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VALIDATED UFLC-MS/MS METHOD FOR THE QUANTITATIVE ESTIMATION OF PALIPERIDONE IN RABBIT PLASMA; ADAPTABILITY TO *IN-VIVO* BIOAVAILABILITY STUDY

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Keywords:

Paliperidone, UFLC-MS/MS, Method validation, Matrix effect, *In-vivo* bioavailability

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ABSTRACT: A simple gradient ultrafast liquid chromatography-tandem mass spectrometry method was developed and validated for the quantitative estimation of paliperidone in rabbit plasma matrix as per USFDA, and EMA guidelines using a stable isotope-labeled paliperidone-d4 was added as an internal standard. The separation was achieved on a Hypersil BDS C18 column (4.6 mm × 10 cm, 5 µm) with the mobile phase composition of 5 mM ammonium formate in HPLC grade water with 0.05% formic acid (Mobile phase A) and acetonitrile with 0.05% formic acid (Mobile phase B) at a flow rate 1 ml/min in gradient mode. A simple single-step protein precipitation extraction method was adapted using acetonitrile. Quantification of analyte was achieved using positive ionization. Mass ion transitions used were 427.0→207.0 for paliperidone and 431.1→211.0 for paliperidone-d4. The linearity curve was obtained, found to be linear, and the regression coefficient was 0.9990 in the concentration range of 0.25-250 ng/ml. The ruggedness and accuracy of all levels of QC were ranged from 0.81 to 6.02% and 94.22 to 104.40%, respectively. The stability study showed that both paliperidone and IS were stable. The novel method developed during the study was specific, precise, and accurate. Hence, this method would be adapted for estimating paliperidone from marketed tablets after oral administration in rabbits.

INTRODUCTION: Paliperidone is a second-generation atypical antipsychotic drug. USFDA was approved in 2006 for the treatment of schizophrenia and schizoaffective disorder. The chemical structure of paliperidone (PP) and paliperidone–d4 (PP-d4) was given in **Fig. 1**.

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PP is chemically known as 3-[2-[4-(6-fluoro-1, 2-benzoxazol-3-yl)piperidin-1-yl]ethyl]-9-hydroxy-2-methyl-6, 7, 8, 9- tetrahydropyrido[1, 2-a] pyrimidin-4-one [1-2] with its molecular formula and molecular weight were $C_{23}H_{27}FN_4O_3$ and 426.484 g/mol, respectively [3].

The specific mechanism of action of PP is unknown. The therapeutic efficacy of the drug possesses both dopamine D2 and 5-HT2A receptor antagonism activity. Presently PP has been formulated as conventional, extended-release tablets and long-acting intramuscular injectable suspension [4-5].

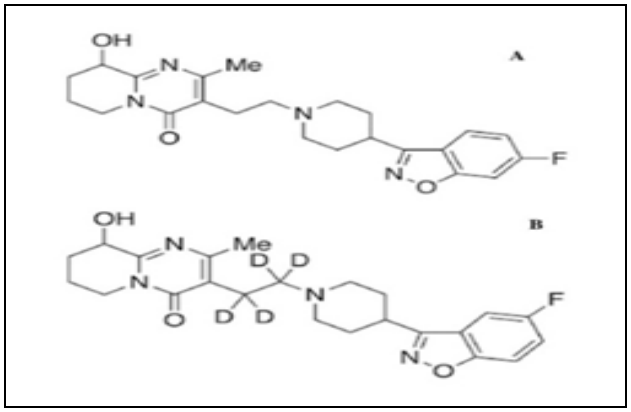

FIG. 1: CHEMICAL STRUCTURE OF (A) PP AND (B) PP-D4

Several bioanalytical methods earlier reported with the literature for quantitative estimation of PP in beagle dog, human blood and urine for solid dosage form and rats and rabbits plasma study with long-acting intramuscular depot injection. Several researchers earlier reported LC-MS/MS methods for quantifying PP in blood and urine. UHPLC-MS/MS method was adapted for the quantification of PP in beagle dog plasma. The separation was achieved using C18 (2.1 × 50 mm, 2.6 µm) under gradient elution with methanol and 0.1% formic acid. 0.3 ml/min flow rate was maintained. A liquid-liquid extraction technique was employed [6]. LC-MS/MS method was developed for the quantification of risperidone and PP in human plasma and urine. The detection was achieved by using the Daicel chiralcel OJ column (50 × 4.6 mm, 10 µm). The composition of the mobile phase was hexane, 0.01M ammonium acetate in isopropanol and 0.01M ammonium acetate in ethanol in the ratio of 80:10:10 at a flow rate of 1 ml/min. Adapted solid-phase extraction technique [7]. The other research group earlier developed LC-MS/MS method using stationary phase has C8 column (100 × 4.6 mm, 5 µm) and the mobile phase composition was methanol: ammonium acetate solution (70:30). The flow rate maintained was 1 ml/min.

A solid-phase extraction technique was employed [8]. LC-MS/MS method was initiated for a pharmacokinetic screening of PP palmitate for long-acting intramuscular depot injection using rats and rabbits. The detection was achieved by using stationary phase ACE C18 column (50 × 4.6 mm, 5 µm) and the mobile phase contains 0.8 mM ammonium formate in HPLC grade water with formic acid (0.05%) (A) and methanol (B), the flow rate was 1 ml/min. Adapted protein precipitation technique [9]. LC-MS/MS method was developed for the quantification of risperidone and 9-hydroxyrisperidone in human plasma. The stationary phase used was Hypersil BDS, C18 column (100 × 4.6 mm, 3 µm) with 0.01 M ammonium formate, pH 4.0 (A), and acetonitrile (B), the flow rate was 0.8 ml/min. The solid-phase extraction technique was employed. 7.5 min total run time was recorded [10]. However, these methods are less suitable for the estimation of PP alone because of the long run time, time-consuming and/or complicated sample extraction procedures.

In the present study, an attempt was made to develop a novel, economical, faster, and simple UFLC-MS/MS method for the quantitative estimation of PP in rabbit plasma using deuterated (PP-d4) internal standard (IS). Validation of the method was done as per USFDA and EMA guidelines. The advantages of the method developed during the study are single step sample extraction procedure was adapted thereby complexity of the extraction procedure was reduced, the sample volume is very low (45.7 µl), recovery is high (>95%), and doesn't require any special treatments thereby this method of sample processing was further economical and simple. 4.5 min of total analysis time was achieved from the present method. This may be attributed to increasing sample turnout, and the method was economical.

MATERIALS AND METHODS:

Materials: The PP (Purity-100.3%) was obtained as a gift sample from Bal Pharma Limited, Bengaluru, India. PP-d4 (Purity-99.82%) was obtained from Eurofins Advinus, Bengaluru, India. PP extended-release tablets were purchased from a local pharmacy. The HPLC grade acetonitrile and methanol were purchased from Merck Specialties Pvt. Ltd., Mumbai, India. Ammonium formate, formic acid, K2EDTA, and dimethylsulfoxide used were analytical grade and purchased from a local supplier, Bengaluru, India.

Animals: Healthy albino rabbits of either sex, 10-12 weeks old, 1.7 to 2.3 kg weight animals were used for *in-vivo* bioavailability study. The experiment was performed by the institutional guidelines, and approval was obtained from the

institutional animal ethics committee (Ref: DCD/ GCP/20/E.C/ADM/2017-2018), Government College of Pharmacy, Bengaluru. Before the experiment started, the animals were fed food and water *ad libitum*.

Methods:

Instrumentation: The prominence UFLC system (Shimadzu Corporation, Japan) is equipped with an API 4000 triple-quadrupole mass spectrometer (ABSCIEX, Concord, Canada) with an electrospray ionization interface.

Liquid Chromatographic Conditions: The chromatographic elution was achieved on a Hypersil BDS C18 (4.6 mm × 10 cm, 5 µm) column and the column oven temperature was maintained at 40 ºC. Gradient elution was performed with mobile phase A: 5 mM ammonium formate in HPLC grade water with 0.05 % formic acid and mobile phase B: acetonitrile with 0.05 % formic acid. The gradient elution programme followed as mentioned in **Table 1** at a flow rate of 1 ml/min and sample cooler temperature was set at 4 ºC. For each analysis, a sample volume of 5 µl was injected.

Mass Spectrometry Conditions: The mass spectrometry condition was achieved using an electrospray ion interface operating atmospheric pressure ionization source in positive mode. The ion source was operated with an ion spray voltage of 5500 V; the source temperature was 550 °C. The curtain and collision gases were 25 and 6 psi, respectively. The ion source gas 1 & 2 (GS1 & GS2) was 40 and 50 psi, respectively. The entrance potential was 10 V for PP and PP-d4.

The MRM mode of the transition state of PP m/z was found to be 427.0→207.0 with a declustering potential of 78 V, collision energy of 42 eV, and collision cell exit potential of 14.5 eV, and MRM mode of the transition state of PP-d4 m/z was found to be 431.1→211.0 with declustering potential of 50 V, collision energy of 25 eV and collision cell exit potential of 10 eV.

Rinsing Solution and Diluents: The rinsing solution was a mixture of methanol and acetonitrile in the ratio of 60:40 v/v, filtered through a 0.45 µm membrane filter and degassed before use. This solution is used for autosampler rinsing to avoid carryover. Dimethylsulfoxide and 80% methanol were used for the stock and working standard preparation, respectively.

Preparation of PP and PP-d4 Stock Solution: The primary stock solutions of PP (1 µg/ml) and PP-d4 (1 µg/ml) were prepared individually using dimethylsulfoxide and sonicated for 10 min to completely dissolve. 200 ng/ml working standard and IS solutions were obtained by further dilution with 80% methanol. These solutions were injected into the mass spectrometer for selecting the precursor and fragment ions.

Preparation of Calibration Curve Standards (CCs) and Quality Control Samples (QCs): The CCs and QCs were prepared independently to evaluate the performance of the proposed method. Nine different concentrations of CCs 0.25, 0.5, 1, 5, 10, 25, 50, 125 and 250 ng/ml and five different concentrations of QCs 0.25 ng/ml (LLOQ), 0.6 ng/ml (LQC), 100 ng/ml (MQC), 200 ng/ml (HQC) and 1000 ng/ml (DQC) were prepared from primary stock solution. To this 47.5 µl blank (untreated/drug-free) rabbit plasma was spiked and mixed well. 20 µl PP-d4 (20 µl of acetonitrile in case of standard blank) and 800 µl acetonitrile were added to all samples. The samples were subjected to vortex for 60 sec followed by centrifugation at 14000 rpm for 10 min set at 4 °C. Transferred approximately 200 µl of supernatant into 1 ml autosampler vials and analyzed using UFLC-MS/MS.

Method Validation: The proposed method for the quantification of PP in plasma has been validated for selectivity, specificity, linearity, precision, accuracy, matrix effect, and stability study as per USFDA and EMA guidelines [11-12] and also based on the papers [13-14] to prove the potential of the developed method.

Carryover Test: The carryover test has been performed by analyzing ULOQ, blank, and Zero samples (blank plasma spiked with IS). (Acceptance criteria: showed no interfering chromatogram peak at the Retention time (RT) of PP and IS and mean analyte and IS response must be ≤20% and ≤5%, respectively of LLOQ) [13].

Selectivity and Specificity: Six different lots of blank rabbit plasma, zero samples, ULOQ and

LLOQ were analyzed to determine the interferences at the RT of PP and IS. Selectivity was determined by comparing chromatograms of the blank plasma with the corresponding spiked plasma with PP and PP-d [4]. Specificity defines the ability to measure the analyte response in the presence of potential impurities and endogenous substances. (Acceptance criteria: accuracy shall not be more than 20% of the nominal concentration for each lot and area response at the RT of PP in the blank matrix was less than 20% of the LLOQ area response and the area response at the RT of PP in the blank plasma was less than 5% of the IS area response for at least 5 out of 6 lots) [13].

Linearity: The linearity was established by injecting triplicate of CCs at 9 different concentration levels ranging from 0.25 to 250 ng/ml.

The standard calibration curve was established by plotting the mean peak area ratio of the PP/PP-d4 versus the nominal concentration (x) of PP in plasma with the least square linear regression analysis with a weighting factor ($1/x^2$). Calculated the correlation coefficient, slope, and intercept using calibration curve (Acceptance criterion: The % accuracy at all levels of QCs should be within 85-115% of nominal concentrations and correlation coefficient (r^2) must be ≥ 0.985) [13].

Precision and Accuracy: The inter and intraday precision and accuracy were determined by analyzing triplicate of QC samples (LLOQ, LQC, MQC, HQC, and DQC) on the same day and three batches on three different days. (Acceptance criteria: The precision and accuracy should be less than 15% except for LLOQ where it should be within 20%) [12].

Matrix Effect and Recovery: A matrix effect check was performed to demonstrate that no considerable endogenous contribution from rabbit plasma affects the measurement of the analyte and IS. Matrix effect check was conducted by analyzing three replicates of low and high-level QCs in six different lots of blank rabbit plasma, one lot of haemolysed plasma, and respective neat solutions. (Acceptance criteria: The deviation of the mean test responses was within 85-115% of the nominal concentration).

Stability Study: The stability study was determined by analyzing low and high-level CCs (0.25 ng/ml and 250 ng/ml) and IS under a different set of conditions: freeze/thaw stability (-20 ºC to ambient temperature, 12 h, three cycles), Benchtop stability (extracted standards were kept at ambient temperature with a duration of 24 h) and autosampler stability (extracted standards were kept in an autosampler at 4 ºC with a duration of 24 h). Finally, stability samples cross-examined with freshly extracted low and high levels of CCs (Acceptance criteria: Mean accuracy should be within 85 to 115% and precision should be less than 15 %) [13].

Adaptability to an *in-vivo* Bioavailability Study: Six healthy albino rabbits were taken and kept for fasting before 24 h of the experiment and then administered orally PP extended-release tablets (1 mg/kg body weight) [15] using an oral gauge. 1 ml of blood samples were withdrawn from marginal ear vein at regular intervals of 0, 1, 2, 4, 8, 12, 18, 20, 22, 24, 36, and 48 h, immediately transferred into a 2 ml Eppendorf tube containing 20 µl K2-EDTA solution (7% w/v), thoroughly mixed and centrifuged at 6000 rpm for 10 min at a temperature of 4 ºC.

Plasma was separated and stored at -20 ºC until analysis. On the day of analysis, the samples were thawed to room temperature and vortexed for 5 min to make a homogeneous solution. A protein precipitation extraction technique was employed to extract the analyte from proteins and other components of plasma. 47.5 µl rabbit plasma, 20 µl IS, and 800 µl acetonitrile was taken, thoroughly mixed, and centrifuged at 14000 rpm for 10 min and temperature maintained was 4 °C. Transferred approximately 200 µl supernatant into a 1 ml autosampler vials and analyzed in UFLC-MS/MS.

RESULTS AND DISCUSSION:

UFLC-MS/MS Method Development: The chromatographic conditions mobile phase composition, stationary phase, gradient elution programme, rinsing solvent, and diluents were finalized after taking various trials to achieve better peak shape, resolution, sensitivity, and appropriate retention time. The satisfactory separation with a sharp peak was achieved with mobile phase 5 mM ammonium formate in HPLC grade water with 0.05

% formic acid (mobile phase A) and acetonitrile with 0.05 % formic acid (mobile phase B) with gradient conditions as mentioned in Table 1 with stationary phase Hypersil BDS C18 column (4.6 mm × 100 cm, 5 µm), flow rate was maintained 1 ml/min and injection volume of 5 µl, 2 min of RT for PP and PP-d4 were obtained, with an overall run time of 4.5 min.

The finalization of mass spectrometry conditions was performed by injecting PP and PP-d4 internal standard solutions into the ESI positive ionization mode. The vital parameters ionization type, MRM transitions, temperature and ion spray voltage, gas parameters such as nebulizer and heater gases, and compound parameters declustering potential, entrance potential, collision energy, and collision cell exit potential were optimized to obtain a better spray and shape for achieving a stable response. **Fig. 2** shows the mass spectrum of PP and PP-d4 of Q1 (precursor) and Q3 (product) ions after fragmentation in the collision cell.

The protein precipitation method was adapted for the extraction of PP rabbit plasma after taking several trials with different solvents tert-butyl methyl ether, diethyl ether, ethyl acetate, acetonitrile, and methanol. Out of these solvents, acetonitrile gave the best recovery.

TABLE 1: GRADIENT PROGRAMME AT A FLOW RATE 1 ML/MIN

Time (min)	Module	Mobile phase A (% v/v)	Mobile phase B (% v/v)
0.01	Pumps	60	40
1.0	Pumps	05	95
3.7	Pumps	60	40
4.5	Controller	Stop	-

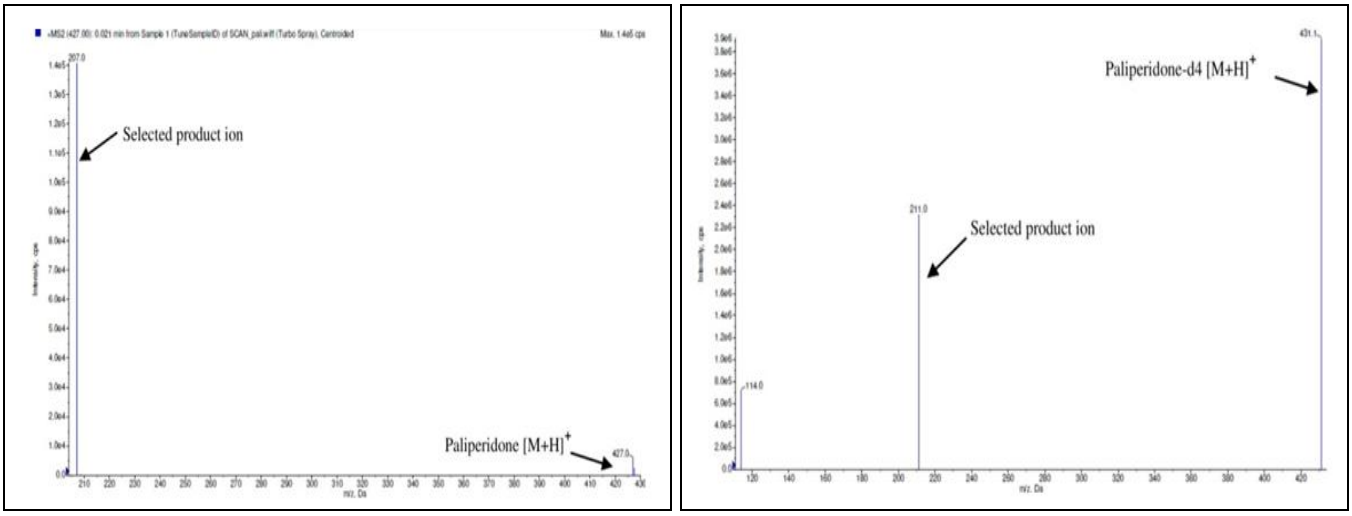

FIG. 2: MASS FRAGMENTATION OF PP AND PP-D4 OF PRECURSOR (Q1) AND PRODUCT (Q3) IONS IN ESI POSITIVE MODE. THE MASS TRANSITION ION PAIR WAS SELECTED AS 427.0→207.0 FOR PP AND 431.1→211.0 FOR PP-D4

Method Validation:

Selectivity and Specificity: The selectivity and specificity of the method were performed by analyzing blank plasma, haemolysed plasma. Zero plasma and LLOQ. The RT of PP and PP-d4 was 2 min **Fig. 3**. indicating no interfering peak of analyte and IS at 2 min of RT. The obtained results of the study revealed that the developed method has good selectivity and analyte-specific.

Linearity: The regression equation from the area response ratio of the peaks of PP/PP-d4 versus concentration **Fig. 4** obtained was y=0.0143x± 0.00013, and the correlation coefficient (r^2) value was 0.9990. The accuracy, precision, and relative error of all levels of CCs of the method were ranged from 97.77 to 103.89%, 0.457 to 3.408%, and 0.30 to 4.00%, respectively. A weighing factor of $1/x^2$ on the standard calibration data was chosen since it resulted in narrow differences between the back-calculated and nominal values at all levels. **Table 2** with RSD and RE below 15%.

The results suggested that the regression curve was linear in the concentration range of 0.25-250 ng/ml and which was within the target concentration of the bioavailability study.

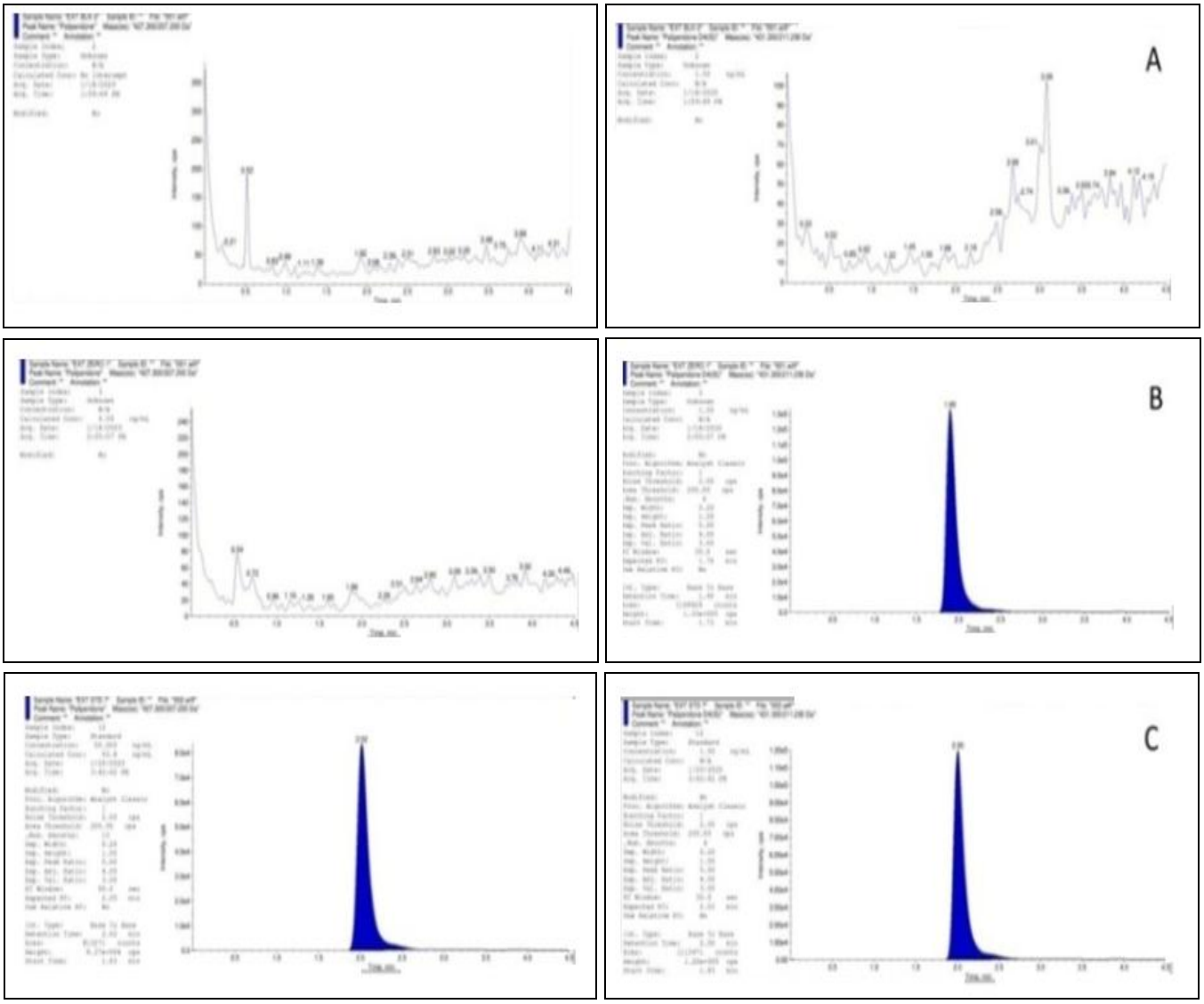

FIG. 3: TYPICAL MRM CHROMATOGRAMS OF (A) BLANK, (B) ZERO SAMPLE AND (C) LLOQ

TABLE 2: MEASURED CONCENTRATION FROM EACH CC's OF PP IN SPIKED RABBIT PLASMA

Nominal conc. (ng/ml)	Mean peak area PP	Mean peak area PP-d4	Area ratio	Measured conc. (ng/ml) ± SD	% RSD	Accuracy (%)	% RE
0.25	3853	1103775	0.00349	0.26 ± 0.009	3.408	103.89	4.00
0.50	7291	1098929	0.00663	0.49 ± 0.008	1.713	97.98	-2.02
1.00	15060	1121567	0.01343	0.96 ± 0.024	2.456	97.77	-2.23
5.00	76787	1100455	0.06978	4.90± 0.076	1.560	97.98	-2.02
10.00	160864	1122695	0.14328	10.03 ± 0.158	1.578	100.31	0.30
25.00	403250	1113440	0.36217	25.27 ± 0.115	0.457	101.43	1.08
50.00	813271	1113471	0.73039	50.40 ± 0.781	1.550	100.78	0.80
125.00	2101550	1126702	1.86522	129.67 ± 1.528	1.178	103.73	3.73
250.00	4137067	1159043	3.56938	248.67 ± 4.155	0.464	99.48	-0.52

Precision and accuracy: The precision and accuracy were assessed by analyzing all levels of QCs triplicates. **Table 3** and **Fig. 5** exhibit related results. The inter-day precision (RSD), accuracy, and relative error at all levels QCs were ranged from 0.81 to 5.93%, 95.25 to 101.90%, and 1.60 to 4.70%, respectively. The intra-day precision (RSD), accuracy, and relative error at all levels QCs were ranged from 1.96 to 6.02%, 94.22 to 104.40%, and 0.83 to 5.26%, respectively. The obtained results of precision and accuracy values were within ±15%. Hence, the developed method has satisfactory accuracy and ruggedness.

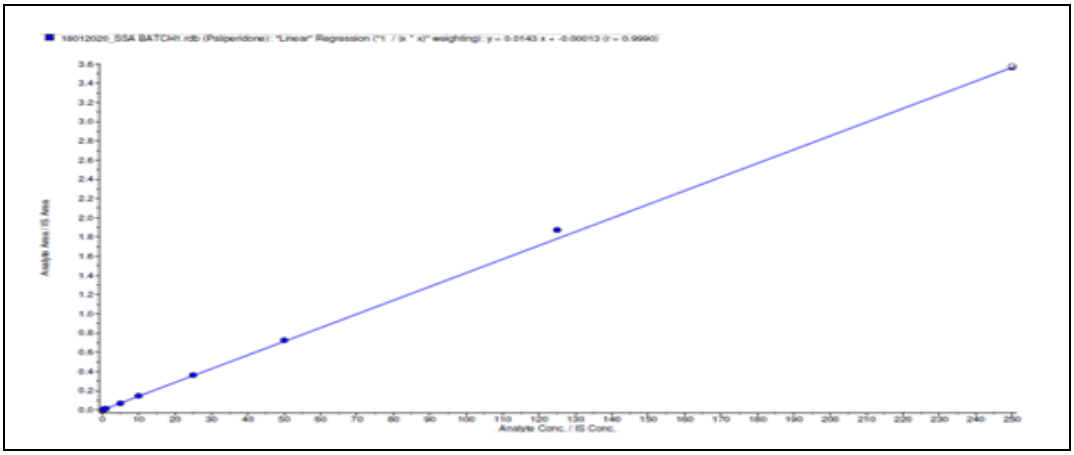

FIG. 4: LINEARITY CURVE OF PP (Y=0.0143X+-0.00013, R2= 0.9990)

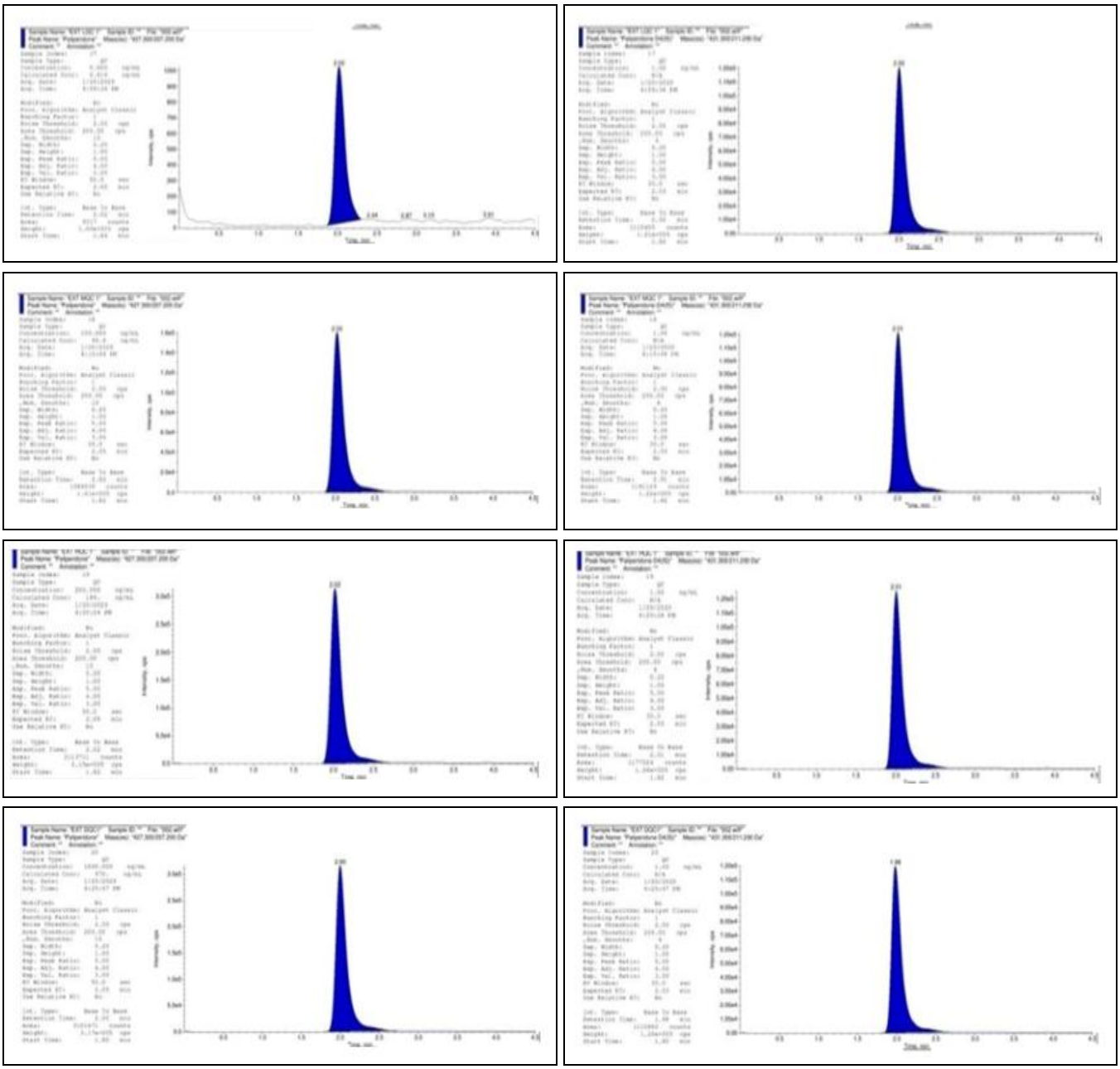

FIG. 5: TYPICAL MRM CHROMATOGRAMS OF PP AND PP-D4 SPIKED IN RABBIT PLASMA AT LQC, MQC, HQC AND DQC

TABLE 3: RESULTS OF INTER-DAY AND INTRA-DAY PRECISION AND ACCURACY

QCs levels	Nominal conc. (ng/ml)	Inter-day				Intra-day			
		Measured conc. (ng/ml) ± SD	Accuracy (%)	% RSD	% RE	Measured conc. (ng/ml) ± SD	Accuracy (%)	% RSD	% RE
LQC	0.60	0.611±0.01	101.90	5.93	1.83	0.605±0.97	100.83	1.96	0.83
MQC	100.00	97.02±0.93	97.04	2.24	-2.96	96.72±1.28	96.72	2.47	-3.28
HQC	200.00	190.60±3.31	95.25	5.21	-4.75	189.48±2.89	94.74	3.12	-5.26
DQC	1000.00	959.20±9.73	97.74	0.81	-2.26	942.24±6.25	94.22	6.02	-5.78

Matrix Effect and Recovery: The mean percentage recovery for PP at 0.6 ng/ml of PP neat and PP with matrix was found to be 99.49 ± 1.11, the recovery of the PP was within acceptable limit ±15%, and no significant matrix effect was observed at the RT of PP and IS indicated that there was no effect on the analyte and IS.

Stability Study: Table 4 consists results of the stability study. The accuracy and RSD at different storage and analytical conditions of low CCs level were ranged from 96.42 to 104.53%, and 2.169 to 7.056%, and accuracy and precision at different storage and analytical conditions of high CCs level were ranged from 91.60 to 102.4% and 2.117 to 5.21%, respectively.

The results suggested that the extracted standards containing PP and PP-d4 were stable in plasma at ambient temperature and in autosampler for 24 h. The obtained results values were within ±15% of the initial (0 h) sample concentrations. Hence, this method was applicable in the long run.

TABLE 4: RESULTS OF STABILITY STUDY

Stability conditions	LLQC (0.250 ng/ml)			HQC (250 ng/ml)		
	Measured conc. (ng/ml)±SD	Accuracy (%)	% RSD	Measured conc. (ng/ml)±SD	Accuracy (%)	% RSD
Freeze-thaw	0.262±2.68	104.53	4.643	249.33±1.15	99.85	3.463
Bench top	0.241±5.24	96.42	7.056	229.12±0.79	91.60	2.117
Autosampler	0.254±0.02	101.57	2.169	256.97±2.56	102.4	5.211

***In-vivo* Bioavailability Study:** The currently developed and validated UFLC-MS/MS method was successfully adapted for the quantification of PP in plasma, followed by the oral route of administration of PP extended-release tablets in rabbits.

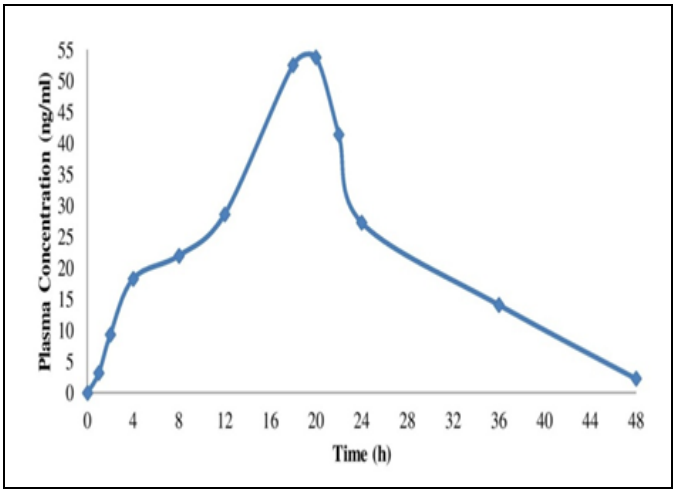

FIG. 6: MEAN PLASMA CONCENTRATION VERSUS TIME PROFILES OF SINGLE-DOSE ORAL ROUTE ADMINISTRATION OF PP EXTENDED-RELEASE TABLETS IN RABBITS

The bioavailability data obtained from the drug in plasma versus the time profile of PP is illustrated in **Fig. 6**. The T_{max}, C_{max}, AUC_{0-t} and AUCo-α were found to be 18 h, 52.60 ng/ml, 890.30 ng.h/ml and 934.53 ng.h/ml. Results suggested that PP reached a peak concentration in plasma about 18 h and it could be quantified up to 48 h in rabbit plasma after oral route administration.

The application to the bio-availability study of PP indicated that this bioanalytical method was suitable and sufficiently sensitive for analyzing PP in rabbit plasma.

CONCLUSION: The present research study could be concluded that, the currently developed and validated UFLC-MS/MS method for the quantification of PP in rabbit plasma has significant advantages as compared to available existing methods. In the present study, an attempt was made to improve the method where it required less or low volume of plasma, simple sample preparation procedure, thereby achieving high percent extraction recovery. Further, the extraction process

was economical. The developed method has a short run time. The stability study results of the proposed method suggested PP and IS were stable in plasma at various storage and analytical conditions. There are few existing methods adapted were liquid-liquid extraction and solid-phase extraction. These methods were not economical, time-consuming, and require special requirements compared to the protein precipitation method. The past method developed by other groups was the adapted protein precipitation method; the sensitivity is less as compared to our method. Hence, the developed method could be adapted to the bioavailability study of PP.

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CONFLICTS OF INTEREST: No potential conflict of interest.

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