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Original Article

High-throughput liquid chromatography tandem mass spectrometry method for simultaneous determination of fampridine, paroxetine, and quinidine in rat plasma: Application to *in vivo* perfusion study

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ABSTRACT

A selective and high-throughput liquid chromatography–mass spectrometry method has been developed and validated for the simultaneous quantification of paroxetine, fampridine, and quinidine in rat plasma using imipramine as an internal standard. Following protein precipitation extraction, the analytes and internal standard were run on XBridge C18 column (150 mm × 4.6 mm, 5 μm) using a gradient mobile phase consisting of 5mM ammonium formate in water (pH 9.0) and acetonitrile in a flow gradient program. The precursor and product ions of the drugs were monitored on a triple quadrupole instrument operated in the positive ionization mode. The method was validated over a concentration range of 0.1–100 ng/mL for all the three analytes, with relative recoveries ranging from 69% to 82%. The intra- and interbatch precision (percent coefficient of variation) across four validation runs were less than 13.4%. The accuracy determined at four quality control (QC) levels (lower limit of quantitation, low QC, medium QC, and high QC) was within ±6.5% of coefficient of variation values. The method proved highly reproducible and sensitive, and was successfully applied in a pharmacokinetic study after single-dose oral administration to rats and also in perfusion study sample analysis.

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1. Introduction

Paroxetine (PRX) is a phenylpiperidine compound that acts as a potent and selective serotonin reuptake inhibitor [1]. Its

action appears to account for the antidepressant activity observed with this class of drugs [2] that are safe and effective for the treatment of depressive and obsessive–compulsive disorders [3]. Fampridine (FMP; 4-aminopyridine) is a selective

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potassium channel blocker. It is used for the treatment of adult patients with multiple sclerosis to improve their walking ability. Considering the mechanism of action of FMP, there is a plausible biological rationale to evaluate its usefulness in symptomatic treatment of multiple sclerosis. Quinidine (QND) is a Class I antiarrhythmic agent, which primarily works by blocking the fast inward sodium current in the heart. Literature reveals that QND in combination with dextromethorphan alleviates symptoms of easy laughing and crying (pseudobulbar affect) in patients with amyotrophic lateral sclerosis and multiple sclerosis [4]. Prescribing a single drug and its administration are not sufficient in neurologic diseases such as multiple sclerosis. Combination therapy, which can decrease the number of medications for a single disease or its associated diseases, is growing enormously. In clinical research, estimation of concomitant drugs plays a key role in the study of drug–drug interactions. Recently, ClinicalTrials.gov has updated information regarding Phase 4 studies of FMP, along with concomitant drugs, in the treatment of multiple sclerosis disease. The research in the current article has been undertaken to provide an accurate method that can be applied to estimate FMP along with concomitant drugs such as PRX and QND, which are prescribed as combination therapy. In line with this, we have developed a method for the simultaneous estimation of PRX, QND, and FMP, which will be highly useful in drug–drug interaction studies and also in developing combined dosage forms. Even though independent high-performance liquid chromatography and liquid chromatography–mass spectrometry (LC–MS) methods are available in the literature for the determination of PRX [5–8], FMP [9,10], and QND [11–14], a technique for simultaneous estimation of these three drugs neither is available nor has been published. The aim of the current study is to develop and validate a sensitive and high-throughput method for simultaneous determination of PRX, FMP, and QND in rat plasma for therapeutic drug monitoring and pharmacokinetic studies and also for *in vivo* perfusion studies in rats. The developed bioanalytical method has been validated according to International Conference on Harmonization (ICH) guidelines [15]. Structural formulas of the analytes are presented in Figure 1.

2. Methods

2.1. Chemicals and reagents

PRX and QND were supplied by Sigma-Aldrich (Shanghai, China). FMP was purchased from Sisco Research Laboratories (Hyderabad, India). Acetonitrile of MS grade and other chemicals of analytical grade were obtained from Merck (Mumbai, India). Water used in the study was prepared using the Milli-Q water purification system (Millipore, Milford, MA, USA). Drug-free rat plasma and rats were obtained from Albino research Labs (Hyderabad, India) and the plasma was stored at -20°C until use.

2.2. Instrumentation

The liquid chromatography tandem-mass spectrometry (LC–MS/MS) analysis was carried out in electrospray ionization

positive mode on a mass spectrometer (API 3000) coupled to a Shimadzu LC system (model: SIL-HTC) operated with Analyst 1.6.1 software. Separation of all the analytes was carried out on an XBridge C18 column (150 mm length $\times$ 4.6 mm internal diameter, and 5 μm particle size). Temperature was set at 30°C . The mobile phase composed of acetonitrile and 0.005M ammonium formate pH 9.0 adjusted with ammonia (gradient mode) in flow gradient of 0.5–2.0 mL/min in a run time of 4 minutes. The injection volume was 10 μL . The full-scan MS and MS/MS spectra of each analyte were obtained by direct infusion of the respective sample solution, at a concentration of 10 $\mu\text{g/mL}$, prepared in methanol. The drugs were analyzed using multiple reaction monitoring mode.

2.3. Mass spectrometric conditions

Turbo ion spray interface operating in positive ionization mode was used to study the parent $\rightarrow$ product ion (m/z) transitions for PRX (330.1 $\rightarrow$ 192.1), FMP (94 $\rightarrow$ 67), QND (325.1 $\rightarrow$ 251.1), and imipramine (281.4 $\rightarrow$ 86.1). Chemical structures of all the four analytes are shown in Figure 1 and the product ion spectra in Figure 2. Declustering potential, entrance potential, collision energy, and collision exit potential were all optimized to allow the highest possible signal transduction with low background noise. Signal optimization was performed by constant infusion of 10 $\mu\text{g/mL}$ drug solutions in 100% methanol at a rate of 50 $\mu\text{L/min}$. The pressure of the drying gas was 35 psi and the temperature was 350°C . The ion spray voltage was set at 4500 V and the pressure of collision gas (nitrogen) was 4 psi. Quadrupoles 1 and 3 were set at unit mass resolution, and each multiple reaction monitoring transition was monitored with a dwell time of 200 milliseconds.

2.4. Standard solutions and fortification

Standard stock solutions of PRX, FMP, and QND were prepared by accurately weighing 10 mg of each standard on a closed electronic microbalance (Sartorius, Goettingen, Germany) and dissolving them separately in 10 mL of methanol. Individual working solutions of analytes were prepared by appropriate dilution of their stock solutions in 50% methanol. All the solutions were stored in a refrigerator at or below 10°C and were brought to room temperature before use. A working solution of then internal standard (ISTD; imipramine 500 ng/mL) was prepared daily in 50% methanol and stored at room temperature. Calibration standards and quality control (QC) samples were prepared by spiking (3%) blank plasma with the working solutions prepared from independent stock weightings. Calibration standards for PRX, FMP, and QND were prepared at concentrations of 0.1 ng/mL, 0.2 ng/mL, 0.5 ng/mL, 1 ng/mL, 5 ng/mL, 10 ng/mL, 50 ng/mL, and 100 ng/mL. QC samples were prepared at 0.1 ng/mL [lower limit of quantitation (LLOQ) QC], 0.3 ng/mL [low QC (LQC)], 40 ng/mL [medium QC (MQC)], and 80 ng/mL [high QC (HQC)] for all the three analytes. Aliquots (0.3 mL) of spiked plasma samples were taken in polypropylene tubes and stored at -70°C . Prior to analysis, all frozen subject samples, calibration standards, and QC samples were thawed unassisted at room temperature and

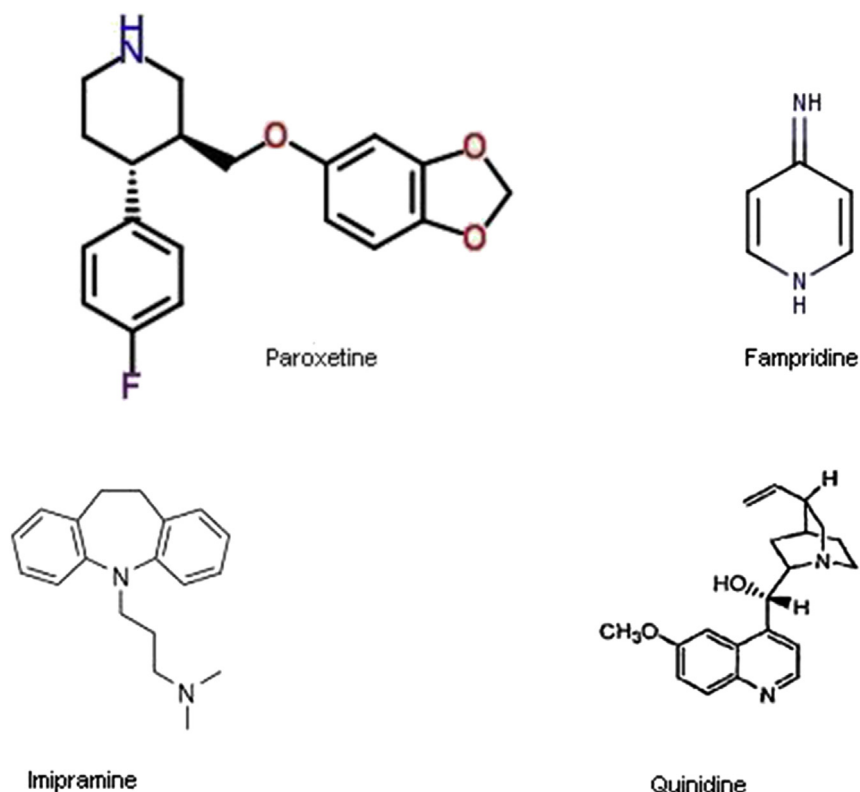


Figure 1 – Chemical structures of four analytes.

subjected to the sample preparation procedure described in the next section.

2.5. Sample preparation

A simple protein precipitation method was used to extract the analytes. Plasma samples stored at around -70°C were thawed, left for 1 hour, and vortexed for 30 seconds at room temperature before extraction to ensure homogeneity. Acetonitrile (150 μL) was added as a protein precipitating agent, vortexed for 1 minute, and then centrifuged at 10,000 rpm for 10 minutes on refrigerated centrifuge at 4°C . The supernatant layer was separated and filtered through 0.45 μm syringe filters, and 10 μL of the solution was injected for LC–MS/MS analysis. Perfusion samples were collected at certain time points, centrifuged at 5000 rpm for 10 minutes, and analyzed.

2.6. Method validation

A complete method validation of PRX, FMP, and QND in rat plasma was done following the United States Food and Drug Administration and European Medicines Agency (EMA) guidelines. Validation runs were performed on 7 separate days to evaluate selectivity, sensitivity, linearity, precision, accuracy, recovery, matrix effect, dilution integrity, and stability of the method. Each validation run was organized with a set of spiked standard samples, blank (with ISTD and without ISTD), and QC samples as per the validation parameters. Standard samples were analyzed at the beginning of the run, and QC samples were distributed consistently throughout the validation runs.

Selectivity of the method toward endogenous and exogenous components of plasma was evaluated in 12 different rat plasma lots. The blank plasma lots were extracted (without addition of ISTD) and injected for LC–MS/MS detection. Later, selectivity in each lot was evaluated by comparing the blank peak responses with the mean peak response observed in plasma spiked LLOQ sample ($n = 6$). The potential for assay interference from concomitant drugs was also investigated by spiking LQC samples with teriflunomide, ibuprofen, tamsulosin, and pioglitazone. Linearity of the method was assessed using four calibration curves analyzed on 3 different days. Each plot was associated with eight-point nonzero concentrations spread over the dynamic range. A linear least squares regression analysis with the reciprocal of drug concentration as a weighing factor ($1/X^2$) was performed on peak area ratios versus analyte concentrations. Peak area ratios for plasma spiked calibration standards were proportional to the concentrations of analytes over the established range.

Intrabatch (within day) and interbatch (between day) precision and accuracy were evaluated at four distinct concentrations (LLOQ, LQC, MQC, and HQC) for each analyte. Precision and accuracy at each concentration level were evaluated in terms of percent coefficient of variation (%CV) and relative error, respectively. The extraction recovery of PRX, FMP, and QND was determined at LQC, MQC, and HQC levels. Relative recoveries were evaluated by comparing the peak areas of extracted samples (spiked before extraction) with those of unextracted samples (blank extracts spiked after extraction). The matrix effect was checked at LQC and HQC levels using six different blank plasma lots (including 1 hemolytic and 1 lipemic lot). Matrix factors for analyte and ISTD

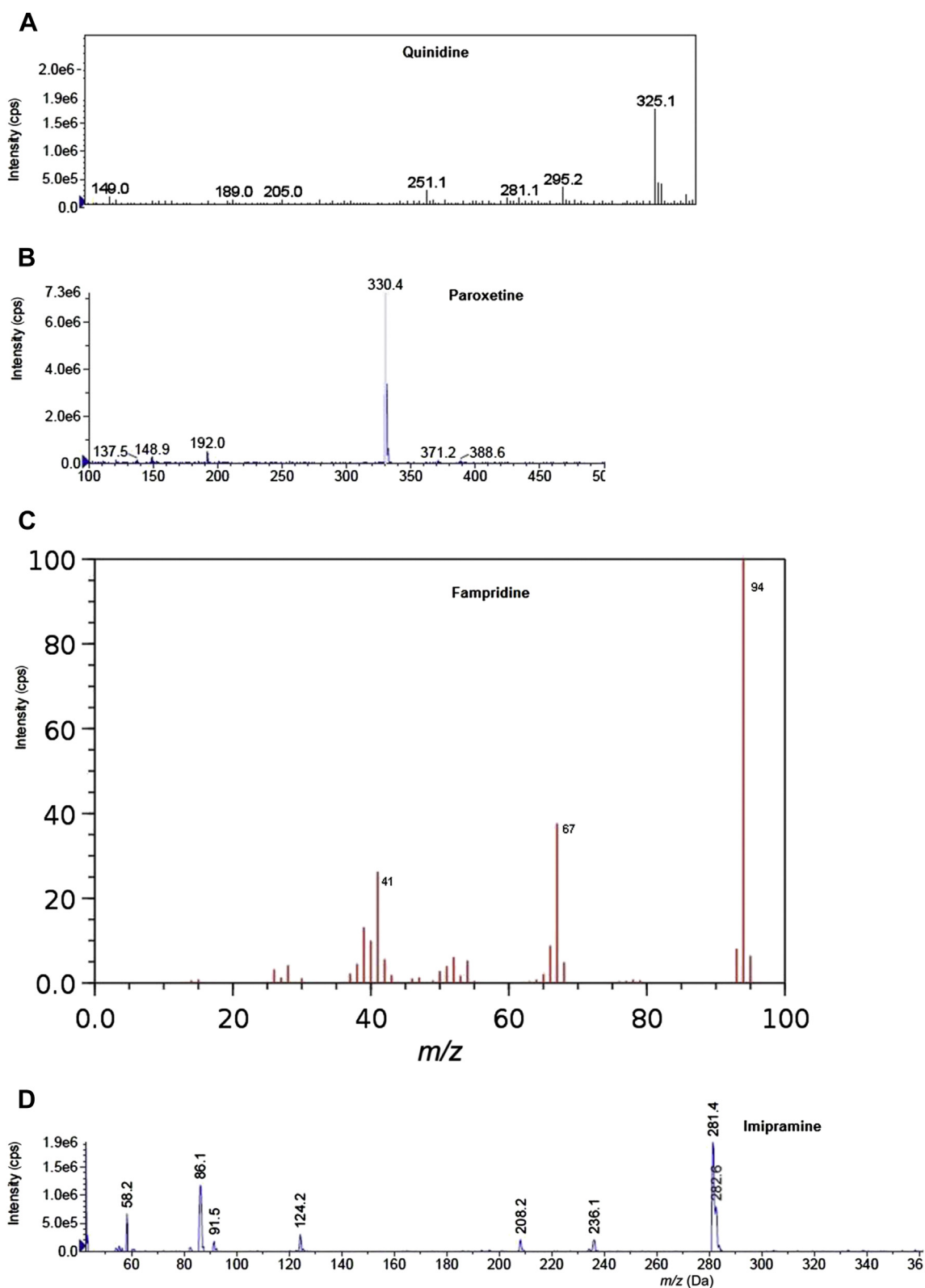


Figure 2 – Product ion spectra of (A) quinidine, (B) paroxetine, (C) fampridine, and (D) imipramine.

were calculated in each lot by comparing the peak responses of postextraction samples (blank extracts spiked after extraction) with the peak responses of equivalent aqueous samples prepared in the mobile phase. ISTD normalized

matrix factor in each lot was later evaluated by comparing the matrix factors of analyte and ISTD. Stability of analytes in both aqueous solutions and biological matrix was evaluated after subjecting them to different conditions and

temperatures that could be encountered during regular analysis. Stability in plasma was evaluated in terms of freeze–thaw stability, bench-top stability, long-term stability, and extracted sample stability. Freeze–thaw stability was evaluated after four freeze (at -70°C)–thaw (at room temperature) cycles. Bench-top stability was assessed at room temperature, and long-term stability was evaluated at both -70°C and -20°C . Stability of extracted samples was determined as autosampler stability at 10°C . Stability in plasma was evaluated at room temperature. All the stability assessments were made at LQC and HQC levels by comparing the stability samples with freshly prepared samples. Stability of analytes in stock solutions and working solutions was assessed at room temperature (short-term stability) and at $1\text{--}10^{\circ}\text{C}$ (long-term stability). All comparisons were made against freshly prepared stock solutions or working solutions. The method was cross-validated for determination of PRX and FMP only (in absence of QND) and for determination of QND only (in absence of PRX and FMP). In cross-validation, two independent precision and accuracy runs, one for the determination of PRX and FMP and the other for the determination of QND only, were evaluated with LQC, MQC, and HQC samples ($n = 6$). During routine analysis, each analytical run was organized with a set of standard samples, a set of QC samples in duplicate, and plasma samples to be determined. Prior to each analytical run, system suitability was evaluated by injecting six replicates of upper limit of quantitation sample and three replicates of LLOQ sample, to check the system precision and accuracy. System suitability was considered acceptable when the CV for response ratios was less than 4.0%.

3. Results

3.1. Method development

For consistent and reliable estimation of analytes, it was necessary to give equal importance to optimization of extraction procedure, and chromatographic and mass spectrometric conditions. Analytes and ISTD were tuned in both positive and negative polarity modes using electrospray ionization technique; however, positive ion mode showed better selectivity and sensitivity for PRX. The Q1 and MS–MS scans were made in infusion mode, and further compound and gas parameters were optimized in flow injection analysis. The $[\text{M} + \text{H}]^{+}$ peaks were observed at m/z values of 330.4, 94.0, and 325.1, for PRX, FMP, and QND, respectively. Most abundant product ions were found at an m/z of 192.1 for PRX, 67.0 for FMP, and 251.1 for QND, by applying sufficient collision-activated dissociation gas and collision energy. An increase in source temperature beyond 350°C augmented the intensity for all analytes except FMP. A 5% change in ion spray voltage and gas parameters did not affect the signal intensity. In the optimization of chromatographic conditions, the isocratic mode was selected as no crosstalk was observed between analytes and ISTD. To facilitate protonation pH adjusted to 9.0 with ammonia to the ammonium formate buffer. Use of methanol over acetonitrile in the mobile phase has shown significant improvement in signal intensities. Replacement of Milli-Q water with 5mM ammonium formate buffer in the

mobile phase resulted in good chromatographic peak shapes, and a further increase in the buffer concentration resulted in a loss of FMP response. A flow gradient of $0.5\text{--}1.5\text{ mL/min}$ was used to minimize the run time without compromising the signal intensity (Table 1). Multiple reaction monitoring chromatograms are presented in Figure 3.

3.2. Selectivity

Selectivity of the method in rat K2EDTA plasma was evaluated in 12 individual matrix lots, including one lipemic and one hemolytic lot. Peak responses in blank lots were compared with the response of the spiked LLOQ, and negligible interference was observed at the retention time of analytes and ISTD.

3.3. Linearity and sensitivity

The linearity of each calibration curve was determined by plotting the peak area ratio (y-axis) of analytes to ISTD versus the nominal concentration (x-axis) of analytes. Calibration curves were linear from 0.1 ng/mL to 100 ng/mL for PRX, FMP, and QND, with r^2 values being more than 0.9962. The r^2 values, slopes, and intercepts were calculated from four intra- and interday calibration curves using weighted ($1/X^2$) linear regression analysis. The observed mean back-calculated concentrations with accuracy (percent relative error) and precision (%CV) are presented in Table 2. The LLOQ was found to be 0.1 ng/mL for all the three analytes, and accuracy was in the range of 69–82%, with a %CV of $\leq 6.5\%$. At LLOQ, the mean signal-to-noise ratios were found to be 26:1, 39:1, and 170:1 for PRX, FMP, and QND, respectively.

3.4. Precision and accuracy

Precision and accuracy were evaluated using four intra- and interday precision and accuracy runs, with each batch consisting of six replicates of QC samples at four concentration levels (LLOQ, LQC, MQC, and HQC). The intra- and interbatch precision were less than 13.4% for FMP, PRX, and QND, with accuracy (percent relative error) between 0.1 and 11.7. Results of precision and accuracy are presented in Table 3.

3.5. Matrix effect

Coeluting matrix components can suppress or enhance ionization, but may not result in a detectable response in matrix blanks due to selectivity of MS detection; however, they can affect the precision and accuracy of the assay. Therefore, the

Table 1 – Gradient program.

Time (min)	Buffer (%)	Acetonitrile (%)	Flow rate (mL/min)
Initial	95	5	1.0
1.0	95	5	1.0
1.2	5	95	2.0
3.0	5	95	2.0
3.2	95	5	1.0
4.0	95	5	1.0

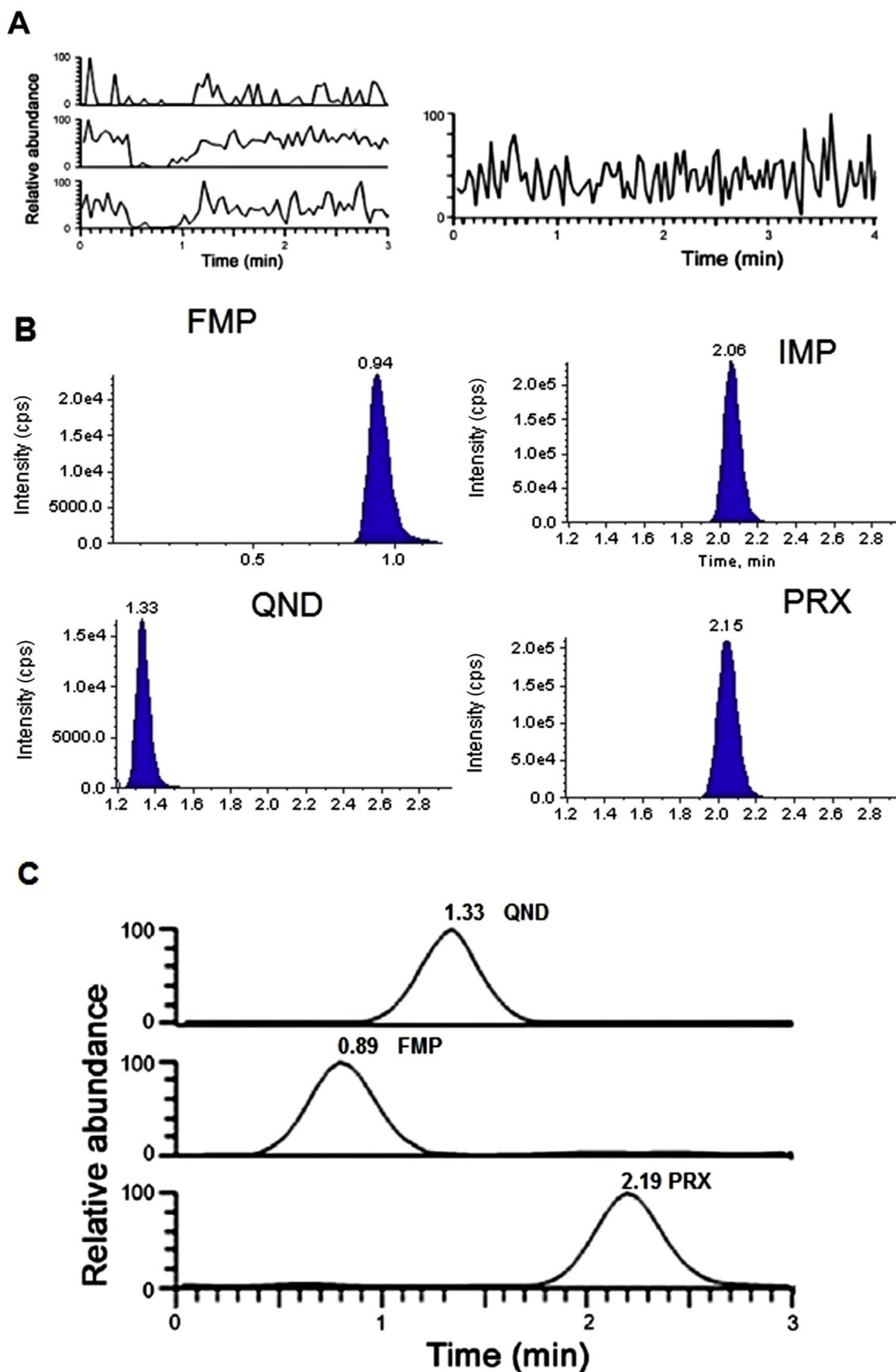


Figure 3 – Extracted ion chromatograms of (A) blank plasma, (B) blank plasma spiked with analytes and Internal Standard (IS), and (C) plasma sample after a single oral dose of three analytes QND, PRX, and FMP. FMP = fampridine; IMP = imipramine; PRX = paroxetine; QND = quinidine.

Table 2 – Summary of calibration standards.

Analyte	Nominal concentration (ng/mL)	Actual concentration (ng/mL) (n = 4)	%CV	%RE
PRX	0.101	0.103	8.7	2.0
	0.203	0.198	13.8	–2.5
	0.507	0.506	11.1	–0.2
	1.015	0.943	5.6	–7.1
	5.074	4.986	5.0	–1.7
	10.147	9.944	3.2	–2.0
	50.737	53.289	2.8	5.0
FMP	101.474	107.730	6.6	6.2
	0.101	0.102	4.3	1.0
	0.202	0.198	7.2	–2.0
	0.505	0.507	7.0	0.4
	1.010	1.003	2.9	–0.7
	5.051	4.962	5.0	–1.8
	10.101	9.921	2.6	–1.8
QND	50.505	51.201	3.0	1.4
	101.011	102.401	7.4	1.4
	0.101	0.103	4.3	2.0
	0.202	0.199	3.8	–1.5
	0.504	0.505	1.3	0.2
	1.000	1.003	1.1	0.3
	5.040	4.990	2.7	–1.0
	10.090	9.979	1.2	–1.1
	50.400	50.455	2.9	0.1
	100.900	100.676	4.5	–0.2

%CV = percent coefficient of variation; FMP = fampridine; PRX = paroxetine; QND = quinidine; %RE = percent relative error.

potential for variable matrix-related ion suppression was evaluated in six independent sources (containing 1 hemolytic and 1 lipemic lot) of rat plasma, by calculating the Internal Standard (IS) normalized matrix factor. The mean IS normalized matrix factor for all the three analytes ranged between 0.9 and 1.1 with a %CV of ≤ 10.7 , as shown in Table 4.

3.6. Extraction recovery and dilution integrity

The extraction recovery of analytes from EDTA plasma was determined by comparing the peak responses of plasma samples ($n = 6$) spiked before extraction with those of plasma samples spiked after extraction. The mean recovery rates of PRX, FMP, and QND were found to be 74.9%, 78.7%, and 76.6%, respectively, with %CV across the three levels ranging between 4.7% and 7.7%, as shown in Table 5. Dilution integrity experiment was carried out at two times the upper limit of quantification concentration for all the three analytes. After 1/2 and 1/4 dilution, the mean back-calculated concentration for dilution QC samples was within 85–115% of nominal value, with a %CV of ≤ 9.0 . Similarly, LQC samples spiked with concomitant drugs were quantified within 15% of nominal value with a %CV of ≤ 6.5 .

3.7. Stability

Stability evaluations were performed in both aqueous and matrix-based samples. The stock solutions were stable for a period of 24 hours at room temperature and for 60 days at 1–10°C. Stability evaluations in matrix were performed against freshly spiked calibration standards using freshly prepared QC samples (comparison samples). PRX, FMP, and QND were stable up to 10 hours on bench top at room temperature and over five freeze–thaw cycles. The processed samples were stable up to 36 hours in the autosampler at 10°C. Long-term matrix stability was evaluated at both –20°C and –70°C over a period of 60 days. No significant degradation of analytes was observed over the stability duration and conditions. The stability results presented in Table 6 were within 85–115%. Stability in rat plasma was evaluated at both LQC and HQC levels by comparing the mean response ratios of stability samples with those of the comparison samples. The stability of analytes at room temperature was within 85–115% for up to 24 hours.

Table 3 – Intra- and interbatch precision and accuracy.

Analyte	Nominal concentration (ng/mL)	Intraday (n = 6)			Interday (n = 24)		
		% Recovery	%CV	%RE	% Recovery	%CV	%RE
Paroxetine							
LLOQQC	0.101	104.12	13.4	1.4	105.01	7.1	3.0
LQC	0.301	101.65	12.3	1.7	101.97	3.3	1.9
MQC	40.118	106.02	4.1	6.0	107.72	3.0	7.7
HQC	80.236	100.67	6.0	0.7	101.55	2.1	1.6
Fampridine							
LLOQQC	0.101	108.18	8.8	8.2	111.68	5.6	11.7
LQC	0.302	105.23	8.0	5.2	104.23	1.9	4.2
MQC	40.221	100.13	4.1	0.1	103.90	6.3	3.9
HQC	80.443	96.41	4.7	−3.6	97.40	2.2	−2.6
Quinidine							
LLOQQC	0.101	105.26	5.1	5.3	102.87	4.78	2.6
LQC	0.302	97.39	5.4	−2.6	98.99	4.33	−1.7
MQC	40.221	107.05	4.3	7.1	108.52	0.56	6.8
HQC	80.443	99.80	3.5	−0.2	99.65	0.89	0.3

%CV = percent coefficient of variation; HQC = high quality control; LQC = low quality control; MQC = medium quality control; %RE = percent relative error.

Table 4 – Matrix effect results.

Analyte	Paroxetine				Fampridine				Quinidine			
	LQC		HQC		LQC		HQC		LQC		HQC	
	MF	ISNMF	MF	ISNMF	MF	ISNMF	MF	ISNMF	MF	ISNMF	MF	ISNMF
Lot 1	1.0602	0.9153	1.1295	1.0119	1.0526	0.9130	1.0708	0.9589	0.9880	0.9333	0.9885	0.9700
Lot 2	1.0381	0.9153	1.0824	0.9783	1.0745	0.9783	1.0170	0.9189	0.9841	1.0000	0.9944	0.9514
Lot 3	1.1619	1.1186	1.1007	0.9257	1.0442	0.9783	1.0985	0.9236	1.0388	1.0000	1.0171	0.9281
Lot 4	1.0471	0.9153	1.1467	0.9856	1.0698	0.9130	1.0478	0.9003	0.9128	0.9333	1.0147	0.9121
Lot 5	1.0855	0.9153	1.1117	0.9949	1.0142	0.8478	1.0916	0.9764	1.0734	0.8667	1.0115	0.9407
Lot 6	1.2419	1.1186	1.1421	1.0187	1.0331	0.9130	1.0474	0.9340	0.8948	0.9333	1.0295	0.9579
Mean		0.9831		0.9858		0.9239		0.9354		0.9444		0.9433
%CV		10.7		3.4		5.3		3.0		5.3		2.2

%CV = percent coefficient of variation; HQC = high quality control; ISNMF = internal standard normalized matrix factor; LQC = low quality control; MF = matrix factor.

Table 5 – Extraction recovery for PRX, FMP, and QND (n = 6).

Analyte	QC level	A	B	% Recovery	Mean % recovery	%CV
Paroxetine	LQC	2099.5	1647.2	78.5	74.9	6.5
	MQC	249,300.8	191,633.2	76.9		
	HQC	512,079.2	354,884.0	69.3		
Fampridine	LQC	3114.5	2556.0	82.1	78.7	4.7
	MQC	431,623.2	342,272.7	79.3		
	HQC	876,272.5	655,047.5	74.8		
Quinidine	LQC	2285.3	1607.5	70.3	76.6	7.7
	MQC	428,802.5	331,623.2	77.3		
	HQC	792,491.2	649,851.5	82.0		

A = mean response in extraction samples; B = mean response in postextraction spiked samples; %CV = percent coefficient of variation; FMP = fampridine; HQC = high quality control; LQC = low quality control; MQC = medium quality control; PRX = paroxetine; QC = quality control; QND = quinidine.

3.8. Application of the method to pharmacokinetic study

In order to verify the sensitivity and selectivity of the developed method in a real-time situation, the developed LC–MS/MS method was successfully applied to a pharmacokinetic study by administration of PRX, FMP, and QND as a single solution to six male Wistar rats by oral route, using a BD syringe attached with an oral gavage needle (size 18) at the dose of 3 mg/kg body weight. Approximately 0.2 mL of blood sample from each anesthetized (isoflurane) rat, at predetermined time intervals, was collected using a capillary tube into prelabeled Eppendorf tubes containing 10% of K2EDTA anticoagulant (20 μ L). The time intervals for the sample collection were 0 hours (predose), 0.5 hours, 1 hour, 2 hours, 4 hours, 6 hours, 8 hours, 12 hours, and 24 hours (postdose). The total blood volume collected from each rat was approximately 1.7–1.9 mL, which does not exceed the maximal recommended blood volume of 20% (2.0 mL for a rat with 200 g body weight). Plasma was obtained by centrifuging blood samples at 4000 rpm for 10 minutes. The obtained plasma samples were transferred into prelabeled microcentrifuge tubes and stored at -50°C . All the samples were analyzed by the developed method, and the mean plasma concentrations versus time profiles of PRX, FMP, and QND are shown in Figure 4. A noncompartmental model was used to estimate the pharmacokinetic parameters in rat plasma. After oral

administration of the three drugs, peak plasma concentrations (C_{max}) were reached at the time to reach maximum concentration (T_{max}) of 2.00 hours, 6.00 hours, and 2.00 hours with an elimination half-life ($t_{1/2}$) of 5.912 ± 0.431 hours, 12.012 ± 2.115 hours, and 6.401 ± 0.885 hours for FMP, PRX, and QND respectively by LC–MS.

3.9. Application of the method to perfusion study

Rats were anesthetized with an intramuscular injection of 1 mL/kg of ketamine–xylazine solution (9:1), placed on a heated surface maintained at 37°C (Harvard Apparatus Inc., Holliston, MA, USA), and a 3 cm midline abdominal incision was made. A proximal 10 cm jejuna segment, starting 2 cm below the ligament of Treitz, was cannulated on two ends and rinsed with blank perfusion buffer. All solutions were incubated in a 37°C water bath. At the starting point of each experiment, the perfusion solution containing the investigated drug, 10mM perfusion buffer (pH 6.5), 135mM NaCl, 5mM KCl, and 0.01 mg/mL phenol red, with an osmolality of 290 mOsm/L, was perfused through the intestinal segment at a flow rate of 0.2 mL/min. The perfusion buffer was perfused for 1 hour without sampling, to ensure steady-state conditions, followed by additional 1 hour of perfusion with samples taken every 10 minutes. The pH of the collected samples was measured at the outlet, to verify that there was no pH change throughout the perfusion

Table 6 – Stability studies of PRX, FMP, and QND in rat plasma at LQC and HQC levels (n = 6).

Stability	Analyte	QC level	A	%CV	B	%CV	% Stability
Bench-top stability (12 h at -25°C)	PRX	LQC	0.29	5.8	0.32	13.3	90.6
		HQC	83.75	2.3	80.66	4.1	103.8
	FMP	LQC	0.29	10.6	0.30	9.0	96.7
		HQC	84.66	1.7	80.17	2.9	105.6
	QND	LQC	0.31	14.7	0.28	12.5	110.7
		HQC	87.9	11.3	87.80	10.0	100.1
Freeze–thaw stability (after 5 th cycle)	PRX	LQC	0.33	12.6	0.32	7.6	103.1
		HQC	79.75	4.3	82.66	4.3	96.5
	FMP	LQC	0.30	5.7	0.31	5.7	96.8
		HQC	79.37	5.2	79.26	2.8	100.1
	QND	LQC	0.30	5.6	0.31	0.51	96.8
		HQC	83.30	4.6	79.75	9.8	104.5
In-injector stability (at 10°C for 36 h)	PRX	LQC	0.33	8.1	0.32	13.1	103.1
		HQC	81.18	4.5	83.39	5.4	97.3
	FMP	LQC	0.30	8.3	0.31	4.1	96.8
		HQC	82.67	4.4	79.89	4.8	103.5
	QND	LQC	0.30	10.0	0.30	8.9	100.0
		HQC	77.27	3.3	79.57	2.1	97.1
Long-term stability (at -20°C for 60 d)	PRX	LQC	0.30	4.2	0.32	13.3	93.8
		HQC	78.10	6.6	80.66	4.1	96.8
	FMP	LQC	0.29	7.1	0.30	9.0	96.7
		HQC	73.70	9.1	80.17	2.9	91.9
	QND	LQC	0.31	4.0	0.28	12.5	110.7
		HQC	77.50	3.4	87.80	10.0	88.3
Long-term stability (at -70°C for 60 d)	PRX	LQC	0.31	0.6	0.32	13.3	96.9
		HQC	77.99	6.6	80.66	4.1	96.7
	FMP	LQC	0.28	4.7	0.30	9.0	93.3
		HQC	74.30	7.4	80.17	2.9	92.7
	QND	LQC	0.32	3.6	0.28	12.5	114.3
		HQC	77.88	8.9	87.80	10.0	88.7

A = mean concentration (ng/mL) of stability samples; B = mean concentration (ng/mL) of comparison samples; %CV = percent coefficient of variation; FMP = fampridine; HQC = high quality control; LQC = low quality control; MQC = medium quality control; PRX = paroxetine; QC = quality control; QND = quinidine.

procedure (pH 6.5). The samples were immediately assayed by LC–MS. The length of the perfused intestinal segment was measured at the end point of the experiment. The effective permeability (P_{eff} , cm/s) through the rat gut wall was determined according to the following equation:

$$P_{\text{eff}} = \frac{-Q \ln(C_{\text{out}}/C_{\text{in}})}{2\pi RL}$$

where Q is the perfusion buffer flow rate (0.2 mL/min), $C_{\text{out}}/C_{\text{in}}$ is the ratio of the outlet concentration to the inlet concentration of drug that has been adjusted for water transport via the nonabsorbable marker phenol red, R is the radius of the intestinal segment (set to 0.2 cm), and L is the length of the perfused intestinal segment. All the samples were analyzed by LC–MS in the proposed method, and the results are shown in Figure 5.

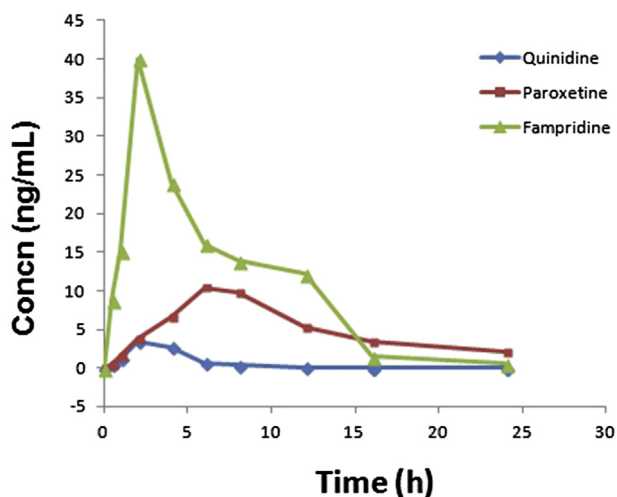


Figure 4 – Mean plasma–time concentration profiles of QND, PRX, and FMP. Concn – concentration; FMP = fampridine; IMP = imipramine; PRX = paroxetine; QND = quinidine.

4. Discussion

A rapid, sensitive, and accurate LC with electrospray ionization tandem mass spectrometry method was developed for simultaneous determination of PRX, FMP, and QND in rat plasma, with a chromatographic run time of 4 minutes. This method offers high selectivity and equal sensitivity to other methods, with a limit of quantitation of 0.1 ng/mL for all the three analytes. The extraction method utilizes a low sample volume of 50 μ L, and has shown consistent and reproducible recoveries for analytes and ISTD with minimum plasma interference and matrix effect. The developed method was successfully applied to a pharmacokinetic study using the test formulation at 3 mg/kg and also for an *in vivo* perfusion study

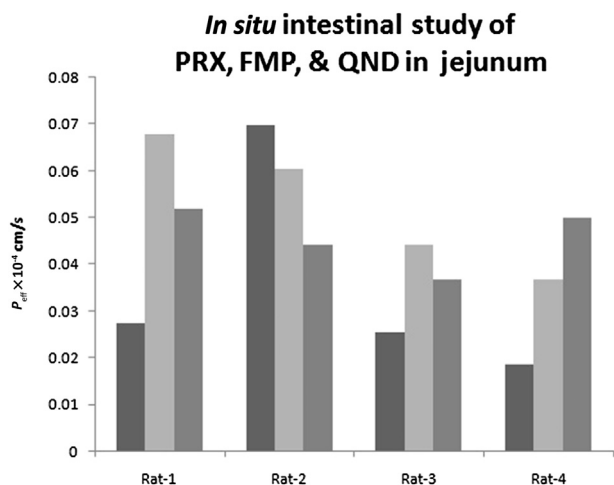


Figure 5 – Perfusion study results. FMP = fampridine; IMP = imipramine; PRX = paroxetine; QND = quinidine.

at 1 mg/mL dose. The concomitant drug analysis along with the target analyte is more advantageous than single compound analysis, and is also useful in drug interaction and toxicology studies. This validated method can be used for the analysis of patient samples receiving PRX, FMP, and QND, to support clinical pharmacokinetic studies.

Conflicts of interest

None.

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