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# Simultaneous determination of Avanafil and Dapoxetine in human plasma using liquid chromatography/tandem mass spectrometry (LC-MS/MS) based on a protein precipitation technique

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A rapid and selective LC-MS/MS method is described for the simultaneous assay of Avanafil and Dapoxetine in human plasma *via* a protein precipitation (PP) sample preparation technique. Tadalafil was chosen as the internal standard reaching good recovery and reproducibility while diminishing the effects of the matrix. An Agilent Zorbax Eclipse XDB $C_{18}$ column (4.6 × 50 mm, 1.8 μm) was used for the chromatographic separation and analysis, while 0.1% formic acid : acetonitrile (60 : 40, v/v) was utilized at a flow rate of 0.5 mL min$^{-1}$. It was revealed that 6 min stop time accomplished the best separation. The assay was linear over the range of 10–6000 ng mL$^{-1}$ for both drugs. The established bio-analytical method validation was demonstrated following US-FDA recommendations including sensitivity, selectivity, linearity, accuracy and precision. Furthermore, other validation parameters were assessed such as the dilution integrity, matrix effect, carryover, and analyte stability during both short- and long-term sample processing and storage. The adopted method was efficaciously applied to a clinical study for the concurrent determination of Avanafil and Dapoxetine in human plasma.

## 1. Introduction

Avanafil, (*S*)-4-((3-chloro-4-methoxybenzyl)amino)-2-(2-(hydroxymethyl)pyrrolidin-1-yl)-*N*-(pyrimidin-2-ylmethyl)pyrimidine-5-carboxamide (Fig. 1a), is a phosphodiesterase type 5 inhibitor with an efficient vasodilation effect. It has been widely used in the treatment of erectile dysfunction and impotence. Moreover, Avanafil action has been investigated for the treatment of pulmonary hypertension.[1] Dapoxetine, (*S*)-*N*,*N*-dimethyl-3-(naphthalene-1-yloxy)-1-phenylpropan-1-amine (Fig. 1b), promotes the obstruction of the serotonin transporter, leading to the enhancement of the effect of serotonin at the post-synaptic cleft, thus, promoting the ejaculatory delay.[2] Combining both drugs in a single dosage form for the treatment

of erectile dysfunction was officially approved by the U.S. Food and Drug Administration (FDA) in April 2012.[3]

A literature survey revealed some analytical techniques for Avanafil and Dapoxetine determination either individually or in
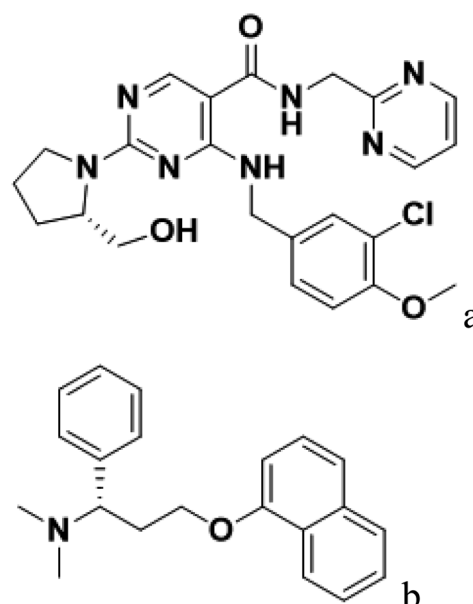
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Fig. 1 Structure of (a) Avanafil and (b) Dapoxetine.

**Table 1** A comparison between literature and the developed method regarding different LCMS/MS methods used for the determination of Avanafil and Dapoxetine

| Analytes | Extraction technique | Stationary phase | Mobile phase | MRM ion transitions $m/z$ with MS detection | Linear range, ng mL$^{-1}$ | Applications | Ref. |
|---|---|---|---|---|---|---|---|
| Avanafil | Solvent extraction | Chromolith® RP-C$_{18}$-18e, (4.6 × 100 mm) | 0.1% formic acid in water : 0.1% formic acid in acetonitrile (75 : 25, v/v) at a flow rate of 0.5 mL min$^{-1}$ | Avanafil 483.95 → (375.1, 155.05, 233.1) | 150–6000 | Pharmaceutical preparations | 21 |
| Avanafil | Solid-phase extraction | Capcell Pak C$_{18}$ (2.0 × 50 mm, 3 μm) | 10 mM ammonium formate, pH 2.5 : acetonitrile (65 : 35, v/v), at a flow rate of 0.3 mL min$^{-1}$ | Avanafil 484.1 → (375.1) | 1–250 | Human plasma | 22 |
| Avanafil | Solvent extraction | Poroshell 120 EC-C$_{18}$ (3.0 × 150 mm, 2.7 μm) | 10 mM ammonium formate and formic acid, pH 4.6 in ultrapure water : 0.1% formic acid in acetonitrile at flow rate of 0.55 mL min$^{-1}$ | Avanafil 484.18566 → (375.1217, 155.0254) | 5–1000 | Urine samples | 23 |
| Dapoxetine | Protein precipitation | Acquity BEH C$_{18}$ (2.0 × 100 mm, 1.7 μm) | Acetonitrile : 0.1% formic acid, pH 6.0 adjusted with ammonium hydroxide (45 : 55, v/v), at a flow rate of 0.2 mL min$^{-1}$ | Dapoxetine 306.2 → (261.1) | 1–500 | Rat plasma | 24 |
| Dapoxetine | Protein precipitation | C$_{18}$ Fortis (2.0 × 50 mm, 1.7 μm) | 0.5% formic acid : acetonitrile (60 : 40, v/v) at a flow rate of 0.3 mL min$^{-1}$ | Dapoxetine 306 → (261) | 1–500 | Rat plasma | 25 |
| Dapoxetine | Liquid–liquid extraction | ACE C$_8$ (4.6 × 50 mm, 5.0 μm) | Acetonitrile : 0.01 M ammonium acetate + 0.02% formic acid solution (85 : 15,v/v) at a flow rate of 0.9 mL min$^{-1}$ | Dapoxetine 306.2 → (157.2) | 5.0–600 | Human plasma | 26 |
| Dapoxetine | Protein precipitation | Ultimate XB C$_{18}$, (2.1 × 50 mm, 5.0 μm) | 0.1% formic acid : acetonitrile containing 0.1% formic acid at a flow rate of 0.8 mL min$^{-1}$ | Dapoxetine 306.2 → (157.2) | 1–500 | Human plasma | 27 |
| Dapoxetine | Protein precipitation | Acquity UPLC BEH C$_{18}$ (2.1 mm × 50 mm, 1.7 μm) | Acetonitrile : 0.1% formic acid at a flow rate of 0.4 mL min$^{-1}$ | Dapoxetine 306.3 → (261.2) | — | Rat plasma | 28 |
| Avanafil, Dapoxetine | Protein precipitation | Zorbax eclipse XDB C$_{18}$ (4.6 × 50 mm, 1.8 μm) | 0.1% formic acid : acetonitrile (60 : 40, v/v) at a flow rate of 0.5 mL min$^{-1}$ | Avanafil 485 → (375, 155), Dapoxetine 306 → (183, 157) | 10–6000 | Human plasma | The developed method |

conjunction with other medicines. These techniques included UV spectrophotometry,[1,2,4–10] spectrofluorimetry[6,11–16] and capillary electrophoresis.[17] Various chromatographic methods were also applied for determination of the cited mixture such as HPTLC[4] and HPLC with different detectors as UV, fluorescence detection[1,8,9,12,16,18–20] and with tandem mass spectrometry.[21–28] A brief comparison between the different LC-MS/MS methods from the previously reported in the literature and the developed one is elucidated in Table 1.

To the best of our knowledge, in spite of the extensive methods previously applied for the analysis of Avanafil and Dapoxetine, there is no LC-MS/MS method for simultaneous determination of both analytes under investigation in human plasma matrix applying Protein Precipitation (PP) technique for sample preparation while using Tadalafil as an internal standard (IS).

Thus, our aim was to establish a selective and sensitive LC-MS/MS method applying a cost effective procedures for sample preparation in order to avoid matrix effect and charge
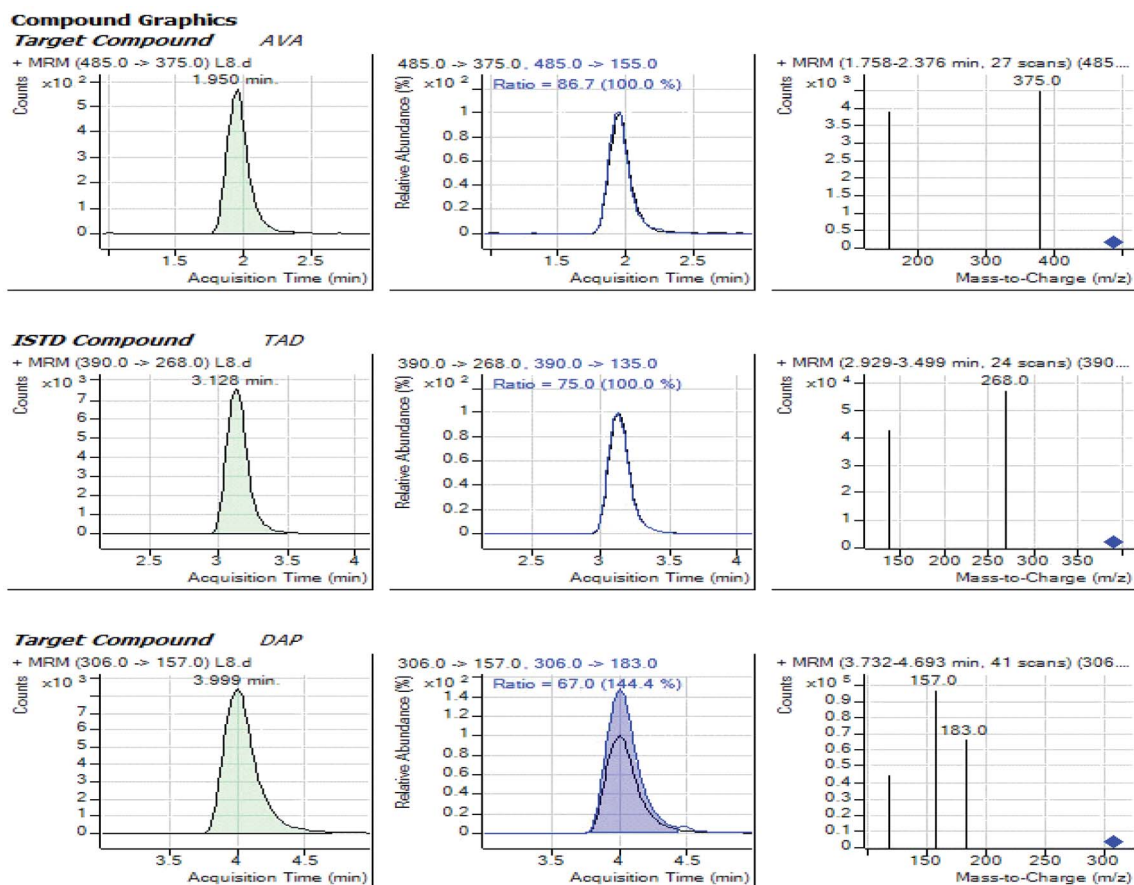
**Fig. 2** Chromatograms of Avanafil, Dapoxetine and Tadalafil.

competition troublesome which represent serious challenges in face of LC-MS/MS method development. Furthermore, the adopted method has been verified and validated following the US-FDA guidelines. The suggested method exhibited important advantages, comprising the shorter run time, wider linearity range and the enhanced sensitivity, accuracy and precision.

## 2. Materials and reagents

Avanafil, purity (99.95%), was acquired from Rakshit Drugs Pvt. Ltd., Hyderabad, India. Dapoxetine HCl, purity (99.97%), was acquired from Synergene Active Ingredients Pvt. Ltd., Hyderabad, India. Tadalafil, purity (99.96%), was acquired from Amoli Organics Pvt. Ltd., Mumbai, India. Ultrapure water ASTM grade I was regularly prepared. Other chemicals comprising HPLC grade solvents, formic acid, acetonitrile, ethyl acetate, and methanol were obtained from Sigma-Aldrich, St. Louis, MO, USA.

### 2.1. Apparatus

The LC-MS/MS assay was conducted *via* Agilent 6460 liquid chromatography coupled with triple quad mass spectrometer (LC-QqQ-MS) (Agilent Technologies, USA), the ion polarity was set in positive mode and Mass Hunter software (version B.03.01, Build 3.1.346.0).

### 2.2. Chromatographic conditions

The analytes were separated on the Agilent Zorbax Eclipse XDB C$_{18}$ (4.6 × 50 mm, 1.8 μm) column with controlled temperature at 25 °C and an isocratic mobile phase of 0.1% formic acid : acetonitrile in ratio of (60 : 40, v/v). An aliquot of 5 μL was used as the injection volume at a flow rate of 0.5 mL min$^{-1}$ using Tadalafil as the IS.

### 2.3. Mass spectrometric conditions

The MS settings were implemented as the followings: Q1 precursor ions (485, 306 and 390); Q3 quantifying product ions (375, 183 and 268) and Q3 qualifying product ions (155, 157 and 135) for Avanafil, Dapoxetine and Tadalafil (IS); respectively. The fragmentation voltage was 135 V for all analytes while the collision energies were 28, 20 and 25 V, for Avanafil, Dapoxetine and Tadalafil (IS); respectively. Polarity was set in electrospray ionization (ESI) positive mode with time filter width equal to 0.07 min. Further MS parameters were costumed including 300 °C desolvation gas temperature, 6 L min$^{-1}$ gas flow rate, 15 psi nebulizer and 4000 V capillary voltage.

### 2.4. Sample preparation and extraction procedures

**2.4.1. Preparation of standard solutions.** Avanafil (500 μg mL$^{-1}$), Dapoxetine (500 μg mL$^{-1}$) and IS, Tadalafil, (25 μg mL$^{-1}$)
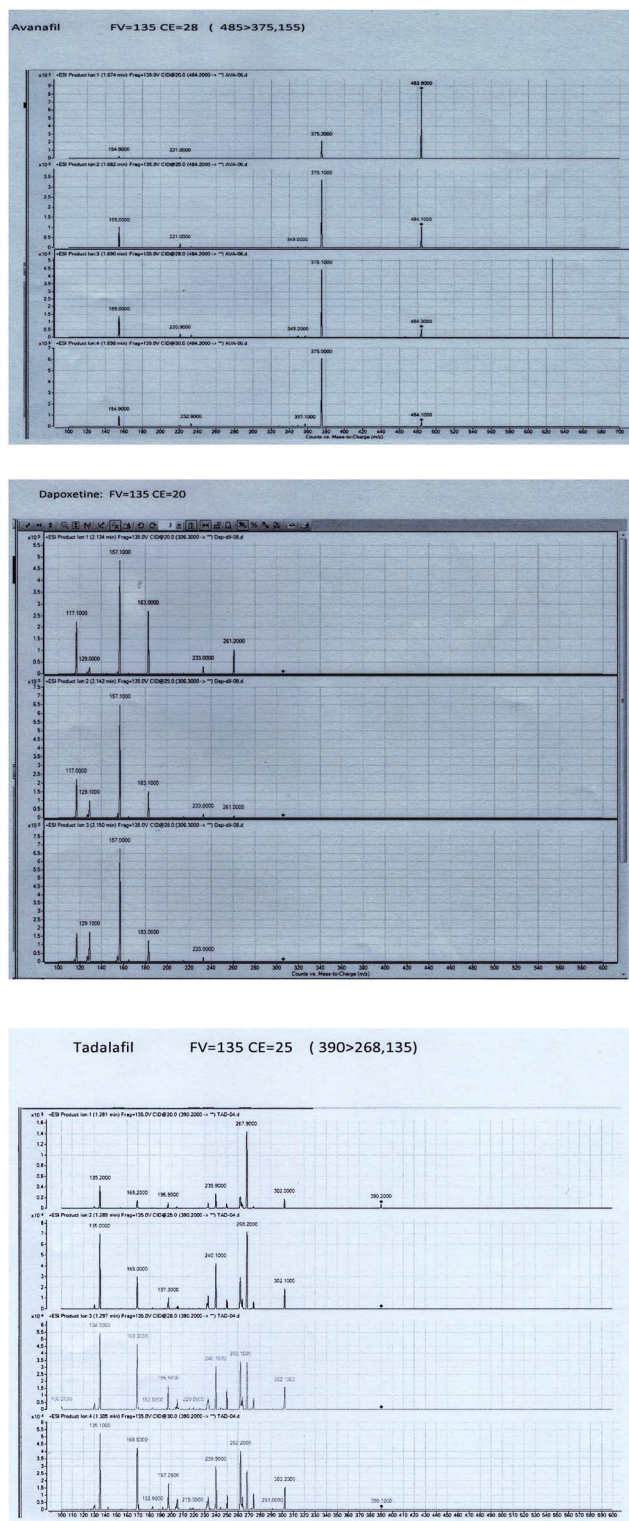
Fig. 3    Mass spectrum for (a) Avanafil, (b) Dapoxetine and (c) Tadalafil.

standard solutions were separately prepared in 100% methanol. Working standard solutions were adequately prepared by an appropriate dilution from their primary stock solutions to prepare the calibration standards and quality control (QC)

samples. All solutions were maintained in freezer at −20 °C once not in use.

**2.4.2. Spiked human plasma sample preparation.** Aliquots of the prepared working solutions were used to spike blank human plasma to prepare calibration standards and QC samples (10, 30, 1500, 5000 ng mL$^{-1}$). Sample preparation was implemented using a one-step PP extraction technique. A 100 µL from Tadalafil (IS) standard solution (25 µg mL$^{-1}$) was added to an aliquot of 250 µL plasma, vortexed for 2 min., then 500 µL acetonitrile was further added for the PP extraction. The prepared mixture was vortexed for 2 min and centrifuged at 6000 rpm for 10 min followed by decantation. A 200 µL was taken from the clear supernatant layer and a 5 µL was injected into the column. The obtained results were interpreted as reported peak areas ratios to IS. Unknown concentrations of Avanafil and Dapoxetine samples were computed by referring to the plotted calibration curves (*i.e.* from computed regression equations).

## 2.5. Bio-analytical method validation characteristics

The adopted method was validated as per the US-FDA guidelines and guidance from the EMA.[3,29] The method was validated for selectivity, sensitivity, linearity, accuracy, precision and carryover in the matrix. The dilution integrity, matrix effect, and analyte stability during both short and long-term sample processing and storage were also investigated.

**2.5.1. Selectivity.** Various sets of blank human plasma from various sources ($n = 6$) were investigated for checking the matrix interference by defining analyte chromatograms in blank plasma and analyte chromatograms at the LLOQ (10 ng mL$^{-1}$). Injection of high concentrations directly after the blank samples was done to ensure the absence of a suggestive carry over.

**2.5.2. Linearity and sensitivity.** The linearity was assessed through measuring nine concentrations as calibration standard samples ranging from 10–6000 ng mL$^{-1}$ in human plasma for both analytes. The calibration curves were plotted using the peak areas ratios of the analytes to IS.

**2.5.3. Accuracy and precision.** The accuracy ($n = 6$) was measured as recovery percent ($R$%) while intra-day ($n = 6$) and inter-day precision ($n = 18$), were measured as (CV%) at the QC levels 10, 30, 1500 and 5000 ng mL$^{-1}$.

**2.5.4. Absolute recovery and matrix effect.** The absolute recovery was measured through comparing the mean peak areas of Avanafil and Dapoxetine spiked human plasma samples at three QC levels, low QC (LQC), medium QC (MQC) and high QC (HQC), with those of the analytes in neat solvent at same concentrations. Furthermore, the matrix effect was assessed by comparing the mean peak areas of Avanafil and Dapoxetine post-preparation spiked human plasma samples with those of the analytes in neat solvent. The variability for both drugs was computed and expressed as (RSD%).

**2.5.5. Stability.** The stability was investigated at LQC (10 ng mL$^{-1}$) and HQC (5000 ng mL$^{-1}$) samples through the comparison of the freshly prepared spiked human samples and the samples exposed to the various stability conditions. The short
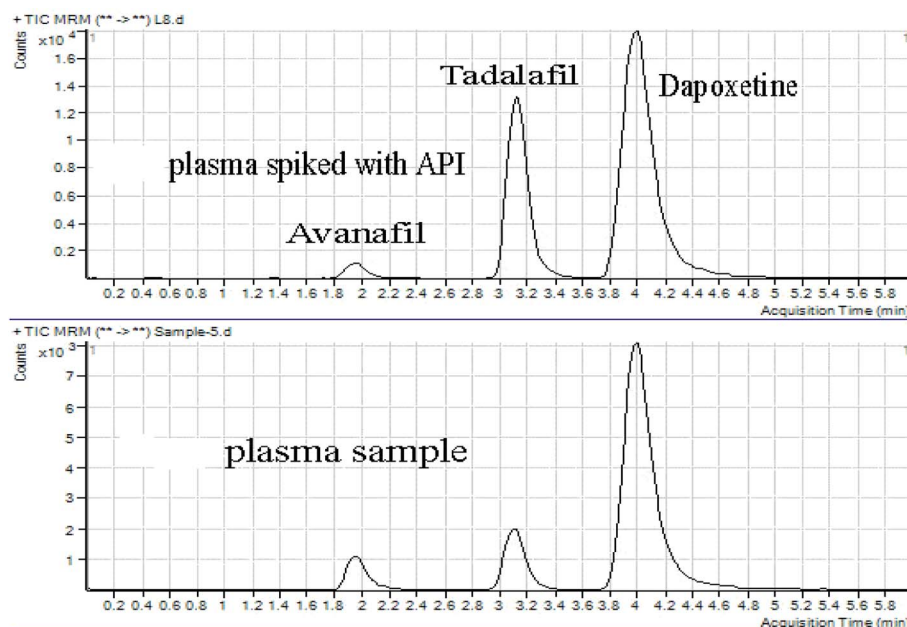
**Fig. 4** Chromatograms of (a) human plasma spiked with (Avanafil & Dapoxetine) standard & Tadalafil (IS), and (b) human plasma sample from real subjects after 2 hours.

**Table 2** Calibration curves for Avanafil and Dapoxetine analytes in human plasma

| Parameters | Avanafil | Dapoxetine |
|---|---|---|
| Linear range | 10–6000 ng mL$^{-1}$ | 10–6000 ng mL$^{-1}$ |
| Linearity equation | $y = 0.000030x + 0.000081$ | $y = 0.000539x - 0.000462$ |
| Slope | 0.000030 | 0.000539 |
| Intercept | +0.000081 | −0.000462 |
| $R^2$ | 0.998156 | 0.999680 |

term stability was assessed at 2–8 °C over 6–12 h and also after leaving the sample in the autosampler over 18–24 h at 2–8 °C. The freeze and thaw stability was evaluated by means of three cycles at −80 °C. The long term stability was evaluated by checking the samples after two weeks while freezing at −80 °C.

**2.5.6. Dilution integrity.** The dilution integrity test was accomplished through diluting the spiked plasma to the upper

limit of quantitation (ULOQ = 6000.0 ng mL$^{-1}$) using the equivalent human blank plasma matrix to accommodate real samples with concentrations of the analytes above the ULOQ. The precision and accuracy of a 2 and 4-fold dilution were calculated.

### 2.6. Real samples and ethical approval

After the approval of the local ethical committee, 5 mL of blood samples from six volunteers were collected 2 h subsequent to the oral administration of the drug (Avanafil/Dapoxetine tablets; 200/60 mg). Then, centrifugation at 4000 rpm was performed to extract the plasma. The plasma was removed, placed in aliquots, then frozen and stored at −80 °C. Ultimately, the extraction procedures were completed using the previously mentioned procedure under Section 2.5.2.

## 3. Results and discussion

The presented study aims are to investigate, develop and verify a simple and an effectively sensitive method with a short run-

**Table 3** Avanafil and Dapoxetine analytes' precisions of in human plasma by means of the adopted PP method

| | Avanafil | | | | Dapoxetine | | | |
|---|---|---|---|---|---|---|---|---|
| QCs | LLOQ | LQC | MQC | HQC | LLOQ | LQC | MQC | HQC |
| Conc. (ng mL$^{-1}$) | 10 | 30 | 1500 | 5000 | 10 | 30 | 1500 | 5000 |
| Intraday precision | | | | | | | | |
| Average (ng mL$^{-1}$) | 10 | 26 | 1635 | 5034 | 9 | 33 | 1608 | 4956 |
| CV% | 13.89 | 6.55 | 0.52 | 0.19 | 10.24 | 5.93 | 3.38 | 0.51 |
| Accuracy R% | 103 | 88 | 109 | 101 | 87 | 109 | 107 | 99 |
| Intraday precision | | | | | | | | |
| Average (ng mL$^{-1}$) | 9 | 29 | 1590 | 5376 | 9 | 30 | 1608 | 4988 |
| CV% | 14.21 | 12.48 | 3.11 | 9.03 | 13.65 | 10.46 | 3.41 | 1.16 |
| Accuracy R% | 90 | 97 | 106 | 108 | 91 | 101 | 107 | 100 |

**Table 4** Matrix effect and recovery of Avanafil and Dapoxetine by means of the adopted PP method

| QCs | Net solution | | Processed plasma | |
|---|---|---|---|---|
| | LQC | HQC | LQC | HQC |
| Conc. (ng mL$^{-1}$) | 30 | 5000 | 30 | 5000 |
| **Avanafil** | | | | |
| Average (peak area) | 171 | 30 276 | 164 | 28 867 |
| RSD% | 0.94 | 2.90 | 1.32 | 2.79 |
| Matrix effect | | | Normalization factor | |
| LQC | 96 | | LQC | 97 |
| HQC | 95 | | | |
| **Dapoxetine** | | | | |
| Average (peak area) | 3139 | 473 010 | 3042 | 469 207 |
| RSD% | 0.87 | 0.08 | 1.75 | 1.15 |
| Matrix effect | | | Normalization factor | |
| LQC | 97 | | LQC | 98 |
| HQC | 99 | | | |
| **Tadalafil IS** | | | | |
| Average | 91 164 | | 89 997 | |
| RSD% | 0.83 | | 2.14 | |
| Matrix effect | | | | |
| LQC | 99 | | | |

time per sample for the quantitative assessment of Avanafil and Dapoxetine at therapeutic concentrations in routine samples.

### 3.1. Optimization of chromatographic conditions

Imperative parameters were evaluated for achieving better sensitivity and chromatographic separation of the analytes. During the chromatographic separation method development,

the adjusted parameters annulled the problems arisen from the charge competition and matrix effect without affecting the sensitivity, accuracy and precision of the method. Different reverse phase columns, such as Inertsil $C_{18}$, Agilent $C_{18}$ and Eclipse Plus, were examined and the Agilent Zorbax Eclipse XDB $C_{18}$ column delivered completely separated peaks with best peak shape. The separation was affected by the use of methanol and acetonitrile and by increasing their percentage up to 85% v/v. Acetonitrile achieved better separation; it exhibits low viscosity and low absorption in the UV region. It enables better mass transfer and better solubility for Avanafil and Dapoxetine.[21,28] Moreover, the use of an ion-suppressing agent, such as acetic acid, ammonium formate or formic acid was tested at various concentrations where the 0.1% formic acid was found to achieve good resolution and also to increase the sensitivity of Avanafil and Dapoxetine. The pH was changed from 2.5 to 7 and different flow rates (0.1–1.0 mL min$^{-1}$) was checked, and it was found that the 0.1% formic acid without the need for pH adjustment and the flow rate of 0.5 mL min$^{-1}$ provided a good resolution and peak shape and afforded a rapid analysis with shorter run time (6 min).

Consequently, the chosen mobile phase was 0.1% formic acid : acetonitrile (60 : 40, v/v) through an isocratic mode, at 0.5 mL min$^{-1}$ as the flow rate while using the Agilent Zorbax Eclipse XDB $C_{18}$ (4.6 × 50 mm, 1.8 µm). Tadalafil at 25 ng mL$^{-1}$ was used as the IS. The adopted method delivered a proper chromatographic separation of the analytes with retention times of 1.95, 3.99 and 3.13 min. for Avanafil, Dapoxetine and Tadalafil IS; respectively, as indicated in Fig. 2. The developed method offered a considerable advantage for a great sample throughput necessary for the routine clinical analysis.

**Table 5** Short term and autosampler stability of Avanafil and Dapoxetine by means of the adopted PP method[a]

| | QCs | LQC | | | HQC | | |
|---|---|---|---|---|---|---|---|
| **Short term stability** | | | | | | | |
| Avanafil | Conc. (ng mL$^{-1}$) | 30 | | | 5000 | | |
| | Sample no. | Initial | After 6 h | After 12 h | Initial | After 6 h | After 12 h |
| | Average | 27 | 26 | 26 | 5144 | 5043 | 5204 |
| | RSD% | 7.62 | 9.56 | 7.69 | 1.83 | 1.03 | 1.51 |
| | Accuracy R% | 91 | 88 | 87 | 103 | 101 | 104 |
| | Stability% | | 96 | 95 | | 98 | 101 |
| Dapoxetine | Average | 31 | 31 | 29 | 5164 | 5096 | 4944 |
| | RSD% | 3.69 | 4.98 | 3.45 | 1.72 | 1.53 | 1.55 |
| | Accuracy R% | 104 | 102 | 97 | 103 | 102 | 99 |
| | Stability% | | 98 | 93 | | 99 | 96 |
| **Autosampler stability** | | | | | | | |
| Avanafil | Sample no. | Initial | After 18 h | After 24 h | Initial | After 18 h | After 24 h |
| | Average | 31 | 32 | 30 | 5422 | 5622 | 5511 |
| | RSD% | 4.88 | 4.82 | 3.33 | 1.97 | 3.40 | 3.43 |
| | Accuracy R% | 104 | 106 | 100 | 108 | 112 | 110 |
| | Stability% | | 101 | 96 | | 104 | 102 |
| Dapoxetine | Average | 34 | 31 | 30 | 5306 | 5134 | 5375 |
| | RSD% | 1.71 | 10.26 | 6.86 | 5.73 | 1.65 | 1.59 |
| | Accuracy R% | 112 | 104 | 101 | 106 | 103 | 108 |
| | Stability% | | 93 | 90 | | 97 | 101 |

[a] Three replicates from one homogenous Q.C sample.

**Table 6** Long term and freeze and thaw stability of Avanafil and Dapoxetine by means of the adopted PP method

| | QCs | LQC | | | HQC | | |
|---|---|---|---|---|---|---|---|
| **Long term stability** | | | | | | | |
| Avanafil | Conc. (ng mL$^{-1}$) | 30 | | | 5000 | | |
| | Sample no. | Initial | 1st day analysis | Last day analysis | Initial | 1st day analysis | Last day analysis |
| | Average | 28 | 30 | 27 | 4870 | 5052 | 5030 |
| | RSD% | 7.14 | 7.02 | 7.41 | 7.69 | 3.34 | 7.27 |
| | Accuracy% | 93 | 99 | 90 | 97 | 101 | 101 |
| | Stability% | | 106 | 96 | | 104 | 103 |
| Dapoxetine | Average | 32 | 30 | 31 | 5175 | 5063 | 4750 |
| | RSD% | 7.95 | 8.48 | 4.98 | 7.13 | 3.61 | 5.50 |
| | Accuracy $R$% | 106 | 99 | 102 | 104 | 101 | 95 |
| | Stability% | | 94 | 97 | | 98 | 92 |
| **Freeze and thaw stability** | | | | | | | |
| Avanafil | Sample no. | Initial | 3rd cycle | | Initial | 3rd cycle | |
| | Average | 28.00 | 29.00 | | 5233.67 | 4880.33 | |
| | RSD% | 7.14 | 3.45 | | 4.11 | 1.18 | |
| | Accuracy% | 93.33 | 96.67 | | 104.67 | 97.61 | |
| | Stability% | | 104 | | | 93 | |
| Dapoxetine | Average | 32.00 | 30.67 | | 5154.00 | 5428.33 | |
| | RSD% | 5.41 | 8.21 | | 1.00 | 2.24 | |
| | Accuracy $R$% | 106.67 | 102.22 | | 103.08 | 108.57 | |
| | Stability% | | 96 | | | 105 | |

### 3.2. Optimization of MS parameters

To obtain better specificity and reproducibility, MS was executed using ESI operated in the positive ion mode where the response for Avanafil, Dapoxetine and Tadalafil (IS) was considerably better than in negative ion mode. The detection of was conducted using the multiple-reaction-monitoring (MRM) mode achieving both high sensitivity and selectivity through using the utmost abundant fragment ions for each analyte. Solid and constant signals of both analytes and IS were denoted for the MRM ion transitions $m/z$ 485 → (375, 155), 306 → (183, 157) and 390 → (268, 135) for Avanafil, Dapoxetine and Tadalafil; respectively as illustrated in Fig. 3.

### 3.3. Optimization of sample preparation

In view of the polarities of both analytes Avanafil (XLogP3-AA = 2.6), Dapoxetine (XLogP3-AA = 5.1), and Tadalafil IS (XLogP3-AA = 2.3),[30–32] PP and liquid–liquid extraction (LLE) techniques using different solvents were investigated for testing the efficiency of the simultaneous extraction of both analytes and IS from human plasma. For LLE, several attempts were tested using ethyl acetate, diethyl ether and tertiary butyl ether as the extracting solvents, to achieve high extraction efficiency while minimizing the matrix effect and getting a substantial quantitative recovery. However, low and non-reproducible recovery were obtained (recovery ± CV%) 45 ± 20%, 55 ± 15%, 65 ± 15%; respectively. For PP technique, acetonitrile and methanol were investigated with different v/v%. It was found that the PP method using acetonitrile was more suitable for the extraction of both analytes and IS based on the higher and better reproducible recovery as 90 ± 7%. Tadalafil IS was used to reach suitable precision and accuracy and to improve reproducibility while diminishing the matrix effect. Thus, the matrix effect became less prominent and the recovery remained directly associated to the method sensitivity".

### 3.4. Selectivity

Selectivity is well-defined as the capability of the chromatographic method to measure the response from Avanafil and Dapoxetine without any effect from the biological matrix. The selectivity was assessed using different sets of blank human plasma ($n = 6$). These samples were extracted, processed and analyzed following the adopted procedure. The results following applying the PP method on blank plasma while comparing to those from the spiked plasma samples at LLOQ (10 ng mL$^{-1}$) displayed the lack of endogenous baseline interferences at the estimated retention times of Avanafil (1.95 min), Dapoxetine (3.99 min), and Tadalafil IS (3.13 min). This demonstrated the significant selectivity of the implemented assay of Avanafil and Dapoxetine in the presence of matrix components as illustrated in Fig. 4.

### 3.5. Linearity and sensitivity

The linearity was estimated using six replicates examination of a blank sample (treated plasma deprived of analytes or IS), a zero sample (treated plasma with only IS), and nine non zero samples (treated plasma samples containing both analytes and IS). Peak area ratios of analytes to the IS were constructed against their corresponding concentrations. The linearity was appraised by least-squares regression analysis and the calibration curves were shown to be linear ($R^2 > 0.998$) within the ranges of 10–6000 ng mL$^{-1}$ for Avanafil and Dapoxetine analytes as shown in Table 2. The regression equations obtained from the mean calibration curves were:

$$y = 0.000030x + 0.000081; R^2 = 0.998156 \text{ (for Avanafil)},$$

$$y = 0.000539x - 0.000462; R^2 = 0.999680 \text{ (for Dapoxetine)},$$

$Y$ represents the peak area ratios to the IS (response) while $X$ represents the drug concentrations (ng mL$^{-1}$).

Blank and zero samples were included in the regression examination in order to confirm the lack of interference. The accuracy (% $R$) of the calibration standards were in the range of (94 to 106%), and (97 to 112%), for Avanafil and Dapoxetine; respectively, as revealed in Table 3. The method sensitivity denoted by the LLOQ (10 ng mL$^{-1}$) was estimated using six replicates assessments within 20% precision and accuracy and also by evaluating the analytes signal-to-noise ratio (S/N).

### 3.6. Precision

The intra-assay precision/repeatability is the extent of the within a day precision by means of the similar investigational variables with the same analyst in a short time period.[33–42] While the inter-assay precision/reproducibility is the extent of the between days or between sets precision and may comprise several variables. Table 3 displays the intraday, analyst-to-analyst and the inter-day precision results needed for the evaluation of both analytes. The data are in accordance with the US-FDA guidelines. The results revealed the high precision of the proposed method.

### 3.7. Absolute recovery and matrix effect

The absolute recovery was computed through using the peak area ratios among spiked samples and relating them to the mean peak area ratios of neat standard solutions with equivalent concentrations and was found to be 90 ± 7% for both analytes. The matrix effect is the variation in signal response due to the occurrence of unintended analytes or interfering substances in the sample. The matrix effect is inspected in order to disclose potential ionization suppression or enhancement triggered by the matrix different components. Adequate recoveries for analytes indicated that the existing sample processing conditions efficaciously withdrawn matrix interference, as shown in Table 4. The matrix effect was studied through the mean peak area ratios between the post-preparation spiked QC samples (LQC and HQC) compared to those acquired from the assessment of neat standards with same concentrations. Ion suppression was detected for both analytes, at all QC concentrations. Mean recovery percentages and % RSD were in the acceptable range, as summarized in Table 4.

### 3.8. Stability

The short term stability is evaluated by comparing the peak areas of the samples which have been prepared and assessed immediately with those obtained after usual sample storage of 6 to 12 h. Nevertheless, the long-term stability test is adopted to assess whether the analyte shows a proper stability in the biological matrices as the human plasma under the sample storage conditions during the time period necessary for the samples produced from a clinical analysis study to be examined (Table 5).

The sample stability was assessed throughout short term storage (24 h at 2–8 °C). Besides, the samples were also evaluated after being stored in the autosampler (at 2–8 °C) for 24 h. The response achieved using the LC-MS/MS assay was matched to that of the freshly prepared solutions and proved an acceptable stability as shown in Table 4.

The freeze and thaw stability test is performed to confirm that the sample exhibits an efficient stability after it is exposed to many freeze and thaw cycles within the study process. This can be accomplished by thawing the samples at LQC and HQC while allowing them to freeze again among 12 to 24 h. The cycle is commonly repeated two times and the sample is analyzed and the results are compared constantly to that of the freshly prepared samples. In the adopted study, thawing of the Avanafil and Dapoxetine frozen samples and retaining them at room temperature for 24 h did not result in the analytes and IS degradation. The results illustrated in Table 6 confirmed that three cycles of freeze and thaw for the LQC and HQC samples did not disrupt both analytes quantification. The QC samples were kept frozen at −80 °C and retained their stability for at least 14 days. Long term stability results are indicated in Table 6 revealing that the analysis of both analytes can be controlled under long term stability settings lacking any significant degradation for both analytes.

### 3.9. Dilution integrity

Dilution of the Avanafil and Dapoxetine samples should not interfere with the accuracy and precision. Dilution integrity can be verified by spiking the matrix with concentrations of the analyte over the ULOQ and then dilution of the samples with blank matrix. The set criteria for the accuracy and precision should be within ±15%. Dilution integrity should cover the practical dilution of the analyzed samples. The precision results (CV%) for a 2 and 4-fold dilution were within 3.1% and 4.7%, while the accuracy results were within 98% and 102%; respectively, acclaiming that a 2 and 4-fold matrix matched dilution integrity are appropriate to preclinical samples with concentrations above the ULOQ.

### 3.10. Application

The maximum serum concentration is reached approximately 0.3–1.15 h and 1–2 h and after the oral administration of Avanafil and Dapoxetine; respectively.[21–28] Hence, the validated bioanalytical method was efficiently applied to analyze Avanafil and Dapoxetine in human plasma from real subjects 2 h following the oral administration of (Avanafil/Dapoxetine tablets; 200/60 mg) showing (average concentrations ± % RSD) of (3000/250 ng mL$^{-1}$ ± 5%), for Avanafil and Dapoxetine; respectively.

## 4. Conclusions

An appropriately fast and accurate LC-MS/MS assay was established and efficiently validated for the simultaneous

determination of Avanafil and Dapoxetine in human plasma by means of a simple and effective PP method. The results of the validation study revealed that the implemented assay was selective, precise, accurate and reproducible over the concentration ranges 10–6000 ng mL$^{-1}$ for both analytes. The adopted method exhibited an appropriate extraction recovery with lack of significant matrix interference. The suggested sample preparation protocol has been successfully applied to conserve the integrity of the studied drugs in bioequivalence studies.

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## Conflicts of interest

There are no conflicts to declare.

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