ISSN : 0974-7419 Volume 10 Issue 10

Trade Science Inc.

Analytical CHEMISTRY

An Indian Journal

Full Paper

ACAIJ, 10(10) 2011 [683-690]

Validated LC method for sildenafil citrate related substances and its potential oxidative degradation product

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Received: 6th April, 2011 ; Accepted: 6th May, 2011

IPDO IPM Communication No: IPDO IPM - 00193

ABSTRACT

A novel stability indicating LC method has been developed and validated for the simultaneous quantitative determination of Sildenafil citrate, its related substances and degradation products. The drug substance was subjected to stress conditions of hydrolysis (acid, base and water hydrolysis), oxidation, photolysis and thermal conditions to assess the degradation. Considerable degradation was observed under oxidative stress conditions. The resulting degradation product under oxidative stress condition was identified by LC-MS. This degradation product was isolated by preparative HPLC and its structure was further confirmed by spectroscopic techniques namely Mass, NMR and FTIR. The stressed samples were assayed against a qualified reference standard and the mass balance was found 99.6%. The developed method is applicable for determination of Assay, related substances and degradation products of Sildenafil citrate. The developed method was validated for linearity, accuracy, precision, range, specificity, ruggedness and robustness by considering Sildenafil citrate, its related substances and degradation products

KEYWORDS

Liquid chromatography;
Sildenafil citrate;
Method validation;
Forced degradation;
Related substance.

INTRODUCTION

1-[[3-(6,7-Dihydro-1-methyl-7-oxo-3-propyl-1*H*-pyrazolo [4,3-*d*] pyrimidin-5-yl)-4-ethoxyphenyl] sulfonyl]-4-methylpiperazine citrate (Figure 1) is commonly known as Sildenafil citrate. It is a novel orally active inhibitor of the type V-cyclic guanosine monophosphate (cGMP)-specific phosphodiesterase (PDE5) on penile erectile activity in patients with male erectile dysfunction, which causes cGMP to accumulate corpus cavernosum[1-4].

No official (pharmacopoeial) method has been reported for the determination of Sildenafil citrate in presence of related substances and degradation products. However, some of the analytical methods describe the determination of Mirodenafil and Sildenafil in the plasma and corpus cavernous of SD male rats by using LC-MS[5], simultaneous determination of Sildenafil and

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desmethyl sildenafil in human plasma by high-performance liquid chromatography method using electrochemical detection with application to pharmacokinetic study[6] and determination of sildenafil citrate and its main metabolite by sample stacking with polarity switching using micellar electro kinetic chromatography[7].

To our knowledge there is no stability indicating LC method available for the simultaneous quantitative determination of Sildenafil citrate and its related substances and degradation products. In this research, we describe the analytical method development and validation for accurate quantification of Sildenafil citrate, its related substances and degradation products in bulk drug samples. Intensive stress studies were carried out on Sildenafil citrate to evaluate the stability indicating nature of the developed method and the method validation was performed as per ICH guidelines[8-10]. This method was conveniently applied for determining the assay and related substances of Sildenafil citrate bulk drug.

EXPERIMENTAL

Chemicals

Samples of Sildenafil citrate, its three related substances namely imp-1, imp-2 and imp-3 were received from process development laboratory, Dr. Reddy's Laboratories Ltd., IPDO, Hyderabad, India. Degradation product, namely imp-4 was isolated by preparative HPLC in Analytical Research Development laboratory of Dr. Reddy's Laboratories Ltd., The structures and chemical names of Sildenafil citrate, its related substances and degradation product shown in Figure 1. HPLC grade Acetonitrile, AR grade Ortho phosphoric acid and Tri ethyl amine purchased from Merck, Darmstadt, Germany. High purity water was prepared by using Millipore Milli-Q plus water purification system.

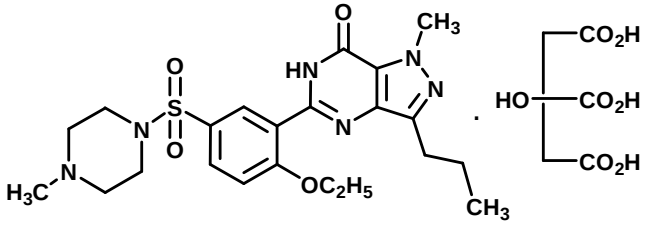


Figure 1 : Sildenafil citrate: 1-[[3-(6, 7-Dihydro-1-methyl-7-oxo-3-propyl-1H-pyrazolo [4,3-d] pyrimidin-5-yl)-4-ethoxyphenyl] sulfonyl]-4-methylpiperazine citrate

Instrumentation

The LC system, used for method development, forced degradation studies and method validation was Waters Alliance system equipped with a Waters 2695 quaternary pump, auto injector and 2998 photo diode array detector and for method validation was Waters Alliance system equipped with a variable wavelength detector. The output signal was monitored and processed by an empower software on Pentium Computer (Digital Equipment Co).

Chromatographic conditions

The chromatographic separation was performed on a 5 µm particles, Waters Symmetry shield RP18 (250 x 4.6 mm) column from Waters Corporation. The Mobile phase A was 0.2 % (v/v) aqueous Triethyl amine Buffer (pH 5.5). The Mobile phase B consisted of Acetonitrile and Buffer in a ratio of 800:200 (v/v). Prior to use, the mobile phase was filtered through a 0.45 µm nylon filter and degassed. The flow rate of the mobile phase was kept at 1.0 mL min^{-1}. A linear gradient was programmed as: time (min)/% Mobile phase B: 0/30, 25/50, 30/50, 40/70, 55/70, 60/30 and 65/30. The temperature of column was maintained at 45°C and the detection was carried out at a wavelength of 225 nm. Injection volume was 10 µL. Mobile phase B was used as a diluent to prepare the sample solutions.

The Chromatographic conditions of preparative HPLC were, Inertsil ODS, 250 x 20 mm column. The Mobile phase was Water and Acetonitrile in the ratio of 50:50 (v/v) with flow rate of 12.0 mL min-1. The eluent was monitored at a wavelength of 225 nm.

Preparation of solutions

Preparation of standard solutions

A stock solution of Sildenafil citrate (2.0 mg mL^{-1}) was prepared by dissolving appropriate amount in the diluent. Working solutions of 500 and 100 µg mL^{-1} were prepared from the above stock solution for related substances determination and assay determination respectively through serial dilutions. A stock solution of impurities blend was prepared (mixture of imp-1, imp-2, imp-3 and imp-4) at a concentration of 500 µg mL^{-1}.

Analytical method validation

The developed chromatographic method was vali-

dated for specificity, sensitivity (limit of detection and limit of quantification), precision (repeatability, intermediate precision and reproducibility), linearity, range, accuracy, solution stability, mobile phase stability, robustness and system suitability.

(A) Specificity

Specificity of the developed method was assessed by performing forced degradation studies. According to ICH[9] guidelines the stress testing of the drug substance can help in assessing the intrinsic stability of the molecule and validate the stability indicating ability of the analytical procedure used. Photo stability testing should be an integral part of stress testing. The standard conditions for photo stability testing are described in ICH Q1B[8]. The specificity of the developed LC method for Sildenafil citrate was established in the presence of related substances namely imp-1, imp-2, imp-3 and degradation product namely imp-4.

(a) Thermal degradation

The drug substance was exposed to heat at 105°C for 7 days. The degraded sample was prepared as per procedure provided in section 2.4.1.

(b) Acid hydrolysis

Solutions for acid hydrolysis were prepared as per test concentration in minimum amount of suitable organic solvent and 5 N hydrochloric acid (10:90, v/v) solution was heated at 70°C temperature with constant stirring for 46 h.

(c) Base hydrolysis

Solutions for base hydrolysis were prepared as per test concentration in minimum amount of suitable organic solvent and 5 N sodium hydroxide (10:90, v/v) solution and was heated at 70°C temperature with constant stirring for 45 h.

(d) Water hydrolysis

Solutions for water hydrolysis were prepared as per test concentration in minimum amount of suitable organic solvent and water (10:90, v/v) solution and was heated at 70°C temperature with constant stirring for 44 h.

(e) Oxidation studies

Solutions for oxidation studies were prepared as per test concentration in minimum amount of organic solvent and 3 % hydrogen peroxide (10:90, v/v) solution and was heated at 70°C temperature with constant stirring for 17 h.

(f) Photolysis

The drug substance was exposed to Ultra Violet and Visible radiation of 254 nm & 365 nm wavelengths in UV cabinet for 7 days. The degraded sample was prepared as per test procedure.

All the stressed samples of Sildenafil citrate were analyzed and peak purities were checked by using Waters 2998 photo diode array detector (PDA).

Assay studies were carried out on stressed samples of drug substance against a Sildenafil citrate qualified reference standard and the mass balance (%assay + % sum of all degradants + % sum of all impurities) was calculated. Assay was performed on bulk drug samples by spiking with related substances and degradation product at different levels (viz.1%, 3% and 5%) with respect to the analyte concentration.

(B) Sensitivity

Sensitivity of the method was proved by establishing the limit of detection (LOD) and limit of quantification (LOQ) for Sildenafil citrate, imp-1, imp-2, imp-3 and imp-4 with a signal to-noise ratios of 3:1 and 10:1 respectively. LOD and LOQ were determined by injecting a series of diluted solutions with known concentrations of drug substance and the impurities. The precision study was also carried out at the LOQ level by injecting six individual preparations of Sildenafil citrate, and imp-1, imp-2, imp-3 and imp-4 at LOQ concentration and by calculating the %RSD for the areas of each peak.

(C) Precision

Precision was determined through repeatability (intra-day) and intermediate (inter-day) precision. Precision of the related substances method was evaluated by injecting six individual preparations of (500 $\mu g\ mL^{-1}$) Sildenafil citrate spiked with 0.15% of each impurity with respect to Sildenafil citrate concentration. The %RSD for each impurity was calculated.

The intermediate precision (ruggedness) of the method was evaluated by different analyst using different column and different instrument in the same laboratory.

Assay method precision was evaluated by carrying out six independent assays of test sample of Sildenafil

citrate against qualified reference standard. The %RSD was calculated for six assays. The intermediate precision of the assay method was evaluated by different analyst, different column and by using different instrument from the same laboratory

(D) Linearity and range

To establish the Linearity of the assay method, calibration solutions were prepared from the stock solution at six concentration levels ranging from 25 to 150% of the analyte concentration. Linearity solutions for the related substances method were prepared by diluting the impurity stock solution to the required concentrations at seven different levels ranging from LOQ to 150% (i.e. LOQ, 25%, 50%, 75%, 100%, 125% and 150%) with respect to 0.15% of the each impurity. The correlation coefficient, slope and y-intercept of the calibration curve were calculated.

(E) Accuracy

For determination of accuracy of the Related substances method, the recovery study was carried out by analyzing the spiked samples. Known amount of impurities were spiked to the previously analyzed samples at different concentration levels of 50, 75, 100, 125 and 150% of the specification levels. The percentage recoveries for imp-1, imp-2, imp-3 and imp-4 were calculated. Each concentration level was prepared in triplicate.

The accuracy of the assay method was evaluated in triplicate at five concentration levels, i.e. 50, 75, 100, 125 and 150 % of Sildenafil citrate assay concentration for bulk sample. The percentage recoveries were calculated.

(F) Robustness

To study the robustness of the method, the experimental conditions were deliberately changed. Critical sources of variability in the operating procedures such as percentage of organic strength of mobile phase, pH of the buffer, temperature of the column and flow rate were identified. By deliberate change in experimental conditions, the resolution between Sildenafil citrate and its related substances and degradation product was evaluated. To study the effect of flow rate on the resolution, it was changed by ±0.2 units, i.e. from 0.8 to 1.2 mL min^{-1}. The effect of pH on the resolution of impurities was studied by varying the pH to ±0.2 pH units with respect to working pH (i.e. the buffer pH altered from 5.5 to 5.3 and 5.7). The effect of column temperature on the resolution was studied at 40°C and 50°C instead of 45°C.

To study the effect of change in mobile phase composition by changing the organic ratio, the organic component (Acetonitrile) was varied by 10% from 90 to 110%, keeping buffer ratio constant.

(G) Mobile phase stability and solution stability

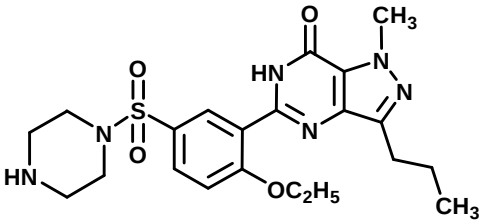
The stability of Sildenafil citrate standard and sample solutions in the assay method and the impurities solutions in the related substances method were carried out by keeping the standard solution, test solution and spiked solution separately in a tightly capped volumetric flasks at room temperature for 48 h. The solutions were analyzed at 6 h interval up to the end of the study period. The mobile phase stability was also carried out by analyzing the freshly prepared standard solution, sample solution and the spiked solution at 6 h interval up to 48 h by keeping same lot of mobile phase for entire study period. The assay and impurity content of Sildenafil citrate were calculated for the study period during mobile phase and solution stability experiments.

RESULTS AND DISCUSSION

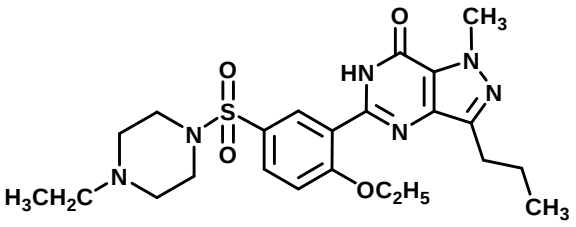
Method development and optimization of chromatographic conditions

The primary objective of this work was to develop a stability indicating LC method for the simultaneous determination and quantification of Sildenafil citrate, its related substances and degradation products. All the impurities along with Sildenafil citrate exhibited a wavelength maximum at about 225 nm (Figure 3). Hence, a detection wavelength of 225 nm was selected for monitoring the drug substance and its related substances. Different stationary phases (C18, C8, Phenyl and Cyano), mobile phases containing different buffers namely phosphate and acetate at different pH ranges from 2 to 8 and different organic modifiers namely Acetonitrile and Methanol in the mobile phase were studied.

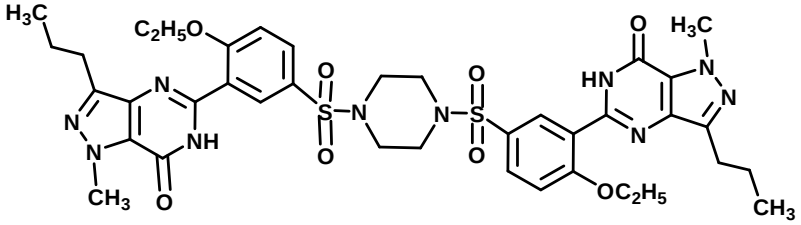
The desired chromatographic separation was achieved on a 5 µm particle size, waters symmetry shield RP18 (250 x 4.6 mm) column having carbon load of 17%.



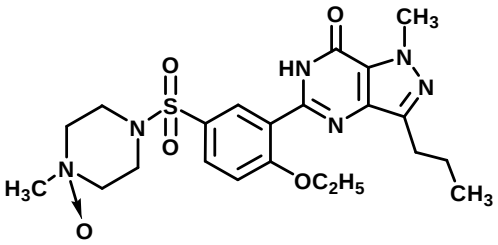
Imp-1 : 5-[2-Ethoxy-5-(piperazine-1-sulfonyl)-phenyl]-1-methyl-3-propyl-1,6-dihydro-pyrazolo[4,3-d]pyrimidin -7-one



Imp-2 : 5-[2-Ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-1-methyl-3-propyl-1,6-dihydro pyrazolo[4,3-d]pyrimidin-7-one



Imp-3 : Bis-1, 4-[4-ethoxy-3-(6, 7-dihydro-1-methyl-7-oxo-3-propyl-1H-pyrazolo[4,3-d]pyrimidin-5-yl)-benzenesulfonyl]piperazine



Imp-4 : 5-[2-Ethoxy-5-(4-methyl-4-oxo-piperazine-1-sulfonyl)-phenyl]-1-methyl-3-propyl-1,6-dihydro-pyrazolo[4,3-d] pyrimidin-7-one

Figure 2 : Impurity structures of sildenafil citrate

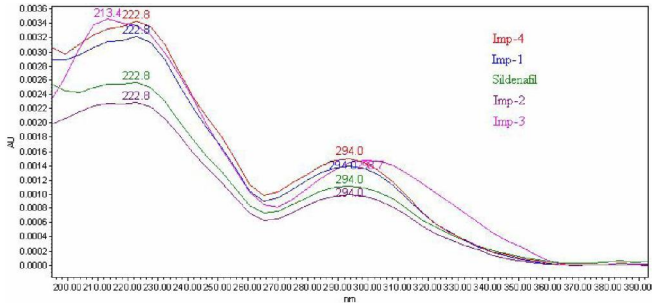


Figure 3 : Typical spectra of sildenafil, its impurities and degradation product

Sildenafil citrate is a basic compound, hence the selection of basic modifier triethyl amine as additive in the mobile phase was imperative to improve the peak symmetry of Sildenafil citrate. The selection of Buffer pH 5.5 and column temperature 45°C played a major role in separating imp-2 from Sildenafil citrate and other unknown impurities, with similar UV spectra. The chromatographic conditions were optimized with respect to sensitivity, selectivity, resolution and analysis time.

In the optimized LC conditions Sildenafil and all its related substances and degradation products were well resolved with a resolution of more than 2.0 and the typical retention times of imp-1, imp-2, imp-3, imp-4 and Sildenafil were about 16.0, 32.1, 49.9, 15.0 and 27.5min respectively (Figure 4). Peaks were symmetrical and the tailing factor for all the peaks of interest was in between 0.9 to 1.3. The results of system suitability parameters are given in TABLE 1.

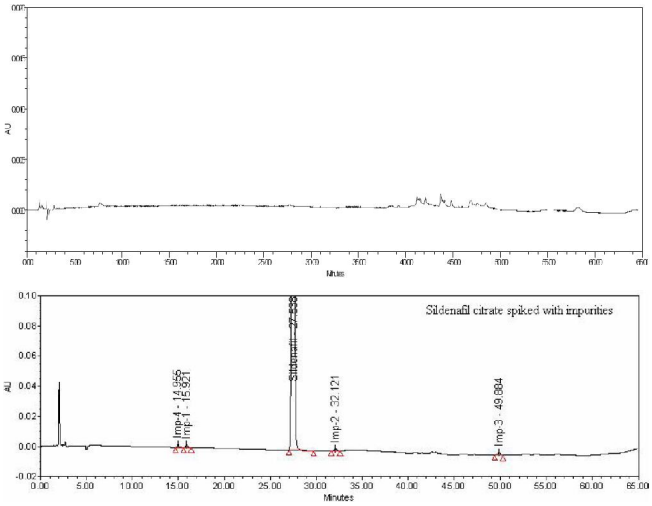


Figure 4 : Typical chromatogram of blank and sildenafil citrate sample spiked with all impurities

TABLE 1 : System suitability report

#	Compound	USP tailing factor	No. of theoretical plates USP tangent method	USP resolution (Rs)
1	Sildenafil	0.9	940004.2	30.9
2	Imp-1	1.3	31191.6	2.8
3	Imp-2	0.9	73135.2	10.8
4	Imp-3	0.9	187607.5	37.4
5	Imp-4	1.1	35338.7	--

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Analysis was performed on different batches of bulk drug samples (n = 3) and the impurity content and assay of the drug substance were assessed (TABLE 2). The same method was used for Stability studies of Sildenafil citrate as per ICH Q1A (R2) guideline.

TABLE 2 : Summary of forced degradation results

#	Stress condition	Time	% Assay of active substance	Mass balance (%Assay+ %impurities+ %degradation products)
1	Thermal (dry heat at 105°C)	7 days	99.5	99.6
2	Acid hydrolysis (5N HCl at 70°C)	46 h	98.3	99.4
3	Base hydrolysis (5N NaOH at 70°C)	45 h	99.4	99.5
4	Water hydrolysis (at 70°C)	44 h	99.6	99.7
5	Oxidation (3% Peroxide at 70°C)	17 h	81.1	99.5
6	UV degradation	7 days	99.5	99.6

Results of forced degradation studies

Stress studies on Sildenafil citrate under different stress conditions mentioned in experimental part have indicated the following degradation behavior of Sildenafil citrate (TABLE 3).

TABLE 3 : Results of intermediate precision

#	Parameter	Variation	%RSD for assay	%RSD for related substances	Resolution between imp-4 and imp-1
1	Different system	a. Waters 2998 PDA	0.3	1.2	>2.0
		b. Agilent 1100 series VWD	0.6	1.9	>2.0
2	Different column	Column-1	0.7	1.2	>2.0
		Column-2	0.7	0.8	>2.0
3	Different analyst	Analyst-1	0.3	1.2	>2.0
		Analyst-2	0.6	1.3	>2.0

The drug substance was stable under the stressed conditions of Thermal, UV, photolysis, Base hydrolysis and water hydrolysis. Moderate degradation of Sildenafil citrate was observed under acid hydrolysis, when vigorous stress conditions were applied (using 5N hydrochloric acid, heated to 70°C with constant stirring for 46 h). Sildenafil citrate has shown a significant sensitivity towards hydrogen peroxide and the oxidative degradation product thus formed was indentified by LC-MS/MS as imp-4. The imp-4 was isolated by isocratic preparative HPLC.

The impurity was characterized by NMR, Mass and FTIR. (Mass: 491.3 (M^{+1}), ^1H NMR: ($CDCl_3$, δ ppm): 1.01 (t, J=7.2 Hz, 3H), 1.56 (t, J=7.2 Hz, 3H), 1.84 (m, 2H), 2.89 (t, J=8.0 Hz, 2H), 3.14 (m, 2H), 3.24 (s, 3H), 3.38 (m, 2H), 3.41 – 3.67 (m, 4H), 4.26 (s, 3H), 4.33 (q, J=7.2 Hz, 2H), 7.23 (d, J=8.8 Hz, 1H), 7.86 (dd, J=8.8, 2.4 Hz, 1H), 8.47 (d, J=2.4 Hz, 1H), 1.57 (br, 1H) and FTIR data: 3308cm^{-1}(NH, stretching), 3108cm^{-1} (Aromatic c-H, stretching), 2962 cm^{-1} (Aliphatic c-H, stretching), 1690 cm^{-1} (Amide C=O, stretching), 1352, 1171 cm^{-1} (SO_2, stretching) & 1248 cm^{-1} (C-N, stretching).

Peak purity results obtained from PDA detector confirmed that Sildenafil citrate peak was homogeneous and spectrally pure in all the stress samples analyzed (Figure 5-8). Mass balance for stressed samples was calculated and found close to 99%. The assay of Sildenafil citrate is unaffected in the presence of imp-1, imp-2, imp-3 and imp-4 and hence stability indicating nature of the developed method was proven (TABLE 2).

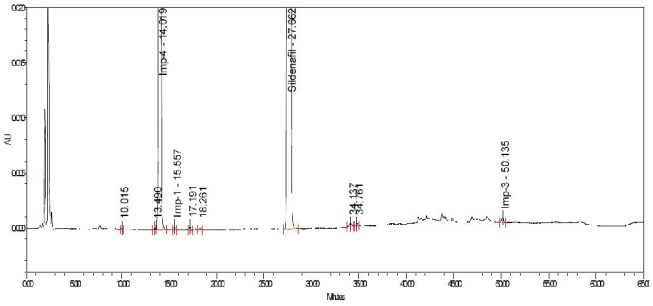

Figure 5 : Typical chromatograms for stressed samples of sildenafil citrate in 3% peroxide at 70°C for 17 h

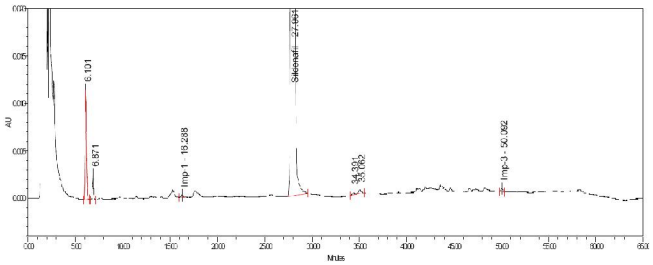

Figure 6 : Typical chromatograms for stressed samples of sildenafil citrate in 5 N HCl at 70°C for 46 h

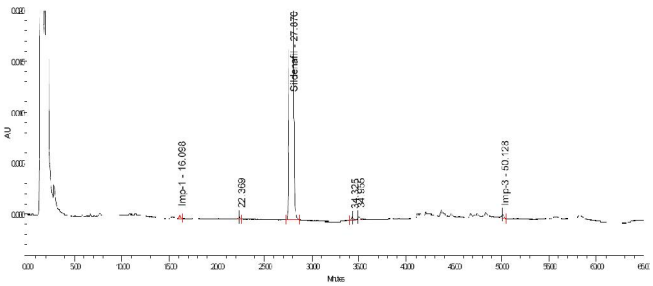

Figure 7 : Typical chromatograms for stressed samples of sildenafil citrate in 5 N NaOH at 70°C for 45 h

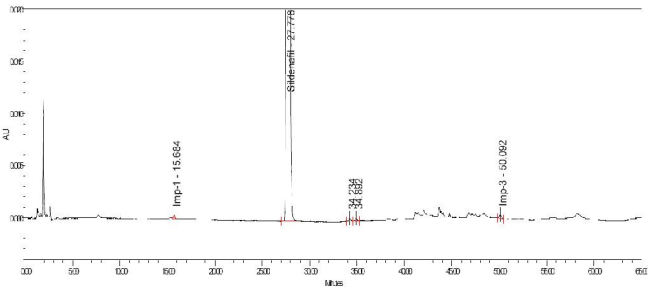

Figure 8 : Typical chromatograms for stressed samples of sildenafil citrate in water at 70°C for 44 h

Method validation

(A) Precision

The %RSD of Sildenafil citrate peak during the assay method precision was within 0.5% and the %RSD for the %area of imp-1, imp-2, imp-3 and imp-4 in the related substances method ranged from 0.3 to 0.8. The %RSD for Sildenafil citrate peak in assay was within 1.0 and the %area of all impurities in related substances method was within 2.0 during intermediate precision confirming good precision of the method (TABLE 3).

(B) Sensitivity

The Limit of detection for Sildenafil citrate, imp-1, imp-2, imp-3 and imp-4 were 0.0084%, 0.0066%, 0.0087%, 0.0075% and 0.0075% respectively with respect to the analyte concentration (i.e. 500 $\mu g\ mL^{-1}$) for 10 µL of injection volume. The Limit of quantitation of Sildenafil citrate, imp-1, imp-2, imp-3 and imp-4 were 0.028%, 0.022%, 0.029%, 0.025% and 0.025% (of analyte concentration, i.e. 500 $\mu g\ mL^{-1}$) respectively for 10 µL injection volume. The %RSD for area of Sildenafil, imp-1, imp-2, imp-3 and imp-4 were below 2.0 for precision at LOQ level.

(C) Linearity

The calibration plot was linear for the assay concentrations over the calibration ranges tested, i.e. 50 – 150 $\mu g\ mL^{-1}$ with a correlation coefficient of greater than 0.999. The slope and y-intercept of the calibration curve were 26884.4 and -0.68 respectively.

The calibration plots for related substances were linear over the calibration ranges tested, i.e. from LOQ to 150% with respect to the impurity specification, for Sildenafil citrate, imp-1, imp-2, imp-3 and imp-4 with correlation coefficient of greater than 0.999.

(D) Accuracy

Accuracy of the method was evaluated as percentage recovery. The percentage recoveries of Sildenafil citrate in bulk drug samples ranged from 99.8 to 100.8%. The percentage recoveries of imp-1, imp-2, imp-3 and imp-4 in bulk drug samples ranged from 98.2 to 102.3%. A typical LC chromatogram of sample spiked with all three impurities and degradation product in Sildenafil citrate bulk drug is shown in Figure 4.

(E) Robustness

In all the deliberately altered chromatographic conditions (flow rate, pH, column temperature and mobile phase organic ratio), there was no significant change in assay values of Sildenafil citrate, resolution between all the impurities and relative retention times of imp-1, imp-2, imp-3 and imp-4, illustrating the robustness of the developed method.

(F) Solution stability and mobile phase stability

The %RSD of Sildenafil citrate assay during solution stability and mobile phase stability experiments was within 1.0. No significant changes were observed in the content of imp-1, imp-2, imp-3 and imp-4 during solution stability and mobile phase stability experiments. The similarity factor in solution stability experiments, for both the solutions of Assay and related substances were 1.00 and 1.03 respectively. All the above results reveal that the sample solutions and mobile phase used during study were stable up to the study period of 48 h.

CONCLUSION

A simple and sensitive stability-indicating reversed-phase LC method was developed and validated according to ICH guidelines, for the simultaneous quantitative determination of Sildenafil citrate, its related substances and forced degradation products in the drug substance. The analytical method was found to be accurate, precise, specific, rugged and robust with a linear dynamic range of LOQ to 150% of specification level of impurities with a regression coefficient of greater than 0.999. This method was conveniently applied for determining the assay and related substances of Sildenafil citrate bulk drug.

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ACKNOWLEDGEMENTS

The authors wish to thank the management of Dr. Reddy's Laboratories Ltd, for supporting this work. Authors wish to acknowledge the process research group for providing the samples. The author also wish to acknowledge Dr.V.S.R.K.Krishna Reddy, for his review and valuable suggestion to improve the quality of this paper.

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