## **Research Article**

## Validation of ultra performance liquid chromatography photodiode array method for determination of mycophenolic acid in human plasma and its application

Rasda Boonprasert<sup>1</sup>, Supawadee Pakdeenukoolkijja<sup>1</sup>, Tippanate Keawvijit<sup>1</sup>, Teera Kolladarungkri<sup>2\*</sup>

<sup>1</sup>Clinical Toxicology Laboratory, Siriraj Poison Control Center, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand <sup>2</sup>Department of Preventive and Social Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand

\***Corresponding author:** Teera Kolladarungkri Teera.kol@mahidol.ac.th

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#### ABSTRACT

Ultra-performance liquid chromatography-photodiode array detection (UPLC-PDA) method is fully validated for measuring mycophenolic acid concentrations in human plasma. The plasma spiking with naproxen, an internal standard, was extracted by solid phase extraction. The eluent solution was diluted with milli-Q and injected into an Ultra Performance LC system. Chromatographic separation was performed on reverse phase column, 1.7 µm (100 mm x 2.1 mm I.D.) and using acetronitrile with 2mM Ammonium acetate in Milli-Q water as a mobile phase. The gradient program of mobile phase was performed at flow rate of 0.45 mL/min with 3 minutes of total run time. Photodiode Array (PDA) Detector was selected at 220 nm. The retention times were 1.43 and 1.75 minutes for mycophenolic acid and naproxen, respectively. This method was fully validated and all accepted in terms of selectivity, accuracy, precision, and stability. The linearity of the method revealed a correlation determination of >0.998 within the concentration range of 0.25 - 20 µg/mL. Recoveries of extraction ranged from 87.3-90.9% with repeatability. A simple, rapid, and reproducible UPLC/PDA method for determination of mycophenolic acid concentrations in human plasma was performed and fully validated. This method was successfully applied to quantify mycophenolic acid concentrations in therapeutic drug monitoring and bioequivalence study.

## 1. INTRODUCTION

Mycophenolic acid (MPA), is an active metabolite of the prodrug mycophenolate mofetil (MMF) and enterocoated mycophenolate sodium (EC-MPS), is an immuno- suppressive drug which having efficacy for renal, liver, heart transplants. Therapeutic drug monitoring (TDM) of MPA is required owing to reported of the inter individual variability in plasma concentrations, the influence of organ failure<sup>1-3</sup> and the probable drug–drug interactions with others drugs<sup>4, 5</sup>.

Although several methods have been reported for the determination of MPA in plasma such as high performance liquid chromatography (HPLC-UV)<sup>6-9</sup> but these method have a long analytic time (4-25 min.) to complete the resolution of all chromatographic peaks.



Figure 1. Chemical structures of mychophenolic acid (A) and naproxen(B).

Liquid chromatography tandem mass spectrometry (LC-MS/MS)<sup>10, 11</sup> has a very short runtime (2.3-6.5 min.) However, the limitation of LC-MS/MS analysis is not only requiring a more expensive instrument than other techniques, but also susceptibility to their matrix effects. Thus, highly specific and highly sensitive methods with short runtime are the requirement of TDM. UPLC is a novel development in the liquid method. It represents a chromatography significant decreasing in total chromatographic time without losing of its efficiency and resolution. Here, we report a UPLC-PDA method with fully validation to determine the MPA plasma level.

The method was fullv validated according to the Guidance for Industry, Bioanalytical Methods Validation, U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (USFDA CDER, 2018, BP)<sup>12</sup>. This method was used in a study of therapeutic drug monitoring and area under curve of MPA in Thai pediatric renal transplant recipients and bioequivalence study of healthy Thai subjects.

## 2. MATERIALS AND METHODS

#### 2.1. Instruments and analytical conditions

Acquity Ultra Performance  $LC^{TM}$ (Waters Corporation, Milford, MA, USA) was used for the separation module. Chromatographic separation was developed on an ACQUITY UPLC<sup>TM</sup> BEH Shield RP column (1.7 µm, 100 mm x 2.1 mm I.D.) (Waters Corporation, Milford, MA, USA). To achieve an optimum result, the mobile phase was performed with a flow rate of 0.45 mL/min and a column temperature of 40°C. The gradient programmed for the mobile phase was optimized using acetronitrile and 2mM ammonium acetate in water. ACQUITY UPLC<sup>TM</sup> Photodiode Array (PDA) Detector (Waters Corporation, Milford, MA, USA) was operated at wavelengths between 200-380 nm and quantitation was optimized on the requirement for high sensitivity and specificity. The auto sampler was conditioned at 8°C and the injection volume was 10µL. Empower 2<sup>TM</sup> software (Waters Corporation, Milford, MA, USA) was used for data management.

#### 2.2. Chemicals and reagents

Mycophenolic acid and naproxen (internal standard, IS) were purchased from Sigma-Aldrich Ltd. (Steinheim, Germany) and the chemical's structure are as shown in Figure 1. HPLC grade acetonitrile and methanol were purchased from Labscan Ltd. (Bangkok, Thailand). Water used for experimentation was produced by Milli-Q® water purification system (EMD Millipore, Billerica, MA, USA). Any other chemicals used were of analytical grade. Drug-free human plasma was obtained from the Department of Transfusion Medicine, Faculty of Medicine Siriraj Hospital.

# **2.3. Standard solution preparation and Calibration curve**

Mycophenolic acid and naproxen standard were accurately weighed. Primary standard stock solutions of both were prepared in Dimethyl sulfoxide (DMSO) with a final concentration of 2,500  $\mu$ g/mL. Working standard solutions were prepared by dilution of stock standard solution with a mixture of methanol and water (50:50, v/v) to a final concentration range of 7.50 – 600  $\mu$ g/mL for used as calibration standards (CS).

The six different concentration levels of the CS were prepared by spiking working standard solutions in drug-free human plasma to the final concentration, which detailed as 0.25, 2.00, 6.00, 10.00, 14.00 and 20.00  $\mu$ g/mL. The QC samples were prepared separately in the same way to create LLOQ, low, medium, and high controls at 0.25, 1.50, 8.00 and 15.00  $\mu$ g/mL, respectively. The IS working standard solution was prepared by diluting the primary standard stock solutions with a mixture of methanol and water (50:50, v/v) to a final concentration of 60  $\mu$ g/mL. All standard stock solutions, working solutions, and QC samples were stored at -20°C until use

## 2.4. Sample preparation

For solid phase extraction (SPE), **OASIS**<sup>®</sup> HLB: Hydrophilic-Lipophilic-Balanced reversed-phase sorbent 30 mg 1 mL (Waters Corporation, Milford, MA, USA) was used for sample preparation. SPE HLB cartridges were initially conditioned with 1 mL of methanol and equilibrated with 1 mL of Milli-O water before use. Twenty  $\mu$ L of IS (60  $\mu$ g/mL) was added into 1.0 mL of plasma samples in 2.0mL micro-centrifuge tube. Twenty µL of orthophosphoric acid was added and mixed immediately. Then, 1 mL of plasma samples were aspirated into the wetted preconditioned SPE HLB cartridges. The plasma components were then washed in two steps with wash solvent, 1 mL of acetic acid in water (2:98, v/v) and 1 mL of acetic acid and 20% methanol in water (2:98, v/v). MPA were subsequently eluted from the dried columns using 0.5 mL of methanol. Two hundred µL of eluent was then diluted with 200 mL of Milli-Q water before being injected into the UPLC system.

## 2.5. Method validation

The UPLC method was fully validated before using the method for quantitative bioanalysis according to the United States Food and Drug: Administration Guidance for Industry: Bioanalytical Method Validation; USFDA 2018<sup>12</sup>. Selectivity was examined using six sources of drug-free plasma, for which the result should not have interfering peaks at the retention times of MPA and IS. Sensitivity at the lower limit of quantification (LLOQ) was also examined by dilution of MPA standard in plasma, which were extracted and then quantified at the lowest detectable concentration with signal to noise ratio (S/N) more than 5.

Linearity and calibration curve were represented by a linear regression model, y=mx+b, where y was the ratio of peak area of analyte to the peak area of IS, x was the concentration at different levels, including 0.25, 2.00, 6.00, 10.00, 14.00 and 20.00 µg/mL. All calibration ranges yielded linear relationships with coefficient of determination ( $r^2$ ) that exceeded 0.995.

Quality control (QC) samples were prepared from spiking MPA standard solution into drug-free plasma for validation studies. Accuracy and precision study were examined by analysis of six replicates of 0.25 (LLOQ), 1.50 (LQC), 8.0 (MQC) and 15.0 (HQC) µg/mL for three consecutive days. Percentage of relative error (%RE) indicating accuracy and percentage of coefficient of variation (%CV) indicating precision were calculated. Both %RE and %CV should be within  $\pm 20\%$  at LLOQ and  $\pm 15\%$  at other concentrations. Extraction efficiency and stability were examined by analysis of three replicates of LQC, MQC, and HQC. The mean absolute recovery of extraction efficiency should be more than 80% and %CV should be within  $\pm 15\%$ . For stability study, the percentage of difference should be within  $\pm 15\%$ .

## 3. RESULTS AND DISSCUSION

## 3.1. Chromatographic separation

To get the optimum results, the gradient of mobile phase were optimized using acetronitrile and 2mM ammonium acetate in water and performed by 45:50 at 0 min., 50:50 at 2-2.5 min. and 45:55 at 3 min. The capacity factor (k') was 1.39 for MPA and 1.91 for IS, the resolution factor was 7.06. Although, the capacity factor is lower than 2 but this method showed a good separation for MPA and IS in 3 minutes as shown in Figure 2. The ACQUITY UPLC<sup>TM</sup> Photodiode Array (PDA) Detector from Waters Co. Ltd. (USA) was used at wavelengths between 200-380 nm. The PDA spectrum in Figure 2 shown that the highest sensitivity of MPA and IS was 215.6 nm and 231.0 respectively. However, the specific nm, wavelength for this method was selected at 220 nm. Wavelength selection was based on the requirement for high sensitivity and specificity for both MPA and IS determination.



Figure 2. The PDA spectrum of mychophenolic acid(MPA) and naproxen(NP).

#### 3.2. Assay performance and validation

#### 3.2.1. Selectivity and sensitivity

Six sources of drug-free plasma were studied for the selectivity. There were no interfering peaks for retention time of MPA and IS in selectivity testing. The representative UPLC-PDA chromatogram obtained from extracted drug-free plasma (Figure 3(A)), calibration standard sample (Figure 3(B)) and clinical plasma sample (Figure 3(C)). The lower limit of quantification (LLOQ) was 0.25  $\mu$ g/mL with 6.65 signals to noise ratio for MPA determination. The developed method is selective and sensitivity for MPA and IS.

#### 3.2.2. Linearity and calibration standard curve

The six-point calibration standard curve was linear over the concentration range of 0.25-20 µg/mL. The calibration standard curve was constructed by plotting MPA to IS peak area ratio (y) against the MPA concentration (x), This method also showed a good linearity, with a coefficient of determination ( $r^2$ ) greater than 0.998 (0.9983, 0.9992, 0.9993 for three consecutive days) as shown in Figure 4 which was within the acceptance limit according to the USFDA guidance. The percentage of accuracy (%RE) and percentage of precision (%CV) was within ±20% at LLOQ and ±15% at other concentrations. The results were summarized in table 1.

## 3.2.3. Extraction efficiency

Extraction efficiency of the method was performed three replicates of LQC, MQC, and HQC by comparing peak areas of extracted samples with peak areas of non-extracted standard solutions at the same concentrations. The mean absolute recovery of LQC, MQC and HQC were 89.1%, 87.3% and 90.9%, respectively for MPA and 86.3% for IS. The results suggested that the sample preparation of this method is consistent, precise and reproducible with %CV were 3.34, 5.14 and 0.28 for LQC, MQC and HQC, respectively.

#### 3.2.4. Accuracy and precision

within-run and between-run The accuracy and precision were examined by analysis of six replicates of LLOQ, LQC, MQC, and HQC for three consecutive days. Method accuracies of within-run and between run were in the range of 96.71 to 109.00 % RE and 99.55 to 105.65%RE, respectively. The within-run and between run precision was also observed, with % CV in the range of 0.20 to 4.08% and 2.78 to 5.13%, respectively. The results were summarized in table 2. These result indicated that the developed method has precision and accuracy to determine MPA in human plasma.



**Figure 3.** The representative UPLC-PDA chromatograms obtained from (A) blank human plasma sample, (B) Calibration standard sample at  $6 \mu g/mL$  (C) clinical plasma sample at 1hr after an oral administration of 500 mg mycophenolate mofetil.

#### 3.2.5. Stability study

Stability of analysis was performed by testing three replicates of plasma spiked with three QC concentrations under various conditions compare with freshly prepared samples. MPA was proved to be stable in human plasma at specified storage conditions for freeze-thaw stability (three cycle), short term stability (4 hrs at room temperature 25 degree Celsius), longterm stability (180 days at -70 degree Celsius) post-preparative stability (10 hrs in autosampler



**Figure 4**. Calibration standard curve of mychophenolic acid (Day 3) was constructed by plotting peak-area ratios of mychophenolic acid plasma concentration to the IS against nominal concentration.

at 8 degree Celsius), re-injection reproducibility (48 hrs in autosampler at 8 degree Celsius). The percentage of differences, accuracy (%RE) and precision (%CV) were within an acceptable range of  $\pm 15\%$ . The results were summarized in table 3. These results indicated that MPA was stable in plasma during sample preparation process and storage conditions.

#### 3.2.6. Application

The validated method has been successfully used quantify MPA to concentrations in study of therapeutic drug monitoring in Thai pediatric renal transplant recipients at Siriraj hospital and bioequivalence study. The study was approved by the Siriraj Institutional Review Board (258/2560(EC3)) of the Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand

## 4. CONCLUSIONS

This UPLC-PDA method is simple, rapid, and reproducible for quantification of mycophenolic acid in human plasma. It was fully validated and within the acceptance limit according to the USFDA standard guideline. It was also successfully applied in therapeutic drug monitoring and bioequivalence study.

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#### **Conflict of interest**

None to declare.

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#### **Ethical approval**

The Institutional Review Board of Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand approved the study protocol. (COA no.Si279/2017).

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