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A SENSITIVE LC-MS/MS ASSAY FOR THE DETERMINATION OF NAFTOPIDIL IN HUMAN PLASMA AND ITS APPLICATION TO A PHARMACOKINETIC STUDY

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Keywords:

Naftopidil, LC–MS/MS, Protein Precipitation, Method validation, Pharmacokinetics

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ABSTRACT: A specific, rapid, and sensitive LC-MS/MS assay was developed and validated for the determination of Naftopidil in human plasma. Naftopidil d7 was used as an internal standard (IS). A faster and one-step protein precipitation (PP) technique was employed for sample preparation. The processed samples were chromatographed on a C_{18} column using ammonium formate (5mM) buffer in combination with acetonitrile as the mobile phase. The mass spectrometer was operated in multiple reaction monitoring (MRM) mode, and the protonated ions were monitored at *m*/*z* 393→190 transitions for the naftopidil and *m*/*z* 400 →190 transitions for the IS. The calibration curve was linear over-concentration of 0.30-300 ng/mL, and the method was fully validated as per the recent U.S. FDA Bioanalytical method validation guideline. The precision (%CV) and accuracy results across five concentration levels in 3 different runs were found to be within the specified limits. The present work complied with our initial research objectives and successfully demonstrated the applicability to pharmacokinetic studies.

INTRODUCTION: Benign prostatic hyperplasia (BPH) is a histological condition and most common urological disorder in elderly men. Hypertension is also a common disease in elderly age adults. Selective α1-adrenoceptor (AR) antagonists are well known to be an effective, non-invasive treatment option for patients with BPH [1, 2]. Naftopidil **Fig. 1** is a novel α1-adrenergic receptor antagonist used to treat lower urinary tract symptoms (LUTS) due to benign prostatic hyperplasia (BPH) [3-6]. The drug is also used to treat hypertension and it acts by blocking α1-adrenergic receptor and Ca^{2+} antagonism in vascular smooth muscle [7, 8].

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Naftopidil is safe and adverse effects by the drug on blood pressure/cardiovascular system are unusual.

In concern with literature, few high-performance liquid chromatography (HPLC) [9-11] methods have been developed to analyze naftopidil in biological samples. In general, HPLC based method are not sensitive enough, specific, and required longer chromatographic run time. Hence, rapid, more specific, and sensitive methods such as LC-MS/MS are needed. So far, only one LC-MS/MS was reported in literature for the determination of Naftopidil. The authors Jain *et al*., 2015 [12] published an LC-MS/MS assay for the quanti-fication of Naftopidil in plasma. The reported LLOQ was 0.495 ng/mL, and Propranolol was used as an internal standard (IS). Samples were prepared by using liquid-liquid extraction (LLE) involved many steps like drying, evaporation, and reconstitution.

Moreover, the method utilized relatively large plasma volumes (>200 µL), which may not be favorable for routine bioanalysis/bioequivalence studies.

The objective of the proposed work is to develop a sensitive LC-MS/MS assay for the quantification of Naftopidil in human plasma. Naftopidil d7 **Fig. 1** was used as an IS to minimize the matrix effect and recovery variation between naftopidil and the IS. Moreover, the present assay utilized relatively smaller plasma volumes (50 µL only) and achieved higher sensitivity (0.30 ng/mL) compared to reported method [12]. With the proposed LLOQ of 0.30 ng/mL, Naftopidil was quantifiable beyond 24 h during pharmacokinetic studies in humans. In the present work, we have demonstrated a fully validated LC-MS/MS assay for the determination of naftopidil in human plasma and its application to a pharmacokinetic study in healthy Indian male subjects under fasting conditions.

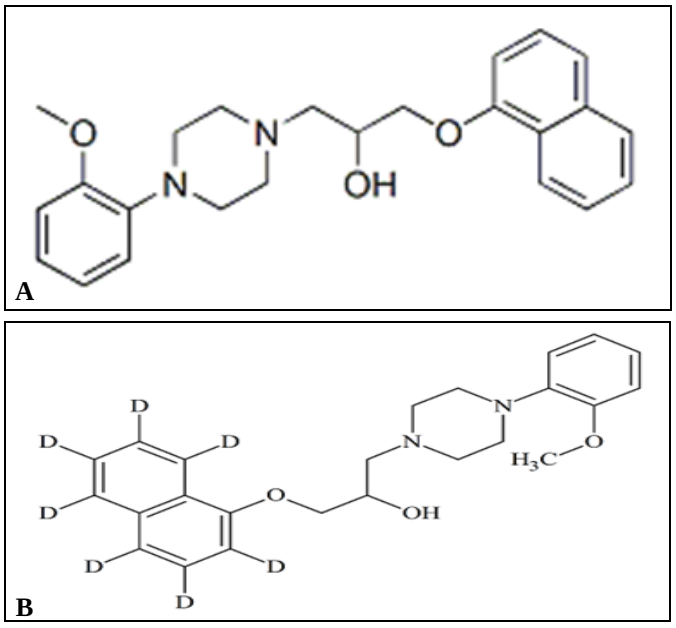

FIG. 1: CHEMICAL STRUCTURES OF A. NAFTOPIDIL AND B. NAFTOPIDIL D7 (IS)

EXPERIMENTAL:

Reagents and Chemicals: Naftopidil reference standard with the purity of >99% was gained from Hetero Labs Limited (Hyderabad, India), whereas and Naftopidil d7 (>99% pure) was gained from Clearsynth Limited (Mumbai, India). LC-MS grade methanol and acetonitrile were obtained from J.T. Baker, Mumbai, India. LC-MS grade ammonium acetate and ammonium formate were purchased from Merck Ltd, Mumbai, India and HPLC grade water was purchased from Rankem Ltd, Hyderabad, India. The control K2 EDTA human plasma was obtained from Deccan's Lab's (Hyderabad, India).

LC-MS/MS Operating Conditions: An HPLC system with LC-20AD binary pump, SIL-HTc autosampler, and a solvent degasser of Shimadzu Corporations (Shimadzu, Kyoto, Japan) connected to a triple quadrupole mass spectrometer of AB Sciex API-4500 (Foster City, CA, USA) with a Turboionspray™ in positive ion mode was used for the present study. The optimized LC-MS/MS operating conditions are listed in **Table 1**.

TABLE 1: OPTIMIZED LC-MS/MS CONDITIONS

Chromatographic conditions		
Mobile Phase	Ammonium formate (5mM) and acetonitrile (15:85, v/v)	
Flow Rate	0.5 mL/min	
Column	Zorbax SB C18, 50*4.6 mm, 3.5µ	
Injection Volume	10 µL	
Run time	2.2 min	
Mass Spectrometric conditions		
Parameter	Naftopidil	IS
Ion transition, m/z	393/190	400/190
Declustering potential, V	100	100
Collision energy, V	45	45
Collision cell exit potential, V	15	15
Entrance potential, V	10	10
Dwell time, msec	200	200
Source parameters		
Source temperature, °C	500	
Ion spray voltage, V	5000	
Curtain gas, psi	30	
Collision gas, psi	5	
Ion source gas 1 (GS1)	40	
Ion source gas 2 (GS2)	40	
Resolution	Unit	

Calibrators and Quality Control (QC) Samples: The stock solutions of Naftopidil and the IS were prepared in methanol. Naftopidil working solutions for spiking into plasma was made in 70% methanol in water. A 200 ng/mL concentration of IS working solution was prepared in acetonitrile and used for the sample preparation. Calibrators were prepared in plasma at a concentration of 0.3, 0.6, 1.5, 7.5, 30, 60, 120, 180, 240 and 300 ng/mL. Similarly, QC samples were prepared at concentrations of 0.3 (LLOQ QC), 0.9 (LQC), 20 (MQC1), 150 (MQC2), and 250 ng/mL (HQC). Calibrators and QC samples were prepared from two independent weighing stock solutions and stored at –70 ± 10 °C until use.

Sample Preparation: A simple PP method was used to extract the analyte from plasma. The frozen plasma samples were allowed to thaw in a water bath and vortexed. Each plasma sample (50 µL) was mixed with 20 µL of ammonium acetate buffer (50 mM). To this, 300 µL of IS dilution (200 ng/mL in acetonitrile) was added and vortexed for 30 s. Then, the samples were centrifuged at 4000 rpm for 5 min, the clear sample was loaded into auto-sampler vials, and 10 µL was injected into the system.

Validation Experiments: We followed bio-analytical method validation guidelines proposed by the U.S. Food and Drug Administration (FDA) [13] and published reports [14, 15] to validate the present method. The validation parameters include system suitability, selectivity, specificity, sensitivity, carryover test, dilution integrity, linearity, precision and accuracy, recovery, and stability.

Pharmacokinetic (pK) Study Design: A pK study in healthy South Indian male subjects (n = 6) was conducted for Naftopidil hydrochloride 50 mg under fasting condition. An age group of 20-45 years with body weight not less than 50 kg, with a body–mass index (BMI) of ≥18.0 kg/m^2 and ≤24.5 kg/m^2 were selected for the study. Consent from all the participants was obtained before registration into the study. Each subject was screened for their healthiness before taken into the study. Venous blood samples were collected into vacutainer tubes (4 mL, K_2 EDTA) at pre-dose and 0.16, 0.33, 0.5, 0.75, 1, 1.25, 1.5, 2, 3, 4, 6, 8, 12, 24, 36, and 48 h post-dose, and the plasma was separated by centrifuging at 4000 rpm for 10 min. The plasma samples were stored at –70 ± 10 °C till their use. Naftopidil pK parameter was calculated using WinNonlin software (Version 5.2) and a non-compartmental model was employed in the calculation of the results.

RESULTS AND DISCUSSION:

Method Development: Nowadays, LC-MS/MS is the most extensively used analytical tool for quantification of drugs in a variety of biological matrices. In the present work, an LC-MS/MS assay was developed to quantify Naftopidil in *in-vivo* samples obtained for pharmacokinetic and bioequivalence studies. Mass spectrometric (MS) conditions were properly adjusted to maximize the analyte signal by infusing the analyte solution.

Naftopidil response was high in the positive ion mode than the negative ion mode. The compound parameters (DP, CE, and CXP) were properly adjusted in multiple reaction monitoring (MRM) mode to get high intense and reproducible product ion spectra. Also, a satisfactory and consistent response was obtained by changing the source dependent parameters (Ion source gas 1 & 2, Source Temperature, and Ion spray voltage). Dwell time for the analyte and the IS was set at 200 ms. The positive ion spray mass spectrum showed a protonated ion by monitoring the transition pairs of *m/z* 393 → m/z 190 (product ion) for naftopidil **Fig. 2a** and *m/z* 400 → m/z 190 (product ion) for the IS **Fig. 2b**. Chromatographic data were processed with Analyst Software™ (version 1.6.1).

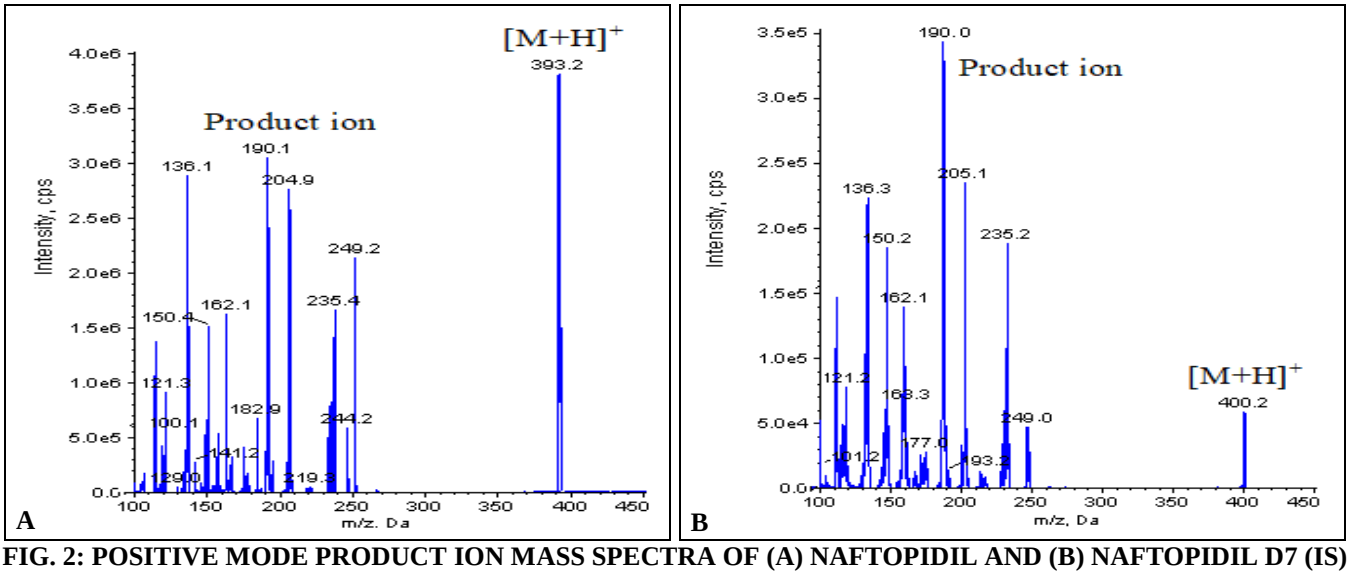

FIG. 2: POSITIVE MODE PRODUCT ION MASS SPECTRA OF (A) NAFTOPIDIL AND (B) NAFTOPIDIL D7 (IS)

Mobile phase combination with a variety of buffers and organic solvents (acetonitrile and methanol) was tested during the method development. A mobile phase composed of methanol and volatile buffers ammonium formate, ammonium acetate, and formic acid resulted in a broad peak for the analyte and longer chromatographic time (>2.5min). Also, the response at the LLOQ level was insufficient to quantify. Hence methanol was replaced with acetonitrile to improve the peak shape and the response. Acetonitrile, in combination with ammonium acetate and formic acid, gave better peak shape and response, but not reproducible. Therefore, an isocratic mobile phase with ammonium formate (5mM): acetonitrile (15:85, v/v) was tested and gave the highest response for the analyte with good peak shape. A variety of HPLC columns packed with C_8 and C_{18} material of different makes (Zorbax, SB C_{18}, 50 × 4.6 mm, 3.5µm; Zorbax XDB-phenyl 75 × 4.6, 3.5µm; Discovery HS C_{18} 50 mm × 4.6 mm, 5 µm; Kromasil 100-5 C_{18}, 50 × 4.6 mm, 5µm; Alltima HP C_{18} 50 × 4.6, 3 µm; Kromasil 100-5C_{18}, 100 × 4.6, 5 µm) were tested to attain acceptable retention time with short run time, symmetric peak shape, better separation from endogenous components and highest response.

The finest chromatography with short retention time (RT) was achieved on Zorbax, SB C_{18} (50 × 4.6 mm, 3.5 µm) column. The mobile phase flow rate was equally important to produce an acceptable peak shape with short run time and was set at 0.5 mL/min. The RT of Naftopidil and the IS was 1.1 min with a total run time of 2.2 min.

The published method [12] utilized LLE procedure to extract the naftopidil from plasma. As an aim to develop a modest and one-step sample extraction procedure, PP was adopted for the present purpose. Moreover, PP is much affordable and easy to perform compared to solid-phase extraction (SPE) or LLE and can easily adapt the method to other laboratories [14-16]. Thus, PP was carried out using acetonitrile and methanol with and without the addition of extraction additives, namely ammonium acetate, formic acid, ammonium formate, *etc*. The extracts were clear, but the recovery was low (50-60%) with both the solvents. The addition of ammonium acetate to the plasma samples helped in refining the extraction recovery. Precipitation with acetonitrile and ammonium acetate as extraction additive gave the highest recovery for the analyte without matrix effect. Stable labeled isotopes as an IS is recommended to overcome the matrix effect in LC-MS analysis [14-16]. Also, these standards will increase the bioanalytical assay precision and accuracy. Hence, Naftopidil d7 was used as an IS and gave good precision and accuracy results.

Method Validation:

Carryover, Selectivity, Specificity and Sensitivity: System suitability test was performed every day before stating the analysis by injecting six consecutive injections of an extracted sample (MQC2) with the IS. The % CV for area ratio was less than 2.0% during the entire course of analysis. Method carryover was critically evaluated by injecting the highest concentration (ULOQ) of the analyte. An extracted blank was injected after injecting the ULOQ sample, and no carryover (0.00%) was found in the blank sample.

Fig. 3 and **4** depict an extracted blank plasma sample and blank sample along with the IS, respectively. The plasma blank sample showed no interference at RT or mass transition of analyte. Similarly, the proposed IS (Naftopidil d7) didn't interfere with the analyte of interest. Six blank plasma lots (4 normal K2 EDTA and one lipemic and one hemolytic plasma) were screened for selectivity test. No interference was detected from plasma components in all the lots tested at the RT of Naftopidil and the IS.

Sensitivity was established at a concentration of 0.30 ng/mL and set as lowest level of quantifiable concentration (LLOQ). At LLOQ, the precision and accuracy of Naftopidil was 1.63% and 106.39%, and the signal-to-noise (S/N) was ≥10. **Fig. 5** depicts a representative MRM-chromatogram of Naftopidil at LLOQ concentration (0.30 ng/mL).

Recovery: The analyte recovery was determined at LQC, MQC2, and HQC concentration levels. The area of extracted samples was compared with the area of post-extraction spiked samples (spiked in the extracted blank sample). The mean recovery (with precision) of Naftopidil was 93.40% (1.65% to 5.09%). The mean recovery of IS (Naftopidil d7) was 96.72%, with a precision ranging from 4.02% to 4.69%.

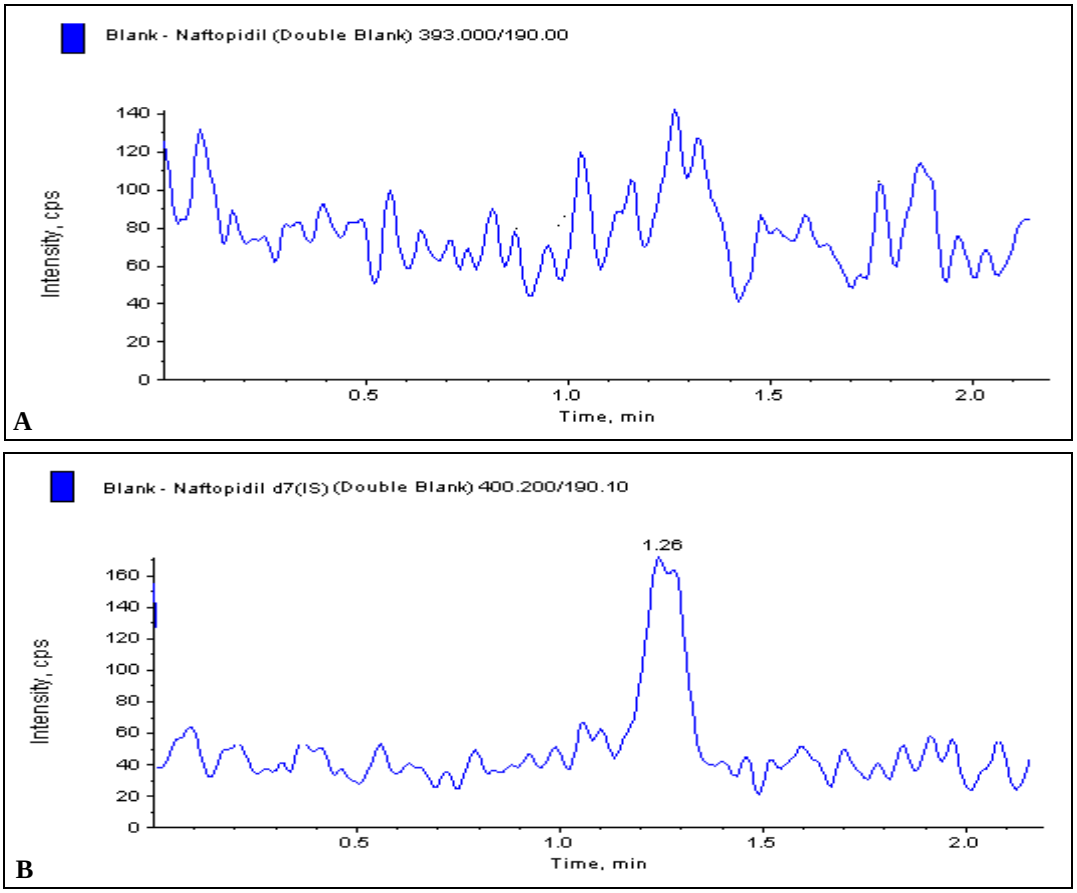

FIG. 3: A MODEL MRM CHROMATOGRAM OF EXTRACTED BLANK PLASMA SAMPLE

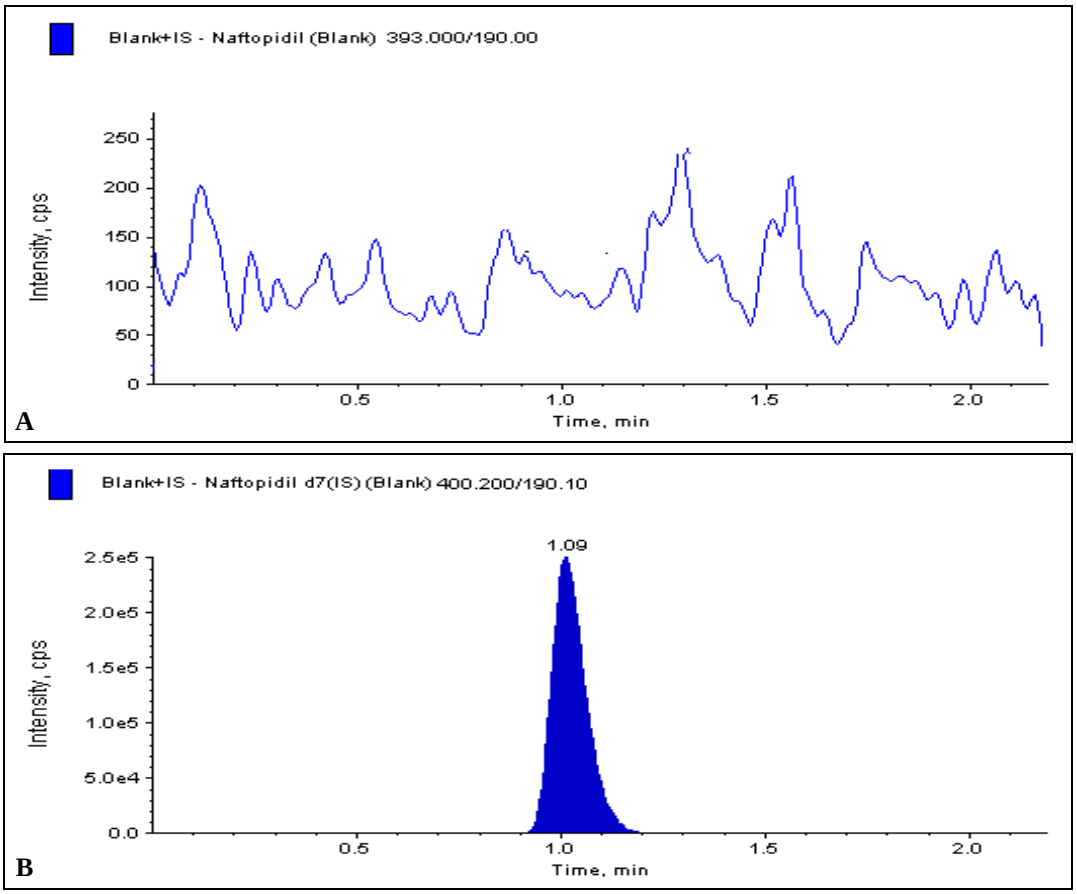

FIG. 4: A MODEL MRM CHROMATOGRAM OF EXTRACTED BLANK SAMPLE WITH THE IS

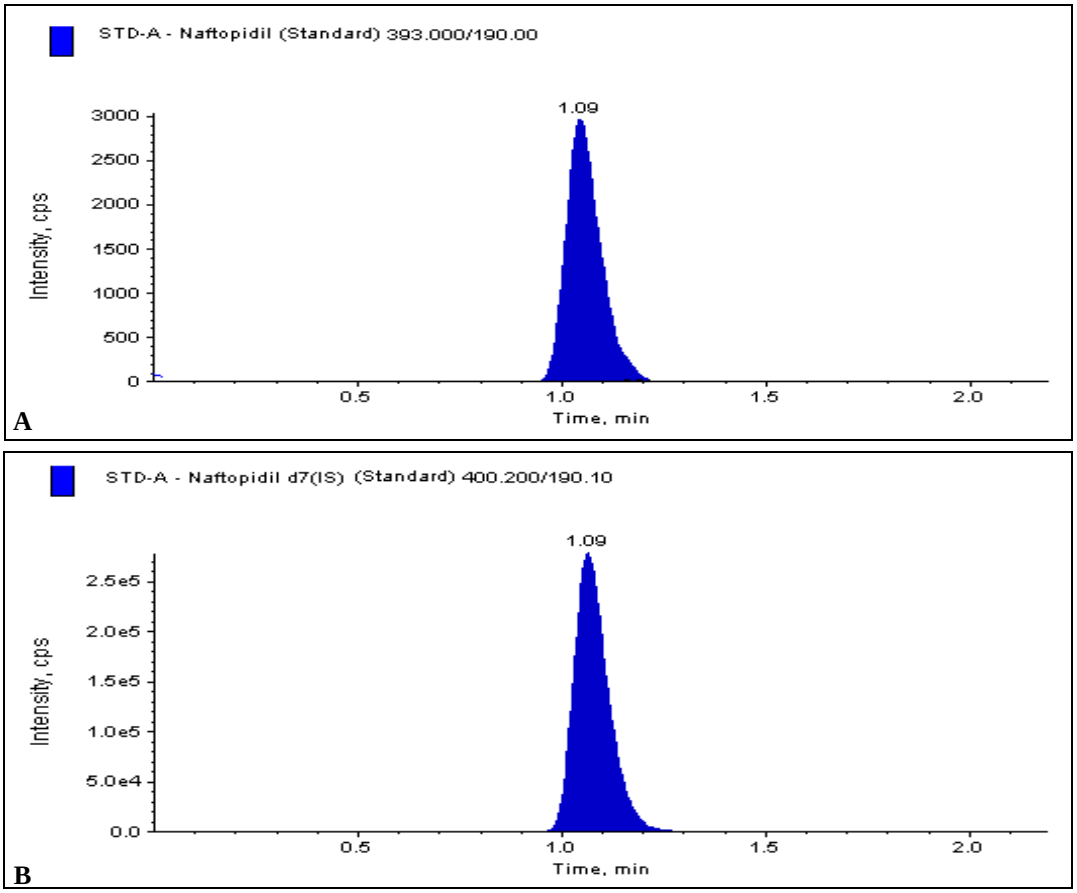

FIG. 5: A MODEL MRM CHROMATOGRAM OF EXTRACTED LLOQ SAMPLE WITH THE IS

Matrix Effect*:* Matrix effect was determined by calculating the IS-normalized matrix factor at LQC and HQC concentration levels. No significant ion suppression or enhancement effect was observed in the tested plasma lots (n=6). The IS-normalized matrix factor valve calculated at LQC and HQC level was 0.99 and 1.01, respectively, which indicated no matrix effect on the proposed assay results **Table 2**.

TABLE 2: MATRIX EFFECT OF NAFTOPIDIL IN DIFFERENT HUMAN PLASMA LOTS

Plasma lot	LQC (0.90 ng/mL)	HQC (250.00 ng/mL)
	IS-normalized MF	IS-normalized MF
1	0.978	0.992
2	0.954	0.972
3	0.949	1.045
4	1.064	1.032
5	1.031	1.053
6	0.968	0.986
Mean	0.991	1.013
S. D	0.0464	0.0341
%CV	4.68	3.36

Linearity, Precision and Accuracy: A total of three standard curves were generated in the concentration range of 0.30-300 ng/mL for Naftopidil in two different days. A regression equation with a weighting factor of $1/x^2$ of the drug to the IS was given the best fit for the concentration–detector response relationship. The correlation coefficient (r^2) for all the batches were ≥0.99. The accuracy and %CV across calibrators ranged from 96.17% to 104.11% and 1.06% to 3.08%.

Intra-day and inter-day precision and accuracy (P&A) were calculated by analyzing three P&A batches in two days. Two batches were run in a day (intra-day), and another batch was analyzed on other day. The results are summarized in **Table 3**.

Sample Dilution Effect: The *in-vivo* study samples concentration above the proposed calibration range can be analyzed by diluting the samples with screened blank plasma. Dilution QC sample (DIQC) was prepared by spiking the 3X concentration of ULOQ (*i.e.*, 900.00 ng/mL). These samples were processed by diluting them two-and-four folds with screened blank plasma. Dilution factor 5 and 10 was applied to quantify these QCs under the undiluted calibration curve standards.

The precision for sample dilution of 5 and 10 times was found to be 1.81% and 1.26%, while the accuracy results were found to be 102.40% and 102.27%, respectively.

TABLE 3: PRECISION AND ACCURACY RESULTS FOR NAFTOPIDIL

QC	Calculated Concentration (mean ± SD; ng/mL)	% Accuracy	%RSD (Precision)
Intra–day (n=12)			
LLOQ	0.33 ± 0.02	108.80	5.85
LQC	0.91 ± 0.05	101.05	5.68
MQC1	21.65 ± 1.19	108.26	5.51
MQC2	155.28 ± 5.52	103.52	3.55
HQC	258.13 ± 9.67	103.25	3.75
Inter–day (n=18)			
LLOQ	0.33 ± 0.02	107.92	5.64
LQC	0.92 ± 0.06	101.85	6.42
MQC1	21.92 ± 1.13	109.23	5.15
MQC2	154.84 ± 6.26	103.23	4.04
HQC	256.56 ± 9.92	102.62	3.86

Nominal concentrations of LLOQ, LQC, MQC1, MQC2 and HQC are 0.30, 0.90, 20.00, 150.00 and 250.00 ng/mL respectively

Stability Studies: The stock solutions of Naftopidil and the IS were found to be stable for 32 days when stored at 2-8 °C and. The % stability (with % RSD range) of Naftopidil and the IS was 101.60 (1.32-1.62%) and 100.64% (0.86-1.26%), respectively.

Also, analyte stability in plasma, as well as in processed samples, were studied under different conditions. Spiked samples were stable at room temperature (benchtop stability) for 8 h, stable up to 4 freeze-thaw cycles, and stable for 75 days at –70 ± 10 °C upon long term storage. Similarly, processed samples were stable in autosampler for 56 h, after 40 h of reinjection and stable 60 h in refrigerator 2-8 °C. The mean % nominal values of the analyte were found to be within ±15% of the predicted concentrations for the analyte at their LQC and HQC levels in **Table 4**.

TABLE 4: VARIOUS STABILITY RESULTS FOR NAFTOPIDIL IN PLASMA (N=6)

Stability experiment	QC (nominal concentration (ng/mL)	Calculated Concentration (ng/mL) Mean ± SD	%Stability /Accuracy	%RSD (Precision)
Autosampler	0.90	0.95 ± 0.09	105.19	9.92
(56 h)	250	265.36 ± 14.49	106.14	5.46
Wet extract stability	0.90	0.87 ± 0.02	96.67	2.72
(60 h)	250	242.28 ± 12.59	96.91	5.20
Bench top (8 h)	0.90	0.88 ± 0.07	97.59	7.84
	250	247.82 ± 7.97	99.13	3.22
Freeze and Thaw	0.90	0.95 ± 0.11	105.74	11.48
(4 Cycles)	250	223.03 ± 25.49	89.21	11.43
Re-injection (40 h)	0.90	0.97 ± 0.05	106.27	5.58
	250	244.78 ± 8.70	97.10	3.55
Long–term	0.90	0.94 ± 0.08	108.27	8.82
(75 days)	250	234.13 ± 6.21	96.50	6.21

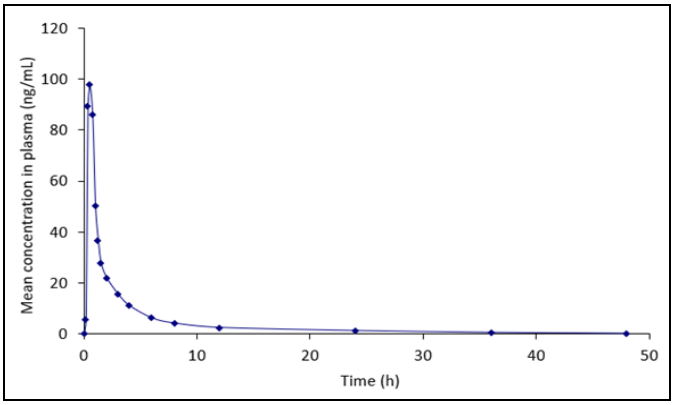

FIG. 6: MEAN PLASMA CONCENTRATION–TIME PROFILE OF NAFTOPIDIL IN HUMANS (N=6)

Pharmacokinetic Results: The suitability of the proposed method for *in-vivo* use was demonstrated by analyzing plasma samples attained for a pK study in healthy subjects. The mean concentration-time profiles of Naftopidil after a single oral dosage of 50 mg Naftopidil hydrochloride is shown in **Fig. 6** and the corresponding pharmacokinetic parameters are listed in **Table 5**.

TABLE 5: PHARMACOKINETIC RESULTS OF NAFTOPIDIL (N=6, MEAN ± SD)

pK Parameter	Mean ± SD
C_{max} (ng/mL)	131.05 ± 15.82
t_{max} (h)	0.50 ± 0.20
AUC_{0-t} (ng h/mL)	251.42 ± 24.43
AUC_{0-inf} (ng h/mL)	255.13 ± 24.12
$t_{1/2}$ (h)	10.21 ± 1.50

CONCLUSION: In conclusion, the present work complied with our initial research objectives and successfully demonstrated the applicability to pharmacokinetic studies. The specific LC–MS/MS assay proposed in this manuscript is simple, fast,

and sensitive concern for the determination of Naftopidil in human plasma. All the validation experiments were conducted as per the recent FDA guidelines. This method makes use of isotope-labeled compound as an IS to avoid possible matrix influence on the results and to limit the variability in recovery between the analyte and the IS. The modest and one step PP procedure gave the highest recoveries for the analyte from plasma.

The standard curve was linear in the range of 0.30-300.00 ng/mL. Naftopidil was stable under various conditions. The method was successfully applied to a pK study of naftopidil in 6 healthy South Indian subjects.

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CONFLICTS OF INTEREST: None

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