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Plasma Mycophenolic Acid Determination by Ultra-high Performance Liquid Chromatography-tandem Mass Spectrometry and Its Application in Pediatric Patients with Nephrotic Syndrome

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Abstract: *Objective:* Mycophenolate mofetil (MMF) is frequently prescribed to pediatric patients with nephrotic syndrome (NS); however, information on the active metabolite mycophenolic acid (MPA) in this population is limited. *Method*: Using ultra-high performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS), we validated a rapid and simple approach to measure MPA in accordance with the Bioanalytical Method Validation Guidance issued by the relevant regulatory authorities. Statistical analyses were performed on 69 pediatric patients with frequently relapsing/steroid-dependent nephrotic syndrome (FRNS/SDNS) who received corticosteroids plus MMF. *Results*: Forty-two patients achieved the endpoints and reported adverse events (AEs). The area under the concentration–time curve of MPA in the AE group was further distributed. Monitoring blood MPA concentrations is critical to prevent AEs during MMF administration in children with FRNS/SDNS. *Conclusion:* UHPLC–MS/MS offers a more accurate reference than immunoassays.

Keywords: Mycophenolic acid; Plasma-drug concentration; Children; Nephrotic syndrome; UHPLC–MS/MS

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1. Introduction

Mycophenolate mofetil (MMF) is a novel immunomodulator obtained from *Penicillium* species [1, 2]. Currently, it is a major immunosuppressive agent used to treat transplant rejection. In vivo, MMF is rapidly transformed into its active metabolite mycophenolic acid (MPA) via esterase-catalyzed hydrolysis (**Figure 1**). This process converts MMF to MPA, which serves as an immunosuppressive metabolite. Subsequently, MPA undergoes glucuronidation, which leads to the formation of its major metabolite, phenolic MPA glucuronide [3]. MPA reversibly and noncompetitively blocks the rate-limiting enzymes involved in purine synthesis, thereby significantly inhibiting the classical guanine nucleotide synthesis pathway, without affecting other synthesis pathways or synthesis via

salvage pathways. MPA does not exert significant effects on the liver, kidney, and bone marrow cells. Therefore, the cytotoxic damage induced by other immunosuppressants can be avoided [4]. However, the individual differences in the pharmacokinetics of MPA and MMF are significant, and the pharmacokinetics of these drugs is related to their anti-immunosuppressive effects and adverse drug reactions.

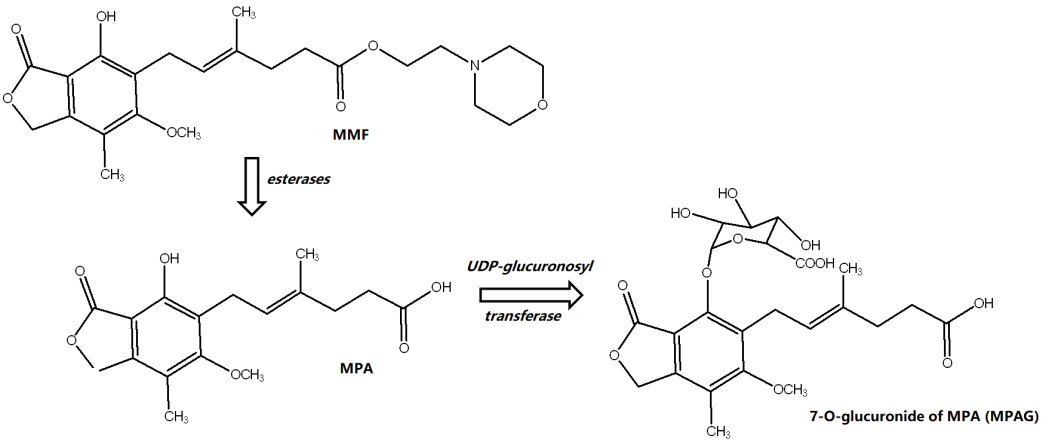

Figure 1. Metabolic pathways of mycophenolic acid in humans.

MMF is frequently used to treat pediatric patients with nephrotic syndrome (NS), and can be used as a first-line treatment for steroid-dependent NS (SDNS) and frequently relapsing NS (FRNS) [5–9]. Moreover, MMF dose adjustment based on plasma MPA concentration can improve therapeutic effects, especially in organ transplantation and lupus [10, 11]. Therefore, a convenient, rapid, and accurate method is required for the detection of plasma MPA levels within the μg range.

Recently, high-performance liquid chromatography (HPLC) and particle-enhanced turbidity suppression immunoassays have been used to determine plasma MPA concentration [12, 13]. Currently, immunoassays are the major detection approach for clinical use because of their automated and convenient features. However, they often overestimate plasma MPA levels because of cross-reactivity and metabolite interference [14]. Therefore, liquid chromatography (LC) may be a better choice for measuring the levels of MPA and its metabolites, including the combined use of ultraviolet detection, mass spectrometry (MS; HPLC–MS), and tandem mass spectrometry (LC–MS/MS) [13, 15–17].

To date, studies have primarily measured MPA concentrations in plasma samples obtained from transplant patients administered MMF in combination with calcineurin inhibitors [18–21]. MPA concentrations have also been determined in pharmacokinetic studies of healthy participants [22, 23]. Data on NS, particularly in pediatric patients, are limited [24, 25]. Pediatric patients with NS may have different metabolic and physiological characteristics compared to adults, which can further complicate the interpretation of pharmacokinetic data and the optimization of dosing regimens. Therefore, the limited data available on pediatric NS patients, represent a new challenge to accurately assess the pharmacokinetic properties and therapeutic efficacy of MMF in this specific population. Therefore, we quantified the levels of the MMF metabolite, MPA, in pediatric patients with NS using ultra-performance LC–MS/MS (UHPLC–MS/MS) to establish a convenient, rapid, and accurate method. We aimed to explore the correlation between MPA plasma levels and symptom relief in pediatric patients with NS treated with MMF, and to provide a basis for individualized and improved treatment in this patient population.

2. Materials and methods

2.1. Chemicals and materials

The following reagents and materials were used in this study: Mycophenolate mofetil (MMF, Cycopin®) was supplied by Hangzhou Zhongmei Huadong Pharmaceutical Co., Ltd (Hangzhou, China). Mycophenolic acid (MPA, 98% purity) and its deuterated analog MPA-d3 (98% purity) were acquired from Toronto Research Chemicals (Toronto, Canada). High-performance liquid chromatography (HPLC)-grade formic acid and dimethyl sulfoxide (DMSO) were sourced from Aladdin (Shanghai, China) and Macklin (Shanghai, China), respectively, while HPLC-grade acetonitrile was obtained from Merck. Ultra-pure water was prepared using a Milli-Q A10 purification system (Millipore, MA, USA). For analytical purposes, drug-free human plasma and K2EDTA-anticoagulated whole blood samples were collected from healthy adult donors.

2.2. UHPLC–MS conditions

UHPLC–MS conditions were developed and modified based on previously published data [26]. The LC–MS/MS equipment comprised an LC-30AD HPLC system (Shimadzu, Japan) and an API 5500 Qtrap mass spectrometer (AB Sciex, USA). Separation was conducted using a C_{18} column (Agilent Eclipse Plus, 2.1 × 50 mm, 1.8 μm). The mobile phase consisted of water (containing 0.1% formic acid) (A) and acetonitrile (B) at a flow rate of 0.35 mL/min. Gradient elution was conducted as follows: 0–0.5 min, 10% B; 0.5–1.5 min, 95% B; 1.5–3.0 min, 95% B; 3.0–3.1 min, 10% B; and 3.1–4 min, 10% B. The column and autosampler temperatures were set at 40 °C and 4 °C, respectively.

MS/MS detection was performed via multiple reaction monitoring (MRM) in the negative electrospray ionization mode for MPA (m/z 319.086 191.000) and the internal standard (IS) (m/z 322.082 191.000) (**Figure 2**). The main operational parameters were as follows: curtain gas at 36 units, ion spray voltage (IS) at −4500 V, ion source interface temperature at 550 °C, collision gas at medium, gas 1 at 50 psi, and gas 2 at 55 psi.

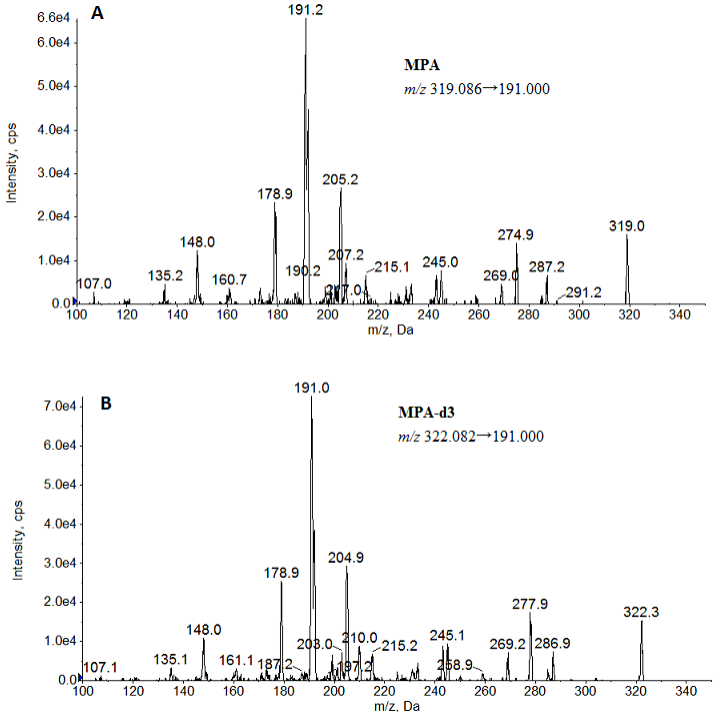

Figure 2. Production spectra of mycophenolic acid (MPA) (A) and MPA-d_3 (B).

Data were acquired and processed using the Analyst SCIEX 1.6.3 software (AB Sciex Pte. Ltd., USA). Linear regression analysis was performed for each calibration curve, employing the concentration as the explanatory variable and the peak area ratio (y) of the analyte to the IS. The calibration procedure utilized a linear curve fit with a weighting factor of $1/x^2$. Acceptance criteria mandated that all calibrator standards must lie within ±15% of their nominal concentrations (±20% for the lower limit of quantification, LLOQ) in each analytical run, with a required correlation coefficient (R^2) of > 0.98.

2.3. Preparation of the calibration standard and quality control (QC) samples

MPA (5 mg) was weighed in duplicate and dissolved in DMSO–methanol (25:75, v/v) at a concentration of 5.00 mg/mL to obtain stock solutions for calibration standards and QC samples. MPA-d_3 (1 mg) was dissolved in DMSO–methanol (25:75, v/v) at a concentration of 1.00 mg/mL. The working solutions for the calibration standards of MPA were prepared by appropriately diluting the stock solution with methanol–water (1:1, v/v) to achieve concentrations of 4, 10, 20, 40, 100, 200, 400, and 1000 μg/mL. The working solutions for QC samples were prepared as previously explained to obtain the concentrations of 4, 8, 80, and 800 μg/mL. Working solutions of IS were prepared by diluting the IS stock solution to 0.5 μg/mL of acetonitrile.

Standard calibration samples were generated by adding 2.5 μL of MPA standard solutions to 47.5 μL of drug-free human plasma, yielding final MPA concentrations of 0.20, 0.50, 1.00, 2.00, 5.00, 10.0, 20.00, and 50.00 μg/mL. Quality control (QC) sampleswere similarly prepared by spiking blank plasma with working solutions at four target concentrations: 0.20 μg/mL (lower limit of quantification, LLOQ), 0.40 μg/mL (low QC, LQC), 4.00 μg/mL (medium QC, MQC), and 40.00 μg/mL (high QC, HQC).

2.4. Sample preparation

The sample was prepared according to a previously published study [27]. First, 500 μL of IS solution was spiked into 50 μL of the plasma sample, followed by vortexing (2500 rpm, 3 min) and centrifugation (21,130 ×*g*, 15 min at 4 °C). Subsequently, 100 μL of the supernatant was transferred to a 1.5 mL centrifuge tube, after which 1 mL of acetonitrile was added. After vortexing and mixing, 1 μL of the obtained sample was injected into the LC–MS/MS system for analyte determination.

2.5. Method validation

The bioanalytical method was fully validated in accordance with the principles outlined in the "Bioanalytical Method Validation Guidance" issued by the Food and Drug Administration, European Medicines Agency, and the Chinese Pharmacopoeia Commission [28–30]. This ensured that all critical parameters, including selectivity, linearity, accuracy, precision, carry-over, matrix effects, recovery, dilution reliability, and stability, were rigorously evaluated and met the required criteria.

2.5.1. Selectivity

Selectivity was assessed by examining endogenous interfering substances in blank plasma samples from six different donors, ensuring no matrix interference signals appeared at the retention times corresponding to MPA and MPA-d3. The selectivity criterion was met if the interference signal remained below 20.0% of the mean LLOQ sample signal for MPA or below 5.0% of the average MPA-d3 signal in the LLOQ sample for internal standard interference.

2.5.2. Carry-over

To evaluate carry-over, blank samples were analyzed following injection of the upper limit of quantification (ULOQ) sample. The measured analyte response in these blank samples must remain below 20% of the lower limit of quantification (LLOQ), while the internal standard (IS) response should not surpass 5% of the LLOQ.

2.5.3. Linearity and sensitivity

Eight different concentrations of MPA in plasma (0.20, 0.50, 1.00, 2.00, 5.00, 10.0, 20.00, and 50.00 μg/mL) were newly prepared in two duplicates. The calibration curves were generated based on the ratios of peak areas (MPA/IS) as "y" versus the analyte concentration as "x," and the weighting factor was $1/X^2$. The correlation coefficient (R^2), which should not be < 0.9800, was used to evaluate goodness of fit. The deviations in the back-calculated concentrations were within 15% of the nominal value and 20% of the LLOQ. At least 75% of the calibrations satisfied the aforementioned acceptance criteria. Points that failed to meet the acceptance criteria were excluded, although the LLOQ or ULOQ of the calibration could not be excluded.

2.5.4. Accuracy and precision

Accuracy and precision were assessed using four levels of QC, namely 0.20 (LLOQ), 0.40 (LQC), 4.00 (MQC), and 400 μg/mL (HQC). Intra- and interbatch accuracy and precision were evaluated in three independent batches over 2 consecutive days. The deviations of each QC should be within ±15% of the nominal concentration and ±20% of the LLOQ. The precision of each QC sample should not exceed 15%, excluding the LLOQ, which should not exceed 20%.

2.5.5. Dilution integrity

Six aliquots of plasma samples were spiked at concentrations exceeding the upper limit of quantification (ULOQ) and subsequently subjected to a 10-fold dilution with blank plasma. For these diluted quality control samples, the mean measured accuracy was required to fall within ±15% of the theoretical concentration, with the inter-assay precision maintained below 15%.

2.5.6. Recovery and matrix effect

The recovery assessment was performed by analyzing the response ratio between extracted quality control (QC) samples and comparative samples spiked with the analyte and internal standard (IS) into blank extracts. These reference samples were prepared at identical concentration levels. The entire process was conducted in six replicates for each of the three tested concentrations: 0.40, 4.00, and 40 μg/mL. The recoveries of the three concentrations should not differ significantly, and the precision of the recovery at the three concentrations should be within 15.0%.

The matrix effect was evaluated by analyzing one replicate each at the LQC, MQC, and HQC, with each replicate prepared using a matrix of six different individuals. Plasma samples were prepared by adding the analyte and IS to blank extracts (A), and solution samples were prepared by adding the analyte and IS to a pure solution (B). The matrix factor (MF) of each lot was calculated by comparing the peak areas of A and B (A/B × 100%). The IS-normalized MF was calculated by dividing the MF of the analyte by that of the IS. The coefficient of variation (%CV) of the IS-normalized MF from the six individuals was within 15%. To further assess potential matrix effects, hemolytic and hyperlipidemic samples were specifically evaluated, as these

conditions were anticipated to arise during the study. The investigation of matrix effects for both hemolytic and hyperlipidemic matrices followed identical analytical procedures. Hemolytic matrices were generated by spiking whole blood into normal matrix specimens, while hyperlipidemic matrices were prepared through the addition of fat emulsion to normal matrix material.

2.5.7. Stability

Six replicates at two concentrations (LQC and HQC) were used to assess MPA stability. Simulating the processing and storage conditions of clinical samples, QC samples were evaluated under different conditions as follows: short-term stability (at room temperature for approximately 8 h), freeze–thaw stability (−80 °C to ambient temperature for five cycles), long-term stability (frozen at −80 °C for 45 days), and autosampler stability (extracted samples kept in the autosampler at 4 °C for 44 h). Additionally, the short-term (room temperature, 8 h) and long-term stability (−20 °C, 62 or 133 days) of stock solution and working solutions was compared to that of freshly prepared stock solution. The analyte was deemed stable if the observed variations remained within a ±15% range, with the precision also required to stay below 15%.

Matrix stability before sample centrifugation was assessed using fresh blood samples. Six replicates of low- and high-concentration blood samples were prepared and incubated at 37 °C for 30 min to allow equilibrium between plasma and blood cells. The blood samples were aliquoted at each concentration level. One part of the samples (control) was immediately centrifuged to obtain plasma, and the other part was centrifuged after incubation at room temperature for 4 h. The analyte was considered stable when the deviations were within ±15%, and the precision should not exceed 15%.

2.6. Application of the method

The bioanalytical method developed in this study was applied to clinical samples collected from 70 patients with FRNS/SDNS enrolled in the STAMP study (NCT04048161) between September 24, 2019 and July 30, 2021. The enrollment criteria were as follows: age 2–18 years, meeting the diagnostic criteria for NS [31], and no history of MMF use within the previous 2 years. This research complied with the ethical principles outlined in the Declaration of Helsinki and received formal approval from the Institutional Review Board at Zhejiang University School of Medicine Children's Hospital (ethics approval no. 2019-IEC-003). Blood samples were collected from pediatric patients before treatment and 0.5 and 2 h after treatment. Following centrifugation, the plasma supernatant was promptly aliquoted and cryopreserved at −80 °C for subsequent analysis. The mycophenolic acid (MPA) concentration-time curve exposure (AUC) was calculated using a validated limited sampling strategy. The formula was as follows:

MPA-AUC = 7.75 + (6.49 × C_{0h}) + (0.7 6 ×$C_{0.5h}$) + (2.43 × C_{2h}) [32, 33].

The plasma MPA-AUC was maintained at 30–50 μg·h/mL for the first 6 months of treatment and at ≤40 μg·h/mL for the next 6 months. The MPA-AUC was measured at the second visit (7 ± 3 days). The medication was adjusted and repeatedly administered until the MPA-AUC was within the concentration range (30–50 μg·h/mL). If the concentration range could not be adjusted, the visit was canceled.

2.7. Data analysis

Data processing and statistical analyses were performed with R software version 4.0.5 (R Core Team, Vienna, Austria), with continuous variables expressed as either medians or mean ± standard deviation values. Intergroup

differences for continuous data were assessed using Student's t-test, while one-way ANOVA served for three-group comparisons, and repeated-measures ANOVA analyzed serial measurements from identical subjects. Kaplan-Meier curve plotting provided detailed visualization of MPA-AUC correlations with clinical endpoints, supplemented by univariate and multivariate Cox regression models evaluating MPA-AUC's predictive capacity for adverse events, with all analyses employing two-tailed testing and statistical significance defined as P-values below 0.05.

3. Results

3.1. Method development

In this study, MPA-AUC needed to be maintained at 30–50 μg·h/mL during the induction period (the first 6 months after drug treatment) and at ≤ 40 μg·h/mL during the maintenance period (gradually reducing drug treatment while ensuring patient stability). A previous study has revealed that the treatment range of AUC_{0-12h} is mostly 30–60 μg·h/mL, with a steady-state valley concentration range of mostly 1.0–3.5 μg/mL [34]. The C_{max} of plasma MPA levels in patients who received kidney transplant is 25.6 ± 22.1 μg/mL [35]. Accordingly, the linear range was set at 0.2–50 μg/mL. Although the method is not particularly sensitive (LLOQ of 0.20 μg/mL for MPA), it was appropriate for clinical practice.

In addition, the test samples used in this study were plasma samples from pediatric patients. For method validation and sample testing, a healthy adult blank plasma matrix was used instead of a pediatric blank plasma matrix to prepare calibration and QC samples for quantification, considering children's health and clinical applicability. To develop this method, adult and pediatric plasma was compared in an experiment. In the analysis batch, calibration samples were prepared using an adult blank plasma matrix, and six replicates of QC samples at four concentration levels (LLOQ, LQC, MQC, and HQC) were prepared using a pediatric blank plasma matrix to evaluate within-run accuracy and precision. The accuracy deviation at each concentration and precision were −5.8%–5.4% and 1.0%–7.1%, respectively, which were both within the acceptance criteria. Additionally, IS responses in zero samples (n = 6) prepared using the two plasma matrices were compared. The deviation in the peak area of the IS in adult and pediatric plasma was 0.5%, with a deviation not exceeding 15%. This indicates that a healthy adult blank plasma matrix can replace a pediatric blank plasma matrix to determine MPA concentration in the plasma of pediatric patients.

3.2. Method validation

3.2.1. Selectivity

Selectivity was evaluated using blank plasma samples from six individuals; the interference of the analyte and IS were < 6.4% and < 0.1%, respectively. Additionally, typical chromatograms of blank plasma samples, blank plasma samples with IS, LLOQ samples, and clinical samples are presented in **Figure 3**, indicating a lack of interference in the MPA analysis.

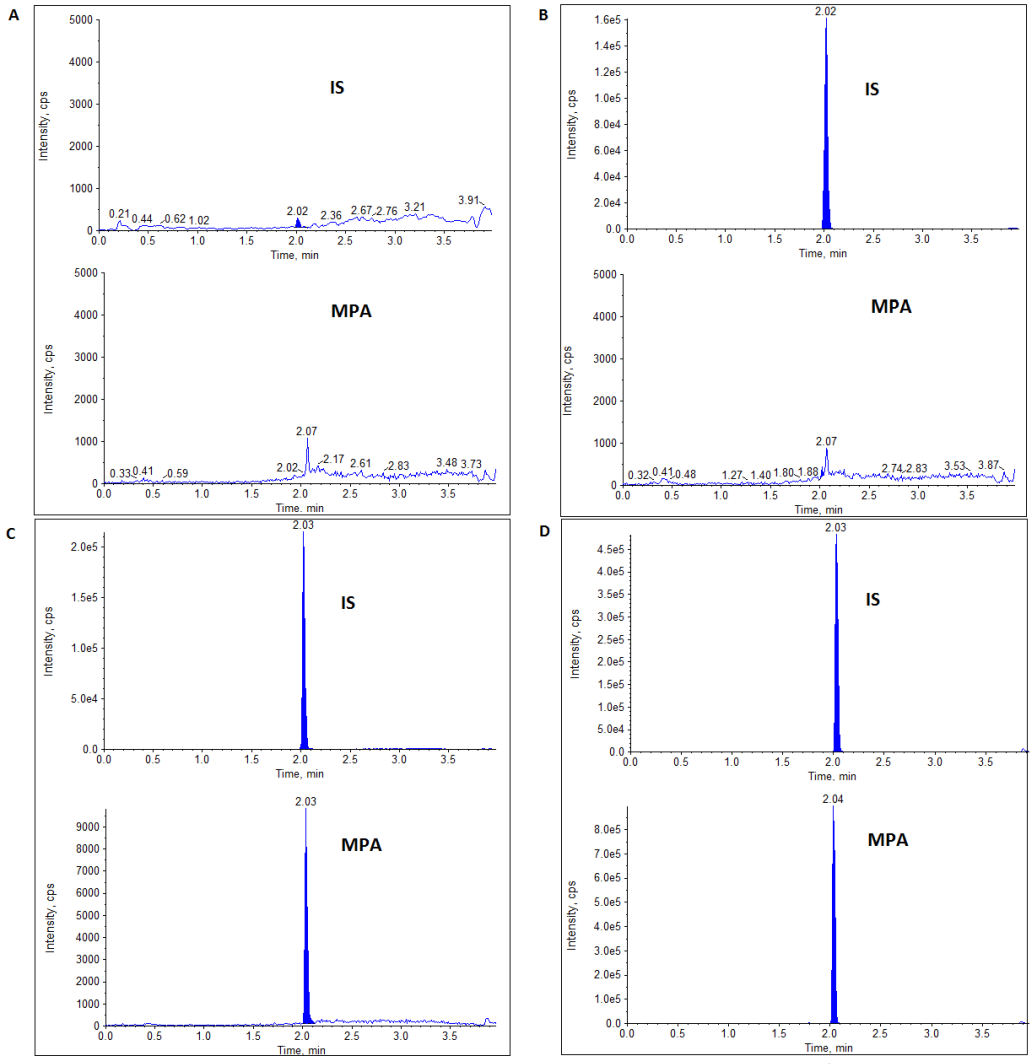

Figure 3. Typical multiple reaction monitoring chromatogram of mycophenolic acid (MPA) and internal standard (IS) in human plasma; (A) Blank plasma sample; (B) Blank plasma sample with IS added; (C) Lower limit of quantification sample; (D) Unknown clinical plasma sample of a patient.

3.2.2. Carry-over

The carry-over in the blank samples following the highest concentration calibration was < 20% and 5% of the LLOQ for the analyte and IS, respectively. Thus, the carry-over did not affect the MPA analysis.

3.2.3. Linearity and sensitivity

Linearity was investigated in each batch, excluding the batch used to evaluate the solution stability. The MPA exhibited good linearity over 0.2–50 μg/mL. The LLOQ of MPA was 0.2 μg/mL.

3.2.4. Accuracy and precision

The intra- and inter-batch accuracies and precision of the MPA are summarized in **Table 1**. For intra-batch accuracy, the deviation of LLOQ ranged from −10.0% to −5.0%, and the deviation of LQC/MQC/HQC ranged from −2.5% to 11.0%. For inter-batch accuracy, the deviation of LLOQ was −7.5%, and the deviation of LQC/

MQC/HQC ranged from −0.1% to 4.7%. The intra- and inter-batch precisions of the LLOQ were within 11.1% and 7.3%, respectively, and those of the LQC/MQC/HQC were within 2.4% and 5.5%, respectively.

Table 1. The accuracy and precision of MPA in human plasma.

Group		LLOQ (0.2μg/ml)	LOQ (0.4μg/ml)	MQC (4μg/ml)	HQC (40μg/ml)
Batch 1 (n=6)	Mean (μg/ml)	0.19 ± 0.02	0.43 ± 0.01	4.05 ± 0.04	44.4 ± 0.32
	RE (%)	-5.0	7.5	1.3	11.0
	CV%	10.5	2.3	1.0	0.7
Batch 2 (n=6)	Mean (μg/ml)	0.19 ± 0.00	0.41 ± 0.01	3.90 ± 0.02	41.87 ± 0.27
	RE (%)	-5.0	2.5	-2.5	4.7
	CV%	0.0	2.4	0.5	0.6
Batch 3 (n=6)	Mean (μg/ml)	0.18 ± 0.02	0.42 ± 0.01	4.04 ± 0.04	39.06 ± 0.23
	RE (%)	-10.0	5.0	1.0	-2.4
	CV%	11.1	2.4	1.0	0.6
Inter-batch (n=18)	Mean (μg/ml)	0.19 ± 0.01	0.42 ± 0.01	4.00 ± 0.08	41.78 ± 2.26
	RE (%)	-7.5	4.7	-0.1	4.4
	CV%	7.3	2.9	1.9	5.5

CV: Coefficient of Variation; HQC: High Quality Control; LLOQ: Lower Limit of Quantification; LOQ: Low Quality Control; MQC: Medium Quality Control; MPA: Mycophenolic Acid; RE: Relative Error.

3.2.5. Dilution integrity

After a 10-fold dilution, the accuracy of the diluted QC samples was 3.8%, and the precision was 0.8%. This proved that a dilution factor of 10 was reliable when the concentration of clinical human plasma exceeded the ULOQ of the calibration.

3.2.6. Recovery and matrix effect

At the three different concentrations, the recoveries of MPA were stable and consistent. The average recoveries of the analytes were 97.5%, 98.1%, and 101.7% at low, medium, and high concentrations, respectively. The recovery precision at these concentrations was 2.3%.

As presented in **Table 2**, the matrix effect was evaluated using IS-normalized MF, and the CV% values of the IS-normalized MFs were within 4.9%, 2.7%, and 4.2% in the normal, hemolytic, and hyperlipid matrices, respectively. The results indicated the absence of a significant matrix effect in different matrices, including the hemolytic and hyperlipid matrices.

Table 2. The recovery and matrix effects of MPA in human plasma (n = 6).

	Recovery		MF (normal)		MF (hemolytic)		MF (hyper-lipid)	
Concentration	Mean (%)	CV%	Mean	CV%	Mean	CV%	Mean	CV%
LQC (0.4 μg/ml)	97.5		0.752900	4.9	0.637041	2.4	0.679417	3.5
MQC (4 μg/ml)	98.1	2.3	0.895183	3.3	0.796858	2.7	0.863574	4.2
HQC (40 μg/ml)	101.7		0.994396	4.8	0.918444	1.4	0.991894	2.4

CV: Coefficient of Variation; HQC: High Quality Control; LQC: Low Quality Control; MPA: Mycophenolic Acid; MQC: Medium Quality Control.

3.2.7. Stability

The stability of MPA in human plasma and solutions was investigated to cover the expected handling process in a clinical analysis, and the results are presented in **Table 3**.

Table 3. The stability of MPA and IS under different conditions.

Matrix	Storage and process condition	Nominal concentration	Average stability (%)	CV%
Plasma	Short-term stability (8 h, room temperature)	LQC (0.4 μg/ml)	100.4	2.5
		HQC (40 μg/ml)	99.4	2.6
	Freeze-thaw stability (5 cycle, -80°C)	LQC (0.4 μg/ml)	106.3	3.6
		HQC (40 μg/ml)	104.2	0.3
	Long-term stability (45 days, -80°C)	LQC (0.4 μg/ml)	107.5	1.5
		HQC (40 μg/ml)	101.7	1.0
	Autosampler stability (44 h, 4°C)	LQC (0.4 μg/ml)	100.4	1.9
		HQC (40 μg/ml)	101.2	0.9
Blood	Blood stability (4 h, room temperature)	0.4 μg/ml	101.3	1.5
		40 μg/ml	98.5	1.3
Solution	MPA stock solution (62 days, -20°C)	5 mg/ml	87.1	0.2
	MPA stock solution (8 h, room temperature)	5 mg/ml	100.2	0.7
	MPA working solution (62 days, -20°C)	4 μg/ml	99.5	0.7
		1000 μg/ml	89.7	1.1
	MPA working solution (8 h, room temperature)	4 μg/ml	105.7	0.6
		1000 μg/ml	100.3	0.7
	IS stock solution (133 days, -20°C)	1 mg/ml	92.9	4.8
	IS stock solution (8 h, room temperature)	1 mg/ml	101.2	2.0
	IS working solution (62 days, -20°C)	0.5 μg/ml	91.3	5.2
	IS working solution (8 h, room temperature)	0.5 μg/ml	102.1	2.5

CV: Coefficient of Variation; HQC: High Quality Control; IS: Internal Standard; LQC: Low Quality Control; MPA: Mycophenolic Acid.

The stock solutions of MPA and IS were stable after storage at −20 °C for 62 and 133 days, respectively. The cells were stable after incubation at room temperature for 8 h. The working solutions of MPA and IS were stable after storage at −20 °C for 62 days or incubation at room temperature. In the whole blood samples, MPA remained stable for 4 h. In addition, the plasma samples of LQC and HQC remained stable at room temperature for 8 h. The extracted samples were stable after storage for 44 h at 4 °C. Plasma samples were subjected to five freeze–thaw cycles (from −80 °C to room temperature) or stored at −80 °C for at least 45 days.

3.3. Analysis of the clinical data from pediatric patients with FRNS/SDNS

Seventy patients were enrolled between November 2019 and July 2021, and one patient was excluded because of lack of information regarding the exact interval of the initial adverse event. Therefore, 69 children were included in the statistical analysis. The patient information is presented in **Table 4**. The follow-up duration ranged from 9 days to 371 days (median, 180 days). Any side effect reported after MMF administration was defined as an MMF-related adverse event, and its occurrence was considered the endpoint.

Table 4. Clinical data of pediatric patients with FRNS/SDNS.

Variables	Values
Gender (male/female)	52/18
Age (years)	5.99 (2.06, 16.53)
BMI	18.43 ± 3.95
Systolic pressure (mmHg)	105.34 ± 10.23
Diastolic pressure (mmHg)	64.33 ± 9.55
Course of disease (months)	14.4 (3.0, 147.6)
Number of recurrences in the previous 1 year	2.73 ± 1.43
Number of recurrences in the previous 6 months	2.31 ± 1.44
eGFR(ml/min/1.73m^2)	162 (98.09, 277.54)
Urine protein creatinine ratio (mg/mg)	0.12 (0, 15.17)
Lymphocyte percentage (%)	38.4 (0.29, 68.7)
White blood cell count (10^9/L)	10.52 ± 3.51
Total cholesterol (mmol/L)	6.43 ± 1.97
Triglyceride (mmol/L)	1.90 ± 1.05
Hemoglobin (g/L)	139.89 ± 20.19
albumin (g/L)	33.79 ± 8.11
24h urinary protein (mg/24h)	89.2 (0, 2321.8)
MPA-AUC (V2 visit)	40.05 ± 15.64

BMI: Body Mass Index; eGFR: Estimated Glomerular Filtration Rate; FRNS: Frequently Relapsing Nephrotic Syndrome; MPA-AUC: Mycophenolic Acid Area Under the Concentration–Time Curve; SDNS: Steroid-Dependent Nephrotic Syndrome.

Among them, 42 reached the endpoint and reported adverse events. Patients were allocated to adverse or non-adverse event groups. The distribution of MPA-AUC in these two groups is shown in **Figure 4**. The MPA-AUC in the non-adverse event group was centered near 30 μg·h/mL, whereas the distribution of MPA-AUC was more dispersed in the adverse event group, suggesting that MPA-AUC is correlated with the onset of adverse events.

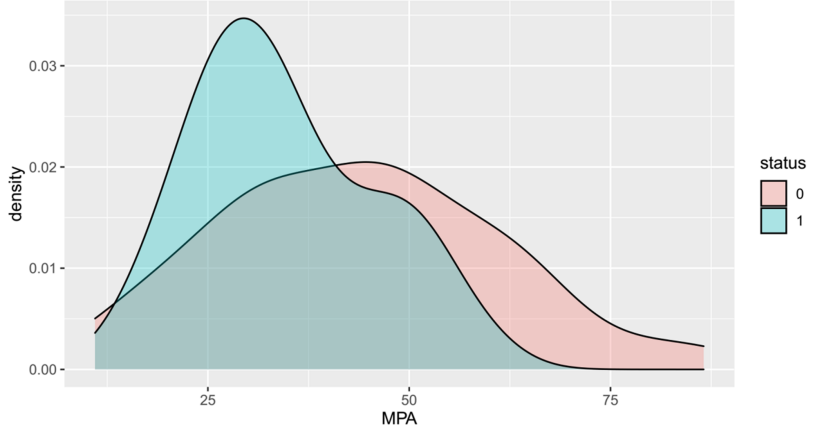

Figure 4. MPA-AUC distribution in the adverse event and non-adverse event groups (0: non-adverse event group; 1: adverse event group). MPA-AUC, area under the concentration–time curve of mycophenolic acid.

3.4. Patient survival analysis

To elucidate the relationship between MPA-AUC and the endpoints in more detail, Kaplan–Meier curves were constructed (**Figure 5**).

The cutoff value was set to 40.05 μg·h/mL based on the average AUC value of MPA. The patients were allocated to low- and high-MPA-AUC groups, where 17 and 24 patients reached the endpoint, respectively.

As shown in **Figure 5**, the incidence of cumulative adverse events differed significantly between the two groups. The log-rank test revealed that the frequency of adverse events was higher in the high MPA-AUC group than in the low MPA-AUC group ($P = 0.035$).

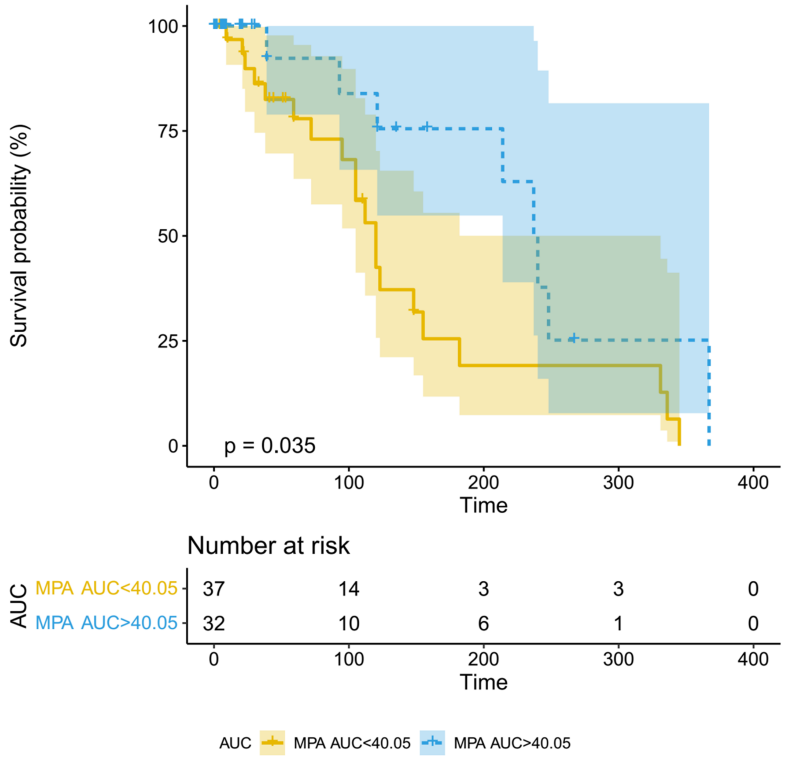

Figure 5. Kaplan–Meier survival curve analysis in the high and low MPA-AUC groups. MPA-AUC, area under the concentration–time curve of mycophenolic acid.

3.5. Univariate and multivariate regression analyses of adverse events in pediatric patients with FRNS/SDNS

To further explore whether the MPA-AUC can be used to predict adverse events, univariate and multivariate Cox regression analyses were performed. In the univariate analysis, the effects of the MPA-AUC and multiple baseline variables on the onset of adverse events is analyzed (**Table 4**). Among all 18 variables, two ($P < 0.05$) significant covariates is obtained, including MPA-AUC ($P = 0.04$, hazard ratio [HR] = 0.4, 95% confidence interval [CI] = 0.17–0.96, **Table 5**). Specifically, a positive relationship was observed between MPA-AUC and the incidence of adverse events. Albumin level was another possible covariate associated with the endpoint ($P = 0.049$; HR = 1.1; 95% CI = 1–1.1).

Table 5. Single-factor Cox proportional hazard regression analysis.

	P value	HR (95%CI)
Gender	0.9	1 (0.9–1.1)
Age (years)	0.99	1 (0.4–2.5)
BMI	0.9	0.99 (0.91–1.1)
Systolic pressure (mmHg)	0.3	0.98 (0.93–1)
Diastolic pressure (mmHg)	0.39	0.98 (0.92–1)
Course of disease (months)	0.84	1 (0.99–1)
Number of recurrences in the previous 1 year	0.46	0.93 (0.76–1.1)
Number of recurrences in the previous 6 months	0.18	0.8 (0.58–1.1)
eGFR (ml/min/1.73m^2)	0.42	1 (0.99–1)
Urine protein creatinine ratio (mg/mg)	0.45	0.85 (0.54–1.3)
Lymphocyte percentage (%)	0.57	0.99 (0.97–1)
White blood cell count (10^9/L)	0.64	1 (0.92–1.2)
Total cholesterol (mmol/L)	0.25	0.89 (0.73–1.1)
Triglyceride (mmol/L)	0.57	0.9 (0.64–1.3)
Hemoglobin (g/L)	0.052	0.96 (0.93–1)
Albumin (g/L)	0.049	1.1 (1–1.1)
24h urinary protein (mg/24h)	0.75	1 (1–1)
MPA-AUC (V2 visit)	0.04	0.4 (0.17–0.96)

BMI: Body Mass Index; CI: Confidence Interval; eGFR: Estimated Glomerular Filtration Rate; HR: Hazard Ratio; MPA: Mycophenolic Acid; MPA-AUC: Mycophenolic Acid Area Under the Concentration–Time Curve.

Multivariate Cox regression analysis (**Table 6**) revealed a stronger correlation with adverse events in the high MPA-AUC group than in the low MPA-AUC group ($P = 0.023$, HR = 0.33, 95% CI = 0.13–0.86). Serum albumin levels also differed significantly between the groups ($P = 0.026$; HR, 1.07; 95% CI = 1.01–1.13).

Table 6. Multivariate Cox regression analysis.

	P value	HR (95%CI)
Albumin (g/L)	0.026	1.07 (1.01–1.13)
MPA-AUC	0.023	0.33 (0.13–0.86)

CI: Confidence Interval; HR: Hazard Ratio; MPA-AUC: Mycophenolic Acid Area Under the Concentration–Time Curve.

A high MPA-AUC independently predicted the risk of adverse events in pediatric patients with FRNS/SDNS, suggesting its use as a reference value to predict adverse event onset (**Tables 5** and **6**).

4. Discussion

Idiopathic NS is a common glomerular disease in children. High MPA levels are associated with an increased

risk of adverse events in children with FRNS/SDNS, indicating that plasma MPA concentrations should be monitored to prevent adverse events [12]. MMF is frequently used to treat pediatric patients with NS [7, 32]. However, the suitable dose of MPA for pediatric patients remains controversial. Accumulating data indicate that MPA underexposure leads to an insufficient treatment response, whereas excessive doses can increase the risk of adverse events (e.g., abdominal pain, diarrhea, and cytopenia) [32, 33, 36–39]. In the present study, a high MPA-AUC was linked to an increased risk of adverse events in children with FRNS/SDNS, which is consistent with previous findings [40]. Therefore, an effective method for monitoring plasma MPA concentration is necessary.

This study successfully developed a convenient and accurate method using UHPLC–MS/MS for plasma MPA determination that is applicable to MPA monitoring in pediatric patients with NS. This method adopts negative ion and MRM mode, with strong specificity and high sensitivity. Using Agilent Elipse Plus C_{18} (2.1 mm × 50 mm, 1.8 μm) as the analytical column for compound separation, the analyte and internal standard can be well separated from interfering impurities on this chromatographic column. The addition of 0.1% formic acid to the aqueous mobile phase and gradient elution improved the peak shape of MPA by precipitating the protein, which was simple and cost-effective. The LLOQ was 0.20 μg/mL, which is a relatively high concentration. Considering the mass spectrometry response of sample detection, after 10-fold precipitation of plasma samples, 100 μL of supernatant was obtained and diluted with 1 mL of acetonitrile to reduce the concentration of the injection solution and obtain a more suitable mass spectrometry response. Future improvements could involve exploring alternative chromatographic conditions or sample preparation techniques to further lower the LLOQ and enhance overall method performance.

UHPLC–MS/MS technology combines the high efficiency of UHPLC with the high sensitivity and specificity of MS/MS, delivering an extremely high separation efficiency and remarkable sensitivity [41]. The improved resolution offered by UHPLC represents a significant advantage when coupled with MS/MS detection. This combination is particularly beneficial when analyzing complex samples such as pediatric NS. While HPLC maybe adequate for many bioassays, UHPLC–MS/MS offers superior resolution, sensitivity, and faster analysis. By contrast, HPLC is more suitable for separating compounds from complex mixtures, although it may be limited in terms of quantitative precision and sensitivity. Immunoassays are based on the specificity of antigen-antibody reactions, offering simplicity and speed. However, sensitivity and specificity are often limited by the quality of the antibody and complexity of the target [42]. As immunoassays are inexpensive, rapid, and convenient, they are widely used in clinical laboratories.

However, plasma MPA levels are often overestimated in immunoassays because of cross-reactivity and metabolite interference [43]. In conclusion, UHPLC–MS/MS excels in analysis speed, specificity, sensitivity, accuracy, stability, and the ability to perform simultaneous multi-analyte analysis, making it the preferred method for the quantitative analysis of small molecules. However, the choice of the method should be based on specific equipment requirements and conditions. As UHPLC is more sensitive and provides higher specificity, it is the preferred method for effectively monitoring plasma MMF concentrations. In this study, a novel method was developed. Negative ion detection was selected, and the standard curve range was 0.2–50 μg/mL. The precision and accuracy of this method were within acceptable ranges, indicating that it is reliable and reproducible for the detection of plasma MPA. Additionally, high accuracy was achieved using samples with high MPA concentrations that were diluted up to 10-fold. The stability test data suggested that MPA was stable over time, demonstrating that this work establishes an effective and applicable method for large-scale sample analysis. Furthermore, this study successfully used the method developed for the pharmacokinetic monitoring

of MPA and therapeutic drug concentrations in biological matrices among adult and pediatric patients with NS.

5. Conclusion

High MPA-AUC was associated with a higher risk of adverse events in children with FRNS/SDNS. This study identified a convenient and accurate LC–MS/MS method for quantifying MPA levels in the plasma of pediatric patients with NS that can facilitate MPA pharmacokinetic studies and therapeutic MPA drug concentration monitoring. This method could provide a basis for the individualized and specialized treatment of NS in children.

Disclosure statement

The authors declare no conflict of interest.

Ethical disclosure

This study was approved by the Medical Ethics Committee of the Children's Hospital affiliated with Zhejiang University School of Medicine (approval no. 2019-IRB-064). This study was conducted in accordance with the principles of the Declaration of Helsinki.

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