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Determination and validation of mycophenolic acid by a UPLC-MS/MS method: Applications to pharmacokinetics and tongue tissue distribution studies in rats

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ABSTRACT

Mycophenolic acid (MPA) has being used clinically for organ rejection prophylaxis. Recent studies have revealed that MPA can also act as a chemo-sensitizing agent when used in combination with various chemotherapeutic agents in a cancer type-specific manner, including with oxaliplatin on oral squamous cell carcinoma (OSCC) cells. To prepare for the analysis of a novel drug delivery route for MPA absorption via oral mucosa as a potential therapeutic product, it is essential to develop and validate a highly sensitive analytical method for the quantification of MPA in biological samples for pharmacokinetic and tissue distribution studies. Herein, we report a sensitive, specific and reproducible UPLC-MS/MS method to do so. Blank rat plasma or tongue tissue homogenates coupled with griseofulvin, as internal standard, was used for generating standard curves ranging from 0.5 to 1000 ng/mL ($r > 0.9990$) for both plasma and tongue tissue homogenates. The chromatographic separation was achieved by a reverse phase ACE Excel 2 Super C_{18} column with a flow rate of 0.4 mL/min under gradient elution. Mass detection was performed under positive ionization electrospray. Inter- and intra-day accuracy and precision of the assay were ≤15% in both plasma and tongue tissue homogenates. The matrix effect was non-significant and extraction recovery rates were within 87.99% and 109.69% in plasma and tongue homogenates, respectively. The validity of this assay has been confirmed by measuring MPA in rat plasma for pharmacokinetics following intravenous administration of 0.5 mg/kg of mycophenolate sodium, as well as monitoring MPA in rat tongues for tissue distribution and detecting MPA that diffused into systemic circulation following a 4-h transmucosal delivery of 357 μg/cm^2 of mycophenolate sodium.

1. Introduction

Mycophenolic acid (MPA) is used to prevent organ rejection after transplant in conjunction with other immunosuppressive agents by oral administration in clinic [1]. MPA acts by inhibiting inosine-5-monophosphate dehydrogenase activities, which is critical in the *de novo* synthesis of purine nucleotides [2]. MPA has also been shown to activate p53 gene, inhibit cyclin D3 and p27, and block Ras-MAPK and mTOR pathways, leading to the inhibition of tumor growth. Interestingly, when combined with oxaliplatin, MPA showed a strong synergistic inhibition effect on oral squamous cell carcinoma (OSCC-25) cells [3]. This finding suggests a potential use of MPA for treating oral precancerous lesions and the possibility of a targeted drug delivery and absorption of MPA via oral mucosa. Thus, there is a need to develop a sensitive and specific analytical method for the quantification of MPA in plasma and tongue tissues for preclinical evaluation of the drug delivery system.

Determination of MPA concentrations in different human biological samples has been reported using liquid chromatography-tandem mass spectrometry (LC-MS/MS) method. Upadhyay *et al.* [4] and Kawanishi *et al.* [5] developed LC-MS/MS methods in human plasma with linear range from 15 to 15,000 ng/mL and 5–200 ng/mL, respectively. Dom *et al.* [6] reported MPA quantification method with 0.6 ng/mL lower limit of quantification (LLOQ) in human kidney and rat kidney. Only a few high performance liquid chromatography (HPLC) methods are available in literature for the analysis of MPA in rat biological samples.

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Jia *et al.* [7] developed a HPLC-UV method for quantifying MPA in rat plasma, stomach, duodenum, jejunum, ileum, colon and rectum with LLOQ at 500 ng/mL. Gao *et al.* [8] reported a HPLC-UV method measuring MPA in rat plasma and bile with LLOQ at 1000 ng/mL and 250 ng/mL, respectively. Ishizaki *et al.* [9] reported a better assay sensitivity of MPA in rat plasma, bile and urine with 90 ng/mL LLOQ. Liu *et al.* and Jiao *et al.* [10,11] was able to accomplish LLOQ of MPA in rat plasma and urine at 2 ng/mL and 100 ng/mL, respectively. However, all of the reported MPA assays in rat biological samples were analyzed by ultra-violet (UV) detection with relatively low sensitivity, at least 20 µL injection volume and more than 12 min running time.

In this study, we report a sensitive, specific and reproducible Ultra Performance LC-MS/MS (UPLC–MS/MS) method for the quantitation of MPA concentration in the rat plasma and tongue tissue samples. The method has a significantly better LLOQ of 0.5 ng/mL in both plasma and tongue homogenates with 5 µL sample injection volume and 4.6 min running time. Furthermore, this method has been proven to be suitable to characterize MPA pharmacokinetics and tissue distribution following the drug administration.

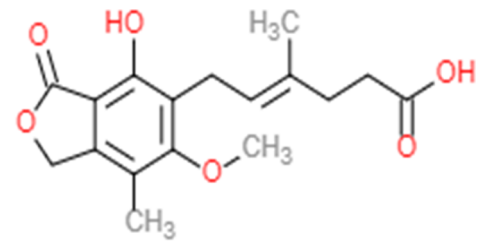
2. Materials and methods

2.1. Chemicals and materials

MPA, griseofulvin (as internal standard, IS) and formic acid were purchased from Sigma Aldrich (St. Louis, MO). Mycophenolate sodium (MPA's salt form) was purchased from USP (Rockville, MD) and used for injection. Acetonitrile and HPLC- grade water were obtained from VWR Chemicals BDH® (Chicago, IL). Blank rat plasma was purchased from Innovative Research (Novi, MI). Male Sprague-Dawley (SD) rats purchased from Envigo RMS (Indianapolis, IN), and blank rat tongue was collected from some of those rats. The chemical structures for MPA and griseofulvin IS are presented in Fig. 1.

2.2. Instruments and conditions

The UPLC-MS/MS system was consisted of a Shimadzu Nexera X2 UPLC system (Columbia, MD) and a 4000 QRRAP® MS/MS system (AB Sciex, Redwood City, CA). The system control and data analysis were performed with Analyst® software 1.6.2 (Sciex, Redwood City, CA).



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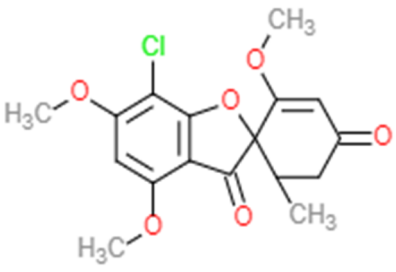


(B)

Fig. 1. Chemical structure of (A) MPA, (B) Griseofulvin (used as IS).

2.2.1. UPLC conditions

MPA separations were carried out on an ACE Excel 2 Super C_{18} column (50 × 2.1 mm, 2 µm, UK). Mobile phase consisted of 0.1% formic acid in water (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B), and ran in a gradient mode at a flow rate of 0.4 mL/min. The gradient program was set as follows: 20% of Solvent B was kept constant for the first 0.2 min and then increased to 98% Solvent B in 2.4 min. The percentage was then kept at 98% Solvent B until 3.4 min. At 3.5 min, Solvent B was reduced back to 20%, followed by an equilibration at 20% Solvent B until 4.6 min. The injection volume of each sample was 5 µL.

2.2.2. MS/MS conditions

Multiple Reaction Monitoring (MRM) data were collected by the MS using a Turbo V™ source with electrospray ionization (ESI) positive mode to detect the specific precursor to product ion transitions, m/z 321.2 → 207.2 for MPA and m/z 353.2 → 285.1 for the IS. The ion spray voltage was set at 4500 V, the source temperature was set at 500 °C. Nebulizer gas and heater gas pressure were 35 psi and 40 psi, respectively. The optimized curtain gas pressure was 25 psi and high collision gas "CAD" pressure was applied. Compound dependent parameters of MPA and IS were optimized and set at 51 and 81 V for declustering potential (DP); 28 and 24 V for collision energy (CE); 9 and 15 V for collision cell exit potential (CXP), respectively.

2.3. Preparation of stock solutions, calibration standards and quality controls

Stock solutions of MPA were dissolved in 50% acetonitrile in water at a concentration of 1 mg/mL. The IS stock solutions were dissolved in acetonitrile at a concentration of 1 mg/mL. Standard and quality control (QC) samples were prepared by spiking MPA into blank rat plasma and rat tongue homogenates, respectively, at concentrations ranging 0.5–1000 ng/mL. Briefly, MPA stock solution was diluted in water to make working solutions for standards (5, 25, 100, 500, 2500, 10,000 ng/mL) and QC samples (8000 ng/mL for high, 250 ng/mL for medium, 10 ng/mL for low, 5 ng/mL for LLOQ). Working solutions were then spiked into blank rat plasma and rat tongue homogenates, respectively, with 10 times dilution to make calibration standards and QC samples. The final concentrations of standards samples were 0.5, 2.5, 10, 50, 250, 1000 ng/mL and QC samples were 0.5, 1, 25, 800 ng/mL. Calibration standards and QC samples were prepared freshly daily.

2.4. Extraction of MPA from plasma and tongue samples

Rat plasma was extracted by protein precipitation method [12]. Briefly, 50 µL of rat plasma samples were extracted by 200 µL of acetonitrile containing 10 ng/mL of IS. After 15 sec vortex, extracted samples were centrifuge at 14,000 rpm for 20 min at 4 °C. The supernatant was then transferred to UPLC-MS/MS injection vials for quantitative analysis.

Each weighted tongue tissue sample was homogenized in a clean scintillation vial with water (1:6, w/v) using Biospec Tissue Tearor™ (Bartlesville, OK) before analysis. Calibration and QC samples were prepared by spiking 45 µL tongue homogenates with 5 µL of spiking solutions to obtain final MPA concentrations ranging from 0.5 ng/mL to 1000 ng/mL. For blank samples, the spiking solution was replaced by 5 µL of water. Tongue homogenates were then extracted by protein precipitation method. Tongue homogenates (50 µL) were extracted by adding 200 µL of acetonitrile with IS in a 1.5 mL centrifuge tube. The mixture was then vortexed for 15 sec and centrifuged at 14,000 rpm for 20 min at 4 °C. Finally, 5 µL of supernatant was injected onto the LC-MS/MS system.

2.5. UPLC-MS/MS method validation

The assay in rat plasma and tongue were validated following U.S. Food and Drug Administration (FDA) Bioanalytical Method Validation Guidelines for Industry [13] with specific aspects described below.

2.5.1. Selectivity and specificity

The selectivity and specificity of the method were evaluated by analyzing six different endogenous sources of blank rat plasma or tongue samples for interference with the analyte and IS. The peak response of the endogenous plasma and tongue with the analytes should be ≤20% of the peak area of the LLOQ standard, the IS should be ≤5% of the average peak area of standard curve and QC samples.

2.5.2. Sensitivity and linearity

Linear calibration curves in rat plasma or tongue homogenates were determined by plotting the peak area ratio of MPA to IS against known standard concentrations of MPA. The slope, intercept, and coefficient of determination were estimated using least squares linear regression method with a weighting of $1/x$. The lower limit of quantification (LLOQ) was estimated based on the signal-to-noise ratio of at least 5:1.

2.5.3. Carryover

The triplicate injections of blank samples were conducted followed by six consecutive injections of upper limit of quantification (ULOQ = 1000 ng/mL) QC samples. The blank sample peak response of the analyte should be ≤20% of the peak area of the LLOQ standard, the IS should be ≤5% of the average peak area of IS throughout the run.

2.5.4. Accuracy and precision

Intra-day accuracy and precision were evaluated by determining the concentrations of LLOQ, low QC (LQC), medium QC (MQC), high QC (HQC) samples on the same day by six replicates of different level of concentration. LQC defined as within three times the LLOQ, MQC defined as mid-range of standard curve, and HQC defined as high-range of standard curve. Inter-day accuracy and precision were determined by the same run on three consecutive days. The relative error (RE%) was used to estimate the accuracy and coefficient of variation (CV%) was used to estimate the precision. The calculated results of inter-day and intra-day precision and accuracy should be ≤15% for three levels of QC and ≤20% for LLOQ. Precision and accuracy samples were determined by comparing to fresh-made calibration curve with known concentration.

2.5.5. Dilution integrity

The dilution integrity of rat plasma or tongue homogenate was performed to determine the accuracy of extended linearity beyond the ULOQ (1000 ng/mL). The effect of 1:10 and 1:5 dilute on determination of MPA in rat plasma was measured by six replicates of spiked dilution quality control (DQC) samples. The concentration of DQC was 5 times of the ULOQ. For rat tongue, six replicates of spiked DQC samples with different dilution factors were determined. The DQC were 5, 10 and 20 times of the ULOQ, in which 5X DQC with 1:5 and 1: 10 dilute, 10X DQC with 1:10 and 1:20 dilute, 20X DQC with 1:20, 1:50 and 1:100 dilute. The accuracy and precision within ± 15% were set as acceptance range.

2.5.6. Extraction recovery and matrix effect

Matrix effects were evaluated by comparing the peak area ratio of the post-extracted blank rat plasma or tongue to the peak area ratio of neat solution at four QC levels (LLOQ, LQC, MQC, HQC) in six replicates. The calculated equation of matrix effect is as follows:

$$\text{Matrix effect } \% = \left[\left(\frac{Response_{post-extraction\ \ spiked\ \ samples}}{Response_{neat\ \ solution\ \ samples}}\right) - 1\right] \times 100$$

The extraction recovery was evaluated by comparing the peak area ratio of the pre-extracted blank rat plasma or tongue QC sets to the peak area ratio of the post-extracted QC sets. The calculated equation of extraction recovery was as followed:

$$\text{Extraction recovery } \% = \left(\frac{Response_{post-extraction\ \ spiked\ \ samples}}{Response_{pre-extraction\ \ spiked\ \ samples}}\right) \times 100$$

2.5.7. Stability

The stability of MPA in rat plasma or tongue was subjected to short-term bench-top, freeze-thaw, post-prepared auto-sampler, and long-term stability studies. All stability experiments were conducted in six replicates of QC samples at four levels (LLOQ, LQC, MQC, HQC).

The short-term bench-top and auto-sampler stability was tested by placed QC samples on the bench-top or auto-sampler for 16 h. Freeze-thaw stability was conducted by three times freeze (at −80 °C) –thaw (at room temperature) cycles. Long-term stability was evaluated by placing the QC samples in −80 °C for 30 days. MPA concentrations in all stability samples were compared to that of freshly made QC samples.

2.6. Pharmacokinetic and tongue distribution studies

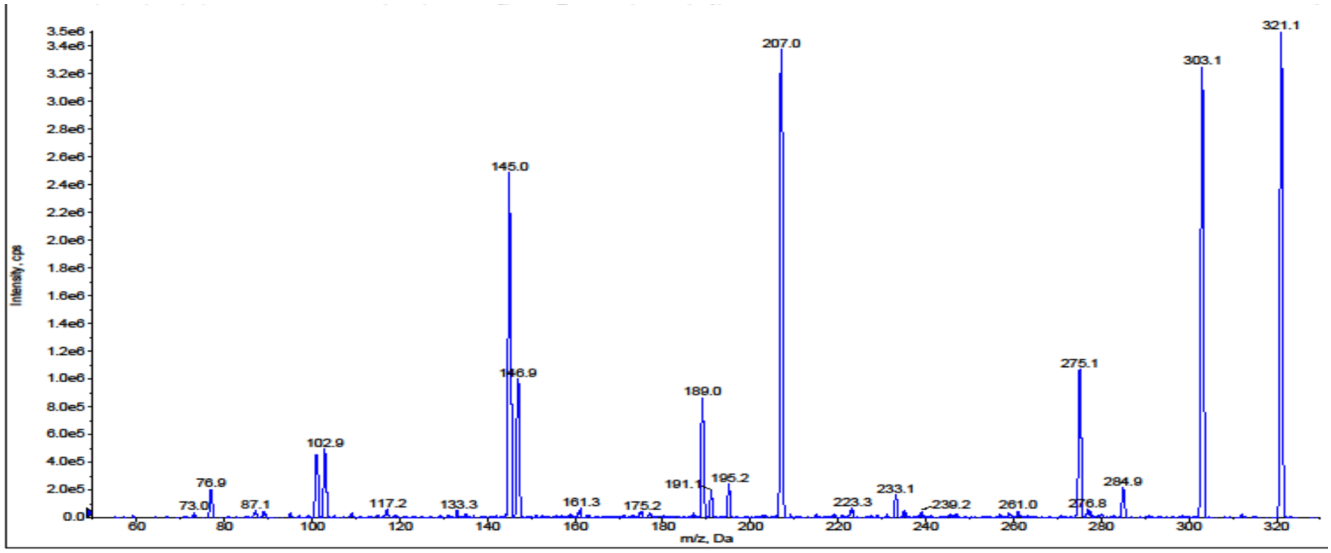
The validated method was applied to investigate the plasma profiles of MPA in rats after intravenous administration of a dose of 0.5 mg/kg of mycophenolate sodium or topical delivery on dorsal tongue with a dose of 0.5 mg/kg mycophenolate sodium. The animal experiment and protocol were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Texas Southern University.

A group of five adult male SD rats were used for the pharmacokinetic studies. The drug formulation used for intravenous administration was prepared in normal saline. Serial blood samples (approximately 150 μL) were collected into heparinized tubes after dosing and for up to 48 h. Plasma was separated immediately by centrifugation of the blood samples at 3000 rpm for 20 min and kept at −80 °C until analysis.

For the tongue tissue distribution and blood diffusion studies, a group of four adult male SD rats were used for the study. A mucoadhesive patch formulation containing mycophenolate sodium was applied on the dorsal tongue surface of a rat for 4 h at a dosage of 357 μg/cm^2 under light anesthesia. Serial blood samples (about 100 μL) were collected at 1, 2, 3 and 4 h and stored into heparinized tubes during the 4-h-patch-application. Rats were sacrificed right after 4-h-blood-drawing. Blood samples were centrifuged at 3,000 rpm for 20 min to separate the plasma samples, which were stored at −80 °C pending MPA analysis. Tongues were removed immediately, rinsed with deionized water for 3 times, sliced into small pieces, and then homogenized in water (1:6 w/v). Blank tongues were prepared separately using drug-free rats.

2.7. Pharmacokinetic data analysis

The pharmacokinetic parameters for each rat were estimated using Phoenix WinNonlin v7.0 software (Pharsight Corporation, Mountain View, CA, USA). Non-compartmental analysis was used to determine the pharmacokinetic parameters of MPA after intravenous administration [14]. The pharmacokinetic parameters including the area under the plasma concentration–time curve during the period of observation (AUC_{0-48}), the area under the plasma concentration–time curve extrapolated to infinity (AUC_{0-inf}), apparent volume of distribution (V_d), the apparent clearance (CL), total body mean residence time (MRT) and terminal elimination half-life were estimated.



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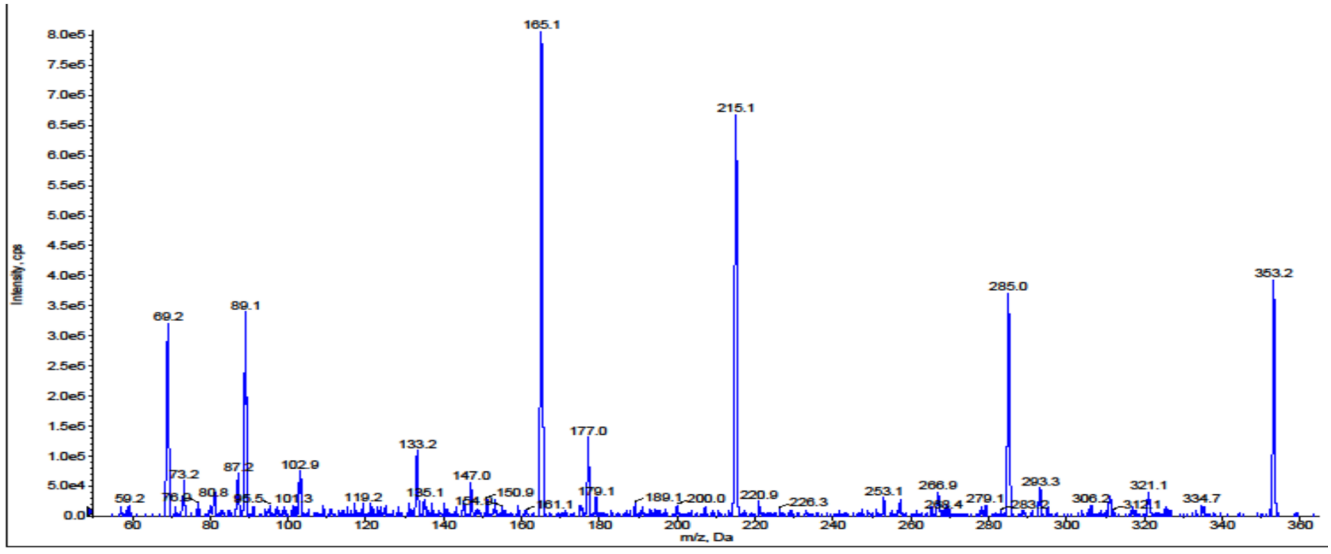

(B)

Fig. 2. Product ion mass spectra of (A) MPA (*m/z* 321.2 → 207.2) and (B) IS (*m/z* 353.2 → 285.1).

3. Results and discussion

3.1. Method development

The method was developed by optimizing both UPLC and MS conditions to obtain the optimal peak shape, chromatographic separation and sensitivity.

3.1.1. Mass spectrometry

Electro spray ionization (ESI) was chosen for this method. MPA showed more response in positive ion mode as compared to the negative ion mode. MPA and IS produced predominantly protonated molecules $[M + H]^+$ at *m/z* 321.1 and *m/z* 353.2, respectively, in Q1 full scan mass spectra. MRM mode was used to identify the molecules by monitoring the transition *m/z* 321.2 → 207.2 for MPA and *m/z* 353.2 → 285.1 for the IS. Transitions were chosen due to their stable and undisturbed features. The product ion mass spectra were shown in Fig. 2 for MPA and IS. Instrument MS source- and compound-dependent parameters were optimized by tuning to improve MPA sensitivity. Curtain gas pressure was set at 25 psi to prevent contamination and high collision gas "CAD" pressure was applied. The ion spray voltage was optimized to 4500 V without losing signal. The source temperature was set at 500 °C according to our flow rate and mobile phase composition. Nebulizer gas was set at 35 psi for best signal stability and sensitivity and heater gas pressure was set at 40 psi for ionization of the sample. Optimized declustering potential (DP) were set at 51 and 81 V for MPA and IS respectively to prevent the ions from clustering together. Collision energy (CE) were optimized to 28 and 24 V for MPA and IS respectively in order to avoid insufficient fragmentation or overmuch fragmentation and collision cell exit potential (CXP) were set at 9 and 15 V for MPA and IS, respectively.

3.1.2. Chromatography separation

The UPLC method was optimized by selecting and testing different column type, mobile phase composition and flow rate in order to obtain good peak shapes, less carry over and higher sensitivity. Several stationary phase columns were tried, including Kinetex® F5 column (50 × 2.1 mm, 1.7 μm), Acquity HSS-T3 (50 × 2.1 mm, 1.8 μm) and ACE Excel 2 Super C_{18} column (50 × 2.1 mm, 2 μm). Finally, an ACE Excel 2 Super C_{18} column (50 × 2.1 mm, 2 μm) was found to be

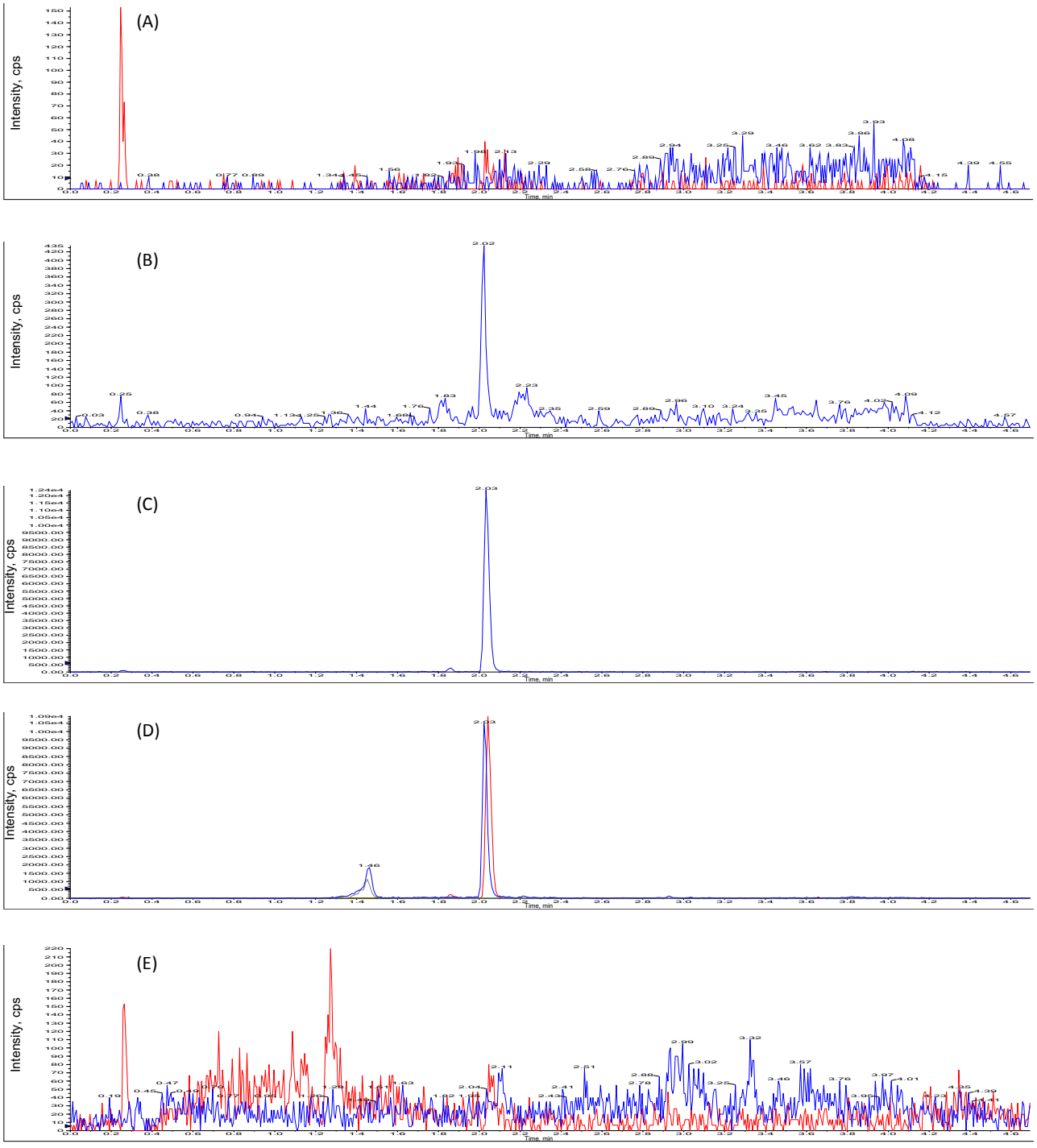

Fig. 3. Representative chromatograms of MPA and IS in blank and spiked rat plasma and tongue: (A) blank plasma, (B) mycophenolic acid spiked in plasma at lower limit of quantification (0.5 ng/mL), (C) blank plasma spiked with internal standard, (D) rat plasma sample at 12 h after 0.5 mg/kg intravenous administration, (E) blank tongue, (F) mycophenolic acid spiked in tongue at lower limit of quantification (0.5 ng/mL), (G) blank tongue spiked with internal standard, (H) rat tongue sample at 4 h after 0.5 mg/kg tongue administration with 100X dilution.

optimal with high sensitivity and good peak shape. This may be attributed to ACE super C_{18} column increases ligand coverage of the silica surface and effectively eliminates the effect of unbonded silanol groups from separations. Initially, a mobile phase consisting of pure water and acetonitrile in varying combination was used, but split peak was observed in the spiked plasma samples. This may be due to inappropriate pH in the mobile phase. Decreasing pH to acidic environment were found to get good symmetrical peak shape. After optimization, acetonitrile with 0.1% formic acid (solvent B) and water with 0.1% formic acid (solvent A) were chosen as mobile phase run in gradient at 0.4 min/mL flow rate to obtain good peak shape. The analysis was 4.6 min per run. Injection volume of each sample was optimized to 5 μL, because 2-μL-injection-volume had relative lower response, while higher injection volume was not necessary to reach the desired response. The retention time was 2.02 min for MPA and 2.03 min for IS (Fig. 3). Since deuterium-labeled IS MPA-d3 was not available when we were developing the method, warfarin and griseofulvin were tested as an IS due to their similar log P (partition coefficient) values as MPA.

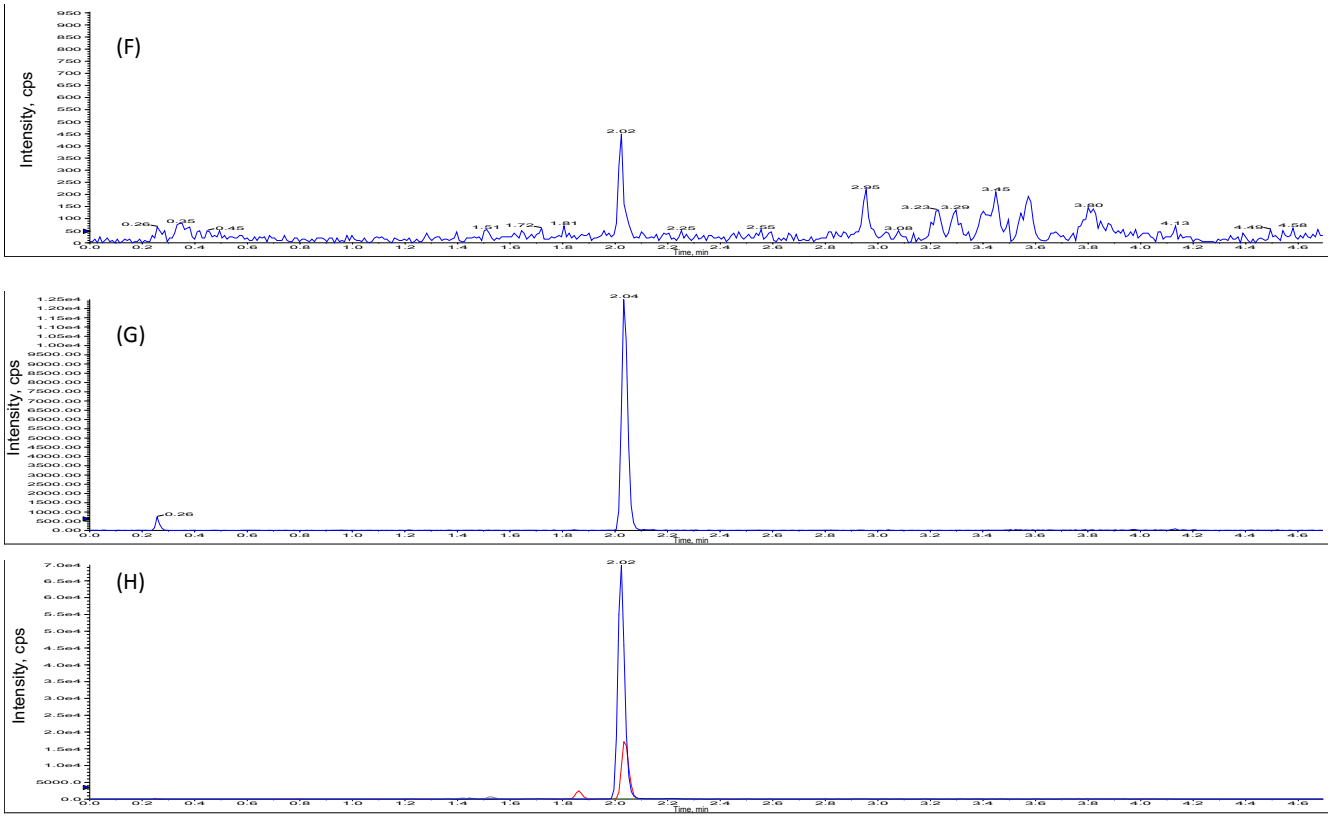

Fig. 3. (*continued*)

Table 1
Intra-day and inter-day accuracy and precision of MPA in rat plasma and tongue homogenates.

Biological samples	Nominal concentration (ng/mL)	Intra-day (n = 6)			Inter-day (n = 18)		
		Observed concentration (mean ± SD)	Accuracy (RE %)	Precision (CV %)	Observed concentration (mean ± SD)	Accuracy (RE %)	Precision (CV %)
Plasma	0.5	0.52 ± 0.04	4.50	7.80	0.51 ± 0.05	−1.04	9.35
	1	1.00 ± 0.10	0.37	10.38	0.98 ± 0.08	1.04	7.87
	25	26.20 ± 0.59	4.80	2.26	25.16 ± 1.93	2.82	7.69
	800	817 ± 45.09	2.13	5.52	785.00 ± 50.67	4.79	6.45
Tongue	0.5	0.52 ± 0.05	9.65	4.58	0.51 ± 0.05	9.55	1.04
	1	0.99 ± 0.04	3.80	−0.98	1.00 ± 0.08	8.50	2.82
	25	25.12 ± 2.04	8.11	0.46	25.48 ± 1.67	6.54	4.79
	800	800.67 ± 13.71	1.71	0.08	798.17 ± 35.34	4.43	4.15

SD, standard deviation; CV, coefficient of variation; RE, relative error.

Table 2
Dilution integrity accuracy and precision of MPA in rat plasma and tongue homogenates.

Biological samples	Original concentration (ng/mL)	Dilution factor	Accuracy (RE %) (n = 6)	Precision (CV %) (n = 6)
Plasma	5000	5	1.89	−11.02
	5000	10	2.43	−13.30
Tongue	5000	5	3.91	−5.10
	5000	10	7.49	7.80
	10,000	10	6.18	7.20
	10,000	20	11.74	1.53
	20,000	10	7.81	6.18
	20,000	20	5.13	−1.46
	20,000	50	2.58	10.58

Table 3
Recovery and matrix effect of MPA in rat plasma and tongue homogenates quality control samples.

Biological samples	Nominal concentration (ng/mL)	Matrix effect (%) (n = 6)	Recovery (%) (n = 6)
Plasma	0.5	13.59 ± 1.93	88.52 ± 4.63
	1	12.08 ± 3.09	87.99 ± 7.70
	25	11.45 ± 6.34	89.00 ± 3.43
	800	5.60 ± 14.68	89.55 ± 13.08
Tongue	0.5	0.58 ± 2.93	106.15 ± 3.33
	1	2.08 ± 6.06	109.59 ± 5.59
	25	1.77 ± 2.57	99.11 ± 7.66
	800	2.28 ± 4.96	101.47 ± 9.43

Table 4
Stability data for MPA in rat plasma and tongue homogenates.

Biological samples	Stability test	Nominal Concentration (ng/mL)	Calculated Concentration (ng/mL)		
			Mean ± SD (n = 6)	CV (%)	RE (%)
Plasma	auto-sampler (16 h)	0.5	0.51 ± 0.04	8.78	2.30
		1	1.03 ± 0.07	6.33	2.80
		25	24.73 ± 1.20	4.84	−1.07
		800	817.83 ± 15.84	1.94	2.23
	short-term (16 h, RT)	0.5	0.51 ± 0.03	6.05	2.47
		1	1.03 ± 0.08	8.22	3.15
		25	27.32 ± 2.40	8.79	9.27
		800	817.5 ± 43.93	5.37	2.19
	freeze and thaw (−80 °C to RT)	0.5	0.50 ± 0.07	14.29	−0.80
		1	0.99 ± 0.08	8.19	−1.12
		25	25.65 ± 2.33	9.09	2.60
		800	801.50 ± 34.03	4.25	0.19
	long-term (−80 °C, 30 days)	0.5	0.46 ± 0.04	9.69	−7.27
		1	1.10 ± 0.04	4.08	10.17
		25	24.65 ± 1.93	7.84	−1.40
		800	745.50 ± 40.65	5.45	−6.81
Tongue	auto-sampler (16 h)	0.5	0.54 ± 0.02	3.54	7.50
		1	1.07 ± 0.06	5.29	7.00
		25	28.03 ± 0.56	1.98	12.10
		800	824.50 ± 41.51	5.03	3.06
	short-term (16 h, RT)	0.5	0.47 ± 0.03	6.17	−6.95
		1	1.04 ± 0.08	7.52	3.50
		25	25.03 ± 1.33	5.30	0.10
		800	847.50 ± 34.63	4.09	5.94
	freeze and thaw (−80 °C to RT)	0.5	0.52 ± 0.03	5.18	4.8
		1	1.04 ± 0.03	2.72	4.00
		25	26.23 ± 1.69	6.43	4.90
		800	762.00 ± 8.04	1.06	−4.75
	long-term (−80 °C, 30 days)	0.5	0.50 ± 0.05	9.62	−0.25
		1	0.93 ± 0.04	3.95	−6.80
		25	23.35 ± 1.82	7.78	−6.60
		800	727.75 ± 30.05	4.13	−9.03

RT, room temperature.

Table 5
Pharmacokinetic parameters of MPA after intravenous administration of 0.5 mg/kg mycophenolate sodium to rats (n = 5).

Parameters	MEAN ± SD
$AUC_{0\rightarrow48}$ (h·ng/mL)	2378.9 ± 356.1
$AUC_{0\rightarrow inf}$ (h·ng/mL)	2424.1 ± 375.3
Half-life (h)	10.5 ± 1.2
CL (mL/h/kg)	209.8 ± 29.0
V_d (ml/kg)	1466.6 ± 376.9
MRT (h)	7.10 ± 1.9

$AUC_{0\rightarrow48}$ = area under the curve from 0 to 48 h; $AUC_{0\rightarrow inf}$ = area under the curve from 0 h to infinity; V_d = volume of distribution; CL = clearance, MRT = mean residence time.

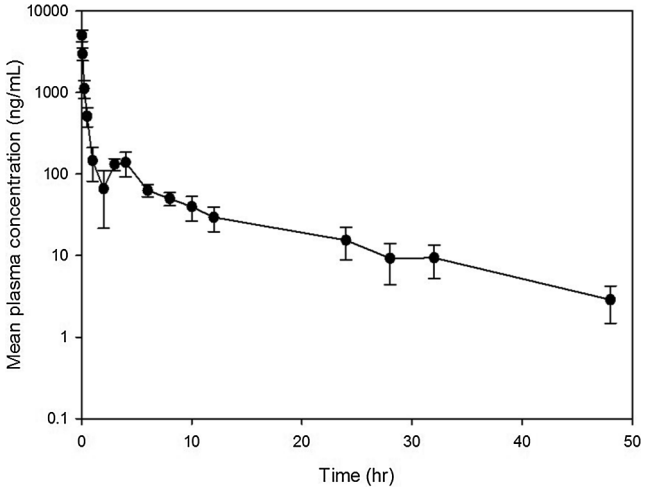

Fig. 4. The profiles of the mean plasma concentration versus time after 0.5 mg/kg mycophenolate sodium intravenous injection to rats (mean ± SD, n = 5).

However, warfarin is a popular anticoagulant drug widely used in clinic, it might be present in human samples and interfere the analysis of clinical samples in the future. Therefore, griseofulvin was selected as the IS with good matrix effect, recovery and close retention time to MPA. No carryover was detected in both blank plasma and tongue homogenate samples after six injection of ULOQ by simple needle washing method. Acetonitrile in water (50% v/v) was used as needle wash solution. Only external rinsing was applied to rinse port, dip time of rinse was 3 sec.

3.1.3. Extraction of MPA from plasma and tongue homogenates

Several methods including protein precipitation, liquid–liquid extraction (LLE) and solid–phase extraction (SPE) have been reported to extract MPA for biological samples [1,15,16]. However, SPE had shown only 77% of recovery for MPA plasma samples. Also, SPE and LLE can be time consuming and involve more complicated procedures. Protein precipitation method was simple, economy and quick if it does not affect selectivity, matrix effect and recovery. With satisfactory matrix effect, recovery and selectivity, one-step protein precipitation method was finally chosen for prepare plasma and tongue homogenate samples.

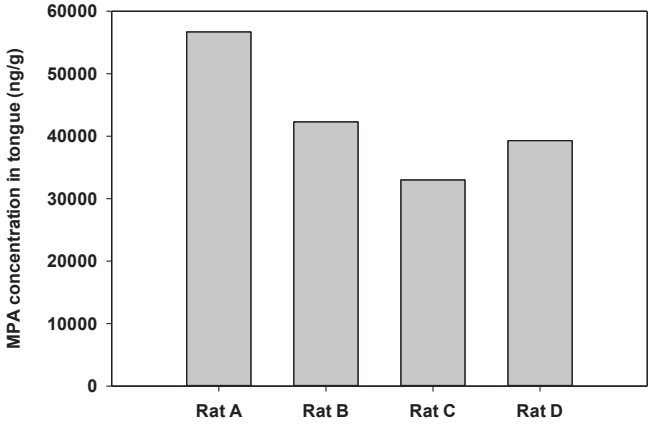

Fig. 5. MPA concentrations in rat tongue tissues (ng/g) immediately after a 4-h mucoadhesive patch application to the tongue surface of 4 SD rats.

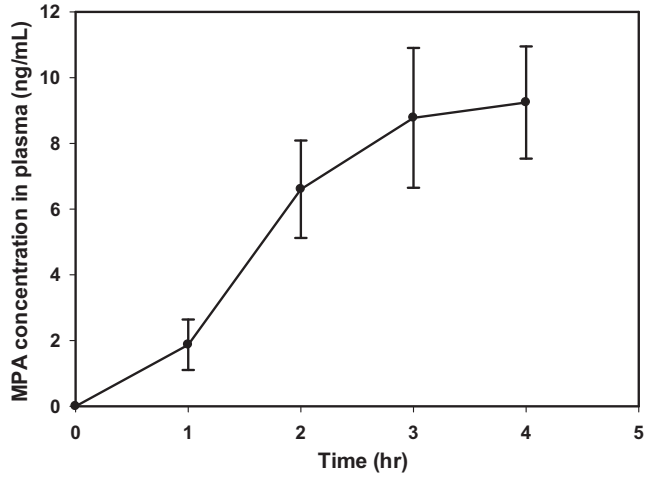

Fig. 6. MPA concentrations in rat plasma (Mean ± SD ng/mL) at various time points during a 4-h mucoadhesive patch application to a rat tongue surface (n = 4).

3.2. Method validation

3.2.1. Selectivity and specificity

There was no interference or significant ion suppression detected from endogenous matrix components. IS also showed no interference with MPA. Chromatography obtained from blank plasma and tongue homogenate extracts were shown in Fig. 3. There was no carryover shown in chromatography for both IS (≤5% of average response) and MPA (≤20% of LLOQ), which met the criteria by FDA's bioanalytical guidelines [13].

3.2.2. Sensitivity and linearity

The standard curves were plotted using peak area ratio (MPA/IS) against its corresponding concentration of standard solutions. The standard curve showed good linearity from 0.5 ng/mL to 1000 ng/mL in both plasma and tongue homogenates. Linear correlation coefficients (r^2) were at least 0.9990 for all calibration curves. The accuracies were within 85% − 115% for plasma and tongue homogenate calibration standards at all concentration levels. LLOQ of the assay was 0.5 ng/mL for both plasma and tongue homogenates, where LLOQ had at least 5:1 signal-to-noise ratio (Fig. 3). We have achieved a significantly lower LLOQ level compared to that of the previously reported HPLC-UV methods (2 ng/mL) [10]. This is critical in terms of successfully characterizing pharmacokinetics of MPA following intravenous and novel patch applications to rat tongue surface.

3.2.3. Accuracy and precision

The inter-day and intra-day accuracy and precision were determined at the LLOQ, LQC, MQC, HQC of 0.5, 1, and 25, 800 ng/mL, respectively, with six replicates in rat plasma and tongue homogenates. Results are presented in Table 1. The intra-day coefficients of variation (CV%) ranged from 7.80% to 10.38% in plasma and −0.98% to 4.58% in tongue homogenates, and the percent relative errors (RE%) range was 0.37% to 4.80% in plasma and 1.71% to 9.65% in tongue homogenates. Inter-day relative error was ≤9.55% and the coefficient of variation was ≤9.83 for QC samples of rat plasma and tongue homogenates. The precision and accuracy were within acceptance range according to FDA bioanalysis guidance. As a result, the assay method of MPA was considered reproducible for quantification.

3.2.4. Dilution integrity

Dilution integrity study was performed to check if samples' dilution would change signals compared to the predicting concentration. The precision (CV%) and accuracy (RE%) of MPA were less than-13.30% and 2.43%, respectively with dilution factor 1/5 and 1/10 in rat plasma. On the other hand, the precision (CV%) and accuracy (RE%) of MPA were less than 10.58% and 11.74%, respectively, with dilution factor 1/5, 1/10, 1/20, 1/50 in rat tongue homogenates. All of them were within ± 15% of nominal concentration (Table 2), indicating the samples higher than ULOQ was reliable for quantification with dilution.

3.2.5. Extraction recovery and matrix effect

The extents of the recovery of MPA and IS were measured to demonstrate the consistent and reproducible of the method. The extraction recovery and matrix effect from different biological matrix are shown in Table 3. Recovery for all QC levels were from 87.99% to 89.55% in rat plasma and 99.11% to 109.59% in rat tongue homogenates. Matrix effect for all QC levels were within ± 15% CVs, suggesting that the enhancement or suppression signals might be negligible.

3.2.6. Stability

MPA concentrations in rat plasma and tongue homogenates were determined by short-term stability, freeze-thaw stability, autosampler stability and long-term stability in sextuplicate at four levels of QCs. Stability results are presented in Table 4. Bench-top stability was performed at room temperature for 16 h with no observed loss of MPA, indicating that the samples were stable under the laboratory handling condition. QCs in autosampler were also stable for 16 h, demonstrating the stability of extracts throughout the process. Three cycles of freeze-thaw stability and long-term stability also demonstrated the reliable storage condition of the samples. CV and RE values for all stability conditions tested were ≤15% (Table 4). Stock solutions for drugs were also stable at room temperature (25 °C) for up to 3 months. The stability study results indicated that MPA was stable throughout the pharmacokinetic and tissue distribution study sampling period and during the LC-MS/MS analysis process.

3.3. Pharmacokinetic and tongue distribution study

The UPLC–MS/MS method was successfully applied to the pharmacokinetics study of MPA following intravenous administration of a single dose of 0.5 mg/kg. The main pharmacokinetic parameters calculated using Phoenix Winnonlin software by non-compartmental analysis are shown in Table 5. Mean plasma concentration vs. time curves (n = 5) are presented in Fig. 4. Following intravenous administration, high MPA concentrations in plasma was observed immediately after the dosing followed by a rapidly decrease. A second peak appeared at around 3 h post dosing, and then a third small peak showed at around 32 h post dosing (Fig. 4). The multiple peaks following intravenous administration are believed to be the result of an enterohepatic circulation of MPA, where MPA was first metabolized to

7-O-MPA-β-glucuronide (MPAG) in the liver, the glucuronide metabolite was then excreted via bile to the gastrointestinal tract, where MPAG was hydrolyzed and converted back to MPA for re-absorption into the systemic circulation. It has been reported that enterohepatic circulation could account for 10%-60% increased MPA systemic exposure [17].

The mean plasma concentration–time curve during the period of observation ($AUC_{0\rightarrow48}$) and area under the plasma concentration–time curve extrapolated to infinity ($AUC_{0\rightarrow inf}$) were 2378.9 ± 356.1 and 2424.1 ± 375.3 h·ng/mL, respectively. The apparent volume of distribution (V_d) was 1466.6 ± 376.9 mL/kg, the apparent clearance (CL) was 209.8 ± 29.0 mL/hr/kg, total body mean residence time (MRT) and terminal elimination half-life were 7.10 ± 1.88 and 10.5 ± 1.2 h, respectively.

The concentrations of MPA in tongue homogenates at 4 h following patch application were shown in Fig. 5. The results showed that a significant amount of MPA was absorbed into the tongue tissue. This result demonstrates that the developed method can also be used to measure the MPA concentration in tissues such as the oral mucosa.

The absorption profile of MPA to systemic circulation during the 4 h patch application was shown in Fig. 6. Plasma MPA concentrations increased slowly during the first hour of patch application, followed by a fast absorption during 1–2 h, and then slowing down from 3 to 4 h. An average of 9.25 ng/mL MPA plasma concentration was observed after 4 h patch application. It is worth to note that MPA concentrations in plasma were much lower than those in tongue homogenates (42,825 + 10,029 ng/g), this might be due to potential slow drug release from keratinized epithelium of dorsal tongue and vehicle components of patch formulation [18,19]. In the meantime, it appears that our study was unique with respect to monitor MPA concentrations in tongue tissue following the patch application while still capable of detecting low levels of MPA in the systemic circulation.

Several drug dosage formulations have been reported for intraoral applications, including the implantable tablet, mucoadhesive patch, film, microsphere, ointment, cream, and hydrogel [20–25]. Most of these applications were instantaneously dissolved with a rapid onset and avoidance of hepatic first-pass effect [26,27]. Patch application to dorsal tongue for sustained drug delivery may be exploited as a new therapeutic modality for intraoral lesions. Further investigation of the novel administration route in mouth cavity using patch formulations are warranted.

4. Conclusion

A sensitive, specific and reproducible UPLC-MS/MS method was developed and validated for the quantification of MPA concentration in rat plasma and tongue homogenates. This method was accurate and precise over the MPA concentration range of 0.5–1000 ng/mL, and showed good recovery and stability without interference from the endogenous components. This assay was successfully applied to a pharmacokinetic study following intravenous administration of the drug using SD rats as an animal model, then was further applied to rat tongue tissue distribution and blood concentration studies following a mucoadhesive patch application on the dorsal tongue surface. Results showed that our detection method was valuable to not only detect MPA concentrations in tongue tissue following the patch application but also track trace amount of MPA in the systemic circulation.

CRediT authorship contribution statement

Xiuqing Gao: Methodology, Conceptualization, Software, Formal analysis, Investigation, Data curation, Writing - original draft. **Robert Y.L. Tsai:** Conceptualization, Funding acquisition, Writing - review & editing. **Jing Ma:** Methodology, Investigation. **Parnit K. Bhupal:** Resources. **Xiaohua Liu:** Conceptualization, Resources. **Dong Liang:** Conceptualization, Writing - review & editing, Funding acquisition, Supervision. **Huan Xie:** Conceptualization, Writing - review & editing, Project administration, Funding acquisition, Supervision.

Declaration of Competing Interest

The authors declared that there is no conflict of interest.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jchromb.2019.121930.

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