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ORIGINAL ARTICLE

Development and validation of analytical method for Naftopidil in human plasma by LC–MS/MS

Pritam S. Jain [a,*], Kajal D. Bobade [a], Pankaj R. Bari [a], Devendra S. Girase [b], Sanjay J. Surana [a,b]

[a] *Department of Pharmaceutical Chemistry, R.C. Patel Institute of Pharmaceutical Education and Research, Karwand Naka, Shirpur, Dist. Dhule 425 405, Maharashtra, India*
[b] *Department of Quality Assurance, R.C. Patel Institute of Pharmaceutical Education and Research, Karwand Naka, Shirpur, Dist. Dhule 425 405, Maharashtra, India*

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Abstract A highly sensitive and simple high-performance liquid chromatographic–tandem mass spectrometric (LC–MS-MS) assay is developed and validated for the quantification of Naftopidil in human plasma. Naftopidil is extracted from human plasma by methyl tertiary butyl ether and analyzed using a reversed-phase gradient elution on a discovery C 18 5 μ (50 × 4.6) column. A methanol: 2 mM ammonium formate (90:10) as mobile phase, is used and detection was performed by MS using electrospray ionization in positive mode. Propranolol is used as the internal standard. The lower limits of quantification are 0.495 ng/mL. The calibration curves are linear over the concentration range of 0.495–200.577 ng/mL of plasma for each analyte. This novel LC–MS-MS method shows satisfactory accuracy and precision and is sufficiently sensitive for the performance of pharmacokinetic studies in humans.

1. Introduction

Naftopidil, a phenylpiperazine derivative, is a novel alpha1-adrenoceptor antagonist and is a new drug for the bladder outlet obstruction in patients with benign prostatic hyperplasia (BPH). Naftopidil is chemically 1-[4-(2-methoxyphenyl)piperazin-1-yl]-3-(1-naphthyloxy)propan-2-ol (Fig. 1) used to treat 'hypertension', Naftopidil exerts its antihypertensive action via alpha1-adrenoceptor blockage and Ca^{2+} antagonism in vascular smooth muscle (Kirsten et al., 1994). Naftopidil competitively inhibited specific [3H] prazosin binding in prostatic membranes of humans. Naftopidil was selective for the alpha 1d-adrenoceptor with approximately 3- and 17-fold higher

* Corresponding author. Tel.: +91 7620227021; fax: +91 02563 251808.
E-mail addresses: pritash79@yahoo.com (P.S. Jain), kajalbobde@rediffmail.com (K.D. Bobade), bpankaj1988@gmail.com (P.R. Bari), girasedevendra@gmail.com (D.S. Girase), sjsurana@yahoo.com (S.J. Surana).

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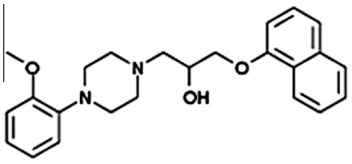

Figure 1 Structure of Naftopidil.

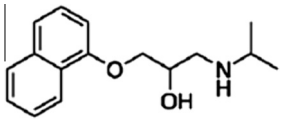

Figure 2 Structure of propranolol.

affinity than for the alpha 1a- and alpha 1b-adrenoceptor subtypes, respectively. In addition to the antagonistic action of this agent on the alpha1 adrenergic receptors of prostatic smooth muscle naftopidil may also act on the lumbosacral cord and thus may improve collecting disorders in patients with benign prostatic hyperplasia (Sugaya et al., 2002). In this way they reduce the pressure on the urethra and so help increase the flow of urine. Newly developed alpha1 adrenoceptor antagonists including naftopidil are free from the ''prazosin-like'' side effect of orthostatic hypotension and associated symptoms (Take et al., 1998). Naftopidil a novel antihypertensive compound, possesses 5HT1 antagonistic properties in addition to being an alpha1 adrenoceptor antagonist (Borbe et al., 1991).

Several chromatographic methods including liquid chromatography–UV (LC–UV) (Jinsong et al., 2000; Kirsten et al., 1994; Li et al., 2006; Sugaya et al., 2002), LC – isotope dilution mass spectrometry (Agostini et al., 1989), and HPLC mass spectrometry (LCMS) (Li et al., 2006) have been developed to measure naftopidil in biological fluids. All these reported methods are inadequate because of insufficient sensitivity, along chromatographic runtime, more plasma volume required for sample processing, and a high injection volume, but still no LC–MS/MS method has been developed and validated in human plasma. (See Figs. 2 and 3).

2. Experimental

2.1. Reagents and chemicals and samples

Pharmaceutical grade naftopidil was supplied by Erregierre Pharmaceuticals (Italy) and was certified to contain 99.8% Naftopidil. Propranolol was supplied by Cadila healthcare limited and was certified to contain 96.9% Propranolol. Both standards were used without further purification. The organic solvents used were of gradient grade and were obtained from Spectrochem. Water was obtained from a Milli-Q Gradient water purification system (Millipore, Barnstead). Ammonium formate, used for mobile phase preparation, was of GR grade and obtained from Merck.

2.2. Chromatographic conditions

A shimadzu UFLC2695LC instrument was used in this study. Separation was carried out on a Discovery C18 column (50 mm_4.6 mm, 5 μ) maintained at 40 °C. The LC mobile phase consisted of methanol: ammonium formate (90:10, v/v). The flow rate was 0.5 mL/min. The injection volume was 10 μL, and the runtime was 3.0 min.

2.3. Mass spectrometry conditions

Detection was carried out by a Applied Biosystem MDS Sciex API 4000 triple-quadrupole MS fitted with an electrospray ionization (ESI) probe and operated in the positive ion mode. The following parameters were optimal: capillary voltage, 5500 V; ion source temperature, 400 °C; GS1, 45; GS2, 55. Detection was carried out in multiple reaction monitoring (MRM) mode. Nitrogen was used as the collision gas. Other parameters are shown in Table 1. Mass spectra of IS (Propranalol) were taken from the literature (Upthagrove et al., 1999).

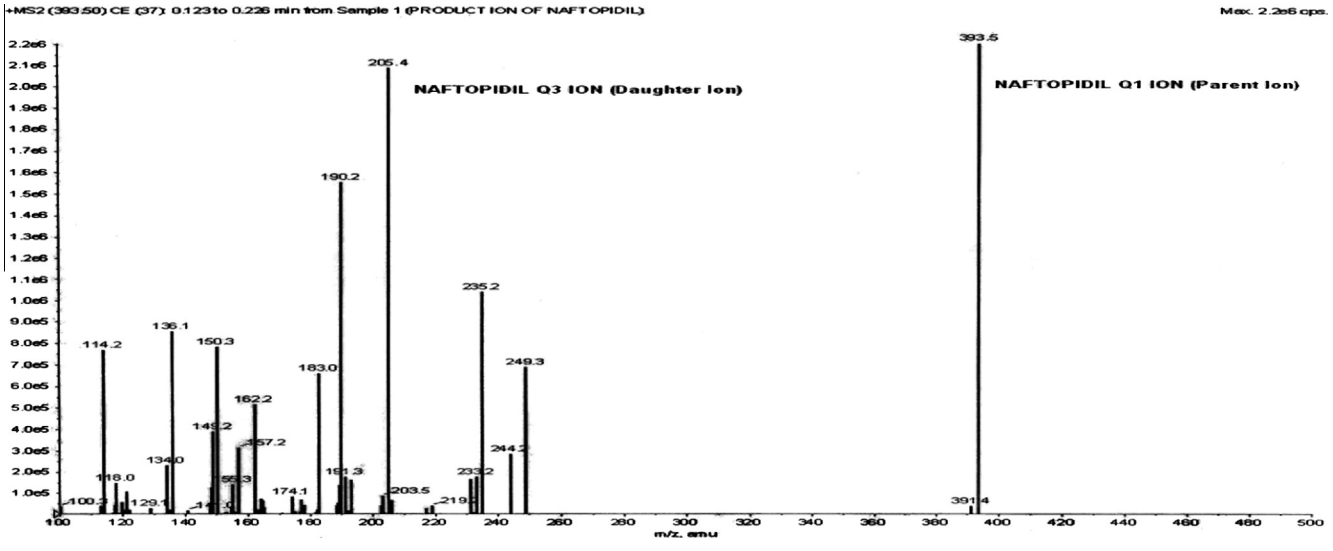

Figure 3 Full scan and product ion spectra of naftopidil.

Table 1 Mass spectrometer parameters.

Parameters	Value
Source temperature (°C)	400
Dwell time per transition (ms)	200
Ion source gas (gas 1) (psi)	45
Ion source gas (gas 2) (psi)	55
Curtain gas (psi)	30
Collision gas (psi)	5
Ion spray voltage (V)	5500
Entrance potential (V)	12 (analyte) and 10 (IS)
Declustering potential (V)	85 (analyte) and 40 (IS)
Collision energy (V)	39 (analyte) and 25 (IS)
Collision cell exit potential (V)	14 (analyte) and 7 (IS)
Mode of analysis	Positive
Ion transition for naftopidil (*m*/*z*)	393.5/205.4
Ion transition for propranolol (*m*/*z*)	260.3/116.2

2.4. Preparation of standard and sample solutions

Standard stock solutions of naftopidil (1 mg/mL) and the IS (1 mg/mL) were separately prepared in 10 mL volumetric flasks with methanol. Working solutions for calibration and controls were prepared from the stock solution by adequate dilution using diluent (methanol: water, 50:50, v/v). The IS working solution (250 ng/mL) was prepared by diluting its stock solution with diluent. Working solutions (20 µL) were added to 980 µL drug-free human plasma to obtain naftopidil concentration levels of 0.495, 0.990, 10.999, 32.350, 111.553, 141.206, 176.508, and 200.577 ng/mL. Quality control (QC) samples were prepared as a bulk based on an independent weighing of standard drug, at concentrations of 0.512 ng/mL (LLOQ), 1.384 ng/mL (low), 98.884 ng/mL (medium) and 170.490 ng/mL (high) as a single batch at each concentration. These samples were divided into aliquots in microcentrifuge tubes (Tarson, 0.3 mL) and stored in the freezer at below −50 °C until analysis.

2.5. Sample preparation

Sample preparation involved a liquid–liquid extraction with Methyl tertiary buty ether. Spiked plasma stability samples of naftopidil were removed from the deep freezer and maintained below −70 C and left at room temperature to thaw. The samples were vortexed, mixed adequately, and centrifuged before pipetting. As soon as the stability samples were thawed, these samples were aliquoted (0.2 ml) and freshly prepared. CS and QC samples were spiked with 50 µL IS (250.0 ng/mL) into pre labeled RIA vials to each tube except blank and mixed for 30 s on vortexer. To this 50 µL 5% ammonia was added to each tube and vortexed for 30 s. 2 ml extraction of solvent methyl tertiary butyl ether was added to each tube and vortexed for 3 min. Samples were centrifuged at 4600 RPM for 5 min at 10 °C. Supernatant layer was separated and evaporated in nitrogen gas up to dryness, reconstituted with 300 µL reconstitution solution (Methanol: 2 mM Ammonium formate (pH-7.00 (90:10) and vortexed for 1 min. The samples were transferred to HPLC vials for analysis.

3. Method validation

3.1. Specificity

To verify the absence of interfering substances around the retention time of analytes, 20 blank samples were analyzed.

3.2. Linearity

Calibration curves were constructed using matrix-matched standard solutions by plotting the peak area of the quantitative ion of each analyte versus concentrations. Concentration range was found to be 5–150 µg/mL and correlation coefficient was 0.999.

3.3. Limit of detection and quantitation

The limits of detection (LODs), defined as the lowest concentration that the analytical process can reliably differentiate from background levels, were estimated for those concentrations that provide a signal-to-noise ratio of 3:1 which was found to be 0.19. The LOQs were estimated at a signal-to-noise ratio of 10:1 found to be 0.57.

3.4. Accuracy and precision

Intra-assay precision and accuracy were calculated at LLOQ (0.512 ng/mL), low quality-control (LQC, 1.384 ng/mL), middle quality-control (MQC, 98.884 ng/mL), and high quality-control (HQC, 170.490 ng/mL) levels for the six replicates, each of the same analytical run. Inter-assay precision and accuracy were calculated after the replicates in six different analytical runs. (Tables 2 and 3).

3.5. Recovery

The recovery of naftopidil was calculated by comparing the peak area of the analyte from the extracted plasma standard with that obtained from an un-extracted standard at the same concentration for the QC samples containing, 1.384, 98.884, 170.490 ng/mL. IS recovery was tested at 500.0 ng/mL by

Table 2 Intra batch accuracy and precision.

Quality control samples	Concentration added (ng/mL)	Concentration found (ng/mL) (mean ± S.D.)	Precision (%) CV	Accuracy (%)
LLOQ QC	0.512	0.567 ± 0.06	10.5	110.7
LQC	1.384	1.586 ± 0.18	11.4	114.6
MQC	98.884	100.490 ± 2.15	2.1	101.6
HQC	170.490	169.979 ± 5.33	3.1	99.7

Table 3 Inter batch precision and accuracy.

Quality control samples	Concentration added (ng/mL)	Concentration found (ng/mL) (mean ± S.D.) (n = 3)	Precision (%) CV	Accuracy (%)
LLOQ	0.512	0.476 ± 0.08	16.3	93.0
LQC	1.384	1.479 ± 0.13	8.8	106.9
MQC	98.884	101.866 ± 4.72	4.6	103.0
HQC	170.490	173.095 ± 8.23	4.8	101.5

n = No. of estimation.

Table 4 Stability data of Naftopidil.

Stability experiments	Recovery (%)	Sample concentration (ng/mL) (n = 6) LQC, HQC	Concentration found (ng/mL) (mean ± S.D.)	% Mean change at quality control level
Three freeze–thaw cycles	After 3rd FT cycle at −(20 ± 5 °C)	1.384	1.475 ± 0.0345	6.6
		170.490	177.679 ± 3.9514	4.2
Autosampler stability for	47 h at 6 °C	1.384	1.383 ± 0.0617	0.0
		170.490	165.218 ± 5.0777	−3.1
Bench top stability for	At room temperature (20 h)	1.384	1.480 ± 0.0253	6.9
		170.490	176.820 ± 2.6191	3.7
Dry extract stability	At −70 °C	1.384	1.357 ± 0.0870	−2.0
		170.490	166.820 ± 4.6241	−2.2

n = No. of estimation.

comparing six extracted and un-extracted samples at each concentration. (Table 4).

3.6. Matrix effect

The matrix effects were investigated for six different samples of plasma, comprising four lots of normal control heparinized plasma, one lot of lipemic plasma, and one lot of hemolyzed plasma. Three samples each at the LQC and HQC levels were prepared from different lots of plasma (i.e., a total of 36 QC samples) and checked for accuracy to see whether the matrix affected the back-calculated value of the nominal concentrations for these different plasma samples. The back-calculated concentrations of all LQC and HQC samples must be within 85–115% of their nominal concentration. At least 67% of QC samples must fall within the above-mentioned criteria at each LQC and HQC levels.

4. Stability

Exhaustive experiments were performed to assess the stability of **naftopidil** in stock solution and in plasma samples under different conditions, simulating the conditions occurring during the analysis of study samples: room-temperature stability, extracted sample stability (process stability), freeze–thaw stability, and the long-term stability of plasma samples, dry extract stability, and stock solution stability.

5. Results and discussion

5.1. Method development and optimization

The chromatographic conditions, especially the composition of mobile phase, were optimized through several trials to achieve good resolution and symmetric peak shapes for the analyte and the IS, as well as short run time. Modifiers such as ammonia and ammonium formate alone or in combination in different concentrations were added. It was found that a mixture of Methanol: 2 mM ammonium formate (90:10, v/v; pH 7.0) could achieve this purpose and was finally adopted as the mobile phase. Ammonia was found to be necessary in order to lower the pH to protonate naftopidil and thus deliver a good peak shape. The percentage of ammonia was optimized to maintain this peak shape while being consistent with good ionization and fragmentation in the mass spectrometer.

The tandem mass spectrometer allows the selective detection of substances with varying masses or fragments without chromatographic separation. The development of the chromatographic system was focused on short retention times in order to assure high throughput, paying attention to matrix effects as well as good peak shapes. The high proportion of organic solvent (Methanol: 2 mM ammonium formate (90:10, v/v; pH 7.0)) eluted the analyte and the IS at retention times of 1.8 and 1.6 min, respectively. A flow-rate of 0.500 mL/min produced good peak shapes and permitted a run-time to 3 min.

Internal standard is necessary for the determination of analyte in biological samples. In the initial stages of this work, several compounds were investigated to find a suitable internal standard and finally Propranolol, structurally related to naftopidil, was found to be the best for the present purpose.

Clean chromatograms were obtained and no significant direct interferences in the MRM channels at the relevant retention times were observed. However, in ESI, signal suppression or enhancement may occur due to co-eluting endogenous components of the sample matrix. These potential matrix effects were evaluated by spiking blank plasma extracts at the low and high QC levels. The resulting chromatograms were compared with those obtained for clean standard solutions at the same concentrations. Three independent plasma

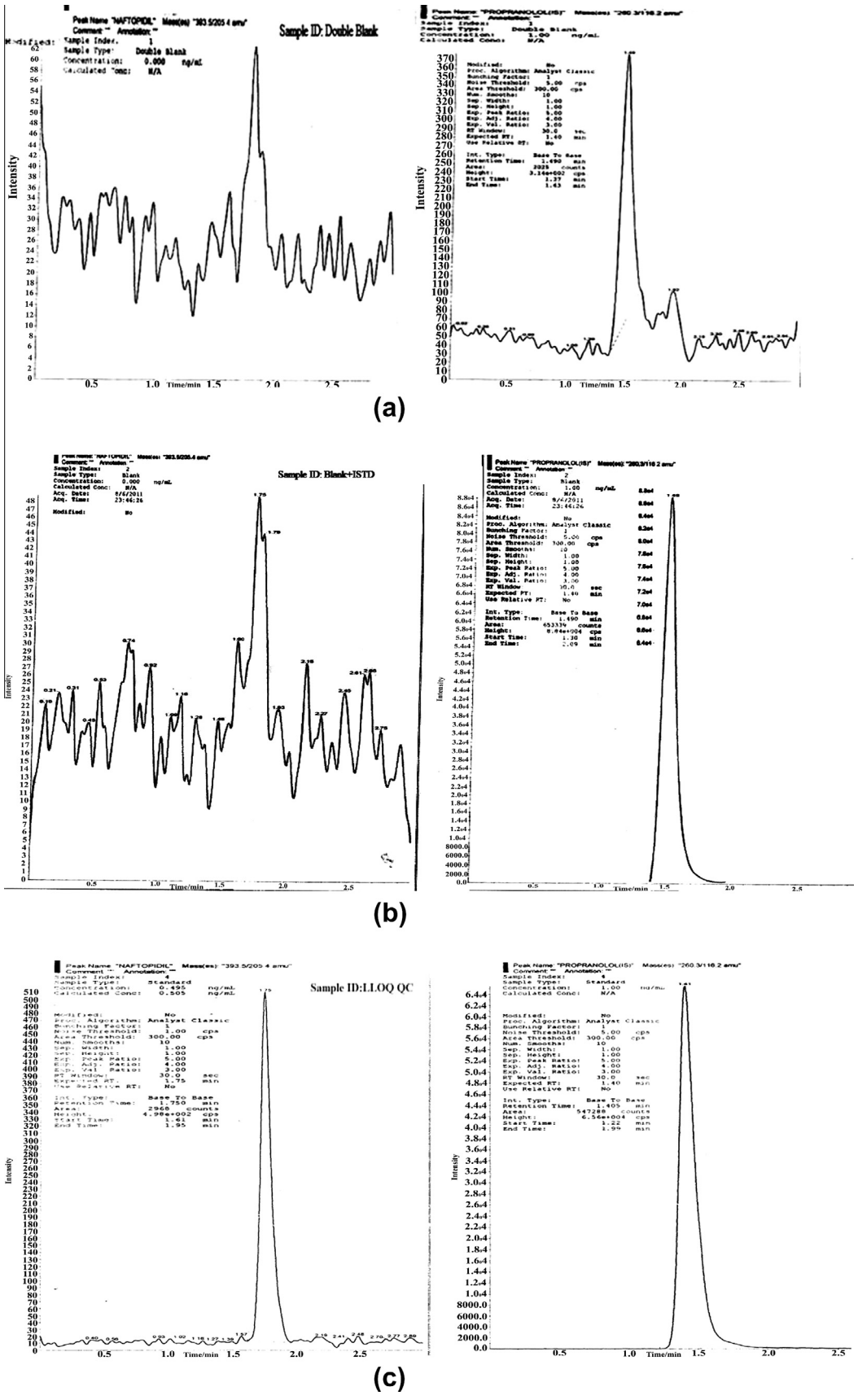

Figure 4 Typical representative chromatograms (a) chromatogram of blank plasma, (b) chromatogram of blank + IS, (c) chromtogram of LLOQ.

lots were used with three samples from each lot. The results (data not shown) showed that there was no significant difference between peak responses for spiked plasma extracts and clean solutions.

5.2. Method validation

The developed method was validated in terms of specificity, linearity, precision and accuracy, recovery, matrix effect, stability, dilution integrity (Yu et al., 1995).

5.2.1. Specificity

The specificity of the method was investigated by comparing chromatograms obtained from six different sources of plasma. The area observed at the retention time of naftopidil was much less than 20% of the LLOQ area (0.512 ng/mL). The representative chromatograms, shown in Fig. 4a and b, indicate that there was no interference with the analyte and IS from endogenous substances in the plasma.

5.2.2. Linearity

The linearity of the method was determined by a weighted least-square linear regression analysis of standard plots associated with eight-point standard calibration curves. Best-fit calibration curves of peak-area ratio against the concentration were drawn. The concentration of naftopidil was calculated from the simple linear equation using a regression analysis of the spiked plasma CSs with a reciprocal of the square of the drug concentration, $1/x^2$, as a weighting factor. The calibration plots were linear from 0.495–200.577 ng/mL with r^2 0.9984. (Fig. 5).

5.2.3. Precision and accuracy

The within batch % coefficient of variation for naftopidil was ranged from 2.0% to 11.4% and % accuracy was ranged from 81.9% to 114.6%. The results are within ±15%. (Tables 2 and 3).

5.2.4. Recovery

The percent mean recovery for naftopidil was observed as 81.91. The mean recovery of IS was 74.4% at a concentration 500.0 ng/mL.

5.2.5. Matrix effect

Processed and analyzed calibration standards in the same matrix which is to be used during validation experiment and three replicates from three different lots of plasma at LQC and HQC levels as per the procedure are described in the sample preparation section. % Nominal concentration was found to be 104% (LQC) and 100.5% (HQC) for the dilutions, which passed the limit of 85–115%.

5.2.6. Dilution integrity

Analyte spiking stock solution was spiked in blank plasma to get concentration equivalent to three times of ULOQ and diluted with blank plasma to get 1/5 and 1/10 concentrations of the spiked sample or as per requirement. Calibration standards and six aliquots each of diluted samples (1/5 and 1/10 dilutions) were processed and analyzed as per the procedure as described in sample preparation. Precision and accuracy of the dilution integrity of the QC's should be ⩽15% and within ±15% of the nominal concentration, respectively. % Nominal concentration was found to be 109.6% (1/5 concentration) and 108.9% (1/10 concentration) for the dilutions, which passed the limit of 85–115%.

5.2.7. Solution stability

The stability of the analyte and IS in human plasma under different temperature and timing conditions, as well as the stability in stock solution, was evaluated as follows. All the stability

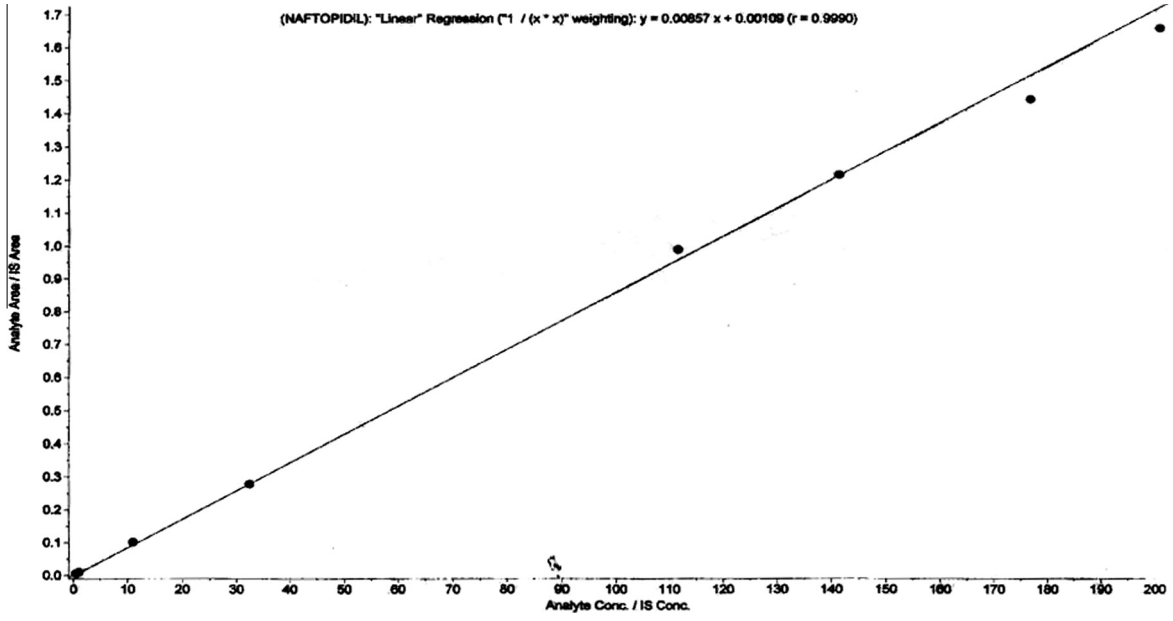

Figure 5 Linearity of Naftopidil.

studies were carried out at two concentration levels (1.384 and 170.490 ng/mL as low and high values) with six determinations for each.

For short-term stability determination, stored plasma aliquots were thawed and kept at room temperature for a period of time exceeding that was expected to be encountered during the routine sample preparation (around 6 h). These results indicate reliable stability behavior under the experimental conditions of the regular analytical procedure.

The stability of QC samples kept in the autosampler for 25 h was also assessed. The results indicate that solutions of naftopidil and IS can remain in the autosampler for at least 25 h, without showing a significant loss in the quantified values, indicating that samples should be processed within this period of time Table 4.

The data representing the stability of naftopidil in plasma at two QC levels over three freeze and thaw cycles are given in Table 3. These tests indicate that the analyte is stable in human plasma for three freeze and thaw cycles, when stored at below −20 °C and thawed to room temperature.

The stability study of naftopidil in human plasma showed reliable stability behavior, as the mean of the results of the tested samples was within the acceptance criteria of ±15% of the initial values of the controls. These findings indicate that storage of naftopidil in plasma samples at below −20 °C is adequate, and no stability-related problems would be expected during routine analysis for pharmacokinetic, bioavailability or bioequivalence studies.

The stability of stock solutions was tested and established at room temperature for 6 h and under refrigeration (2–8 °C) for 8 days. The recoveries for naftopidil and IS were 77.3% (low Q.C), 69.2% (middle Q.C), 59.5% (higher Q.C) and 74.4% respectively. The results revealed optimum stability for the prepared stock solutions throughout the period intended for their daily use.

6. Conclusion

In summary, the LC–MS/MS method for the quantitation of naftopidil in human plasma was developed and fully validated as per FDA guidelines. This method offers significant advantages over those previously reported, in terms of improved sensitivity and selectivity, faster run time (3 min) and lower sample requirements. Thus the volume of samples to be collected per time point from an individual during trial is reduced significantly, allowing inclusion of additional points. With dilution integrity up to 10-fold, we have established that the upper limit of quantification is extendable up to 200.577 ng/mL. Hence, this method is useful for single and multiple ascending dose studies in human subjects. The current method has shown acceptable precision and adequate sensitivity for the quantification of naftopidil in human plasma samples obtained for pharmacokinetic, bioavailability or bioequivalence studies. The desired sensitivity of naftopidil was achieved with an LLOQ of 0.495 ng/mL, which has a within and between-batch CV of 10.5 and 16.3%, respectively. The sensitivity could be further improved by sample concentration. The simplicity, liquid–liquid extraction and sample turnover rate of 2 min per sample, make it an attractive procedure in high-throughput bioanalysis of naftopidil. The validated method allows quantification of naftopidil in the 0.495–200.577 ng/mL range.

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