American Journal of Analytical Chemistry, **2012, 3, 59-66**
http://dx.doi.org/10.4236/ajac.2012.31009 Published Online January 2012 (http://www.SciRP.org/journal/ajac)

A Validated Rapid Stability-Indicating Method for the Determination of Related Substances in Vardenafil Hydrochloride by Ultra-Performance Liquid Chromatography

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Received September 25, 2011; revised October 31, 2011; accepted November 14, 2011

ABSTRACT

A novel, sensitive, stability indicating RP-LC method has been developed for the quantitative determination of Vardenafil and its related impurities in both bulk drugs and Pharmaceutical dosage forms. Effective chromatographic separation was achieved on a C18 stationary phase with simple mobile phase combination delivered in a simple gradient programme and quantitation was by ultraviolet detection at 210 nm. The mobile phase consisted of a buffer and acetonitrile delivered at a flow rate 0.25 ml·min^{-1}. Buffer consisted of 20 mM Ammonium bi carbonate, pH adjusted to 5.0 by using ortho Phosphoric acid. In the developed UPLC method the resolution (Rs) between vardenafil and its four potential impurities was found to be grater than 2.0. Regrreation analysis showed an r value (correlation coefficient) grater than 0.999 for vardenafil and its four impurities. This method was capable to detect all four impurities of vardenafil at a level of 0.25 µg.mL^{-1} with respect to test concentration of 500 µg·ml^{-1} for a 2 µl injection volume. The inter and intra day precision values for all four impurities and for vardenafil was found to be with in 2.0% RSD. The method showed good and consistent recoveries for vardenafil in bulk drugs (98.8% - 100.9%), pharmaceutical dosage forms (100.5% - 101.5%) and its all four impurities (99.8% - 102.5%). The test solutions was found to stable in acetonitrile for 48 h. The drug was subjected to stress conditions of hydrolysis, oxidation, photolysis, and thermal degradation. Considerable degradation was found to occur in peroxide hydrolysis. The stress samples were assayed against a qualified reference standard and the mass balance was found close to 99.9%. The developed RP-LC method was validated with respect to linearity, accuracy, precision and robustness.

Keywords: Vardenafil; Validation; Impurities and Degradation Products

1. Introduction

Vardenafil HCl, 2-[2-ethoxy-5(4-ethyl-piperazine-1-sulphonyl)-phenyl]-5-methyl-7-propyl-3H-imidazo [5,1-f] [1-3] triazin-4-one hydrochloride (**Figure 1**) is used to treat erectile dysfunction. Vardenafil and other ED drugs inhibit phosphodiesterase type 5 (PDE-5) enzyme, which in turn maintains higher levels of cyclic guanosine monophosphate, which relaxes smooth muscles, promotes penile blood flow, and enhances erectile function [1,2]. Pharmalogical studies indicate that interaction between PDE-5 inhibitors and certain drugs containing nitrates may drastically lower blood pressure [4]. Despite the efficacy of PDE-5 inhibitors as a treatment for ED, their draw backs are also notable. Adverse effects such as head ache, facial flushing, dyspepsia, visual disturbances and muscle aches have been reported [3]. Recently, reports of blind ness have implicated Viagra [5]. A few HPLC methods determination of vardenafil in bulk drug and biological samples were reported in the literature [6-10].

All the above methods developed (references [6-10]) for the quantification of vardenafil and other PDE-5 inhibitors employed complex analytical instruments such as mass spectrophotometer for their estimation mainly in dietary supplements and bulk drug powders. As far as we are aware there is stability indicating UPLC method for

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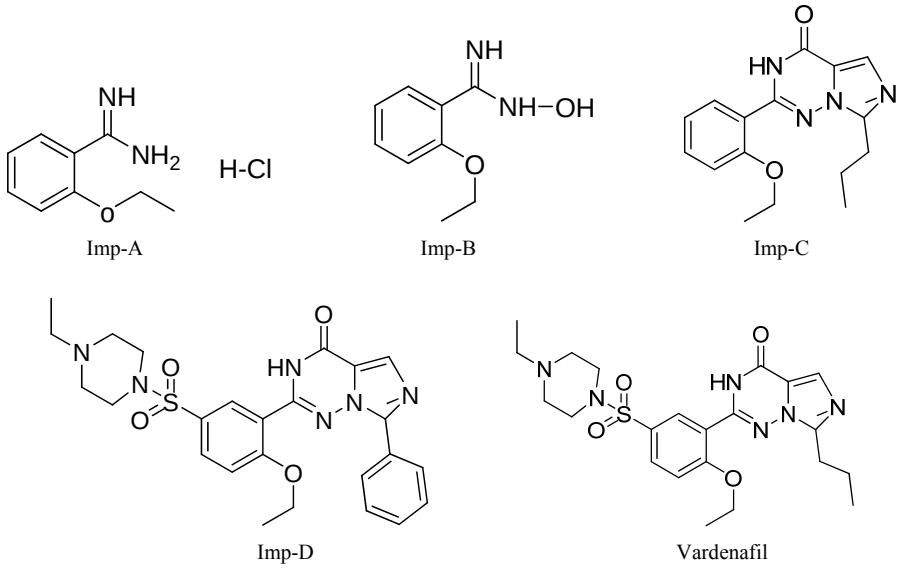


Figure 1. Vardenafil and its impurities.

the determination of related substances and for quantitative estimation of vardenafil.

Hence, an attempt has been made to develop an accurate, rapid, specific and reproducible method for determination of vardenafil and all four impurities in bulk drug samples and in pharmaceutical dosage forms along with method validation as per ICH norms [11-13]. The stability tests were also performed on both drug substances and drug products as per ICH norms [14-16].

1.1. Experimental

Chemicals

Samples of vardenafil HCl and its related impurities were received from Chiral Solutions private Limited. Hyderabad, India (**Figure 1**).Commercially available 20 mg vardenafil tablets (Levitra) were purchased. LC grade acetonitrile was purchased from Merck, Darmstadt, Germany. Analytical reagent grade Ammonium Bi carbonate were also purchased from Merck. High purity water was prepared by using a Millipore Milli-Q plus water purification system. All samples and impurities used in this study were greater than 99.5% purity analyse by UPLC.

1.2. Equipment

The UPLC system, used for method development, forced degradation studies and method validation was a Waters Accquity Quaternary pump plus auto sampler and a photodiode array detector (MA, USA).The out put signal was monitored and processed using Empower software on Pentium computer (Digital Equipment Co). Photo stability studies were carried out in a photo stability chamber (sanyo, Leicestershine, UK).Thermal stability studies were performed in a dry air oven (Merck Pharmatech, Hyderabad, India).

1.3. Chromatographic Conditions

The chromatographic column used was a Zorbax Extended C18 (100 × 2.1) mm with 1.8 µm particles. The mobile phase consisted of a mixture of buffer, acetonitrile. Buffer consists of 20 mM Ammonium bi carbonate pH adjusted to 5.0 with Ortho Phosphoric acid. The column temperature was maintained at 25˚C and the detection was monitored at a wave length of 210 nm .The injection volume 2 µl. Acetonitrile was used as a diluent.

1.4. Preparation of Solutions

Preparation of Standard Solutions

A stock solution of vardenafil (500 $\mu g \cdot ml^{-1}$) was prepared by dissolving an appropriate amount in acetonitrile. Working solutions were prepared from above stock solution for related substances determination and assay determination, respectively. A stock solution of impurities (mixture of imp-A, imp-B, imp-C and imp-D) at a concentration of 0.3 $mg \cdot ml^{-1}$ was also prepared in acetonitrile.

1.5. Preparation of Sample Solutions

Vardenafil tablets contained 20 mg of vardenafil. The inactive ingredients present in vardenafil were lactone monohydrate, sodium lauryl sulphate, microcrystalline, cellulose, silicon dioxide and magnesium stearate. Twenty vardenafil tablets (20 mg) were weighed and the average weight was calculated. The tablets were powder equivalent to 50 mg of active pharmaceutical ingredient (vardenafil) was transferred in to a 100 ml volumetric flask. Approximately 75 ml acetonitrile were added and the flask

was placed on rotary shaker for 10 min to dissolve the material completely. The solution was then diluted to 100 ml and centrifuged at 3000 rpm for 10 min. The supernatant was collected and filtered through a 0.45 µm pore size nylon 66-membrane filter. The filtrate was used as sample solution.

1.6. Specificity

Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities. Stress testing of the drug substance can help to identify the likely degradation products, which can in turn help to establish the degradation path ways and the intrinsic stability of the molecule and validate the stability indicating power of the analytical procedure used.

The specificity of the vardenafil in the presence of its impurities namely imp-A, imp-B, imp-C and imp-D and degradation products was determined by the developed UPLC method. Forced degradation studies were also performed on vardenafil to provide an indication of the stability indicating property and specificity of the proposed method. The stress conditions employed for degradation study included light (carried out as per ICH Q1B), heat (60˚C), acid hydrolysis (0.1N HCl), base hydrolysis (0.1N NaOH), water hydrolysis (room temperature for 48 h) and oxidation (3% H_2O_2).For heat and light studies, the study period was 7 days where as for acid, base, peroxide and water hydrolysis the test period was 48 h. Peak purity of stressed samples of vardenafil was checked by using photo diode array detector of waters corporation, MA, USA.

2. Analytical Method Validation

The developed chromatographic method was validated for linearity, precision, accuracy, sensitivity, robustness and system suitability.

Precision

The precision of the related substance method was checked by injecting six individual preparations of (500 $\mu g \cdot ml^{-1}$) vardenafil spiked with 0.2% each imp-A, imp-B, imp-C and imp-D. Each %RSD area of imp-A, imp-B, imp-C and imp-D was calculated. Precision study was also determined by performing the same procedures on a different day (inter-day precision).

The intermediate precision (rugged ness) of the method was also evaluated by a different analyst, different column and different instrument in the same laboratory.

Assay method precision was evaluated by carrying out six independent assays of test samples of vardenafil against qualified reference standard. The %RSD of six assay value obtained was calculated. The intermediate precision of the assay method was evaluated by different analyst and by using different instrument from the same laboratory.

2.1. Sensitivity

Sensitivity was determined by establishing the limit of detection (LOD) and limit of quantification (LOQ) for imp-A, imp-B, imp-C and imp-D estimated at a signal to noise ratio of 3:1 and 10:1 respectively, by injecting a series of diluted solutions with known concentrations. The precision study was also carried out at the LOQ levels by injecting six individual preparations of imp-A, imp-B, imp-C and imp-D, the %RSD for the areas of each impurity was calculated.

2.2. Linearity and Range

Linearity test solutions for the assay method has prepared from stock solution at five concentration levels from 50 to 200% of assay analyte concentration (250, 375, 500, 750 and 1000 $\mu g \cdot ml^{-1}$).

A linearity test solutions for related substances method was prepared by diluting the impurity stock solution to required concentrations. The solutions were prepared at six concentration levels. From LOQ to 200% of the permitted maximum levels of the impurity (*i.e.* LOQ (0.05%), 0.10%, 0.20%, 0.30% and 0.40%) was subjected to linear regression analysis with the least squares method. Calibration equation obtained from regression analysis was used to calculate the corresponding predicted responses. The residuals and sum of the residual squares were calculated from the corresponding predicted parameters.

Linearity was checked for three consecutives days in the same concentration range for both assay and related substances method and the %RSD value of the slope and Y-intercept of calibration curve were calculated. Upper and lowers levels of range were also established.

2.3. Accuracy

The accuracy of the assay method was evaluated in triplicate at five concentration levels *i.e.* 250, 375, 500, 750 and 1000 $\mu g \cdot ml^{-1}$ in the bulk drugs and pharmaceutical dosage forms. At each concentration, three sets were prepared and injecting in triplicate. The percentage recovery was calculated at each level.

The bulk sample shows the percentage of imp-D at a level of the 0.052% and 0.08% of total impurities (limit not more than 0.2% for a single known impurity, for total impurities the limit was 0.50%). The study was carried out in triplicate at 0.10%, 0.20% and 0.30% of the analyte concentration (500 $\mu g \cdot ml^{-1}$). The percentage of recoveries for imp-A, imp-B, imp-C and imp-D were calculated.

2.4. Robustness

To determine the robustness of the developed method, experimental conditions were deliberately changed and

the resolution (Rs) bet ween vardenafil, imp-A, imp-B, imp-C and imp-D were evaluated. The flow rate of the mobile phase was 0.25 ml·min^{-1}. To study the effect of the flow rate on the developed method, 0.1 units of flow were changed (*i.e*. 0.2 and 0.3 ml·min^{-1}). The effect of column temperature on the developed method was studied at 20˚C and 30˚C. The effect of pH on resolution of impurities was studied by varying ± 0.1pH units (*i.e*. 4.9 and 5.1). In all the above varied conditions, the component of the mobile phase was held constant.

2.5. Solution Stability and Mobile Phase Stability

The solution stability of vardenafil in the assay method was carried out by leaving the test solutions of samples in tightly capped volumetric flasks at room temperatures for 48 hours. The same sample solutions were assayed at 6 hours interval up to the study period against freshly prepared standard solution. The mobile phase stability was also carried out by assaying the freshly prepared sample solution against freshly prepared reference standard solutions at 6 h intervals up to 48 h. Mobile phase prepared was kept constant during the study period. The %RSD of assay of vardenafil was calculated for the study period during mobile phase and solution stability experiments.

The solution stability of vardenafil and its related impurities was carried out by leaving both spiked and un spiked sample solution in tightly capped volumetric flasks at room temperature for 48 h. Content of imp-A, imp-B, imp-C and imp-D were determined at 6 h interval, up to the study period.

Mobile phase stability was also carried out for 48 h by injecting the freshly prepared sample solutions for every 6 h interval. Content of imp-A, imp-B, imp-C and imp-D was checked in the test solutions. Mobile phase prepared was kept constant during the study period.

3. Results and Discussion

3.1. Method Developed and Optimization

The main objective of the chromatographic method is to separate vardenafil from imp-A, imp-B, imp-C and imp-D. Impurities were co eluted using different stationary phases such as C8, Phenyl and cyano as well as different mobile phases. The Chromatographic separation was achieved on a Zorbax Extended C18 (100 × 2.1 mm, 1.8 µm), flow rate of 0.25 ml·min^{-1}. The peak shape of vardenafil was found to be symmetrical. In optimised chromatographic conditions the peak shape of vardenafil was found to be symmetrical and impurities imp-A, imp-B, imp-C and imp-D were separated with a resolution grater than 2, typical retention times of Vardenafil, imp-A, imp-B, imp-C and imp-D were about 2.9, 5.5, 6.8, 7.1 and 7.5 respectively (**Figure 2**). The system suitability results are given in **Table 1** and developed UPLC method was found to be specific for vardenafil and its four impurities namely imp-A, imp-B, imp-C and imp-D (**Figure 2**).

3.2. Results of Forced Degradation Studies

Degradation Behaviour

LC studies on vardenafil hydrochloride under different stress conditions suggested the following degradation behaviour.

Degradation in basic solution: in 1N NaOH at room temperature after 48 h, no major degradation was observed. Minor degradation was observed when more stressed conditions were applied (1N NaOH reflux at 60˚C). A minor degradation product was observed at 0.93 min retention time.

Degradation in acidic solution: In 1N HCl at room temperature no degradation was observed. The drug was also stable in 1N HCl on heating at 60˚C for 48 h. The drug was slightly degraded in acid hydrolysis.

Oxidative hydrolysis: the drug was exposed to 3% hydrogen peroxide at room temperature for 48 h. The drug gradually underwent degradation with time in 3% hydrogen peroxide and prominent degradation was observed. A major degradation product was observed (7.2%) at 4.2 min retention time.

Degradation in neutral (water) solution: No major degradation products were observed after 48 h at room temperature. The drug was also stable in water on heating at 60˚C for 4 h. The drug was stable to water hydrolysis.

Photolytic conditions: When the drug powder was exposed to light for an overall illumination of 1.2 million lux hours and an integrated near ultraviolet energy of 200 watt hours/square meter (w/m h) (in a photo stability chamber), no degradation was observed.

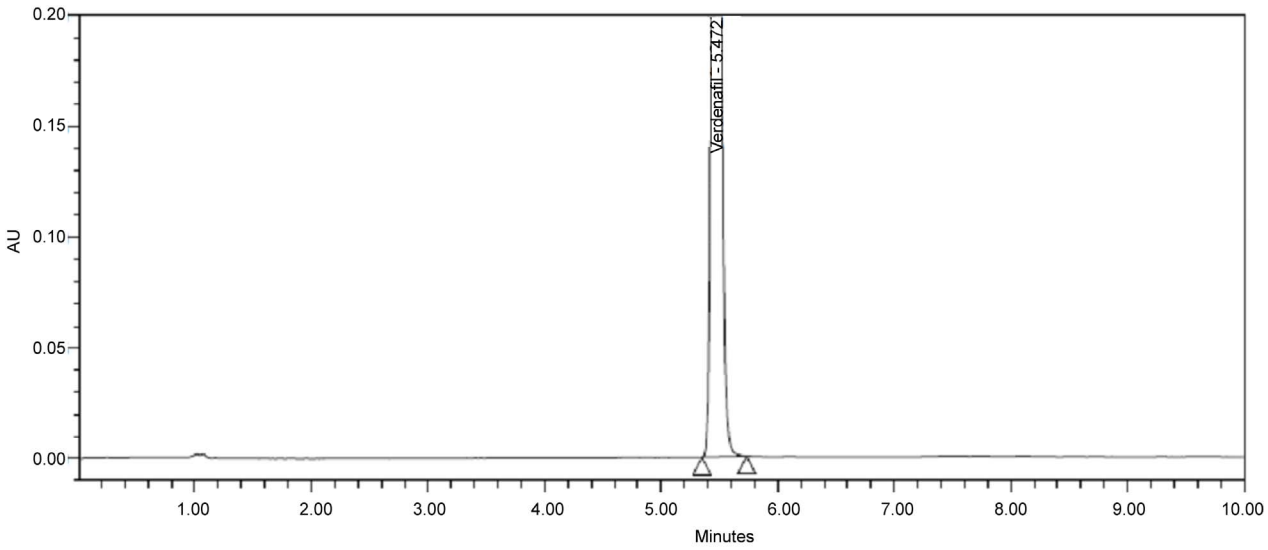
The mass balance of stressed samples was close to 99.4% (**Table 1**). The assay of vardenafil is unaffected in the presences of imp-A, imp-B, imp-C and imp-D and its degradation products confirm the stability indicating power of the developed method.

3.3. Precision

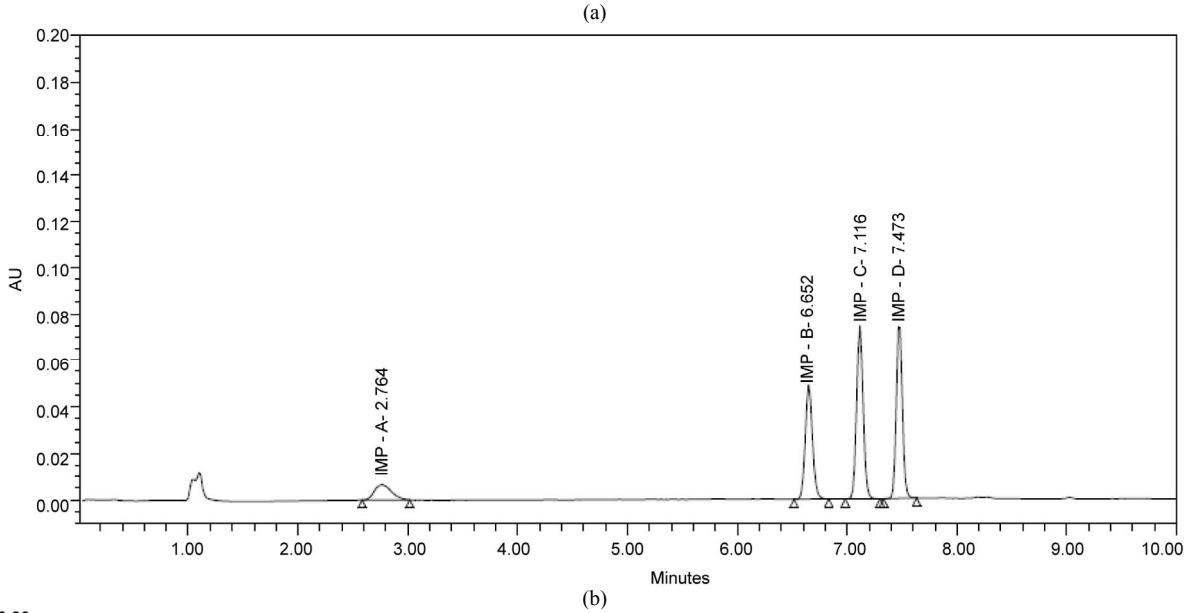
The %RSD of the assay of vardenafil during the assay method precision study was within 0.05% and the %RSD for the area imp-A, imp-B, imp-C and imp-D in related substances method precision study was within 1.1%. The %RSD of the assay results obtained in the intermediated precision study was within 0.1% and the %RSD for the area of imp-A, imp-B, imp-C and imp-D were well within 0.6%, conforming good precision of the method.

3.4. Limit of Detection and Limit of Quantification

The limit of detection of all impurities namely imps-A,



(a)



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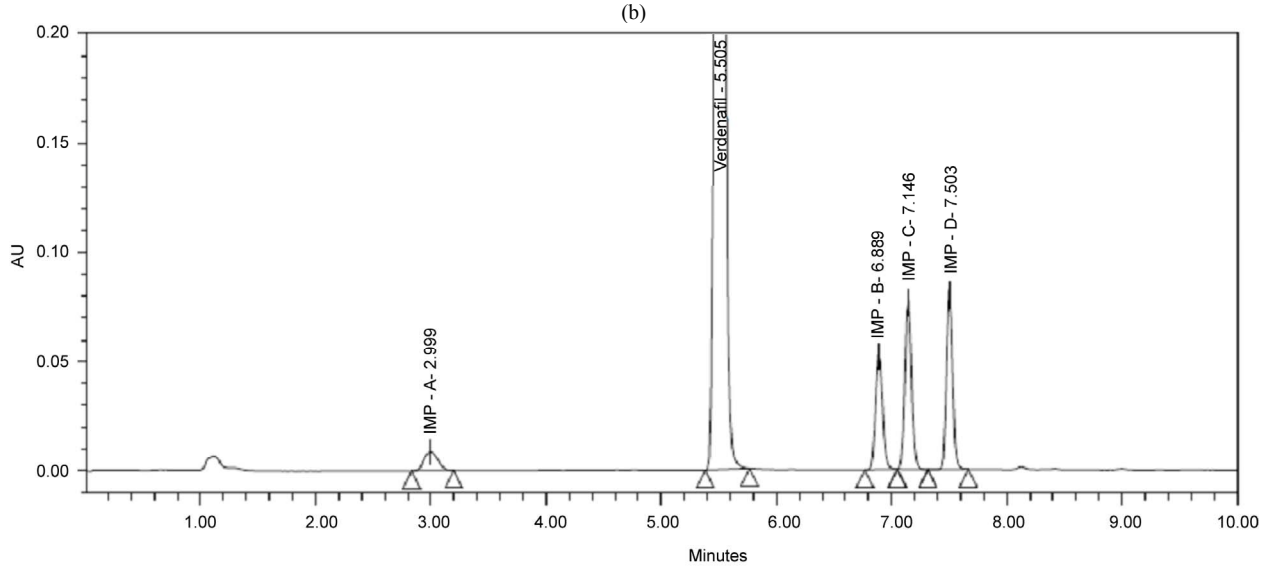

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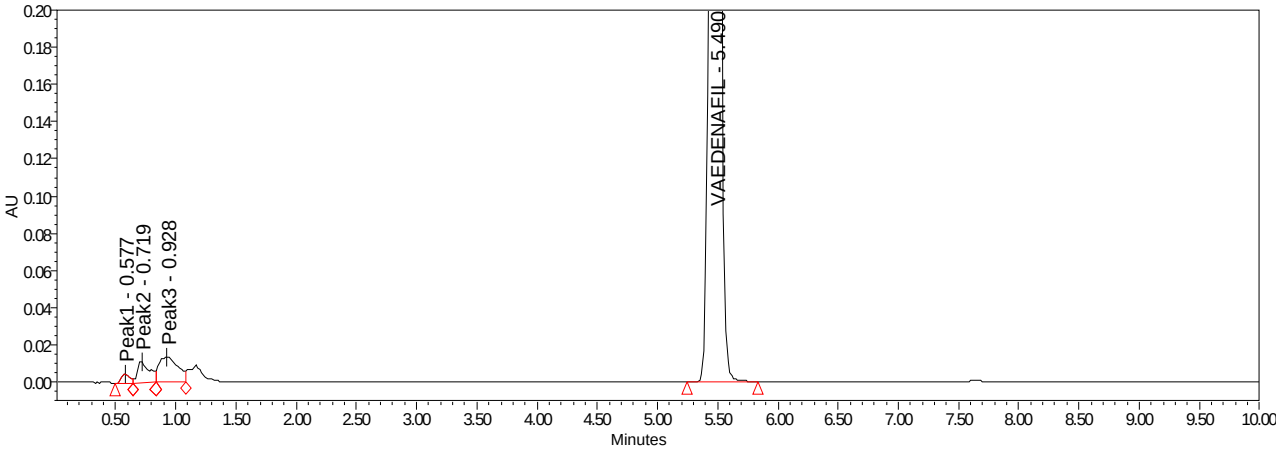

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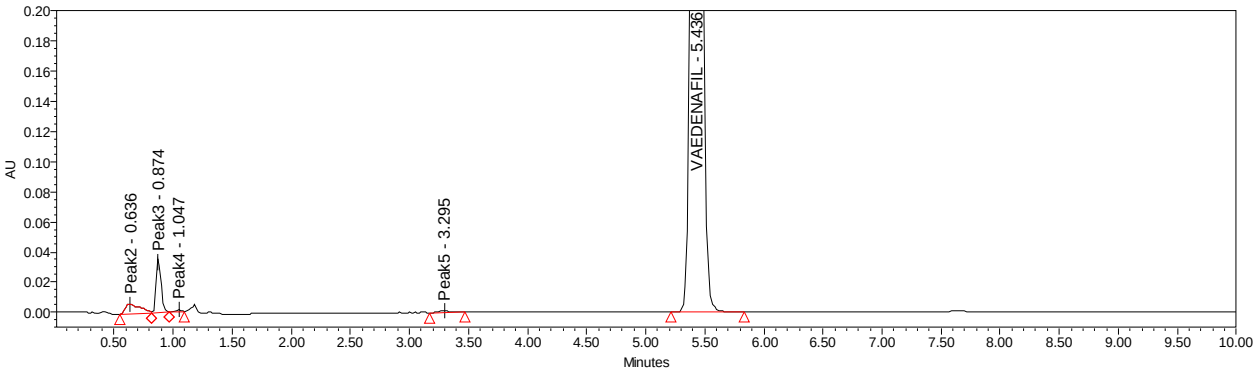

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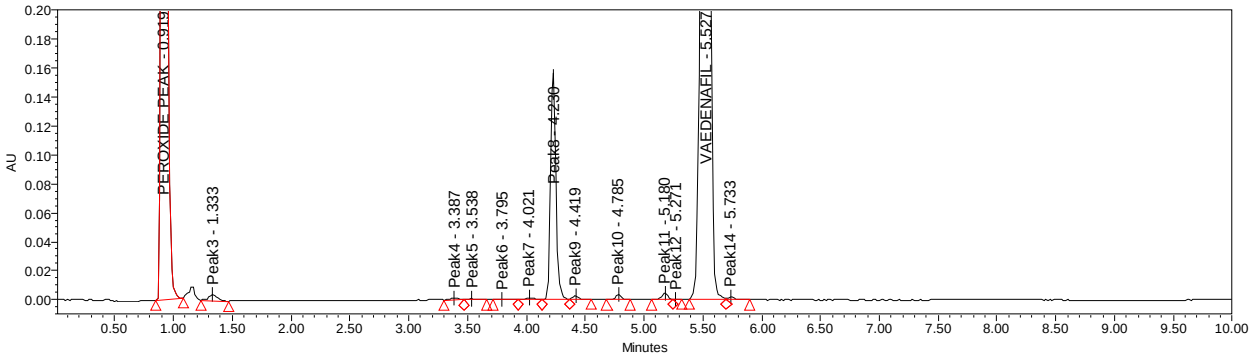

(f)

Figure 2. Typical HPLC chromatograms of Vardenafil HCl under stress conditions (a) pure Vardenafil HCl bulk sample; (b) spiked with imp-A, imp-B, imp-C and imp-D at 0.3% level; (c) spiked with imp-A, imp-B, imp-C and imp-D at 0.3% level in Vardenafil HCl sample; (d) Base hydrolysis; (e) acid hydrolysis; (f) Peroxide hydrolysis.

Table 1. Summary of forced degradation.

Stress condition	Time	% assay of active substance	Mass balance (% assay + % impurities + % degradation products)
Acid hydrolysis (1N HCl,)	48 h	96.51%	99.8%
Base hydrolysis (1N NaOH)	48 h	95.53%	99.5%
Oxidation (3% H_2O_2)	4 h	82.62%	99.6%
UV, 254 nm	24 h	99.21%	99.5%
Thermal 60˚C	24 h	99.10%	99.4%

imp-B, imp-C, imp-D and Vardenafil were achieved at 0.016%, 0.017%, 0.017%, 0.016%, and 0.017% for 2 µl injection volume. The limit of quantification for all impurities namely imps-A, imp-B, imp-C imp-D and Vardenafil was achieved at 0.051%, 0.053%, 0.054% 0.052% and 0.05% for 2 µl injection volume. The precision at LOQ concentration for imp-A, imp-B, imp-C, and imp-D were below 2.4%.

3.4.1. Linearity

The linearity calibration plot for the assay method was obtained over the calibration ranges tested, *i.e*. 250 - 1000 $\mu g \cdot ml^{-1}$ and correlation coefficient obtained was greater than 0.999. The results showed that an excellent correlation existed between the peak area and concentration of the analyte.

Linear calibration plot for the related substances method was obtained over calibration ranges tested, *i.e*. LOQ (0.05%) to 0.4% for imp-A, imp-B, imp-C imp-D and Vardenafil. The correlation coefficient obtained was greater than 0.999. The above results showed that an excellent correlation existed between the peak area and the concentration of imp-A, imp-B, imp-C, imp-D and Vardenafil.

3.4.2. Accuracy

The percentage recovery of Vardenafil in bulk drug sample ranged from 99.8% to 100.8%. The percentage recovery of impurities in Vardenafil sample varied from 98.7% to 103.5%.

3.4.3. Robustness

In all the deliberate varied chromatographic conditions (flow rate and column temperature), the resolution between the critical pairs, *i.e*. imp-B and imp-C was greater than 4.0, illustrating the robustness of the method.

3.4.4. Solution Stability and Mobile Phase Stability

The %RSD of the assay of Vardenafil during the solution stability experiments were within 0.2%. No significant changes were observed in the content of impurities namely imp-A, imp-B, imp-C and imp-D during solution stability and mobile phase stability experiments when performed using the related substance method. The solution stability and mobile phase stability experiment data confirms that the sample solution and mobile phases used during assay and the related substance determination were stable for at least 48 h.

4. Conclusion

The gradient UPLC method developed for quantitative and related substance determination of vardenafil HCl in both bulk drug and pharmaceutical dosage form was precise, accurate and specific. The method was completely validated showing satisfactory data for all the method validation parameters tested. The developed method is stability indicating and can be used for the routine analysis of production samples and also to check the stability of vardenafil HCl sample.

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