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Short communication

Determination of mycophenolic acid glucuronide in microsomal incubations using high performance liquid chromatography–tandem mass spectrometry

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ABSTRACT

A sensitive and specific HPLC–MS/MS method was developed for the analysis of mycophenolic acid glucuronide (MPAG) in incubations with human liver microsomes. Incubation samples were processed by protein precipitation with acetonitrile. MPAG and the internal standard phenolphthalein glucuronide were chromatographed on a C18 Synergi Fusion-RP column (100 mm × 2 mm, 4 μm) using gradient elution with a mixture of 1 mM acetic acid in deionized water and 1 mM acetic acid in acetonitrile at a flow rate of 0.22 mL/min. The mass spectrometer was operated with negative electrospray ionization and analysis was carried out in the single reaction monitoring (SRM) mode using the mass transitions of m/z 495 → 319 and m/z 493 → 175 for MPAG and phenolphthalein glucuronide, respectively. The MPAG calibration curve was linear over the concentration range of 1.0–20 μM. The within-day and between-day relative standard deviations ranged from 1.1 to 7.9% and accuracy was within 8%. The simple and reproducible method is suitable for measuring mycophenolic acid glucuronidation in microsomal incubations.

1. Introduction

Mycophenolic acid (MPA) is an immunosuppressant drug that has been widely and successfully used in transplant recipients as well as in patients with immune disorders [1,2]. MPA is administered as either an ester prodrug or a sodium salt and is extensively metabolized by UDP-glucuronosyltransferases (UGTs) to two glucuronidated metabolites; MPA-7-*O*-glucuronide (MPAG) is the main metabolite of MPA (Fig. 1). Plasma concentrations of MPAG are typically 20–100-fold higher than MPA in patients receiving mycophenolate therapy. MPAG is approximately 82% bound to plasma albumin and is mainly excreted in the urine constituting the main elimination pathway for MPA [1]. Other minor MPA metabolites include the acyl glucuronide, 7-OH glucose conjugates, and 6-*O*-desmethyl-MPA [3,4].

Formation of MPAG is carried out by multiple UGT isoforms. The main UGT isoforms involved are UGT1A7 and UGT1A9, while UGT1A8 and UGT1A10 play a smaller role in MPAG formation [5]. Plasma concentrations of MPA and MPAG vary widely within and between patients, which can directly affect clinical outcomes [6]. *In vitro* studies with human liver microsomes, a commonly used approach in drug metabolism and interaction studies, may provide some clues to understanding this variability. Previous studies have used microsomal incubations with MPA to characterize the UGT isoforms involved in its glucuronidation and study the interaction potential of MPA with other drugs [7–12]. Multiple reports have described assays to measure MPAG in human plasma and urine [13–27]. MPAG has been measured in microsomal incubations by thin layer chromatography [10,12], HPLC with UV detection [4,7,11,28,29], and LC–ion trap MS [8]. MPAG has also been measured by LC–MS/MS after solid phase extraction [9] and protein precipitation [30]. However, there is no detailed method description and validation reported. Thus, this paper describes an HPLC–tandem mass spectrometry assay for the quantitative determination of MPAG in human liver microsomal incubations.

2. Experimental

2.1. Chemicals and reagents

MPA and mycophenolic acid β-D-glucuronide (MPA-7-*O*-glucuronide; MPAG) were purchased from Toronto Research Chemicals (North York, Ontario, Canada). Potassium phosphate dibasic, uridine diphosphate glucuronic acid, magnesium chloride, alamethicin, phenolphthalein β-D-glucuronide (PG; internal standard), and glacial acetic acid were purchased from Sigma–Aldrich (St. Louis, MO, USA). Pooled human liver microsomes were purchased from In Vitro Technologies Inc. (Baltimore, MD, USA). Acetonitrile and methanol were purchased from EMD Chemicals Inc. (Gibbstown, NJ, USA). All chemicals used were of the highest

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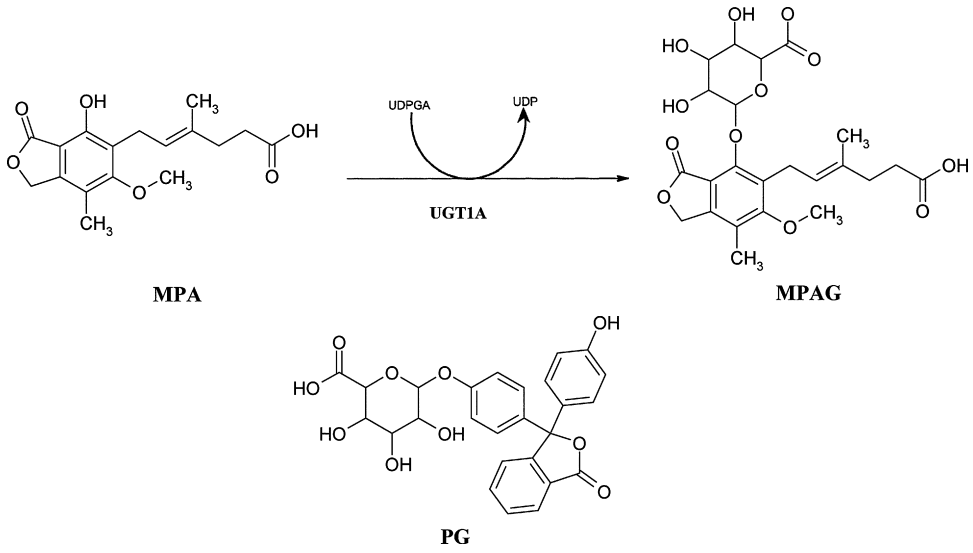

Fig. 1. Structures of mycophenolic acid (MPA), mycophenolic acid glucuronide (MPAG), and the internal standard phenolphthalein glucuronide (PG).

purity available for analytical research. Deionized water was prepared using a Barnstead Nanopure Diamond UV Ultrapure Water System (Dubuque, IA, USA).

2.2. Chromatography conditions

MPAG and the internal standard (IS) PG were chromatographed with a ThermoFinnigan Surveyor series HPLC system consisting of a Surveyor Plus autosampler and Surveyor MS pump (Thermo Corp., San Jose, CA, USA). Gradient chromatography was carried out at ambient temperature on a reversed-phase Phenomenex (Torrance, CA, USA) Synergi Fusion-RP (C18) column (100 mm × 2 mm, 4 μm). The two mobile phases consisted of (A) 1 mM acetic acid in deionized water and (B) 1 mM acetic acid in acetonitrile. Gradient elution at a flow rate of 0.22 mL/min was employed with the following steps: at start of the run, 30% B for 1 min, then increased to 90% B in 0.75 min, held at 90% B between 1.75 and 3.1 min, and from 3.6 to 6.5 min, the column was re-equilibrated at 30% B. The total run time was 6.5 min. The temperature of the autosampler was maintained at 10 °C and the injection volume was 5 μL. A divert valve was used to divert flow to waste from 0 to 2 min and from 4.5 to 6.5 min.

2.3. Mass spectrometry conditions

The LC–MS/MS analysis was carried out on a TSQ Quantum triple quadrupole mass spectrometer (Thermo Corp., San Jose, CA, USA), equipped with an electrospray ionization (ESI) source operated in the negative ion mode. Detection of MPAG and PG was performed for their $[M-H]^-$ ions. Analysis was carried out in the single reaction monitoring (SRM) mode using the mass transitions of m/z 495 → 319 and m/z 493 → 175 for MPAG and phenolphthalein β-D-glucuronide, respectively. MPA was also monitored at a mass transition of m/z 319 → 191. The mass spectrometer settings included a capillary temperature of 350 °C, spray voltage of 3.0 kV, and source collision induced dissociation (CID) of 5 V. Nitrogen was used as the sheath and auxiliary gas set to 35 and 15 (arbitrary units), respectively. The argon collision gas pressure was set to 1.5 mTorr (0.2 Pa) and the collision energy was set to 30 eV for MPA, 22 eV for MPAG, and 25 eV for PG. The peak full width at half maximum (FWHM) was set at 0.2 m/z and 0.7 m/z for Q1 and Q3, respectively, and the scan time was set to 250 ms. Data acquisition and analysis were performed with Xcalibur software version 1.4 (Thermo Corp., San Jose, CA, USA).

2.4. Stock solutions, standards, and quality controls (QCs)

Stock standard solutions of MPAG (0.2 and 2 mM) were prepared by dissolving the appropriate amount of MPAG in methanol. A series of MPAG standards (concentrations: 0, 1, 2, 4, 10, 15, and 20 μM) and quality control samples (concentrations: 2.5, 7.5, and 16 μM) were prepared by subsequent dilution of the stock standard solutions in 0.1 M phosphate buffer, pH 7.1. Working solutions of MPA (6 mM) and the internal standard PG (1 mM) were prepared in methanol.

2.5. Microsomal incubation conditions and sample preparation

The incubation conditions were optimized with respect to time of incubation and microsomal protein concentration. Stock solutions of UDPGA (25 mM) and $MgCl_2$ (5 mM) were prepared in phosphate buffer. Alamethicin (0.2 mg/mL) was prepared in phosphate buffer containing 10% ethanol. The incubation mixture (final volume, 105 μL) consisted of MPA, 1 mM $MgCl_2$, 0.1 M potassium phosphate buffer (pH 7.1), 0.16 mg/mL microsomal proteins, and 16 μg/mL alamethicin (100 μg alamethicin/mg microsomal protein). MPA concentrations used for the kinetic study were 50, 100, 300, 500, 1000, 1500, 2000, and 2500 μM. The mixture was preincubated on ice for 15 min. The reaction was started by adding UDPGA (final concentration, 1 mM). After the mixture was incubated for 30 min at 37 °C, the reaction was stopped by adding 315 μL ice-cold acetonitrile and 20 μL internal standard, vortex-mixing, and placing tubes on ice. Tubes were centrifuged for 10 min at 20,817 × g. The supernatant was diluted in a ratio of 1:5 with purified water and 5 μL was injected into the HPLC system.

2.6. Method validation

The method was validated for selectivity, linearity, sensitivity, precision, accuracy, recovery, matrix effect, and stability according to the guidelines issued by the Food and Drug Administration (FDA) for the validation of bioanalytical methods [31].

2.6.1. Calibration, precision, and accuracy

Calibration curves were constructed using six different concentrations of MPAG prepared in incubation buffer. Curves were obtained daily for 3 days by calculating peak area ratios of MPAG to PG. Data points were fitted using linear regression and a $1/y^2$ weighting-scheme. The precision and accuracy of the assay was determined using quality control (QC) samples of known MPAG concentrations (2.5, 7.5, and 16 μM) prepared in incubation buffer and processed in the same manner as standards and incubations samples. Six replicates of each QC were analyzed on 3 days, after which the inter- and intra-day precision values (R.S.D.%) were calculated using one-way ANOVA using day as the grouping variable as described previously [32]. Accuracy was calculated as the percent error in the calculated mean concentration relative to the nominal MPAG concentration (R.E.%). For the assay to be considered acceptable, the precision and accuracy determined at each QC concentration level was required to be within 15%.

2.6.2. Extraction recovery, absolute matrix effect, and stability

Extraction recovery, absolute matrix effect, and stability were evaluated for MPAG samples prepared at concentrations of 2.5 and 16 μM and for the internal standard PG at a concentration of 50 μM. Each set of samples was analyzed in triplicate. Extraction recovery was determined by comparing peak areas of the standards extracted from spiked matrix (phosphate buffer containing microsomal proteins) to matrix extracted in the same manner and spiked after extraction with the same standard concentration. Matrix effect on ionization was evaluated by comparing the MPAG peak areas of samples spiked post-extraction with corresponding peak area ratios of standards prepared in the injection solution. Processed stability was evaluated by re-injecting the samples after keeping them in the autosampler at 10 °C for 36 h. Comparison of MPAG and PG peak areas before and after 36-h storage provided a measure of stability under normal operating conditions.

2.7. Data analysis

To estimate precision, one-way ANOVA analysis was performed using JMP IN 5.1.2 (SAS Inc., Cary, NC, USA). Data were fitted to the Michaelis–Menten equation and the apparent kinetic parameters of K_m and V_{max} were determined by non-linear regression analysis (Prism 4.0, GraphPad software, San Diego, CA, USA).

3. Results and discussion

3.1. Chromatographic method and development

A variety of reversed-phase columns were tried including phenyl-hexyl (Luna) and multiple C18 columns (Gemini, Synergi Hydro-RP, and Synergi Fusion-RP). The Synergi Fusion-RP column has a polar-embedded group that is designed to give enhanced retention of polar molecules and balanced compound retention; this was ideal for the current application because of the polarity differences between MPAG and MPA. Symmetrical peaks with appropriate retention times were achieved with gradient elution on this column. The MPAG, internal standard PG, and MPA were separated within 4 min of the chromatographic run. Representative extracted LC–MS/MS chromatograms of processed microsomal incubations are shown in Fig. 2. The retention times for MPAG, PG, and MPA were 3.35, 3.41, and 3.75 min, respectively. Chromatographic separation of MPAG and MPA is critical with LC–MS/MS-based plasma assays because MPAG concentrations are up to 100-fold higher than MPA and in-source fragmentation of MPAG to MPA can result in significant over-estimation of MPA concentrations if MPAG is not separated [33,34]. A small peak at the retention time for MPAG can be seen in the extracted LC–MS/MS chromatogram for MPA (Fig. 2E and inset); in-source fragmentation does not affect the current method because MPAG is separated and the enzyme substrate MPA is not quantified. The MPAG peak was detected only when substrate, enzyme, and co-substrate were added. Chromatograms of double blank incubations, which contained all incubation constituents except MPA, did not show any interfering peaks at the retention times of either PG or MPAG.

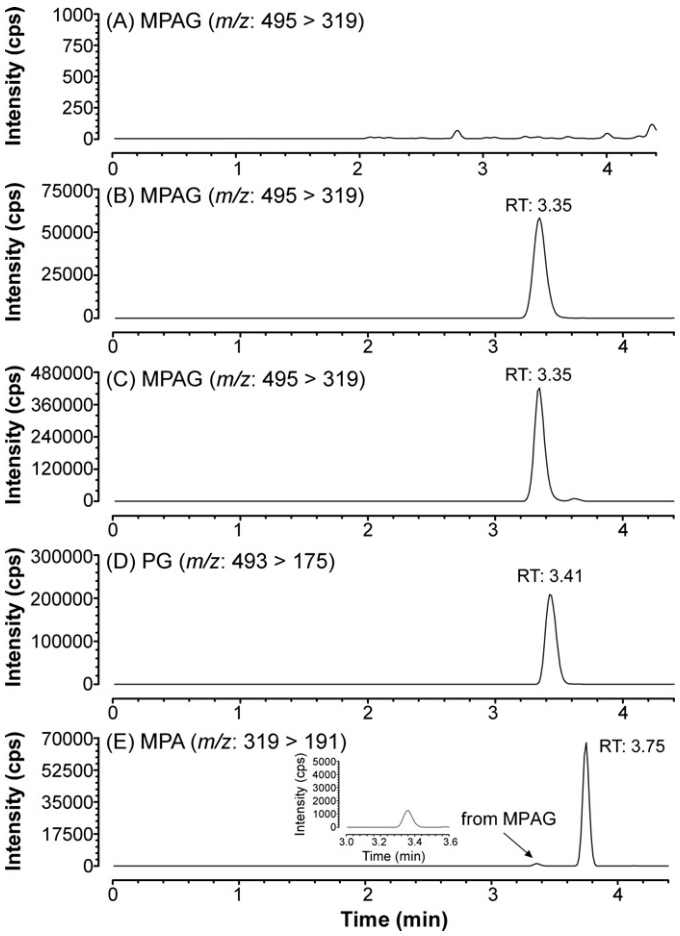

Fig. 2. Extracted HPLC–MS/MS chromatograms of (A) microsomal incubations in absence of MPA and PG, (B) spiked lowest MPAG standard (1 μM), (C) MPAG in microsomal incubation (estimated concentration is 10 μM), (D) representative chromatogram of PG (50 μM) as the internal standard, and (E) MPA (50 μM) in microsomal incubation. The small peak at 3.35 min in (E) (enlarged in inset) is from in-source fragmentation (loss of glucuronide) of MPAG to MPA.

3.2. Calibration, precision, and accuracy

Standard curves for MPAG were linear over the range of 1–20 μM. The mean correlation coefficient (r^2) for the standard

Table 1
Precision (R.S.D.%) and accuracy (R.E.%) for MPAG in microsomal incubations (six replicates per day for 3 days)

Concentration (μM)		R.S.D. (%)[a]		R.E. (%)
Nominal	Measured (mean)	Intra-day	Inter-day	
2.50	2.70	5.6	8.9	8.0
7.50	7.40	3.9	5.1	−1.3
16.0	16.5	6.1	6.8	3.1

[a] Estimated using one-way ANOVA.

Table 2
Assessment of extraction recovery, matrix effect, and stability

Nominal MPAG concentration (μM)	Extraction recovery[a] (%)	Matrix effect[b] (%)	Stability[c] (%)
2.5	83.3	88.0	105.3
16	91.6	87.3	109.7
50 (PG)	103.4	96.9	107.0

PG, phenolphthalein glucuronide (internal standard).
[a] Extraction recovery was calculated using the following formula: recovery (%) = [(mean raw peak area)$_{\text{pre ext. spike}}$/(mean raw peak area)$_{\text{post ext. spike}}$] × 100.
[b] Matrix effect was calculated using the following formula: matrix effect (%) = [(mean raw peak area)$_{\text{post ext. spike}}$/(mean raw peak area)$_{\text{neat}}$] × 100.
[c] Stability was calculated using the following formula: stability (%) = [(mean raw peak area)$_{\text{after 36 h}}$/(mean raw peak area)$_{\text{initial run}}$] × 100.

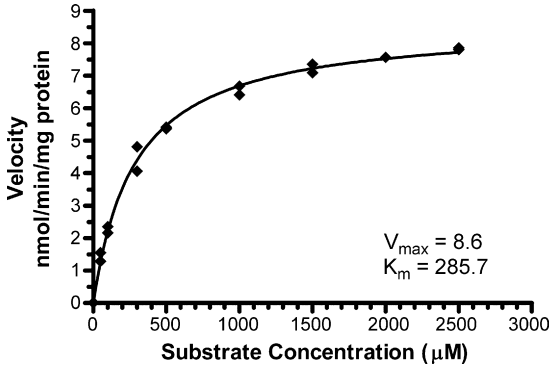

Fig. 3. Determination of apparent K_m and V_{max} for MPAG formation in human liver microsomes.

curve was at least 0.99. Intra- and inter-day R.S.D.% for MPAG QC samples were less than 10% and all calculated concentrations were within 8% (R.E.%) of the actual concentration (Table 1).

3.3. Extraction recovery, matrix effect, and stability

Table 2 shows the results from the assessment of extraction recovery, matrix effect, and stability for MPAG and PG. Average extraction recovery for MPAG was 87.4%. There was no significant matrix effect as the average suppression of ionization by matrix was 12.3%. MPAG and PG were stable in the processed incubation mixtures as well as in the reconstitution solution for at least 36 h (<10% change in measured concentration).

3.4. Characterization of K_m and V_{max}

The enzyme kinetic parameters for MPAG formation were estimated by incubating different concentrations of MPA (50–2500 μM) with human liver microsomes (Fig. 3). MPAG formation was consistent with Michaelis–Menten kinetics. The apparent K_m and V_{max} were 285.7 μM and 8.6 nmol/min/(mg of protein), respectively. Previous kinetic studies on MPAG formation *in vitro* reported values for K_m and V_{max} ranging from 95 to 351 μM and from 2.5 to 20.5 nmol/min/(mg of protein), respectively [7–10,12,28]; the values determined using this assay are within these ranges.

4. Conclusion

This paper describes a specific and sensitive HPLC–tandem mass spectrometry assay for measuring MPAG in human liver microsomes with a run time of 6.5 min. Although several reports have described assays for MPAG in plasma and urine, this is the first detailed report of a validated method to determine MPAG concentrations in human liver microsomes. The validated assay is a precise (R.S.D. <10%) and accurate method for determining MPAG in microsomal incubations over a range of 1–20 μM. The method is reproducible and subject to minimal matrix effect (Tables 1 and 2). Thus, the assay described is suitable for *in vitro* pharmacogenetic and interaction studies of MPA metabolism.

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