Simultaneous determination of mycophenolic acid and its acyl and phenol glucuronide metabolites in human serum by capillary zone electrophoresis

Kaname Ohyama[a]*, Naoya Kishikawa[b], Hiroo Nakagawa[a], Naotaka Kuroda[b], Masaharu Nishikido[c], Mugen Teshima[a], Hideto To[a], Takashi Kitahara[a], Hitoshi Sasaki[a]

[a] Department of Hospital Pharmacy, Nagasaki University Hospital of Medicine and Dentistry

[b] Graduate school of Biomedical Sciences, Course of Pharmaceutical Sciences, Nagasaki University

[c] Division of Nephro-Urology, Department of Translational Medicine, Graduate school of Biomedical Sciences, Nagasaki Univeristy

* Corresponding author. Department of Hospital Pharmacy, Nagasaki University Hospital of Medicine and Dentistry, Nagasaki, Japan. Tel.: +81-95-819-7248; fax: +81-95-819-7251.   E-mail address: k-ohyama@nagasaki-u.ac.jp

Abstract

A simple capillary electrophoretic method was developed for simultaneous determination of mycophenolic acid (MPA) and its metabolites, acyl glucuronide (AcMPAG) and phenol glucuronide (MPAG), in human serum. The method utilized only running buffer in the separation, prior acidification of serum, and extraction with ethyl acetate. Separation was performed by capillary zone electrophoresis using 20 mM sodium acetate-acetic acid (pH 4.9) as running buffer, applied voltage of 15 kV, and UV detection at 217 nm. Each electrophoretic run was completed within 14 min. The optimized method demonstrated good performance concerning specificity, linearity ($r > 0.998$), sensitivity (limit of detection: for MPA, 0.10 μg/ml; for AcMPAG, 0.10 μg/ml; MPAG, 0.42 μg/ml), accuracy (87-108%) and precision (< 9.3%). This method was successfully applied to measurements of MPA, AcMPAG, and MPAG in renal transplant patient samples and could be a useful alternative to HPLC-based methods.

1. Introduction

Mycophenolic acid (MPA) is widely used as an immunosuppressive agent in the therapeutic protocol after organ transplantation and in treatment of autoimmune disease. To improve its oral absorption, MPA is administered as an ester prodrug mycophenolate mofetil (MMF, Cellcept®, Roche Pharmaceuticals) that is completely absorbed and readily hydrolyzed by esterases to MPA. MPA non-competitively, selectively and reversibly inhibits the enzyme inosine monophosphate dehydrogenase and guanosine monophosphate synthesis [1].

MPA undergoes metabolism primarily by the uridine diphosphate glucuronosyl transferase enzyme family at liver, kidney, and intestine [2]. The phenolic MPA glucuronide (MPAG), major metabolite of MPA, is pharmacologically inactive, but can be hydrolysed back to MPA during entrohepatic recirculation [3]. Two further metaolites have been identified, namely the acyl glucuronide (AcMPAG) and the phenolic glucoside of MPA and, of these metabolites, only AcMPAG was found to hold similar concentration-dependent pharmacological action to MPA [4, 5]. Therefore, besides MPA, the quantification of MPAG and AcMPAG is important for monitoring of MMF therapy.

A number of high-performance liquid chromatography (HPLC) methods, using UV detection or mass spectrometry, for determination of both MPA and MPAG have been published [6-13]. Now the papers focused on the quantification of AcMPAG in addition to MPA and MPAG are increasing [14-18] and further rise in attention to simultaneous determination of these three analytes is ensured.

Because of a marked difference in polarity of MPA and its metabolites and a

potential interference from endogenous compounds in blood sample, HPLC methods often required a gradient elution [8, 11-13, 17, 19, 20] or an ion-pair reagent [6, 7, 21], which made the separation condition and the preparation of mobile phase complicated. Additionally, a slow column equilibration and less reproducibility of separation in HPLC are sometimes caused by the mobile phase including an ion-pair reagent. On the other hand, transplant patients receiving MMF therapy are usually in co-medication with many drugs such as other immunosuppressive drugs, antifungal drugs, and antimicrobial drugs. The interference of these agents with the peaks of MPA and the glucuronide metabolites in chromatogram can occur. From these points of view, a novel assay method, which is based on the different separation principle with liquid chromatography, will be desired.

Capillary electrophoresis (CE) is rapidly developing as an alternative analytical tool with different separation mechanism to HPLC [22]. The main attractions of CE are its high efficiency, no need of organic solvent for separation, extremely low solvent consumption compared with HPLC, small sample volume requirement, and ease of automation. Among commonly used modes of CE, capillary zone electrophoresis (CZE) is the simplest mode and can be accessed by only altering buffer composition. Up to date, only one group reported a clinical analysis of MPA and MPAG by CE [23], however, the quantification of AcMPAG was out of scope in this work. Besides its pharmacological activity, AcMPAG has the toxicological effect that may contribute to the gastrointestinal side effects such as diarrhea [24] and the different pharmacokinetics with MPA [17]. Therefore, determination of AcMPAG is informative for not only improving outcome but also reducing side effects in immunosuppressive therapy by

MMF.

The present study proposed the CZE method for simultaneous determination of MPA, AcMPAG, and MPAG in human serum.   Except for HPLC methods, this is the first report demonstrating simultaneous determination of these three analytes.

2. Materials and methods

2.1. Chemicals

MPA, AcMPAG, MPAG, and the carboxybutoxy ether of MPA (MPAC, internal standard [IS]) were generous gifts from Roche Palo Alto LLC (Palo Alto, CA, USA). Sodium tetraborate, potassium dihydrogen phosphate, sodium acetate, acetic acid, sodium hydroxide, phosphoric acid, hydrochloric acid, and sodium dodecyl sulfate were purchased from Wako (Osaka, Japan). Blank human serum was purchased from Funakoshi (Tokyo, Japan).   Acetonitrile and ethyl acetate was obtained from Nacalai Tesque (Kyoto, Japan).   All chemicals were of analytical grade.   Distilled water was obtained using Simpli Lab-UV (Millipore Corporation, Bedford, MA, U.S.A.). Buffers were filtered through a 0.45-µm membrane filter (Millipore).

2.2. Apparatus and electrophoretic conditions

All the CE experiments were performed on a CAPI-3200 system equipped with a photodiode array detector (Otsuka Electronics, Osaka, Japan).   Fused-silica capillaries (75 µm i.d. x 375 µm o.d.) were obtained from Polymicro Technologies (Phoenix, AZ, USA).   Total and effective length (from inlet of capillary to detection window) of capillaries used here were 60 cm and 50 cm, respectively.   All the buffers used in this

study were borate buffer (sodium tetraborate-hydrochloric acid), phosphate buffer (potassium dihydrogen phosphate-sodium hydroxide) and acetate buffer (sodium acetate-acetic acid).　New capillaries were first rinsed with 0.1M hydrochloric acid for 10 min, 0.1M sodium hydroxide for 10 min, and 20 mM borate buffer (pH 9.0) for 20 min and then left in borate buffer overnight for the equilibration of capillary.　At the beginning of each day's work, the capillary was rinsed with 0.1M sodium hydroxide for 5 min and a running buffer for 10 min.　To achieve reproducible results, the capillary was rinsed with 0.1M sodium hydroxide for 1 min and running buffer for 2 min between runs and, especially in serum sample analyses, the capillary was rinsed with sodium dodecyl sulfate for 2 min before sodium hydroxide.　Separation conditions were as follows: applied voltage, 15 kV; detection wavelength, 217 nm; temperature, 25℃; sample injection, vacuum at 50 kPa for 1 s.

2.3. Stock solutions, calibration standards and quality controls

Stock solutions of MPA (2 mg/ml), AcMPAG (1 mg/ml), and MPAC (0.5 mg/ml) were prepared in acetonitrile and stored at -20℃.　MPAG stock solution (5 mg/ml) was prepared in acetonitrile-water (50:50, v/v) and stored at -20℃.　These stock solutions were diluted by acetonitrile for MPA and AcMPAG and with acetonitrile-water for MPAG to prepare working solutions.

540 µl Drug-free serum acidified with 10 µl phosphoric acid (850 g/l) was transferred into Eppendorf tube and then, was spiked with each working solution (total volume of 600 µl) to yield calibration standards.

The preparation of quality control samples at three different concentrations (QC$_{low}$,

QC$_{med}$, QC$_{high}$: MPA, 0.835, 9.98, 46.4 μg/ml; AcMPAG, 0.835, 5.01, 17.5 μg/ml; MPAG, 8.35, 99.8, 234 μg/ml) was performed in the same way as calibration standards but they were independent of calibration standards.

2.4. Clinical samples

The applicability of the proposed method was assessed on two stable renal transplant patients (Pt. 1 and Pt. 2) receiving MMF therapy (Pt. 1, 250 mg twice a day; Pt. 2, 500 mg at morning and 250 mg at evening). This application study was approved by Ethics Committee of Nagasaki University Hospital and written informed consent was obtained from the patients. Blood samples were collected at 2 h after the administration of MMF at morning. After the collection, the samples were left for 30 min and centrifuged at 15,000 rpm for 5 min. A 600-μl aliquot of the supernatant (*i.e.* serum) was transferred into Eppendorf tube and acidified with phosphoric acid. The serum samples were stored at -20℃ until analysis.

2.5. Sample preparation

To calibration standards, QC samples, and patient serum samples, 6 μl of MPAC (IS, 0.5 mg/ml) was added followed by 1.2 ml of ethyl acetate as a liquid-liquid extraction solvent. The tubes were briefly vortex mixed and centrifuged for 5 min at 15,000 rpm. The supernatant was then transferred into a clean tube and evaporated to dryness. The residue was reconstituted with 200 μl of acetonitrile-water mixture (50:50, v/v) and an aliquot of it was injected onto CE system.

2.6. Assay validation

Specificity was determined by comparing the migration times of each analyte against the electropherograms of extracted blank serum sample and blank serum sample spiked with only IS.

The linearity of the method was evaluated by processing a six-point calibration curves (n = 5) in the range of 0.40-53 μg/ml for MPA, 0.40-20 μg/ml for AcMPAG, and 5.0-267 μg/ml for MPAG.   The peak area ratios between each analyte and IS were plotted against nominal concentrations.   Regression analysis was conducted to determine slope, intercept and correlation coefficient for the evaluation of linearity. The limit of detection (LOD) was defined at signal-to-noise ratio of 3:1.

The precision and accuracy of the method were determined by the analyses of QC samples.   Precision (%) is expressed as coefficient of variation (CV), while accuracy (%) is expressed as ([measured concentration/nominal concentration] x 100).   The recovery of the extraction procedure was assessed by comparing the peak areas of the extracted QC samples (QC_{med} and QC_{high}) with peak areas of reference standards prepared in acetonitrile-water mixture (50:50, v/v).

3. Results and discussion

3.1. Separation conditions (buffer selection / applied voltage)

Manipulation of buffer pH is a key strategy for optimization of the separation of ionizable analytes in CZE because buffer pH determines the extent of the ionization of each analyte and the magnitude of electroosmotic flow (EOF).   In this study, due to the deprotonation of carboxylic acid, MPA, MPAG, AcMPAG, and MPAC are negatively

charged at pH>4.5. The separation based on the difference of the charge to mass ratio between the analytes can be obtained above pH 4.5. For EOF, the occurrence of EOF at pH 4 and its magnitude is elevated by an increase in pH. The running buffers such as borate buffer (20 mM, pH 9.0), phosphate buffer (20 mM, pH 6.5), and acetate buffer (20 mM, pH 4.9) were evaluated (Fig. 2). Interestingly, the peak of AcMPAG was shortened when using borate buffer. This phenomenon could be attributed to the hydrolysis of AcMPAG at alkaline condition, which was indicated by Shipkova et al. [14]. To prevent the hydrolysis of AcMPAG, Shipkova and Armstrong insisted of the prior acidification of plasma samples before sample preparation [14, 18] while two groups did not recognize a need of it [15, 17]. Taking into consideration the results obtained by using borate buffer, the current study employed the prior acidification of the serum samples to prevent degradation of AcMPAG. The separation order was dramatically altered by the change of buffer pH and the increase in pH made the peaks sharp. All running buffers provided good separation while MPAC and MPAG peaks migrated closely at pH 6.5. Finally, acetate buffer (20 mM, pH 4.9) was selected for this study.

The effect of applied voltage was studied at 10, 15, and 20 kV using 20 mM acetate buffer at pH 4.9. The elevation of voltage led to shorter analysis time and sharper peaks, however, the peaks of AcMPAG and MPAC were very close at 20 kV. The applied voltage was set at 15 kV.

3.2. Electropherograms

Fig. 3 (A) and (B) show representative electropherograms of blank serum and blank

serum containing the IS.   The peaks of each component in blank serum were well resolved and also there was no interference from endogenous compounds at the migration times of analytes.   Fig. 3 (C) and (D) show representative electropherograms of blank serum spiked with MPA (5.0 μg/ml), AcMPAG (3.33 μg/ml), and MPAG (66.7 μg/ml) and serum sample of renal transplant patient (Pt. 1), in which MPA of 5.83 μg/ml, AcMPAG of 1.33 μg/ml, and MPAG of 38.9 μg/ml was found, respectively. The peaks were identified by spiking the serum samples with each standard solution. Current medication to each patient was recorded at the time of blood sample collection and included: tacrolimus, methylprednisolone, famotidine, fluvastatin, valsartan, amoxicillin, and nateglinide.   No electrophoretic interference was detected from any of these medications.

3.3. Linearity, sensitivity

Calibration curves in human serum were linear over the concentration range, based on the peak area ratio of MPA, AcMPAG, or MPAG to the IS.   The correlation coefficient (*r*) was greater than 0.998 in all calibration curves (n = 5).   The assay parameters consisting of calibration range, slope, intercept, correlation coefficients, and LOD are given in Table 1.   The sensitivity of the proposed method was comparable to previously reported HPLC method [15].

3.4. Accuracy, precision, recovery

Table 2 summarizes the results for intra- and inter-day accuracy and precision. Assay accuracy and precision were determined by six replicate analyses of QC samples

(QC$_{low}$, QC$_{med}$, and QC$_{high}$) on a single day (*i.e.* intra-day) and over six consective days (*i.e.* inter-day).   During the course of method validation, intra- and inter-day precision was less than 10%.   The accuracy of MPA, AcMPAG, and MPAG concentrations ranged from 87-102%, 88-108%, 89-105%, respectively.   These data show that the method is both precise and accurate in human serum analysis and met the requirements for bioanalytical analysis [25].   The recoveries (%) of MPA, AcMPAG, and MPAG were as follows: for MPA, 82.4 (QC$_{med}$), 78.5 (QC$_{high}$); for AcMPAG, 83.3 (QC$_{med}$), 80.3 (QC$_{high}$); for MPAG, 58.1 (QC$_{med}$), 57.5 (QC$_{high}$).   The lower recovery of MPAG by the extraction procedure may be attributed to the relatively hydrophilic nature of MPAG, which was pointed out by Bahrami and Mohammadi [6].

3.5. Clinical application

The concentrations (μg/ml) of MPA, AcMPAG, and MPAG in serum samples obtained from two renal transplant patients were 5.88, 1.33, and 38.9 for Pt. 1 and 2.80, 0.41, and 10.9 for Pt. 2, respectively.   The levels of each analyte after the administration of MMF was closely related to kidney function expressed as creatinine clearance (Ccr) [1, 26-28].   In the current study, the concentration of all the analytes in Pt. 1 were higher than those in Pt. 2, which was consistent with the lower value of Ccr in Pt. 1 (Ccr calculated by Cockcroft-Gault Fomula: Pt. 1, 27 ml/min; Pt. 2, 38 ml/min).

4. Conclusions

A simple CZE method allowed simultaneous determination of MPA, AcMPAG, and MPAG in human serum and also, was successfully applied for the analysis of serum

samples from renal transplant patients.   This method is a cost-effective way of measuring MPA and its two metabolites in a single run and could be potentially useful as an alternative to HPLC-based methods.

Acknowledgements

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Figure caption

Fig. 1 Chemical structures of mycophenolic acid (MPA), carboxybutoxy ether of MPA (MPAC, IS), and glucuronide metabolites of MPA: acyl glucuronide (AcMPAG) and phenol glucuronide (MPAG).

Fig. 2 Effect of buffer pH on the separation. Running buffers (20 mM) were as follows: pH 9.0, borate buffer; pH 6.5, phosphate buffer; pH 4.9, acetate buffer. Other conditions as in section 2.2. Peaks: 1, MPA; 2, AcMPAG; 3, MPAC (IS); 4, MPAG.

Fig. 3 Electropherograms: (A) human blank serum; (B) human blank serum containing IS; (C) human blank serum spiked with MPA (5.0 µg/ml), AcMPAG (3.33 µg/ml), and MPAG (66.7 µg/ml); (D) renal transplant patient serum sample (Pt. 1) at 2 h after MMF oral administration. Running buffer: 20 mM acetate buffer (pH 4.9). Other conditions as in section 2.2. Peaks: 1, MPA; 2, AcMPAG; 3, MPAC (IS); 4, MPAG.

Table 1   Assay parameters for determination of MPA, AcMPAG, and MPAG in human serum

	MPA	AcMPAG	MPAG
Calibration range [μg/ml]	0.40-55.3	0.40-20.0	5.00-267
Linearity			
n	5	5	5
Slope*	0.154±0.002	0.096±0.002	0.118±0.002
Intercept*	0.028±0.022	0.004±0.008	0.450±0.150
Correlation coefficient	0.9991	0.9994	0.9981
LOD [μg/ml]	0.10	0.10	0.42

* Mean±standard error

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Table 2 Intra-day (n = 6) and inter-day (n = 6) accuracy and precision for

determination of MPA, AcMPAG, and MPAG in human serum

Sample Analyte	Intra-day		Inter-day	
	Accuracy (%)*	Precision (CV, %)	Accuracy (%)*	Precision (CV, %)
MPA				
QC_{low}	87.1±0.45	1.3	102±3.3	8.2
QC_{med}	93.4±0.19	0.5	96.3±1.2	3.1
QC_{high}	97.1±0.34	0.9	96.9±1.6	4.1
AcMPAG				
QC_{low}	88.4±0.71	2.0	98.5±2.4	6.2
QC_{med}	100±0.32	0.8	102±2.0	5.0
QC_{high}	108±0.46	1.1	103±2.4	5.8
MPAG				
QC_{low}	88.5±1.8	5.1	94.3±3.5	9.3
QC_{med}	97.9±0.67	1.7	102±2.1	5.3
QC_{high}	105±0.34	0.8	102±2.9	7.0

* Mean ± standard error

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Fig. 1

MPA

Glucuronide
Conjugation

Enterohepatic
recirculation

Glucuronide
Conjugation

AcMPAG

Pharmacologically active

MPAG

Pharmacologically inactive

MPAC (IS)

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Fig. 3

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Fig. 3 (continued)