Stability-Indicating HPLC Method for Simultaneous Determination of Degradation Products and Process-Related Impurities of Avanafil in Avanafil Tablets

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The objective of the current research is to understand the degradation behavior of avanafil under different stress conditions and to develop a stability-indicating high-performance liquid chromatography (HPLC) method for simultaneous determination of degradants observed during degradation. Avanafil tablets were exposed to acid, base, water, oxidative, thermal, and photolytic degradation conditions. In acid, oxidative, thermal, and humidity degradation, significant degradation was observed. All the degradants observed during degradation were separated from known impurities of avanafil by using reverse-phase (RP)-HPLC. Mobile phase A, 0.1% trifluoro acetic acid and triethylamine in water, and mobile phase B, water and acetonitrile in the ratio of 20:80 (v/v), were used at a flow rate of 1.2 mL/min in gradient elution mode. Separation was achieved by using Inertsil ODS 3 column (3 μm, 4.6 mm × 250 mm) at 45 °C. Peak responses were recorded at 245 nm. Method capability for detecting and quantifying the degradants, which can form during stability, was proved by demonstrating the peak purity of avanafil peak in all the stressed samples. Mass balance was established by performing the assay of stressed sample against reference standard. Mass balance was found >97% for all the stress conditions. The developed analytical method was validated as per International Conference on Harmonization (ICH) guidelines. The method was found specific, linear, accurate, precise, rugged, and robust.

Keywords: Avanafil, tablets, HPLC, degradation products, impurities, validation

Introduction

Avanafil is a selective inhibitor of cGMP-specific type 5 phosphodiesterase. It is used for erectile dysfunction. Avanafil is available under the brand name of STENDRA. STENDRA was developed by Vivus Inc. It is available in 50 mg, 100 mg, and 200 mg strengths tablets. The recommended starting dose is 100 mg, but based on individual efficacy and tolerability, the dose can be increased to 200 mg. Maximum daily dose is 200 mg/day. It has the molecular formula $C_{23}H_{26}ClN_7O_3$ and molecular weight of 483.95 [1].

Based on the literature search, it was found that a colorimetric method [2] for determination of avanafil in bulk and finished dosage form and a stability-indicating high-performance liquid chromatography (HPLC)–diode array detector method [3] for avanafil analysis were reported. Some other methods were also reported for the estimation of avanafil and depoxentine in the bulk drug and formulated drug product by liquid chromatography [4], dual wavelength spectrophotometry [5], ultraviolet (UV) chemometrics [6], and by using fluorescence detector [7]. One application note from Waters Inc. for screening of herbal/dietary supplements [8] and a review paper describing review of analytical methods for the determination of four new phoshodiesterase type 5 inhibitors in biological samples and pharmaceutical preparations [9] are also available in public domain. Some more research articles related to formulation of avanafil were also found [10, 11].

No pharmacopoeial method is available for estimation of avanafil and its impurities [12, 13]. To the best of our present knowledge, no literature was reported about the degradation studies and the simultaneous determination of degradation products, impurities in avanafil, and its formulated drug product.

The present paper describes degradation behavior of avanafil and the development of a stability-indicating HPLC method for determination of degradants and known impurities of avanafil in avanafil tablets.

The developed method can separate and quantitate the degradants and other known impurities of avanafil, namely, deschloro impurity, acid impurity, dichloro impurity, dimer impurity, and diamine impurity. Deschloro and acid impurity are degradants. Based on maximum daily dose, limit for impurities in avanafil tablet is 0.2% [14].

Experimental

Materials. Trifluoro acetic acid and triethylamine were procured from Merck, Mumbai. Acetonitrile used in the experiment was of HPLC grade and also procured from Merck, Mumbai. Avanafil tablets, its impurities deschloro, acid, dichloro, dimer, and diamine, and avanafil reference standard were supplied by Dr. Reddy's, India. The chemical structures of (*s*)-4-[(3-chloro-4-methoxybenzyl) amino]-2[2-(hydroxymethyl)-1-pyrrolidinyl]-*n*-(pyrimidinylmethyl)-5-pyrimidinecarboxamide (avanafil) and its impurities (*S*)-2-(2-(hydroxymethyl)pyrrolidin-1-yl)-4-(4-methoxybenzylamino)-*N*-(pyrimidin-2-ylmethyl)pyrimidine-5-carboxamide (deschloro impurity), (*S*)-4-(3-chloro-4-methoxybenzylamino)-5-carboxy-2-(2-hydroxymethyl-1-pyrrolidinyl)pyrimidine (acid impurity), (*S*)-4-(3,5-dichloro-4-methoxybenzylamino)-2-(2(hydroxymethyl) pyrrolidin-1-yl)-*N*-(pyrimidin-2-ylmethyl)pyrimidine-5-carboxamide (dichloro impurity), 2,4-bis(3-chloro-4-methoxybenzylamino)-*N*-(pyrimidin-2-ylmethyl)pyrimidine-5-carboxamide (dimer impurity), and *N*-(3-chloro-4-methoxybenzyl)-4-(3-chloro-4-

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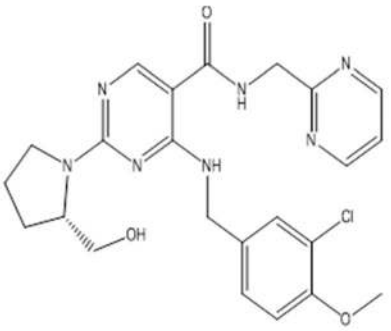
methoxybenzylamino)-2-((*S*)-2-(hydroxymethyl)pyrrolidin-1-yl)-*N*-(2-((*S*)-2-(hydroxymethyl)-pyrrolidin-1-yl)-5-(pyrimidin-2-ylmethylcarbamoyl)-pyrimidin-4-yl)-pyrimidine-5-carboxamide (diamine impurity) are shown in Figure 1.

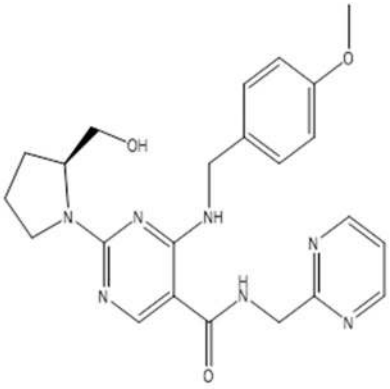
Instrumentation. Water, used in preparation of diluent and mobile phases, was purified by a water purification system (Milli-Q, Millipore, Bedford, MA, USA). The analysis was conducted on two different Waters Alliance HPLC system equipped with quaternary solvent delivery pump, an autosampler and photodiode array (PDA) UV detector. Two lots (020236159 and 020236281) of Inertsil ODS 3 column (3 μm, 4.6 mm × 250 mm) were procured from GL Sciences Inc., USA.

Method

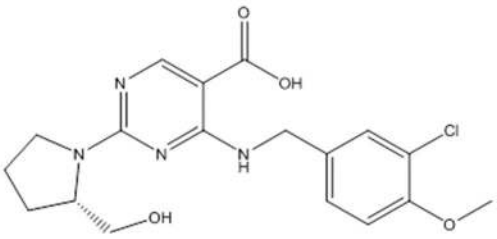
Chromatographic condition. The chromatographic separation was performed by using Inertsil ODS 3 column 4.6 mm × 250 mm, 3 μm. Mobile phase consists of mixture of mobile phase A and mobile phase B. Mobile phase A is 0.1% trifluoro acetic acid and triethylamine in water, and mobile phase B consists of water and acetonitrile in the ratio of 20:80 (v/v). The gradient program *T* (min)/% B: 0/15, 5/15, 13/34, 27/38, 35/50, 45/50, 60/70, 65/70, 66/15, and 75/15, with a flow rate of 1.2 mL/min, was used. Ten microliters of each solution was injected into liquid chromatograph, while



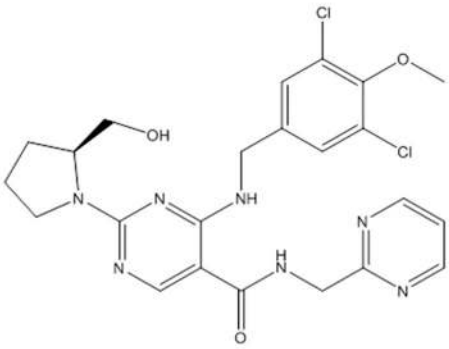
Avanafil



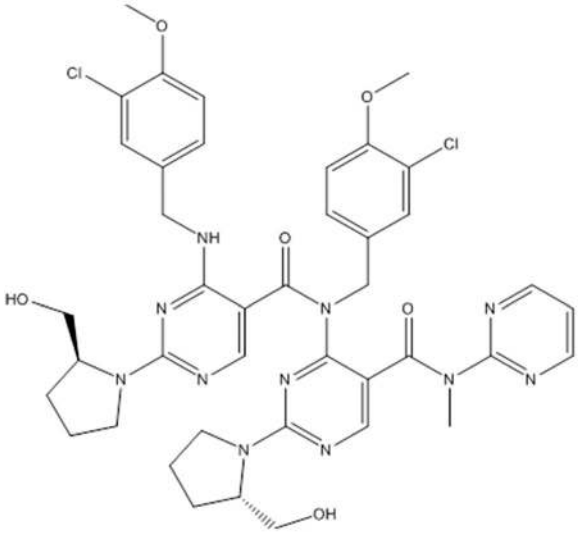
Impurity: Deschloro



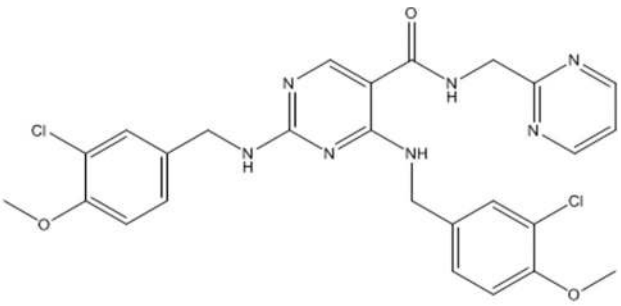
Impurity: Acid impurity



Impurity: Dichloro



Impurity: Diamine



Impurity: Dimer

Figure 1. Structures of avanafil and its impurities

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peak responses were recorded at 245 nm. Column oven temperature was kept as 45 °C.

Solution preparation. Water and acetonitrile were mixed in the ratio of 50:50 v/v to prepare the diluent.

Standard solution preparation. Appropriate amount of avanafil working standard was dissolved in diluent to prepare avanafil standard solution at a concentration level of 0.75 μg/mL

Sample preparation. Twenty avanafil tablets were transferred to a mortar and pestle. The tablets were crushed to a fine powder. Avanafil tablet powder equivalent to 75 mg of avanafil was weighed and transferred to a 200 mL volumetric flask. About 140 mL of diluent was added, and solution was sonicated for about 20 min with intermittent shaking. Solution was made up to the volume with diluent. A portion of the solution was centrifuged at 10,000 rpm for 10 min. Concentration of avanafil in final preparation was 375 μg/mL.

Method validation. For the proposed method, the following validation parameters were performed as per ICH guidelines: specificity, precision, accuracy, limit of detection, limit of quantification, linearity, range, ruggedness, and robustness [15–18].

System suitability. System suitability parameters were measured to check the system performance. System precision was determined on three replicate injections of standard preparation containing avanafil at a concentration level of 0.75 μg/mL. The acceptance criteria were less than 5.0% relative standard deviation (RSD) for avanafil peak areas, and the United States Pharmacopeia (USP) tailing factor was less than 2.0 for avanafil peak from standard solution.

Forced degradation study. To understand the degradation behavior of avanafil, forced degradation studies were conducted. The stress studies were conducted separately on avanafil tablets 200 mg and its placebo. The stress conditions included acid hydrolysis (5 N HCl, 65 °C, 24 h), base hydrolysis (5 N NaOH, 65 °C, 24 h), water hydrolysis (65 °C, 24 h), oxidation (5% H_2O_2, 25 °C, 5 h), thermal (105 °C, 6 h), humidity (90% RH for 15 days), and photolytic (1.2 million lux hours visible light and 200 Wh/m^2 UV light, 16 h) [19, 20].

The stressed samples were then analyzed by the proposed method. Peak purity test was carried out, and mass balances were calculated for stressed samples. Placebo interference was performed by analyzing the placebo as per the proposed method.

Precision. The precision of the test method was demonstrated by doing repeatability and intermediate precision. Avanafil tablets (unspiked preparation) contain impurities, but these are present below reporting threshold (<0.1%). The repeatability of test method was evaluated by analyzing six samples of avanafil tablets 200 mg by spiking the impurities deschloro impurity, acid impurity, dichloro impurity, dimer impurity, and diamine impurity (0.2% of impurities with respect to 375 μg/mL avanafil). % RSD for content of each impurity was calculated. Intermediate precision was demonstrated by using different analyst, different instrument, different column, and performing the analysis on different days.

Limit of detection (LOD) and limit of quantitation (LOQ). Limit of detection and limit of quantification for avanafil and its impurities (deschloro impurity, acid impurity, dichloro impurity, dimer impurity, and diamine impurity) were established based on signal-to-noise ratio method. Limit of detection was determined by identifying the concentration at which the signal to ratio was achieved close to 3. Limit of quantification was determined by identifying the concentration, where impurity and avanafil peak signal-to-noise ratio was found close to 10.

Precision of avanafil and impurities at about limit of quantification was conducted. Six test preparations of avanafil tablets 200 mg placebo, having avanafil and its impurities at the level of Limit of quantification, were prepared and injected into the system. The % RSD for six replicate preparations was calculated.

Linearity. Linearity was determined by plotting a graph of concentration versus peak area of avanafil and its impurities (deschloro impurity, acid impurity, dichloro impurity, dimer impurity, and diamine impurity). The solutions were prepared at seven concentration levels ranging from limit of quantification level to 150% of the target concentration (about 0.75 μg/mL for deschloro impurity, acid impurity, dichloro impurity, dimer impurity, diamine impurity, and avanafil) and injected into the HPLC system. The correlation coefficient value, slope, *y*-intercept, and bias at 100% level were calculated.

Accuracy. Accuracy study for avanafil and its impurities (deschloro impurity, acid impurity, dichloro impurity, dimer impurity, and diamine impurity) were conducted by spiking impurities on test preparation of avanafil tablets 200 mg. Samples were prepared in triplicate at different concentration levels ranging from LOQ to 150% of specification (LOQ, 50%, 100%, and 150% for deschloro impurity, acid impurity, dichloro impurity, dimer impurity, and diamine impurity).

Robustness. Experiments were performed by deliberately altering the conditions to establish the robustness of the developed method. System suitability parameters were the major evaluation criteria for this study. The variables evaluated in this study include change in column temperature from 40 °C to 50 °C (±5 °C), change in column flow rate from 1.0 mL/min to 1.4 mL/min (±17%), and change in aqueous phase in mobile phase B 90% to 110% (±10%).

Solution stability and mobile phase stability. Solution stability of standard and spiked test preparation was determined by keeping the test and standard solutions on bench top at room temperature for 48 h. The samples were injected after a time interval of 24 h, and the impurity levels were estimated against a freshly prepared standard solution. The stability of mobile phase was also established by keeping the mobile phase in tightly closed condition on bench top for 48 h at room temperature. The freshly prepared sample and standard were injected by using the stored mobile phase at a time interval of 24 h.

Results and Discussion

Method development and validation

Optimization of chromatographic conditions. The main objective of this study was to understand the degradation behavior of avanafil and to develop a stability-indicating HPLC method to determine the degradants observed during degradation. Degradation studies were conducted, and the degradants and other known impurities were separated by using high-performance liquid chromatography. Based on the solubility of avanafil, diluent was optimized as water–acetonitrile (5:5). A solution containing all the impurities (0.75 μg/mL) and avanafil (375 μg/mL) was prepared in the diluent. Maximum absorption wavelength was selected as 245 nm, based on the intersecting value observed from the UV absorption spectra of avanafil and its impurities.

Based on the p*K*a of avanafil (5.5 and 12.5), initially, a buffer for mobile phase was chosen as potassium dihydrogen phosphate (pH 3.5; 0.01 M) containing 0.5% of triethylamine. Mobile phase A was prepared by mixing buffer and acetonitrile in the ratio of 8.5:1.5 v/v. Water and acetonitrile were mixed in a ratio of 2:8 v/v to make mobile phase B. Gradient program was chosen as *T* (min)/% B: 0/10, 5/10, 13/20, 30/40, 35/100, 45/100, 46/10, and 50/10. Mobile phases were delivered at a flow rate of 1.0 mL/min. Column screening was done by using different columns such as X terra RP18 (4.6 × 150 mm 5 μm) and Inertsil ODS 3 (4.6 × 150 mm, 5 μm). Based on peak shapes and separation, Inertsil ODS 3 (4.6 × 150 mm, 5 μm)

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was selected for further optimization trials. Column length and particle size were further optimized to get the optimum separation between known impurities and degradation products. At the retention time of Dimer impurity, one hump was also observed in diluent, to remove the hump at the retention time of dimer impurity, the buffer for mobile phase was changed to 0.1% of each of trifluoroacetic acid and triethylamine in water.

Gradient program was further optimized to get the optimum separation between unknown degradants and known impurities (deschloro, acid impurity, and dimer impurity). Finally, the mobile phases, mobile phase A, containing buffer (0.1% v/v, trifluoro acetic acid and triethyl amine in water), and mobile phase B consisting of water and acetonitrile in the ratio of 20:80 (v/v) were found suitable. The gradient *T* (min)/% B: 0/15, 5/15, 13/34, 27/38, 35/50, 45/50, 60/70, 65/70, 66/15, and 75/15, with flow rate of 1.2 mL/min was finalized. The injection volume was finalized as 10 μL, while detector was set at 245 nm. The column temperature was finalized as 45 °C.

The relative retention times for deschloro impurity, acid impurity, dichloro impurity, dimer impurity, and diamine impurity against avanafil were 0.84, 0.94, 1.31, 1.58, and 1.63 respectively. The relative response factor for deschloro impurity, acid impurity, dichloro impurity, dimer impurity, and diamine impurity against avanafil were 1.06, 1.14, 0.78, 0.95, and 0.82 respectively.

Method validation. The developed HPLC method was validated as per ICH guidelines with respect to specificity, precision, accuracy, LOD/LOQ, linearity, ruggedness, and robustness.

System suitability. System suitability parameters were measured to verify the system performance. The system suitability was established based on RSD (%) for avanafil peak areas from three standard replicates (≤5.0) and tailing factor (≤2.0) for avanafil peak from standard preparation. RSD (%) of avanafil peak areas and tailing factor for avanafil peak were found to be 0.5 and 1.1, respectively. System suitability parameters were found within the acceptance limits.

Specificity. The specificity studies were performed to study the degradation behavior of avanafil. Placebo interference was evaluated by analyzing the placebo prepared as per test method. No peak was observed in placebo at the retention time of deschloro impurity, acid impurity, dichloro impurity, dimer impurity, diamine impurity, and avanafil. Stressed samples were injected into the HPLC system with photodiode array detector by following test method conditions. All degradant peaks were resolved from avanafil and known impurities peaks. The chromatograms of the stressed samples were evaluated for peak purity of avanafil using Waters Empower networking software. Assay of all the stressed samples was performed against reference standard to calculate the mass balance (% Assay + Impurities + % degradants).

Avanafil was found stable under base hydrolysis (5 N HCl, 65 °C, 24 h), water hydrolysis (65 °C, 24 h), and photolytic stress (200 Wh/m^2, 16 h). Degradation was observed mainly in acid stress (5 N HCl, 65 °C, 24 h), oxidation (5% H_2O_2, 25 °C, 5 h), thermal (105 °C, 6 h) stress, and humidity stress (90% RH for 15 days) study. In acid stress, acid impurity was one of the major degradant observed. An unknown impurity was observed at the relative retention time of about 0.70 during oxidative stress. During humidity and heat stress, unknown impurities at the relative retention time of 0.81 and 1.11 were also observed. The retention times of known impurity in stressed samples were confirmed by injecting the standards. To correlate the degradation behavior observed during stress study and the real-time stability (accelerated stability condition up to 6 months), analysis of in-house avanafil tablets 200 mg was done. These unknown impurities were not forming in stability samples, so it was not required to identify these impurities. The proposed method was validated for avanafil also to ensure that unknown degradants can be quantified against avanafil, with desired accuracy and precision.

For all forced degradation samples, the purity angle was found less than purity threshold. Mass balance results were calculated for all stress conditions and were found >97% (Table 1). This indicates that there is no interference and co-elution from degradants in quantification of impurities in drug product.

Precision. The % RSD for the content of deschloro impurity, acid impurity, dichloro impurity, dimer impurity, diamine impurity, and avanafil in repeatability study was less than 3.8, and in intermediate precision study, it was less than 4.3, which confirm that the method is precise. The % RSD values are presented in Table 2.

LOD and LOQ. Limit of detection and limit of quantification for avanafil and its impurities (deschloro impurity, acid

Table 1. Summary of forced degradation results

Stress condition	Purity angle	Purity threshold	Purity flag	Degradation	Mass balance (%)
Sample unstressed	0.024	0.237	No	NA	NA
Acid hydrolysis (5 N HCl, 65 °C, 24 h)	0.033	0.261	No	2.6117	99.3
Base hydrolysis (5 N NaOH, 65 °C, 24 h)	0.030	0.270	No	0.7403	99.8
Oxidation (5% H_2O_2, 25 °C, 5 h)	0.064	0.260	No	2.5342	97.5
Water hydrolysis (65 °C, 24 h)	0.102	0.270	No	0.0513	99.8
Thermal (105 °C, 6 h)	0.026	0.267	No	7.3103	99.0
Humidity (90% RH, 15 days)	0.062	0.253	No	2.9363	104.6
Photolytic (1.2 million lux hours visible light and 200 wh/square m^2 UV light)	0.047	0.247	No	0.1387	102.4

Table 2. LOD/LOQ, linearity, and precision data

Parameter	Des[a]	Acid[b]	Dichloro	Dimer	Diamine	Avanafil
LOD (μg/mL)	0.0361	0.0352	0.0341	0.0356	0.0318	0.0348
LOQ (μg/mL)	0.1083	0.1056	0.1022	0.1069	0.0995	0.1037
Correlation coefficient	0.999	0.999	0.999	0.999	0.999	0.999
Intercept (a)	−122.299	−35.059	−158.772	−86.939	−7.136	1472.560
Slope (b)	49,092.346	51,639.610	38,000.409	44,071.738	38,225.056	44,682.865
Bias at 100% response	0	0	1	0	0	2
Precision (RSD [%])	2.8	2.9	3.7	2.0	2.6	3.0
Intermediate precision (RSD [%])	3.0	2.4	1.5	1.2	4.2	NA
Precision at LOQ (RSD [%])	1.9	3.0	8.5	2.9	2.1	5.2

[a]Deschloro impurity.
[b]Acid impurity.

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impurity, dichloro impurity, dimer impurity, and diamine impurity) were established based on signal to ratio method. The limit of detection, limit of quantification, and the precision at LOQ values are reported in Table 2.

Linearity. Linearity was established for deschloro impurity, acid impurity, dichloro impurity, dimer impurity, diamine impurity, and avanafil, from concentration levels ranging from limit of quantification level to 150% of the target concentration (about 0.75 µg/mL). The correlation coefficient value was more than 0.999, and bias at 100% level was less than 5%, for avanafil and its impurities (Table 2).

Accuracy. The percentage recoveries of deschloro impurity, acid impurity, dichloro impurity, dimer impurity, diamine impurity, and avanafil were found ranging from 87.4% to 109.2%. The

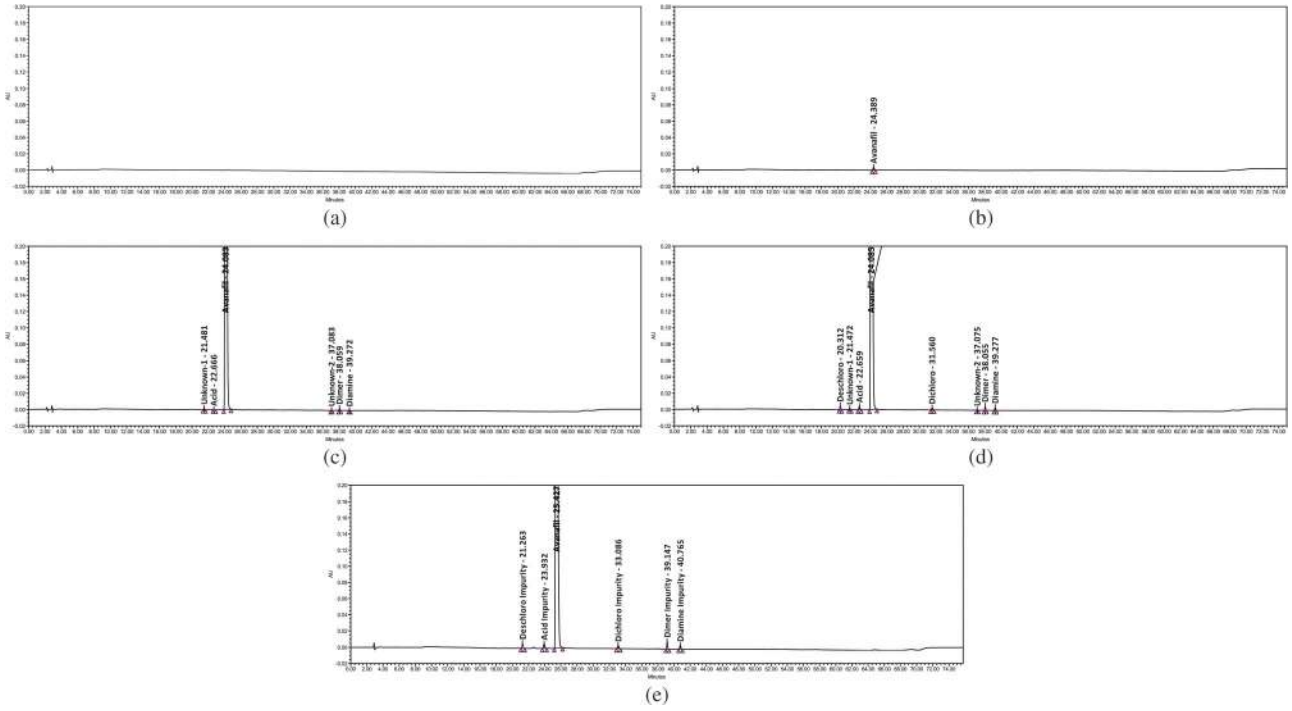

Figure 2. Representative chromatograms of avanafil tablets: (a) blank preparation, (b) standard preparation, (c) unspiked test preparation, and (d) test preparation spiked with impurities at 0.2% level with respect to 375 µg/mL of avanafil (e) test preparation spiked with impurities at 150% spike level

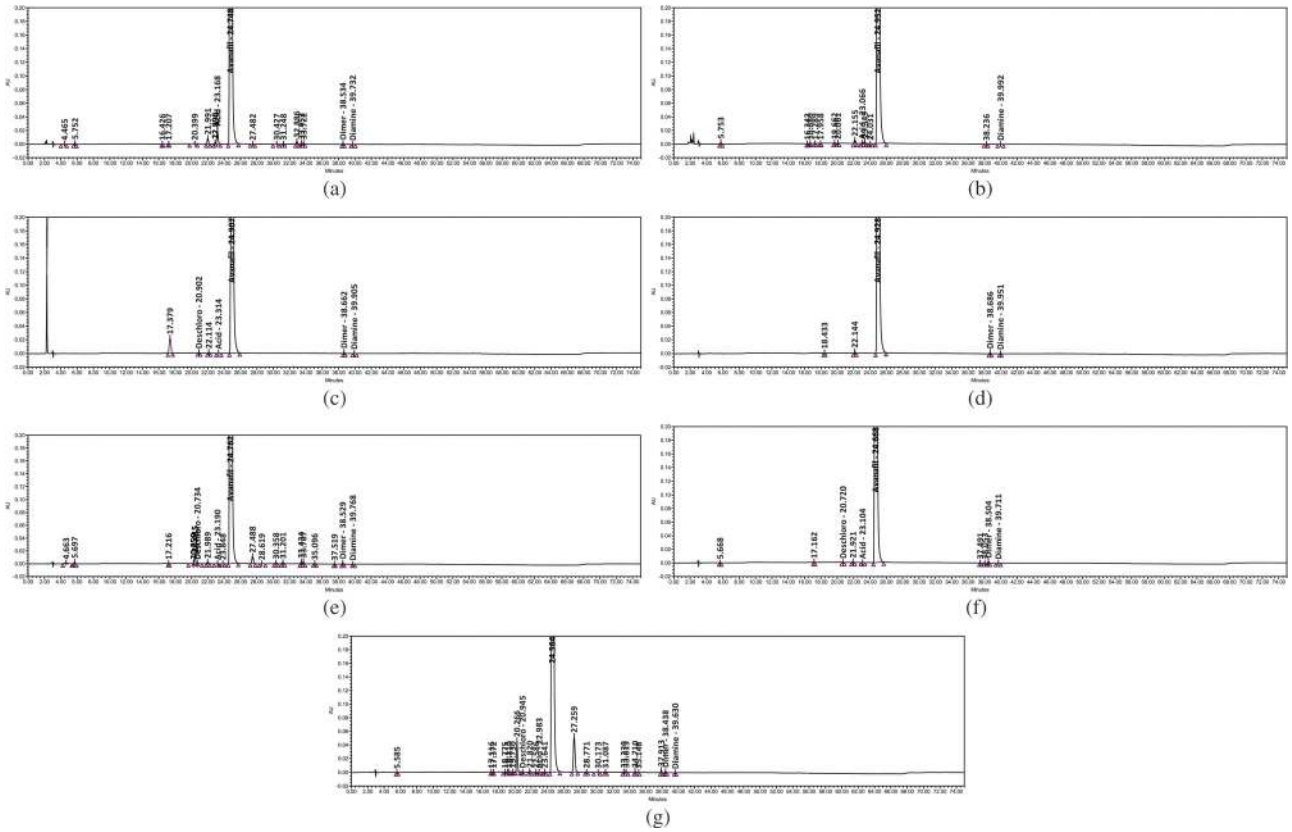

Figure 3. Representative chromatograms of avanafil tablets: (a) acid degradation, (b) base degradation, (c) peroxide degradation, (d) water degradation, (e) humidity degradation, (f) UV degradation, and (g) thermal degradation

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Table 3. Recovery results

%Recovery[a]						
Amount spiked	Deschloro	Acid impurity	Dichloro	Dimer	Diamine	Avanafil
LOQ	92.4 ± 5.2	102.9 ± 7.5	97.6 ± 3.9	109.2 ± 6.1	101.8 ± 8.0	99.3 ± 5.1
Level 1 (50%)	96.0 ± 0.0	96.1 ± 0.6	95.5 ± 0.6	92.0 ± 0.6	95.3 ± 0.0	106.1 ± 5.1
Level 2 (100%)	91.3 ± 1.1	90.8 ± 1.5	87.4 ± 0.6	88.7 ± 0.7	90.3 ± 1.7	102.9 ± 0.9
Level 3 (150%)	94.7 ± 0.6	94.9 ± 0.8	94.2 ± 0.4	93.7 ± 0.5	95.0 ± 0.6	98.1 ± 2.3

[a]Mean ± RSD (%) for three determinations.

Table 4. Robustness results

Stress condition	Observed system suitability parameters	
	USP Tailing ≤2.0	Area (RSD [%], [n = 3] ≤ 5.0)
Column temperature 40 °C	1.1	0.1
Column temperature 50 °C	1.1	0.5
Column flow 1.0 mL/min	1.1	1.2
Column flow 1.4 mL/min	1.1	0.9
Aqueous 90%	1.1	1.5
Aqueous 110%	1.1	0.3

LC chromatogram of spiked sample (at 0.2% level for deschloro impurity, acid impurity, dichloro impurity, dimer impurity, and diamine impurity) is shown in Figures 2 and 3. The % recovery values for avanafil and impurities are presented in Table 3.

Robustness. In all the deliberate varied chromatographic conditions (flow rate, column temperature, and composition of aqueous), all analytes were adequately resolved and elution orders remained unchanged. The tailing factor for avanafil peak was less than 1.1, and RSD for peak areas was less than 1.5% (Table 4).

Solution stability and mobile phase stability. The variability in the estimation of all five impurities was within ±15% during solution stability and mobile phase stability. The results from solution stability and mobile phase stability experiments confirmed that standard solutions, test preparations, and mobile phase were stable up to 48 h on bench top.

Conclusion

Based on the forced degradation studies, it was found that avanafil is prone to acid, oxidative, thermal, and humidity stress conditions. To quantify the degradants observed during stress studies and other known impurities of avanafil in pharmaceutical dosage forms, a simple and efficient reverse-phase HPLC method was developed and validated. The method was found specific, precise, accurate, rugged, robust, and linear. This is a stability-indicating method and can be used for routine analysis of production samples to check the impurity contents.

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