to separate plasma which was transferred to polypropylene tubes and stored at −20 °C until analysis. For analysis, the plasma samples were thawed and an aliquot of 100 μl each was then treated using the procedure described above for the calibration standards of plasma samples, except that the spiking of 10 μl each of MPA and MPAG working solutions was replaced by the spiking of 20 μl of methanol–water (8:2, v/v) for standardization.

2.5.4. Clinical samples for pharmacokinetics application

The applicability of the developed assay for pharmacokinetics studies was assessed by carrying out pharmacokinetic investigation of MPA and MPAG in a stable renal transplant patient (female, Chinese, age: 42 years) who had been receiving 500 mg MMF (CellCept®) twice daily for more than 3 months. The study was approved by the Ethics Committee of Singapore General Hospital and written informed consent was also obtained from the patient prior to the study. Venous blood samples (3 ml each) for pharmacokinetic analysis were collected into EDTA Vacutainer tubes over a 6 h period at 0 (pre-dose), 0.5, 1, 1.5, 2 and 6 h after MMF administration. The blood samples were then processed and treated using the same procedure as described above for analysis. The concentrations of MPA and MPAG in the plasma samples obtained were calculated by reference to calibration

curves generated from calibration standards analyzed along with these samples.

Urine sample was also collected from the patient incrementally over 12 h from the time MMF was administered to the time the next dose was administered. The total volume of urine collected over the 12 h period was recorded, and a 50 ml aliquot was removed and stored at −20 °C until analysis. For analysis, the urine sample was thawed and an aliquot of 25 μl was then treated using the procedure described above for the calibration standards of urine samples, except that the spiking of 25 μl each of MPA and MPAG working solutions was replaced by the spiking of 50 μl of methanol–water (8:2, v/v) for standardization. The concentrations of MPA and MPAG in the urine sample were calculated by reference to calibration curves generated from calibration standards that were analyzed along with the urine sample.

2.5.5. Data analyses

The peak areas of MPA and MPAG were used for quantitative computations. Calibration curves were constructed by non-weighted least-squares linear regression of peak areas versus concentrations spiked to drug-free biological samples. All experiments were performed in triplicate unless otherwise stated. Routine clinical samples were analyzed once. The concentrations of MPA and MPAG in the clinical samples were calculated from the peak areas on the basis of the equations for the standard calibration curves.

3. Results and discussion

3.1. Chromatographic separation

The marked difference in the polarity of MPA (relatively non-polar, $\log P = 3.88 \pm 0.38$ for uncharged form) and MPAG (relatively polar, $\log P = 0.49 \pm 0.52$ for uncharged form) posed a problem in determining both drugs simultaneously in a single analytical run. Hence, previous published non-ion-pair reversed-phase HPLC assays using UV detection involved either two sets of chromatographic conditions for separate runs to analyze each analyte [17–20] or the use of gradient elution method for simultaneous analysis [21]. However, the former methods would prove to be inefficient for the analysis of the two analytes when a large number of samples were to be involved while the latter gradient elution method would also be time-consuming as extra time would be necessary for baseline equilibration between consecutive runs. Therefore, preliminary attempts to utilize reversed-phase chromatography (in the absence of ion-pair reagent) with isocratic elution method to separate both MPA and MPAG in a single run were carried out in this study. Although three previous published reversed-phased HPLC methods (non-ion-pair) reported the feasibility of simultaneous determination of MPA and MPAG using mobile phase at acidic pH of around 2 [22–24], preliminary results by adopting these reported chromatographic conditions were unsatisfactory due to interferences to the MPAG peak from peaks of endogenous plasma substances. Attempts to increase the retention of MPAG on the column by reducing the acetonitrile composition of the mobile phase in order to separate it from the plasma interferences did not provide much improvements but resulted in unsatisfactory longer run time as the retention of the relatively more non-polar MPA on the column was much increased. Despite further attempts to resolve the above problems by variations in pH of the running buffer (pH 2.4, 4.4, 7.0 and 7.5) together with variations in the acetonitrile composition of the mobile phase, results were still unsatisfactory (data not shown).

Hence, isocratic ion-pair chromatography for the simultaneous determination of both MPA and MPAG was employed by the addition of an ion-pairing reagent to the mobile phase to reduce the difference in polarity between the two analytes, so as to improve chromatographic separation [25]. This is achieved by the addition of an ion-pairing reagent, TBA-HS in this case, to the mobile phase (pH above pK_a of the analytes) to reduce the difference in polarity between the two analytes by the association of positively-charged TBA cations with negatively-charged MPA and MPAG anions to form non-polar, uncharged ion-pairs. The use of the ion-pairing reagent thus served to retain MPAG for improved resolution from interfering peaks due to endogenous water-soluble plasma proteins that elute before MPAG. In order to optimize the chromatographic conditions for determining MPA and MPAG in human plasma in a single analytical run, the effects of the following factors were investigated: UV detection wavelength, sample preparation method, pH of running buffer and mobile phase composition.

3.1.1. Selection of the detection wavelength

UV absorption spectra revealed absorption maxima at 215, 250 and 304 nm for MPA, and 215, 251 and 295 nm for MPAG. Although higher sensitivity was attained at 215 and 250 nm as compared to 304 nm for both compounds, more significant interferences from endogenous plasma substances and other possible co-administered medications rendered the two former detection wavelengths inappropriate in the analysis of plasma samples. Hence, detection was performed at 304 nm, the third absorption maxima of MPA, for increased specificity of the simultaneous MPA and MPAG assay in plasma samples. The third absorption maxima of MPA was chosen instead of that for MPAG because the plasma concentrations of MPAG were usually very much higher than that of MPA [4], so this slight shift in the detection wavelength from the third absorption maxima of 295 nm for MPAG would not compromise the detection sensitivity of MPAG to any great extent. As for the analysis of MPA and MPAG in urine samples, the absence of interferences in urine samples at 215 nm made it possible to employ 215 nm as the detection wavelength for increased sensitivity in order to ensure that MPA, a minor urinary excretion product of MMF (only around 0.6% of the administered

dose of MMF is excreted as MPA), could be adequately quantified.

3.1.2. Sample preparation method

Among the published methods to-date for the determination of both MPA and MPAG in human plasma, sample preparation methods for plasma samples include solid-phase extraction (SPE) for the simultaneous extraction of both analytes [17,22,25] and protein precipitation with [24] or without [21,23,26] evaporation to dryness. The multi-step SPE method was not only work-intensive and time-consuming, but also not cost-effective due to the use of additional expensive SPE columns. Comparatively, liquid-liquid extraction (LLE) method would be relatively more cost-effective and LLE extractions would be as clean as that of SPE. Hence, one-step and two-step LLE methods using eight different organic solvents or mixtures (diethyl ether, ethyl acetate, chloroform, diethyl ether/ethyl acetate 1:1 (v/v), hexane/ethyl acetate 1:1 (v/v), ethyl acetate/acetonitrile 7:3 (v/v), butanol/chloroform 2:8 (v/v), dichloromethane/dichloroethane 1:1 (v/v)) were tested for the simultaneous extraction of both MPA and MPAG from human plasma. However, due to the marked difference in polarity of the two compounds, none of the above organic solvents or mixtures were adequate in providing sufficient extraction yields for both MPA and MPAG simultaneously to allow proper pharmacokinetics application (data not shown).

Hence, protein precipitation using acetonitrile was chosen as the sample preparation procedure for plasma samples in this assay. Although protein precipitation does not provide clean extractions like SPE and LLE, its main advantage over the latter two techniques is its universal applicability to all types of samples, regardless of the nature of the drug to be analyzed. This is especially important in the present assay that requires a sample preparation technique that enables the simultaneous analysis of two analytes with markedly different polarities. In addition, the use of protein precipitation for sample preparation of plasma samples is much simpler, faster and less costly as compared to SPE and LLE, and thus, is particularly advantageous for clinical pharmacokinetics studies or routine drug monitoring that involve analyses of large batches of patient samples.

To date, only three HPLC assay papers reported the determination of MPA and MPAG in urine samples [18,20,24]. In the assays reported by Wiwattanawongsa et al. and Teshima et al., urine samples were all diluted 20-fold using 25% methanol in 10 mM trifluoroacetic acid [24] and water [20], respectively. Bullingham et al. divided each urine sample into two aliquots and diluted each sample with acetonitrile–water (10:90, v/v) by factors of 10 for MPA analysis and 50 for MPAG analysis separately [18]. This was not required in the present assay that could determine both MPA and MPAG simultaneously. It was also assessed that a 20-fold dilution carried out by Wiwattanawongsa et al. [24] and Teshima et al. [20] was unnecessary in this assay as a 10-fold dilution was able to provide detection over the desired concentration ranges for both MPA and MPAG with good linearity. Direct injection of undiluted urine was also assessed and found to be inappropriate as linearity was unattainable at the higher MPAG concentrations of the same desired concentration range. Hence, a simple 10-fold dilution was employed as the sample preparation method of urine samples. No endogenous interfering peaks that eluted at the same time as the MPA and MPAG peaks were observed during urinary analysis. This sample pretreatment method by just a 10-fold dilution of urine using only water as a diluent, instead of a more complex mixture with organic solvents, is thus much simpler and efficient than that employed in the three previous reported methods.

3.1.3. Effect of pH of running buffer

The use of isocratic ion-pair chromatography was first reported by Huang et al. but the method involved time-consuming and tedious SPE for sample preparation with the addition of two internal standards, as well as a long run time of 20 min [25]. More recently, a faster isocratic ion-pair chromatographic method was reported by Hosotsubo et al. that could be completed in 8.5 min and involved only protein precipitation as a simple sample preparation method [26]. However, the aqueous component of the mobile phase reported in this method did not involve a buffer system but simply a 40 mM tetrabutylammonium bromide solution without pH control (no report on the pH of the mobile phase used) [26] and this resulted in irreproducible retention times, particularly for MPA, when preliminary studies were carried out to adopt this reported method. Retention times were observed to shift from 11.1 to 8.2 min for MPA (R.S.D. = 9.84%) and from 5.0 to 4.7 min for MPAG (R.S.D. = 1.82%) after 11 consecutive runs ($n = 11$) over a total period of 165 min. During these preliminary studies, the pH of the 40 mM tetrabutylammonium bromide solution was measured to be 4.7 (EcoMet pH meter, Istek, Seoul, Korea) and this is very close to the pK_a of the aliphatic carboxylic acid groups (pK_a 4.5) present in MPA and MPAG. Hence, any slight shift in the pH of this mobile phase during sample injections would drastically affect the ionization of the analytes and hence their retention on the column. Therefore, the observed irreproducibility in retention times, especially for MPA, could most likely be attributed to the lack of a buffer in the mobile phase. Thus, improvement to the ion-pair chromatographic method reported by Hosotsubo et al. was made in this study by employing the use of a buffer system to control pH for enhanced reproducibility.

In the present assay, 40 mM TBA-HS was used as the ion-pairing reagent in a 20 mM phosphate buffer solution for the aqueous component of the mobile phase. For the analysis of compounds like MPA and MPAG that possess ionizable carboxylic acid groups (pK_a of the aliphatic carboxylic acid group: 4.5), pH is an important factor affecting chromatographic separation. In reversed-phase ion-pair chromatography, pH should be selected to achieve maximal ionization of analyte molecules and ion-pairing reagent molecules for the

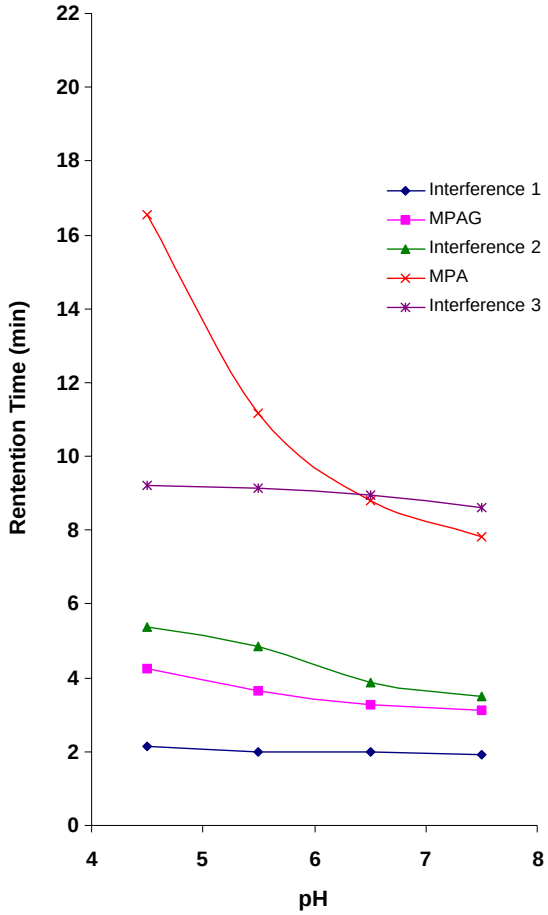


Fig. 2. The influence of pH of running buffer on the qualitative retention of MPA, MPAG and endogenous plasma interferences. Chromatographic conditions: 27% (v/v) of acetonitrile; running buffer solution of 40 mM TBA-HS in 20 mM potassium dihydrogen phosphate at various pH; flow rate of 1.0 ml/min; UV detection wavelength of 304 nm.

formation of ion-pairs [27]. The pH of the running buffer was initially selected at 7.5 at which both MPA and MPAG would be 99.9% ionized. However, significant endogenous interfering peaks that overlapped with MPA and MPAG peaks were observed at this pH for plasma samples. Investigations were thus carried out within the buffer pH range of 4.5–7.5 to optimize the pH in order to attain chromatographic separation with no interferences to MPA and MPAG peaks in plasma samples. As shown in Fig. 2, the influence of pH on the retention times of the plasma interfering peaks was minimal as compared to that of the MPAG peak and in particular, the MPA peak. Hence, pH is an important determinant for the optimization of chromatographic separation in this case. At pH 6.5, similar interferences were observed to the MPA and MPAG peaks as at pH 7.5 (Fig. 2). When pH of the running buffer was lowered to 5.5 and 4.5, the interfering peaks were not observed to elute near the MPA and MPAG peaks (Fig. 2). To facilitate the formation of ion-pairs, analyte molecules should be maximally ionized and therefore, pH 5.5 was more desirable than pH 4.5 since the two compounds of interest were 90.9% ionized at pH 5.5 instead of being only 50% ionized at pH 4.5. Moreover, each analytical run could be completed within a shorter run time at pH 5.5 as compared to that at pH 4.5. Hence, pH 5.5 was selected as the optimal buffer pH at which good chromatographic separation with no plasma interference to MPA and MPAG peaks could be attained within a reasonable analysis time.

In this set of experiments, it was also found that the potential problem of irreproducible retention times, which was observed when Hosotsubo's method was initially adopted, was solved by ensuring the control of pH. The present assay thus benefits from the use of a buffer in the mobile phase system for pH control to ensure reproducibility.

3.1.4. *Effect of acetonitrile composition of mobile phase*

Attempt was made to further separate the MPA and MPAG peaks from the significant endogenous plasma peaks described in the previous section by studying the effect of acetonitrile composition of the mobile phase at pH 5.5. The influence of acetonitrile composition on qualitative retention of MPA, MPAG and endogenous plasma peaks was presented in Fig. 3. Although the resolution between the MPAG

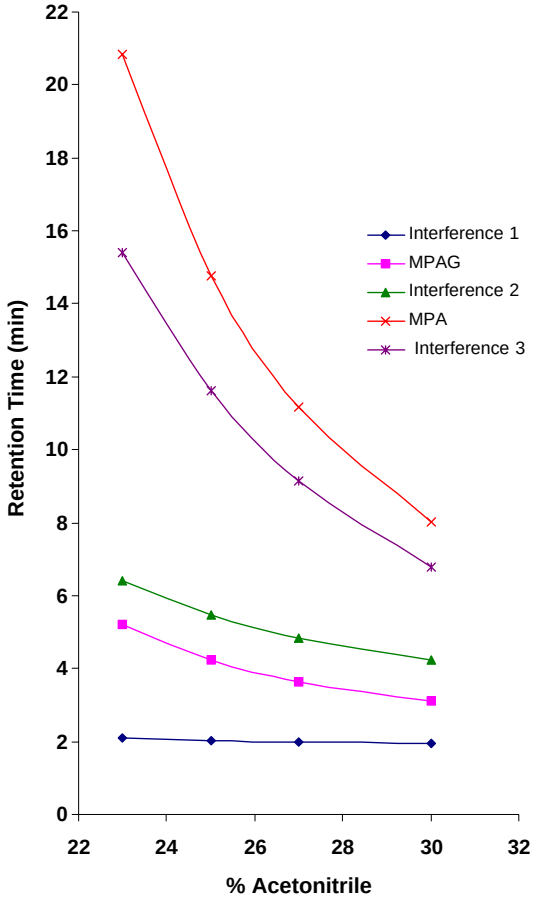


Fig. 3. The influence of acetonitrile composition of mobile phase on the qualitative retention of MPA, MPAG and endogenous plasma interferences. Chromatographic conditions: running buffer solution of 40 mM TBA-HS in 20 mM potassium dihydrogen phosphate at pH 5.5; flow rate of 1.0 ml/min; UV detection wavelength of 304 nm.

peak and the first endogenous peak, as well as that between the MPA peak and the third endogenous peak, were increased with a decrease in acetonitrile composition, the total analytical run time was also increased. Hence, acetonitrile at a composition of 27% was determined as optimal for adequate chromatographic separation within a reasonable run time.

3.2. Optimal conditions and assay validation

Under the fully optimized chromatographic conditions for the simultaneous MPA and MPAG assay, representative chromatograms obtained from (A) blank pooled human plasma; (B) blank pooled human plasma spiked with 10 mg/l MPA and 200 mg/l MPAG; (C) plasma sample from a renal transplant patient under immunosuppressive therapy with MMF obtained 1 h after MMF administration; and (D) plasma sample from a renal transplant patient under immunosuppressive therapy without MMF are shown in Fig. 4. Representative chromatograms of (A) blank human urine; (B) blank human urine spiked with 25 mg/l MPA and 250 mg/l MPAG; and (C) a 12 h urine sample from a renal transplant patient under immunosuppressive therapy with MMF are presented in Fig. 5. For both analyses of plasma and urine samples, MPAG and MPA peaks were well resolved, with retention times of around 3.8 and 11.4 min, respectively. Each chromatographic run was completed within 15 min. As the sample preparation procedure only involved a simple protein precipitation step for plasma samples and a dilution step for urine samples, the use of an internal standard was therefore not required and quantitation of MPA and MPAG was based on absolute peak area measurements.

3.2.1. Specificity and selectivity

The specificity and selectivity of the assay were examined in order to validate that the assay can reliably quantitate both MPA and MPAG in the presence of other constituents in clinical samples, especially plasma samples. This investigation is of particular importance as polypharmacy is common in transplant patients. Potential chromatographic interferences by commonly administered concomitant drugs, as well as endogenous plasma interferences, were evaluated by the analysis of several patient plasma specimens received for

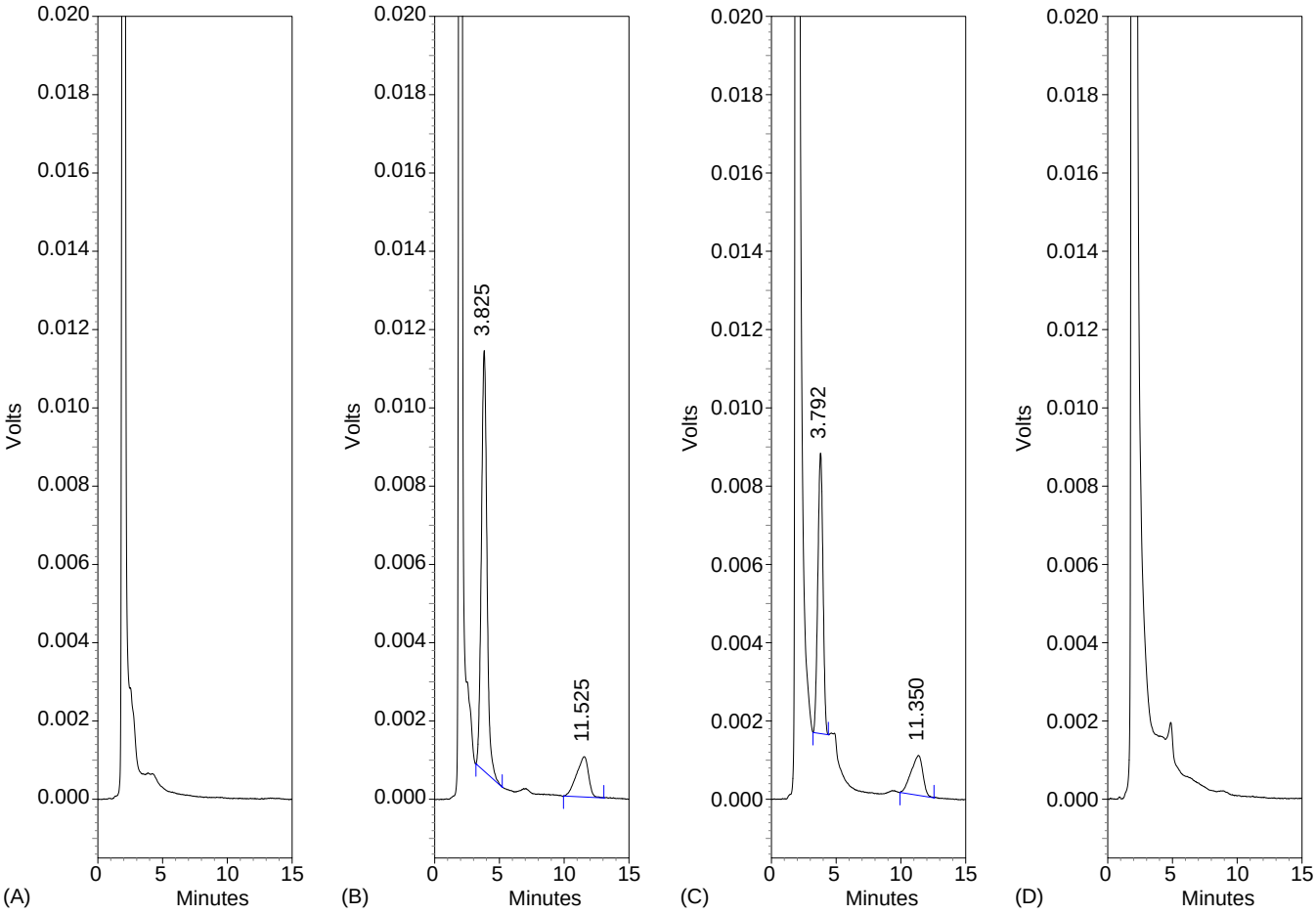


Fig. 4. Representative chromatograms showing the simultaneous analysis of MPA and MPAG in human plasma: (A) blank pooled human plasma; (B) blank pooled human plasma spiked with MPA (10 mg/l) and MPAG (200 mg/l); (C) plasma sample from a stable renal transplant patient under chronic immunosuppressive therapy with MMF obtained 1 h after MMF administration (MPA: 8.97 mg/l, MPAG: 111 mg/l); (D) plasma sample from a renal transplant patient under immunosuppressive therapy without MMF. Retention times: MPAG ∼3.8 min, MPA ∼11.4 min.

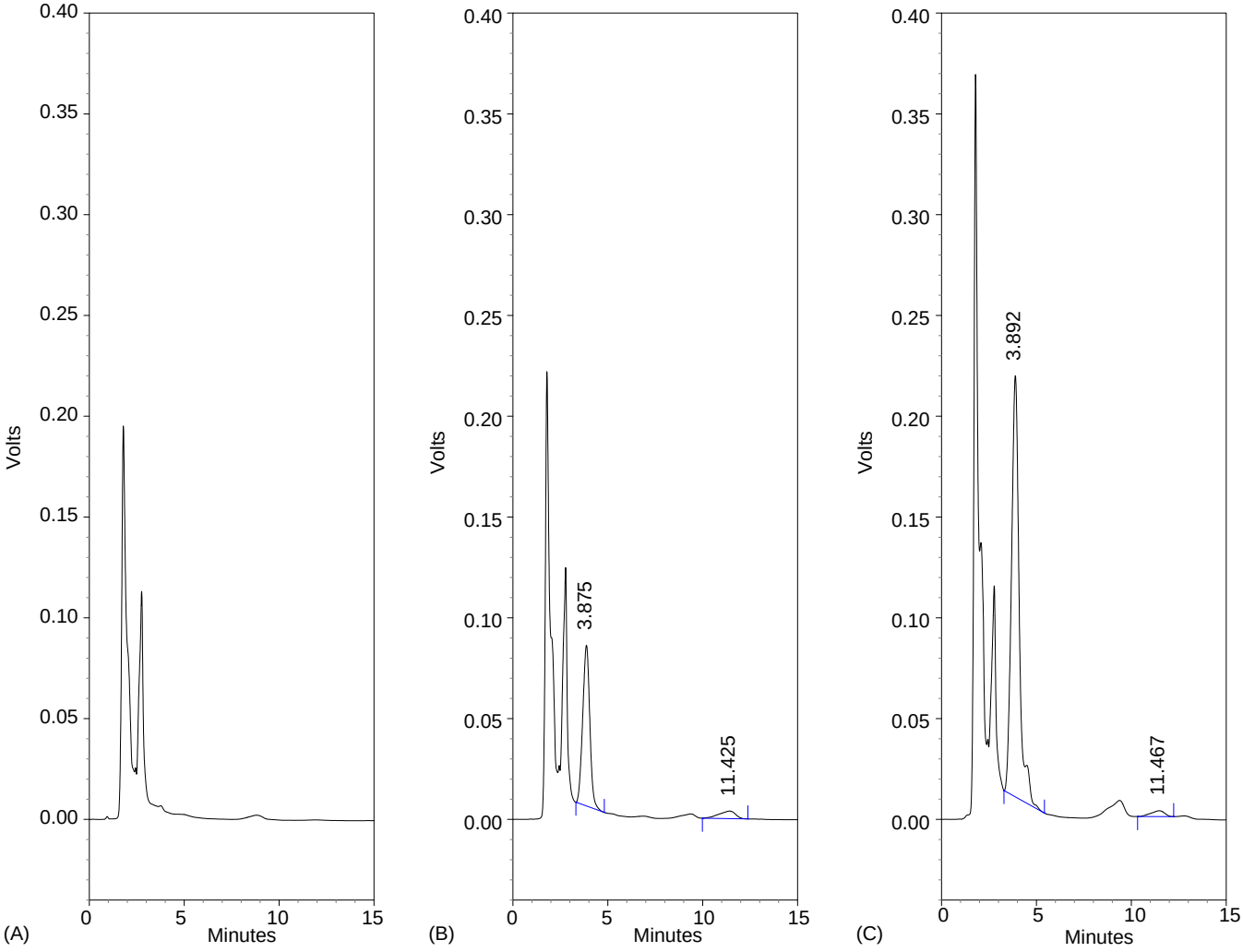


Fig. 5. Representative chromatograms showing the simultaneous analysis of MPA and MPAG in human urine: (A) blank human urine; (B) blank human urine spiked with MPA (25 mg/l) and MPAG (250 mg/l); (C) 12 h urine sample (collected at the time the evening dose of MMF was administered to the time the next morning dose was administered) from a stable renal transplant patient under chronic immunosuppressive therapy with MMF (MPA: 17.0 mg/l, MPAG: 565 mg/l). Retention times: MPAG ∼3.8 min, MPA ∼11.4 min.

routine drug monitoring. These clinical samples were from renal transplant patients who were on routine follow-up at Singapore General Hospital and under immunosuppressive therapy with and without MMF. Screening of these plasma samples did not show any interfering peaks from common co-administered drugs (Table 1) where the MPAG and MPA peaks eluted (Fig. 4).

3.2.2. Linearity

Assay linearity was evaluated based on the analysis of calibration standards prepared as described above. Each calibration concentration was assayed in triplicates and quantitation was based on absolute peak area measurements. For plasma samples, six-point calibration curves were linear over the range of 0.5–40 mg/l MPA ($y = 7311.1x - 497.35$, $r^2 = 0.9987$) and over the range of 10–400 mg/l MPAG ($y = 2312.7x - 478.37$, $r^2 = 0.9994$). A high degree of correlation was also demonstrated in urine samples, with linearity data of $y = 12981x - 50301$ ($r^2 = 0.9988$) for MPA in the range of 5–500 mg/l; and $y = 8805.8x - 48158$ ($r^2 = 0.9987$) and $y = 13460x - 1085057$ ($r^2 = 0.9996$) for MPAG in the ranges of 20–250 and 250–4000 mg/l, respectively.

3.2.3. Limits of detection and quantitation

Limit of detection (LOD) was defined as the analyte concentration with a signal-to-noise ratio of three. The limit of quantitation (LOQ) was determined as the lowest point on the calibration curve that could be analyzed within 20% of the nominal value (absolute percentage error within 20%).

For plasma samples, the LOD values were found to be 0.1 mg/l for MPA and 3 mg/l for MPAG, and LOQ values were evaluated as 0.5 mg/l for MPA and 10 mg/l for MPAG. The sensitivity of the present assay was compared with that of previous published studies for simultaneous determination of MPA and MPAG in plasma samples that similarly

Table 1
Drugs that did not show interferences to MPAG and MPA peaks under the optimized chromatographic conditions

Immunosuppressives	Antibiotics
Azathioprine	Sulfamethoxazole-trimethoprim
Cyclosporine	Cardiac drugs
Methylprednisolone	Amlodipine
Prednisolone	Atenolol
Rapamycin (Sirolimus)	Captopril
Tacrolimus	Diltiazem
Gastro-intestinal drugs	Enalapril
Esomeprazole	Nifedipine
Famotidine	Simvastatin
Omeprazole	Others
Anti-diabetic drugs	Alpha-calcidol
Tolbutamide	Calcium carbonate
Hypnotic	Erythropoietin
Zopiclone	Ferrous fumarate
Anti-viral	Folic acid
Ganciclovir	Renalvite
	Vitamin B complex

Table 2
Intra- and inter-day precision (quantitation based on absolute peak areas) of the simultaneous MPA and MPAG assay in human plasma and urine

	Nominal drug concentration (mg/l)	Imprecision (R.S.D.) (%)	
		Intra-day ($n = 3$)	Inter-day ($n = 3$)
Plasma samples			
MPA	1	0.42	4.42
	25	2.49	8.60
	40	2.48	7.45
MPAG	10	2.38	7.89
	200	8.14	9.97
	400	4.30	7.50
Urine samples			
MPA	5	3.04	4.00
	50	3.04	3.16
	500	1.82	0.36
MPAG	50	3.27	6.01
	500	3.34	2.11
	4000	1.39	1.41

employed protein precipitation without evaporation to dryness [21,23,26]. The LOD values of MPA and MPAG were poorer than those reported by Shipkova et al. (LOQ values not reported) [21] while the LOQ values were not as good as those by Svensson et al. (LOD values not reported) [23] because lower detection wavelengths (215 and 254 nm) were used in these previous studies for increased sensitivity. However, as discussed earlier, due to the interferences that were found at lower wavelengths in the present assay, sensitivity had to be slightly compromised for increased selectivity in this case. When compared to the method presented by Hosotsubo et al. [26] that employed the same 304 nm detection wavelength, the LOD and LOQ values of MPA were comparable but that of MPAG were not as good. Nevertheless, sensitivity in this present assay was sufficient for the determination of the MPAG concentrations in clinical plasma samples as the plasma concentrations of MPAG were usually relatively high.

The LOD and LOQ values in urine samples were assessed to be 1 and 5 mg/l, respectively, for MPA, and 7 and 20 mg/l, respectively, for MPAG. Three assay papers on the analysis of MPA and MPAG in urine samples were previously reported [18,20,24], and the LOD and LOQ values in this study were more or less comparable to those reported. In this present study, the sensitivity of the assay for the urinary analysis of MPA and MPAG was demonstrated to be more than sufficient for quantitating the total cumulative amounts of both analytes in 12 h urine samples of stable patients on chronic MMF therapy.

3.2.4. Precision and accuracy

Intra-day precision of the assay was studied by triplicate analyses ($n = 3$) of samples at each of three spiked concentrations within the same day. Inter-day precision of the method was determined at the same three concentrations used in the study of intra-day precision, with samples at each concentration being analyzed in triplicates over three consecutive days ($n = 3$). The precision of the plasma sample analysis of MPA and MPAG was studied using pooled blank human plasma spiked with MPA at concentrations of 1, 25 and 40 mg/l, and MPAG at concentrations of 10, 200 and 400 mg/l. The relative standard deviation (R.S.D.) values for absolute peak areas of MPA and MPAG in plasma samples were less than 2.5 and 8.2%, respectively, for intra-day precision, and not more than 8.6 and 10.0%, respectively, for inter-day precision (Table 2). For the analysis of MPA and MPAG in urine samples, precision of the assay was evaluated using blank urine samples spiked with MPA at concentrations of 5, 50 and 500 mg/l, and MPAG at concentrations of 50, 500 and 4000 mg/l. The R.S.D. values for absolute peak areas of MPA and MPAG in urine samples were less than 3.1 and 3.4%, respectively, for intra-day precision, and not more than 4.0 and 6.1%, respectively, for inter-day precision (Table 2).

The accuracy of the assay was also assessed by evaluating the analytical recoveries (defined as the analyzed concentration expressed as a percentage of the actual spiked concentration) and mean absolute percentage errors (defined as the absolute percentage of difference between the analysed and the spiked concentration over that of the spiked value) of MPA and MPAG concentrations in both plasma and urine samples over the linearity ranges, with each concentration being assayed in triplicates. As shown in Tables 3 and 4, the mean analytical recoveries of MPA and MPAG in plasma samples were within 86.0–104 and 89.9–115%, respectively, and those in urine samples were within 92.4–116 and 95.0–118%, respectively. The mean absolute percentage errors of MPA and MPAG concentrations in plasma and urine were not more than 18.0% for the concentrations at the quantitation limits (LOQ) and not more than 10.1% for the other concentrations examined (Tables 3 and 4).

Table 3
Accuracy of the simultaneous MPA and MPAG assay in human plasma

	Drug concentration (mg/l)		R.S.D. (%)	Mean absolute percent error between spiked and analyzed concentrations	Mean analytical recovery (%)
	Spiked	Analyzed[a] (mean ± S.D.)			
MPA	0.5	0.43 ± 0.02	5.52	14.0	86.0
	1	0.95 ± 0.04	3.94	5.00	95.0
	5	5.19 ± 0.18	3.41	3.80	104
	10	9.55 ± 0.23	2.20	4.50	95.5
	25	26.0 ± 1.81	6.60	4.16	104
	40	39.4 ± 0.98	2.48	1.40	98.6
MPAG	10	11.5 ± 0.27	2.34	15.2	115
	25	22.5 ± 0.84	3.73	10.1	89.9
	50	50.5 ± 3.82	7.57	0.90	101
	200	204 ± 19.2	9.39	2.19	102
	300	293 ± 2.00	0.68	2.27	97.7
	400	403 ± 17.3	4.30	0.74	101

[a] Average of three replicates.

3.2.5. Stability

The stability of both MPA and MPAG in biological fluids has been well established in literature. As reported by Tsina et al. [17], MPA and MPAG were stable in whole blood and plasma for at least 4 h at 20–23 °C, at least 8 h at 1–4 °C and at least 11 months at a freezer temperature of −20 °C. MPA and MPAG were also reported to be stable in plasma after three freeze–thaw cycles [17]. After sample preparation of spiked plasma samples by protein precipitation using acetonitrile, MPA and MPAG were demonstrated to be stable in the extract for 24 h at room temperature (25 °C) [26]. As for urine samples, Bullingham et al. [18] reported that MPA and MPAG were stable in urine at room temperature (21–24 °C) for at least 8 h, refrigerated (1–4 °C) for at least 15 days, frozen (−20 °C) for at least 6 months, and also after three freeze–thaw cycles.

3.3. Clinical application

The applicability of the present methodology for pharmacokinetic studies is illustrated in Fig. 6, which shows the 12 h pharmacokinetic profiles of MPA and MPAG in plasma from an individual stable transplant patient. Although plasma levels were analyzed up to 6 h after drug administration, the levels of MPA and MPAG at 12 h ($t = 12$) after drug administration (also considered 0 h of the next dose administration) could also be plotted on the profiles by safely assuming these levels to be the same as that analyzed at 0 h. This assumption could be made confidently as this patient has been on MMF for more than 3 months, so her plasma levels of both analytes were at steady-state; and moreover, her morning and evening doses of MMF were the same, so it could be safely assumed that the trough levels (at 0 and 12 h after drug administration)

Table 4
Accuracy of the simultaneous MPA and MPAG assay in human urine

	Drug concentration (mg/l)		R.S.D. (%)	Mean absolute percent error between spiked and analyzed concentrations	Mean analytical recovery (%)
	Spiked	Analyzed[a] (mean ± S.D.)			
MPA	5	5.81 ± 0.06	1.01	16.2	116
	10	9.70 ± 0.54	5.53	3.00	97.0
	25	23.1 ± 0.59	2.57	7.64	92.4
	50	49.4 ± 1.38	2.80	1.20	98.8
	100	94.0 ± 2.15	2.29	5.96	94.0
	250	263 ± 1.15	0.44	5.28	105
	500	495 ± 8.95	1.81	1.05	99.0
MPAG	20	23.6 ± 0.39	1.65	18.0	118
	50	49.6 ± 1.45	2.91	0.74	99.3
	100	95.0 ± 0.55	0.58	5.01	95.0
	250	242 ± 2.06	0.85	3.30	96.7
	500	535 ± 15.2	2.83	7.06	107
	2000	1950 ± 5.74	0.29	2.32	97.7
	4000	4020 ± 54.8	1.37	0.48	101

[a] Average of three replicates.

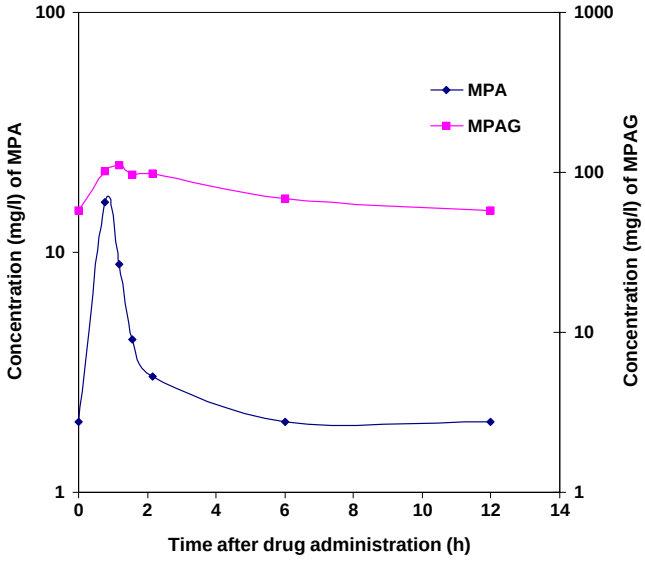


Fig. 6. Pharmacokinetic profiles of MPA and MPAG of an individual stable renal transplant patient under chronic immunosuppressive therapy, receiving 500 mg MMF (CellCept®) twice daily.

of both MPA and MPAG would be constant. For this individual stable patient receiving 500 mg MMF (CellCept®) twice daily, the results indicated that the plasma trough levels (C_{min}) of MPA and MPAG were 1.96 and 57.9 mg/l, respectively. The maximum plasma concentration (C_{max}) of MPA was 16.2 mg/l at peak time (t_{max}) of 45 min after drug administration and C_{max} of MPAG was 111 mg/l at t_{max} of 70 min after drug administration. The calculated area under the plasma concentration-time curve at steady-state (AUC_{ss}) was 38.3 mg h/l for MPA and 906.9 mg h/l for MPAG. The assay was also successfully applied for the determination of the concentrations of MPA and MPAG of the same patient's 12 h urine sample (Fig. 5c). The cumulative amounts of MPA and MPAG excreted into urine by the patient during the 12 h after drug administration were 13.6 and 452.0 mg, respectively.

4. Conclusion

The simplicity of the sample preparation procedures, requirement of small plasma and urine volumes and capability of determining both MPA and MPAG in a simultaneous chromatographic run render the reversed-phase ion-pair chromatographic assay presented in this study desirable for its practical application in clinical pharmacokinetic studies. To date, this is the third analytical method using reversed-phase ion-pair chromatography for the simultaneous determination of MPA and MPAG. This present method employs the use of a buffer system to control the pH of the mobile phase for enhanced reproducibility in retention times, which is an improvement over the ion-pair assay by Hosotsubo et al. in which the chromatographic analyses were carried out in the absence of a running buffer, without pH control [26]. The present assay thus benefits in this aspect. In addition, as compared to the other reported ion-pair method established by Huang et al. [25], the present assay appeared to be superior in terms of its much simpler sample preparation procedure and faster analysis time. Furthermore, the present method offers a simplified sample pretreatment procedure of urine samples by only a 10-fold dilution with water alone, instead of a 20-fold dilution using 25% methanol in 10 mM trifluoroacetic acid as employed by Wiwattanawongsa et al. [24] and using water by Teshima et al. [20] or the much more complex procedure comprising of separate dilutions of two aliquots of each urine sample using acetonitrile–water (10:90, v/v) by factors of 10 for MPA analysis and 50 for MPAG analysis as reported by Bullingham et al. [18]. The optimized analytical method in this study showed good performance in terms of selectivity, sensitivity, linearity, precision and accuracy for the simultaneous quantitation of MPA and MPAG in both human plasma and urine. Furthermore, clinical applicability of this assay for pharmacokinetic studies of MPA and MPAG was also demonstrated. Hence, this present analytical method is currently being applied to an ongoing clinical pharmacokinetic study.

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References

[1] European Mycophenolate Mofetil Cooperative Study Group, Lancet 345 (1995) 1321.
[2] H.W. Sollinger for the US Renal Transplant Mycophenolate Mofetil Study Group, Transplantation 60 (1995) 225.
[3] The Tricontinental Mycophenolate Mofetil Renal Transplantation Study Group, Transplantation 61 (1996) 1029.
[4] R.E.S. Bullingham, A. Nicholls, M. Hale, Tranplant. Proc. 28 (1996) 925.
[5] K. Budde, K.P. Braun, P. Glander, T. Bohler, P. Hambach, L. Fritsche, J. Waiser, I. Mai, H.H. Neumayer, Transplant. Proc. 34 (2002) 1748.
[6] R.E.S. Bullingham, A.J. Nicholls, B.R. Kamm, Clin. Pharmacokinet. 34 (1998) 429.
[7] B. Fulton, A. Markham, Drugs 51 (1996) 278.
[8] L.M. Shaw, D.W. Holt, M. Oellerich, B. Meiser, T. van Gelder, Ther. Drug Monit. 23 (2001) 305.
[9] W.K. Tsang, K.L. Tong, S. Yeung, W. Lee, H.W. Chan, Transplant. Proc. 32 (2000) 1755.
[10] X. Wang, X. Tang, D. Xu, Transplant. Proc. 30 (1998) 3573.
[11] S.M. Suhail, A. Vathsala, H.X. Lou, K.T. Woo, Transplant. Proc. 32 (2000) 1757.
[12] M. Mourad, P. Wallemacq, J. Konig, E.H. de Frahan, D.C. Eddour, M. de Meyer, J. Malaise, J.P. Squifflet, Clin. Pharmacokinet. 41 (2002) 319.
[13] R.J. Bopp, R.E. Schirmer, D.B. Meyers, J. Pharm. Sci. 61 (1972) 1750.

[14] N. Sugioka, H. Odani, T. Ohta, H. Kishimoto, T. Yasumura, K. Takada, J. Chromatogr. B 654 (1994) 249.
[15] G. Seebacher, G. Weigel, E. Wolner, R. Mallinger, M. Grimm, G. Laufer, I. El Menyawi, A. Griesmacher, M.M. Muller, Clin. Chem. Lab. Med. 37 (1999) 409.
[16] U.D. Renner, C. Thiede, M. Bornhauser, G. Ehninger, H.M. Thiede, Anal. Chem. 73 (2001) 41.
[17] I. Tsina, F. Chu, K. Hama, M. Kaloostian, Y.L. Tam, T. Tarnowski, B. Wong, J. Chromatogr. B 675 (1996) 119.
[18] R. Bullingham, S. Monroe, A. Nicholls, M. Hale, J. Clin. Pharmacol. 36 (1996) 315.
[19] A. Jain, R. Venkataramanan, I.S. Hamad, S. Zuckerman, S. Zhang, J. Lever, V.S. Warty, J.J. Fung, J. Clin. Pharmacol. 41 (2001) 268.
[20] D. Teshima, K. Otsubo, N. Kitagawa, S. Yoshimura, Y. Itoh, R. Oishi, J. Clin. Pharm. Ther. 28 (2003) 17.
[21] M. Shipkova, P.D. Niedmann, V.W. Armstrong, E. Schutz, E. Wieland, L.M. Shaw, M. Oellerich, Clin. Chem. 44 (1998) 1481.
[22] C.E. Jones, P.J. Taylor, A.G. Johnson, J. Chromatogr. B 708 (1998) 229.
[23] J.O. Svensson, C. Brattstrom, J. Sawe, Ther. Drug Monit. 21 (1999) 322.
[24] K. Wiwattanawongsa, E.L. Heinzen, D.C. Kemp, R.E. Dupuis, P.C. Smith, J. Chromatogr. B 763 (2001) 35.
[25] J.J.Z. Huang, H. Kiang, T.L. Tarnowski, J. Chromatogr. B 698 (1997) 293.
[26] H. Hosotsubo, S. Takahara, Y. Kokado, S. Permpongkosol, J.D. Wang, T. Tanaka, K. Matsumiya, M. Kitamura, A. Okuyama, H. Sugimoto, J. Chromatogr. B 753 (2001) 315.
[27] M. Waksmundzka-Hajnos, J. Chromatogr. B 717 (1998) 93.