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Determination of 5-HT receptor antagonists, MEFWAY and MPPF using liquid chromatography electrospray ionization tandem mass spectrometry in rat plasma and brain tissue

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ABSTRACT

A simple, selective, and sensitive liquid chromatography electrospray ionization tandem mass spectrometry (LC–ESI-MS/MS) method was validated for the determination of 4-fluoromethyl-N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-N-(2-pyridyl)cyclohexane-1-carboxamide (MEFWAY) and 4-fluoro-N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-N-(2-pyridyl)benzamide (MPPF) in rat plasma and brain samples, respectively. Plasma and brain samples were extracted with a mixture of acetonitrile and methanol (1:1, v/v) and then separated on a C_{18} column (Gemini 3 μm 110 Å, 50 × 2.00 mm ID, Phenomenex, USA). Quantitation was performed using LC–ESI-MS/MS in multiple-reaction monitoring (MRM) mode with positive ion electrospray ionization (ESI). The limit of quantification (LOQ) of 5 ng/mL and 1 ng/mL were obtained in 50 μL brain homogenate and plasma, respectively. The analytical linear ranges of this method were 1–4000 ng/mL in plasma and 5–4000 ng/mL in brain homogenate with a correlation coefficients (R^2) greater than 0.9993. The intra- and inter-day precision and accuracy values were within the assay validation guideline (lower than 13.0%). The analytes in plasma and brain samples were stable after three freeze–thaw cycles, long-term storage (one month at −80 °C), and short-term (4 h) storage at room temperature. The present method was successfully applied to plasma-brain pharmacokinetic studies to investigate brain penetration of a single dose of MEFWAY and MPPF in rats.

1. Introduction

The successful treatment of central nervous system (CNS) disorders is associated with a variety of factors, including the complexity of the brain, the probability that CNS drugs may cause side effects, and the requirement of CNS drugs to cross the blood–brain barrier (BBB) [1]. It has been challenging to determine the concentration of new chemical entities (NCEs) in the brain to establish the physiological relationship between blood and regional brain levels in preclinical and clinical pharmacokinetic/pharmacodynamics (PK/PD) studies. Because transport across the BBB does not occur instantaneously [2], a highly sensitive and selective method is required to monitor therapeutic efficacies and drug levels in both the brain and blood *via* their simultaneous determination.

The liquid chromatography–tandem mass spectrometry (LC–MS/MS) for determination of small molecules in biological fluids has become a useful method in preclinical and clinical stages of drug discovery and development [3–5]. During the last decade, electrospray ionization (ESI)-MS has become a common analytical tool following liquid chromatography (LC) separation due to its sensitivity, broad availability, and applicability [6]. However, the acceptability of the method should be evaluated based on accuracy, precision, selectivity, sensitivity, reproducibility, stability, and application [7].

It is known that 4-fluoromethyl-N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-N-(2-pyridyl)cyclohexane-1-carboxamide (MEFWAY) and 4-fluoro-N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-N-(2-pyridyl)benzamide (MPPF) are 5-HT_{1A} receptor antagonists that permit the determination of 5-HT_{1A} receptor density and the identification of the initial stages of psychiatric symptoms in the CNS [8–10]. To compare the brain distribution and efflux transport between MEFWAY and MPPF, a highly sensitive and selective analytical method for brain tissue is required. To the best of our knowledge, high-performance liquid chromatography (HPLC) or LC–MS/MS have not been applied to monitor MEFWAY or MPPF. In this study, we developed and validated a specific, selective, and reliable LC–ESI-MS/MS analytical method to measure MEFWAY and MPPF concentrations. The method is simple,

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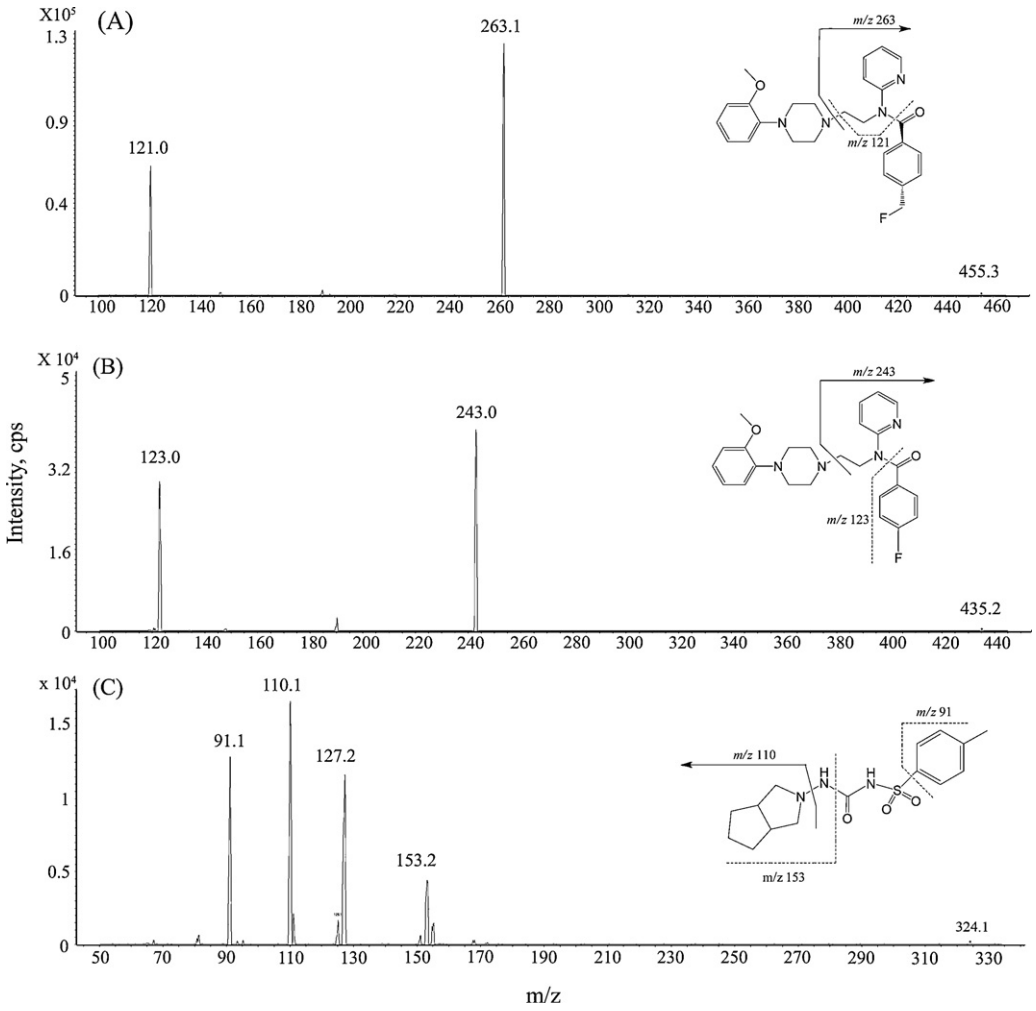

Fig. 1. Product ion mass spectra of MEFWAY ([M+H]$^+$ m/z 455.3 → 263.1) (A), MPPF ([M+H]$^+$ m/z 435.2 → 243.0) (B) and gliclazide (IS) (C), ([M+H]$^+$ m/z 324.1 → 110.1) in positive ionization mode.

sensitive, and rapid and uses plasma and brain homogenates. The applicability and reliability of LC–ESI-MS/MS analysis was successfully evaluated *in vivo* in pharmacokinetic and brain distribution studies using both MEFWAY and MPPF.

2. Materials and methods

2.1. Chemicals and reagents

MEFWAY (purity > 98%) and MPPF (purity > 98%) were synthesized by the department of nuclear medicine (Yonsei University, College of Medicine, Seoul, Korea). The internal standard gliclazide was purchased from Sigma–Aldrich (St. Louis, MO, USA). The structures of all compounds are shown with their mass spectra in Fig. 1. HPLC-grade acetonitrile, methanol, and water were purchased from J.T. Baker (Phillipsburg, NJ, USA). Pooled blank rat plasma containing EDTA as an anticoagulant was purchased from BioChemed (Winchester, VA, USA).

2.2. In vivo pharmacokinetic study

Fifteen male Sprague-Dawley rats (7 weeks of age, body weight 251 ± 4.2 g, NARA-Bio Company, Pyungtaek, Korea) were divided into five groups of three for each compound. The rats were housed under standard laboratory conditions (temperature 24 ± 3 °C, humidity 50 ± 3%, 12-h day/night cycles). Prior to the experiments, the animals were allowed to acclimate to the facility for 1 week and were provided with a standard chow diet and drinking water. The rats were anesthetized by intraperitoneal injection of Zoletil (Virbac, 50 mg/kg) and underwent surgery for catheterization. A catheter (polypropylene tubing, 0.58 mm ID, 0.965 mm OD; PE50, Becton Dickinson, Parsippany, NJ) was inserted into the left femoral vein of the rat. This intravenous catheter was used for intravenous administration of the compound and for blood aspiration. After a 1-day recovery period, a single dose of 5 mg/kg of MEFWAY and MPPF in a vehicle solution containing dimethylsulfoxide (DMSO), PEG400, and water (5:40:55, v/v/v) was intravenously administered in the animals. The femoral vein blood (about 0.2 mL) and brain were collected at intervals of 2 min, 0.5, 1, 2, and 4 h after oral dosing. The venous blood samples were centrifuged at 13,000 rpm for 3 min at 4 °C and then the plasma samples were collected. The isolated, clean brains were washed three times using cold saline solution, blotted dry with tissue, weighed, and then stored at −80 °C until LC–MS/MS analysis. All animal procedures were approved by the KRICT Animal Care and Use Committee.

2.3. LC–MS/MS analytical system

The LC system for LC–ESI-MS/MS analysis consisted of a pump, an autosampler, and a system controller (Agilent 1260 Series system; Agilent Inc., Santa Clara, CA, USA). The separation was performed on a Gemini C_{18} column (3 μm,

50 × 2.00 mm; Phenomenex, Torrance, CA, USA) using a mixture of acetonitrile–methanol and water (40:40:20, v/v/v) at a flow rate of 0.3 mL/min. The column and autosampler tray were maintained at 40 and 4 °C, respectively. The analytical run time was set at 1.8 min, and the analytes and eluent were introduced directly into the tandem quadrupole mass spectrometer (Agilent 6460A QQQ LC-MS/MS; Agilent Inc.). The system was controlled using MassHunter software (Agilent Inc.). Nitrogen was used as the nebulizer gas; the nebulizer pressure was set at 15 psi, and the sheath gas temperature was set at 400 °C. Desolvation gas (nitrogen) was heated to 350 °C and delivered at a flow rate of 8 L/min. Capillary voltage was 3500 V for each compound. The optimized collision energies of 22 and 14 were used for both target compounds and gliclazide as an internal standard. Typical product ion scan spectra for the MEFWAY, MPPF, and internal standard are shown in Fig. 1. Quantification was performed using the multiple-reaction monitoring (MRM) mode at m/z 455.3 → 263.1 for MEFWAY, m/z 435.2 → 243.0 for MPPF, and m/z 324.1 → 110.1 for the internal standard. The fragmentor energy of the MS was set at 155 and 115 V for both target compounds and gliclazide, respectively.

2.4. Preparation of stock solutions and standards

The standard stock solutions of both target compounds and the internal standard were prepared in DMSO to final concentrations of 1 mg/mL. Working standard solutions of compounds were prepared by diluting each primary solution with 1× phosphate buffered saline (PBS). A working solution of the internal standard (20 ng/mL) was prepared by diluting an aliquot of stock solution with acetonitrile. All prepared solutions were stored at −20 °C in polypropylene bottles in the dark until they were used. The two calibration standards, 1, 5, 10, 50, 100, 500, 1000, and 4000 ng/mL (plasma) and 5, 10, 50, 100, 500, 1000, and 4000 ng/mL (brain), were prepared by spiking appropriate amounts of the working standard solution into drug-free rat plasma and brain homogenate. Quality control (QC) samples at 5, 50, 500, 1000, and 4000 ng/mL were prepared in bulk by adding the appropriate working standard solution to pooled drug-free rat plasma. The QC samples were aliquoted (50 μL) into glass tubes and stored at −20 °C until analysis.

2.5. Sample preparation

2.5.1. Plasma samples

For sample preparation by protein precipitation, aliquots of precisely 50 μL of blank rat plasma, calibration standards, or QC samples were directly added with 350 μL of acetonitrile–methanol mixed solution (1:1, v/v) containing 20 ng/mL of the internal standard, gliclazide. The mixture was then vortex-mixed for 5 min and centrifuged at 13,000 rpm for 5 min. Then, 100 μL from the supernatants were transferred into a new 96-well plate, and 5 μL of the solution were injected into the LC–ESI-MS/MS analytical system.

2.5.2. Brain samples

Brain flesh was cleaned by replacing the blood with a perfusate of 1× PBS. The brain was washed three times with water and the clean brain tissue was prepared by homogenizing in 1× PBS buffer (1:3, w/v). Homogenization was conducted in an ice bath using an ultrasonic probe for 20 s. The homogenization aliquots were stored at −80 °C prior to analysis. To extract both target compounds from the brain homogenate, an cold acetonitrile–methanol (1:1, v/v) precipitation procedure was used. A total of 50 μL of homogenate was added to a 10 times volume of acetonitrile–methanol (1:1, v/v), vortexed for 5 min and centrifuged. Supernatant samples (100 μL) were carefully transferred onto a 96-well plate for LC/MS/MS analysis.

2.6. Method validation

According to the FDA guidance for bioanalytical method validation, the method was validated for specificity, sensitivity, linearity, intra- and inter-day precision and accuracy, and stability [11].

2.6.1. Specificity and sensitivity

The signal-to-noise (S/N) ratio was evaluated by analyzing five separate blank sample matrices for interference. The limit of quantification (LOQ) was defined as the lowest concentration of standard that yielded an S/N ratio ≥ 3 with allowable accuracy and precision (<15%). The LOQ was determined by analyzing five replicates of samples spiked with each analyte.

2.6.2. Precision and accuracy

To evaluate the precision and accuracy of the method, two QC samples at 5, 50, 500, 1000, and 4000 ng/mL (low, middle, and high concentrations) in plasma and brain homogenate were analyzed in sets of six replicates to evaluate the intra- and inter-day precision and accuracy. The percentage deviation of the mean from the estimated concentration, expressed as the relative error (RE) and the coefficient of variation (CV), serve as measures of accuracy and precision, respectively.

2.6.3. Stability

The purpose of stability testing was to investigate the changes in target compound concentration under all possible conditions during shipping and handling. Five replicates of two QC samples (low and high) in plasma and brain homogenate were subjected to three freeze–thaw cycles or were stored at room temperature for 1 day before processing to evaluate the effects of three freeze–thaw cycles and room-temperature matrix stability. The long-term stability was evaluated by measuring three aliquots of QC samples stored at −80 °C for one month. To assess the post-preparative stability, three replicates of QC samples at each of the concentrations were processed and stored under autosampler conditions at 4 °C for 1 day.

2.6.4. Pharmacokinetic data analysis

Pharmacokinetic calculations were performed on each individual set of data using the pharmacokinetic software WinNonlin standard edition version 5.3 (Pharsight Corp., Mountain View, CA, USA) using a non-compartmental method. Pharmacokinetic parameters C_{max} (maximum concentration), $t_{1/2}$ (terminal half-life), T_{max} (time to reach C_{max}), $AUC_{0\text{-time}}$ (areas under the curve from time 0 to the last measured concentration calculated), and CL (clearance) were obtained in rat plasma and brain. The brain/plasma ratio was calculated as $(AUC_{brain,\ 0\text{-time}})/(AUC_{plasma,\ 0\text{-time}})$.

3. Results and discussion

3.1. Method development

For optimization of sample preparation procedures, liquid–liquid extraction (LLE), solid-phase extraction (SPE), and liquid–liquid extraction (LLE) combined with solid-phase extraction (SPE) methods were also applied and compared with the protein precipitation method. In all methods, the recoveries of MEFWAY and MPPF were over 80% and ethyl acetate extraction method combined with SPE (Oasis HLB μElution Plate 30 μm; Waters Corp., Milford, MA, USA) showed highest recoveries (up to 99%, data not shown). However, this method is expensive and time-consuming although it was useful for measuring both MEFWAY and MPPF. For the easy sample preparations, one-step protein precipitation using acetonitrile has been applied to extract

Table 1
Recovery for MEFWAY, MPPF and gliclazide in quality control samples.

Analytes		Plasma (ng/mL)		Brain (ng/mL)	
		5	500	5	500
MEFWAY	A	61,092	2,307,058	55,679	1,834,353
	B	66,521	2,380,512	65,809	2,387,926
	Recovery[a] (%)	92	91	84	82
	CV[b] (%)	1.3	1.7	4.0	1.7
MPPF	A	32,663	1,436,266	29,184	1,240,264
	B	35,400	1,501,898	36,357	1,490,295
	Recovery[a] (%)	92	95	80	83
	CV[b] (%)	3.2	1.0	3.5	2.0
Gliclazide (20 ng/mL)	A	19,690,256		18,883,746	
	B	24,291,238		25,555,762	
	Recovery[a] (%)	81.2		74.1	
	CV[b] (%)	6.3		4.3	

A, mean area response of extracted analyte; B, mean area response of non-extracted analyte.
[a] Recovery (%) = $A/B \times 100$.
[b] CV (%) = standard deviation of the concentration/mean concentration × 100.

many compounds from plasma and urine and has been used to optimize the mobile phase composition for separation of low molecular weight molecules [12,13]. However, extraction using ice-cold acetonitrile is a less specific, but highly rapid, method. In this study, we applied this technique to precipitate proteins from brain tissue homogenates. The most efficient recovery and cleanest extraction of the target compounds from the brain were obtained by combining acetonitrile and methanol precipitation (1:1, v/v) (Table 1). In the present study, we used acetonitrile–methanol (1:1, v/v) for protein precipitation because it produced a clean chromatogram for a blank plasma and brain sample, with extraction recoveries of more than 91% and 80% from 50 μL of plasma and brain homogenate, respectively. Also, a Gemini C_{18} column and a mixture of acetonitrile–methanol and water (45:45:10, v/v/v) were used for chromatographic separation, which produced symmetric peaks of the analytes and the internal standard and reduced the run time. Gliclazide (20 ng/mL) was used as an internal standard to quantify both MEFWAY and MPPF. Fig. 2 shows the representative LC–ESI-MS/MS MRM chromatograms: blank, spiked at the LOQ level (1 ng/mL in plasma and 5 ng/mL in brain), and the samples obtained 30 min after intravenous administration of both MEFWAY and MPPF to Sprague-Dawley rats in the plasma and brain. Based on these results, both target compounds and the internal standard were eluted at 0.7 and 0.9 min with apparently symmetrical peaks, respectively, and with an end time of 1.8 min. There were no significant interfering peaks from reduction in the matrix or constituents of the drug-free plasma and brain.

3.2. Specificity, sensitivity, linearity, precision, and accuracy

Calibration curves were obtained over the concentration range of 1–4000 ng/mL in plasma and 5–4000 ng/mL in brain homogenates for both MEFWAY and MPPF. Calibration curves for both the plasma and brain had good linearity based on linear

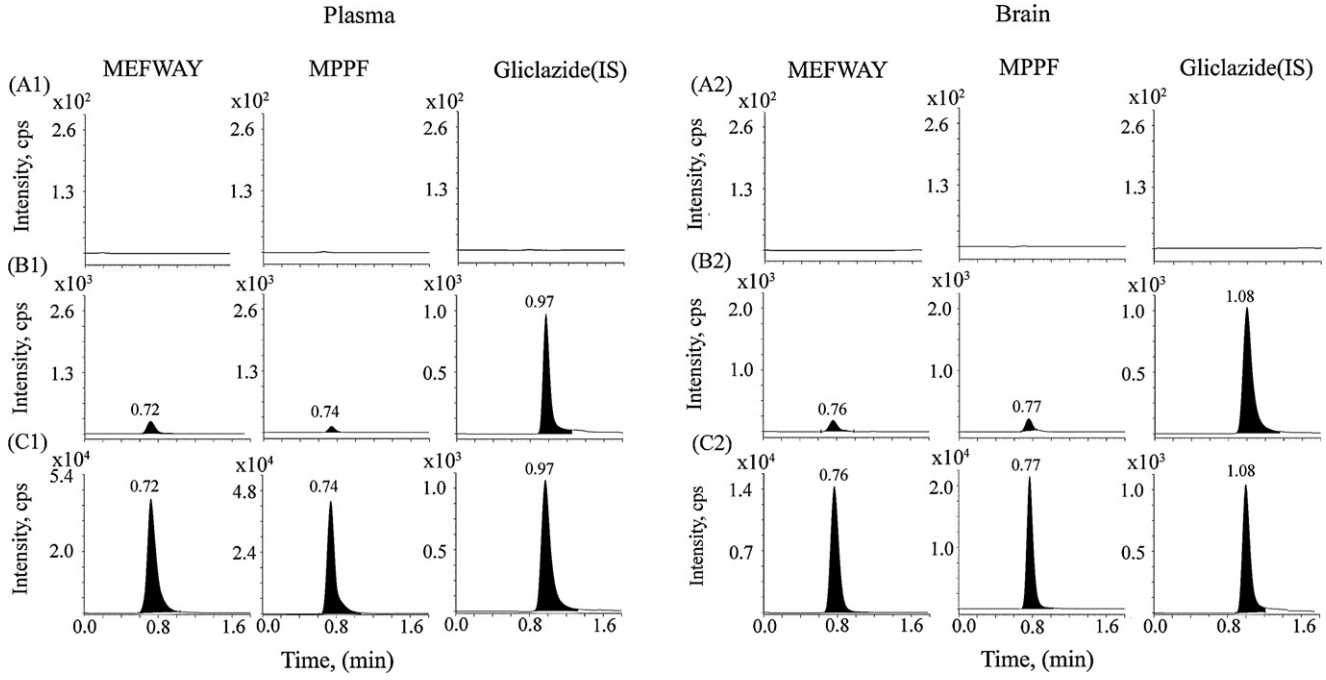

Fig. 2. Representative MRM chromatograms of MEFWAY, MPPF and gliclazide (IS) in rat plasma (A1, B1 and C1) and brain (A2, B2 and C2). (A) Blank samples from plasma (A1) and brain (A2); (B) samples at an 5 ng/mL (LLOQ) with both target compounds added at an 1 ng/mL (LLOQ) in plasma (B1) and brain (B2), respectively; and (C) plasma (C1) and brain (C2) samples at 30 min after an intravenous administration of 5 mg/kg both target compounds to rats, respectively.

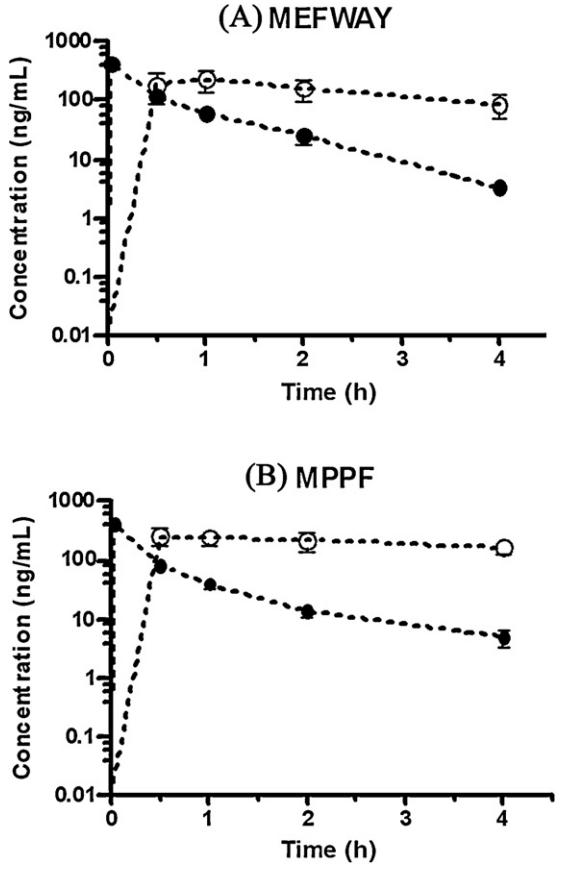

Fig. 3. Mean plasma (●) and brain (○) concentration–time plots after intravenous administration of MEFWAY and MPPF at a dose of 5 mg/kg to rats, respectively. Each point represents the mean ± SD ($n = 3$).

regression analysis with a weighting of 1/concentration. The correlation coefficients (R^2) for MEFWAY and MPPF were greater than 0.9993 in the plasma and brain, which suggested good repeatability. The LOQ and S/N ratio were set at 1 ng/mL (S/N = 91) and 5 ng/mL (S/N = 102) for MEFWAY and 1 ng/mL (S/N = 82) and 5 ng/mL (S/N = 96) for MPPF in plasma and brain homogenates, respectively. The LOQ values were sufficiently sensitive to determine the pharmacokinetic profile of an intravenous dose of 5 mg/kg of both target compounds. Both intra- and inter-assay accuracies and CV values for both target compounds were calculated (Table 2). These results indicated that the present method had acceptable accuracy and precision. To assess the stability of both target compounds, three MEFWAY and MPPF samples that underwent freeze–thaw cycles, short-term temperature storage, and long-term stability handling were evaluated. The results showed that three freeze–thaw cycles, long-term storage for one month at −80 °C, and short-term (4 h) storage at room temperature did not affect the quantification of the QC samples at low and high concentrations before injection (Table 3).

3.3. Application to an in vivo BBB study

The proposed method was successfully applied to analyze the levels of both MEFWAY and MPPF *in vivo* in rats following intravenous administration at 5 mg/kg. MEFWAY and MPPF were detected during the plasma-brain pharmacokinetic (PK) study. The time-dependence of concentration (five different time points) curves for both target compounds in the plasma and brain are shown in Fig. 3, and the main PK parameters derived from these profiles are presented in Table 4. The results showed similar

Table 2
Accuracy and precision of MEFWAY and MPPF in quality control samples.

Concentration (ng/mL)		Intra-batch ($n = 6$)						Inter-batch ($n = 3$)					
		MEFWAY			MPPF			MEFWAY			MPPF		
		Mean (ng/mL)	RE[a] (%)	CV[b] (%)	Mean (ng/mL)	RE[a] (%)	CV[b] (%)	Mean (ng/mL)	RE[a] (%)	CV[b] (%)	Mean (ng/mL)	RE[a] (%)	CV[b] (%)
Plasma	1	0.9	−11	4.6	0.8	−13	6.0	0.9	−13	6.7	0.9	−11	4.1
	50	47	−5.6	2.4	47	−5.3	2.6	48	−3.3	1.2	52	3.3	5.6
	500	493	−1.3	0.5	494	−1.3	0.6	497	−0.7	0.6	500	0.1	1.0
	1000	1003	0.3	0.3	1005	0.5	0.1	1007	0.7	0.3	1010	1.0	0.1
	4000	3976	−0.6	1.0	3975	−0.6	0.9	4008	0.3	0.1	4016	0.4	0.7
Brain	5	4.5	−10	5.4	4.5	−11	5.1	4.5	−10	6.7	4.4	−12	3.9
	50	47	−4.5	3.9	47	−6.7	4.8	47	−5.3	2.4	47	−6.7	1.2
	500	491	−1.6	0.5	490	−1.8	0.4	495	−0.9	0.1	493	−1.2	0.7
	1000	1007	0.7	0.5	1008	0.8	0.4	1012	1.2	0.8	1013	1.3	0.6
	4000	4009	0.3	0.1	4010	0.3	0.0	4008	0.3	0.1	4009	0.3	0.1

[a] RE (%) = (calculated concentration − theoretical concentration)/theoretical concentration × 100.
[b] CV (%) = standard deviation of the concentration/mean concentration × 100.

Table 3
Stability of MEFWAY and MPPF in plasma and brain.

Statistical variable			Plasma				Brain			
			Autosampler stability	Freeze/thaw stability	Short-term stability	Long-term stability	Autosampler stability	Freeze/thaw stability	Short-term stability	Long-term stability
MEFWAY	5.0 ng/mL	Mean	4.7	4.8	4.8	4.7	5	4.9	4.9	4.9
		RE[a] (%)	−6.7	−3.3	−4.0	−6.0	−0.2	−2.1	−2.8	−1.7
		CV[b] (%)	2.5	1.2	5.5	3.7	2.4	5.9	6.3	5.6
	500 ng/mL	Mean	511	503	503	504	500	502	498	502
		RE[a] (%)	2.2	0.6	0.6	0.9	0.0	0.3	−0.3	0.4
		CV[b] (%)	0.2	1.0	2.2	1.4	0.4	1.8	2.2	1.5
MPPF	5.0 ng/mL	Mean	4.6	4.7	4.7	4.8	4.7	4.7	4.8	4.8
		RE[a] (%)	−8.8	−6.7	−6.3	−4.4	−6.3	−5.2	−4.9	−4.2
		CV[b] (%)	1.8	3.6	7.9	4.9	5.1	2.9	3.4	7.2
	500 ng/mL	Mean	507	496	499	503	503	498	495	494
		RE[a] (%)	0.6	1.2	1.7	1.9	0.7	−0.5	−0.9	−1.1
		CV[b] (%)	0.1	0.6	0.5	0.6	1.3	0.8	0.5	1.0

[a] RE (%) = (calculated concentration − theoretical concentration)/theoretical concentration × 100.
[b] CV (%) = standard deviation of the concentration/mean concentration × 100.

Table 4
Pharmacokinetic parameters of MEFWAY and MPPF in brain and plasma following an intravenous administration at a dose of 5 mg/kg in rats ($n = 3$).

Parameter	MEFWAY		MPPF	
	Plasma	Brain	Plasma	Brain
$AUC_{0-4\,h}$ (h × μg/mL)	0.26 ± 0.06	0.49 ± 0.38	0.21 ± 0.03	0.80 ± 0.41
C_{max} (μg/mL)	–	0.20 ± 0.16	–	0.29 ± 0.13
T_{max} (h)	–	0.67 ± 0.29	–	0.67 ± 0.29
$t_{1/2}$ (h)	0.71 ± 0.13	4.32 ± 3.42	1.10 ± 0.39	6.26 ± 3.41
CL (L/h/kg)	20.0 ± 4.98	–	23.8 ± 4.02	–
$AUC_{brain,\,0-4\,h}/AUC_{plasma,\,0-4\,h}$	1.90 ± 1.17		4.02 ± 2.32	

time-concentration curves for MEFWAY and MPPF because they have similar structures. The concentrations of both target compounds in the brain homogenate were higher than that of the plasma at the same time point. In case of MPPF, the maximum brain concentration (C_{max}) of MPPF reached 0.29 ± 0.13 μg/mL after intravenous dosing, and the biological brain half-life ($t_{1/2}$) was 6.26 ± 3.41 h. The area under the brain concentration–time curve ($AUC_{0-4\,h}$) was 0.80 ± 0.41 μg h/mL. For the PK profile of MEFWAY, the maximum brain concentration (C_{max}) of MEFWAY reached 0.20 ± 0.16 μg/mL, similar to MPPF, while the biological brain half-life ($t_{1/2}$) was 4.32 ± 3.42 h. The area under the brain concentration–time curve ($AUC_{brain,\,0-4\,h}$) was 0.49 ± 0.38 μg h/mL. This suggests that high concentrations of both target compounds rapidly distributed to the brain by 30 min after intravenous administration and then slowly decreased after the time to reach maximum brain concentration. Brain-to-plasma (B/P) ratios were 1.90 and 4.02 for MEFWAY and MPPF, respectively, which suggested that MPPF may be more highly distributed to the brain than MEFWAY. Based on our results, both compounds had high brain penetration.

4. Conclusions

A simple, rapid, and sensitive LC–MS/MS method was validated for the determination of MEFWAY and MPPF in rat plasma and brain homogenate. A small volume of plasma and brain homogenate combined with one-step protein precipitation using acetonitrile and methanol (1:1, v/v) was used for sample preparation, which yielded excellent extraction recovery and produced a clean chromatogram. No significant interference peaks were observed in the blank chromatograms from the plasma and brain. The method was subsequently validated for selectivity, linearity, accuracy, precision, and stability. The intra- and inter-day measurements of the analytes had a higher requirement for accuracy and precision. The applicability of this method was successfully demonstrated by measuring single-dose MEFWAY and MPPF concentrations in the plasma-brain PK study in rats. A simple, fast, and low-cost LC–MS/MS method will be applicable to PK–PD brain studies of both MEFWAY and MPPF.

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