Development and Validation of a Stability-Indicating LC Method for the Assay of Lodenafil Carbonate in Tablets

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Abstract

A stability-indicating liquid chromatographic method has been developed for the quantitative determination of lodenafil carbonate in tablets. The method employs a Synergi Fusion C18 column (250 × 4.6 mm, i.d., 4 µm particle size), with mobile phase consisting of a mixture of methanol–acetic acid 0.1% pH 4.0 (65:35, v/v) and UV detection at 290 nm, using a photodiode array detector. A linear response (r = 0.9999) was observed in the range of 10–80 µg/mL. The method showed good recoveries (average 100.3%) and also intra and inter-day precision (RSD < 2.0%). Validation parameters as specificity and robustness were also determined. Specificity analysis showed that no impurities or degradation products were co-eluting with the lodenafil carbonate peak. The method was found to be stability-indicating and due to its simplicity and accuracy can be applied for routine quality control analysis of lodenafil carbonate in tablets.

Introduction

Erectile dysfunction (ED) is inability to achieve erection, an inconsistent ability to do so, or a tendency to sustain only brief erections. The ED is a haemodynamic phenomenon depending on the integrity of neurological, vascular, endocrinological, tissue (corpora cavernosa), psychological and relational factors; changes in any one of these components may lead to erectile dysfunction (1).

The prevalence of ED in Brazil has been estimated to 65.6 cases per 1000 person-years and the studies demonstrated that although incident ED is associated with age, it is not an inevitable outcome of the aging process (2). The identification of medical and behavioral risk factors for ED is essential to determine which factors could be modified for prevention efforts. The projection of one million new cases annually in Brazil adds to the public health importance of this condition (2, 3).

The phosphodiesterase type 5 inhibitors (PDE5) have revolutionized the way in which men with ED can be treated (4–6). New chemical entities can be welcome in this field whenever presenting a different pharmacological profile or expanding the access to developing countries' populations (7). The physiological process of erection involves the release of nitric oxide (NO) in the corpus cavernosum of the penis, which facilitates the conversion of guanosine triphosphate to cyclic GMP (cGMP) and leads to a cascade of events culminating in decreased intracellular calcium and resultant smooth muscle relaxation. At the same time, cGMP is broken down to GMP by the enzyme phosphodiesterase (PDE), the type 5 isoform, which is found in relatively high concentrations in corpora cavernosa. PDE5 acts at this step, slowing the breakdown of cGMP, resulting in a higher concentration and a longer duration of this natural second intracellular messenger (8). The higher cGMP level, therefore, acts as amplifier of the normal erectile physiology and is dependent on factors that must be present in normal erectile function as intact libido, sexual stimulation, and sensory pathways (9, 10).

Lodenafil carbonate, bis-(2-{4-[4-ethoxy-3-(1-methyl-7-oxo-3-propyl-6,7-dihydro-1H-pyrazolo[4,3-d]pyrimidin-5-yl)-benzenesulfonyl]piperazin-1-yl}-ethyl) carbonate, is a new PDE5 inhibitor developed in Brazil, which is a dimer formed by two lodenafil molecules linked by a carbonate bridge (Figure 1). After ingestion, the bridge is broken delivering the active compound lodenafil. Therefore, lodenafil carbonate is a prodrug, which relaxes insulated human and animal cavernous tissues through inhibition of cyclic guanosine monophosphate hydrolysis (11).

Lodenafil carbonate is currently available in tablets, but at the moment, there are no methods published for the quantitative analysis of this drug as active pharmaceutical ingredient or in a

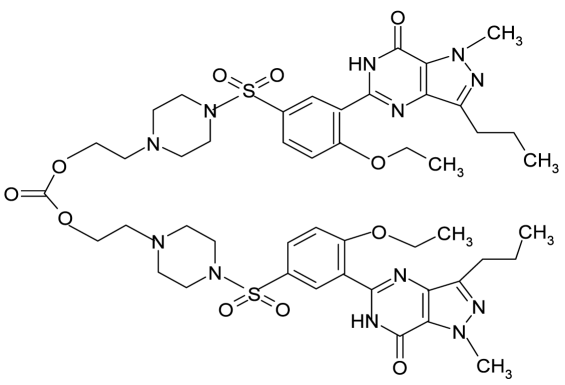

Figure 1. Chemical structure of lodenafil carbonate.

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finished product. As far as could be determined, there is one paper describing pharmacokinetics studies using a liquid chromatography coupled to tandem mass spectrometry (LC–MS–MS) (11). This method was developed for lodenafil carbonate in biological fluids and there were no stability indicating method for the determination of lodenafil carbonate in the presence of degradation products. Besides, this method has difficult detection (LC–MS–MS) making it unsuitable for routine analysis. The purpose of this study was to develop a suitable stability-indicating reversed-phase liquid chromatography method using UV detection for the quantitative analysis of lodenafil carbonate in solid pharmaceutical dosage forms. Moreover, the method was validated with respect to specificity, linearity, precision, accuracy, robustness and forced degradation studies to show the stability indicating nature of the method (12).

Experimental

Samples

Lodenafil carbonate reference substance (assigned purity of 100.54%) was kindly supplied by Cristália Laboratories, São Paulo, Brazil. Lodenafil carbonate tablets (Helleva, 80 mg) were obtained from commercial sources within their shelf life period. The excipients include calcium phosphate, dibasic dihydrate, povidone, lactose, colloidal silicon dioxide, sodium croscarmellose, microcrystalline cellulose and magnesium stearate.

Reagents and solvents

All chemicals used were of pharmaceutical or special analytical grade. Methanol (LC grade) was from Tedia (Fairfield, OH) and sodium hydroxide was purchased from Merck (Darmstadt, Germany). Ultra-pure water was obtained from a Water Purification Unit from Labconco (Kansas City, MO).

Instrumentation and analytical conditions

The development and validation of the assay was performed on a Shimadzu LC system (Kyoto, Japan) comprising an LC-10 ADVP pump, an SPD-M 10 AVP diode array detector, an SCL-10 AVP system controller, SIL-10 ADVP auto injector and a degasser module. The detector was set at 290 nm and peak areas were integrated automatically by computer using a Class VP V6.14 software program. The experiments were carried out with Phenomenex Synergi C18 column (250 mm × 4.6 mm i.d.; 4 µm particle size, 80 Å) at room temperature. The LC system was operated isocratically using a mobile phase consisting of a mixture of methanol–acetic acid 0.1% pH 4.0 (65:35, v/v), which was filtered through a 0.45 µm membrane filter (Millipore, Bedford, USA) and run at a flow rate of 1.0 mL/min. The injection volume was 20 µL for both standard and samples. Peak identity was confirmed by both, retention time comparison and comparison of spectra obtained from the photodiode array detector (PDA detector). The quantitation was performed using the absolute area of the peak.

Preparation of lodenafil carbonate reference standard

The stock solution of lodenafil carbonate reference substance was prepared by weighing 10 mg of reference material into a 100 mL volumetric flask and diluting to volume with sodium hydroxide 0.1 M, obtaining a concentration of 100 µg/mL. The working standard solution (40 µg/mL) was obtained by dilution of the stock solution in mobile phase.

Preparation of sample solution

To prepare the sample solutions, tablets containing 80 mg of lodenafil carbonate were accurately weighed and crushed to fine powder. A quantity of the powdered tablets, equivalent to 10 mg of lodenafil carbonate, was transferred into a 100 mL volumetric flask with 50 mL of sodium hydroxide 0.1 M and kept in an ultrasonic bath for 10 minutes. After that solvent was added to make up the volume. The solution was diluted with mobile phase, giving a final concentration of 40 µg/mL.

Validation study

The method was validated using samples of the pharmaceutical formulation with lodenafil carbonate evaluating following parameters: specificity, linearity, precision, accuracy, robustness, stability, and system suitability, in accordance with the International Conference on Harmonization (ICH) guidelines (12).

Linearity

Linearity was evaluated by constructing three analytical curves each one with eight reference substance concentrations of lodenafil carbonate in the range of 10–80 µg/mL prepared by the dilution of appropriate amounts of standard stock solution with mobile phase. Triplicate 20 µL injections were made for the standard solution to verify the reproducibility of the detector response at each concentration level. The linearity of the calibration curve was then determined for intra- and inter-day experiments on three different days. The peak area of lodenafil carbonate was plotted against the respective concentrations to obtain the analytical curve. The results were subjected to regression analysis by the least squares method to calculate calibration equation and correlation coefficient. An ANOVA statistical evaluation was performed.

Specificity

Chromatographic runs of a placebo solution and forced degradation studies were performed to evaluate the specificity of the method. The stress conditions employed were: acid, alkali, oxidation, heat and light. The reference standard solution (200 µg/mL) of lodenafil carbonate was prepared by diluting the stock solutions with acid, alkaline, or oxidative solutions. After the degradation treatments were completed, the samples were allowed to equilibrate at room temperature, neutralized with acid or base (when necessary), and diluted with mobile phase to 40 µg/mL. The samples were analyzed against a freshly prepared control sample (with no degradation treatment). Specific conditions are described below:

Hydrolytic studies: Solution of lodenafil carbonate in 1.0 M hydrochloric acid was exposed at room temperature for 24 h and also to 60°C for 5 h. The studies in alkaline conditions were done at room temperature in 1.0 M sodium hydroxide for 24 h and the solution was also submitted to 60°C for 5 h.

Oxidative studies: Oxidative conditions were obtained by

treating the reference standard solution with 3.0% hydrogen peroxide at room temperature for 2 min, protected from light.

Photolytic studies: Photolytic studies were induced by exposing the reference standard solution in a photostability chamber to 200 watt hours/square meter of near ultraviolet light (254 nm) for 15 min. The chamber (17 × 17 × 100 cm) was internally coated with mirrors, in order to distribute the light uniformity. The intensity of the UV radiation was determined using a luximeter.

Thermal studies: To study thermal degradation, the reference standard solution was heated at 50°C for 24 h.

Precision

The precision of the analytical procedure was evaluated by the determination of repeatability of the method (intra-day precision) and intermediate precision (inter-day precision) of the sample solutions. Repeatability was calculated by assaying six samples prepared as described in sample preparation, during the same day, under the same experimental conditions. The intermediate precision was studied by comparing the results of the assay on different days (3 days). The relative standard deviations (RSD) of the peaks were calculated.

Accuracy

To confirm the accuracy of the method, recovery was determined at three concentrations (28, 40, and 52 µg/mL), in accordance with the recommendations of the ICH guidelines (12), by adding known amounts (8, 20, and 32 µg/mL) of the reference substance at the beginning of the process, corresponding to 70%, 100%, and 130% of the nominal analytical concentration (40 µg/mL). Each level was made in triplicate.

Robustness

Method robustness was assessed by changing experimental conditions such as the column (Phenomenex Synergi C18, 150 mm × 4.6 mm i.d.; 4 µm particle size, 80 Å), flow rate (0.8 and 1.2 mL/min), composition (70:30 and 60:40, v/v), and pH (pH 3.8 and 4.2) of the mobile phase. Samples were analyzed at each modified condition, to assess any impact on assay results.

Stability of sample solution

To assess the stability of sample solutions of the pharmaceutical formulation of lodenafil carbonate, the tested samples were maintained at room temperature for 24 h. The stability of these solutions was studied by performing the experiment and observing any change in the chromatographic pattern, compared with freshly prepared solutions (13).The stability of lodenafil carbonate in 0.1 M sodium hydroxide was studied. Stock sample solution of lodenafil carbonate (100 µg/mL) was prepared and stored at 2–8°C for 18 days.

System suitability test

System suitability parameters were measured using three injections of a reference solution containing 40 µg/mL of lodenafil carbonate to verify the system performance. Relative standard deviations of the retention time, tailing factor, number of theoretical plates, peak area, and capacity factor were measured to test system suitability.

Results

Method development

The peak of lodenafil carbonate is well resolved with retention time ~7.0 min.

Method validation

Linearity

The calibration curve for lodenafil carbonate was constructed by plotting the peak areas versus concentration. It was linear in the 10–80 µg/mL range with a correlation coefficient of 0.9999 and the representative linear regression equation was y = 32.335x – 10.179.

Specificity

When submitted to acid conditions, no degradation peaks were detected but the area of lodenafil carbonate decreased at room temperature and with heat at 60°C by an extent of 3.6% and 6.7%, respectively. Only 7.6% of lodenafil carbonate degraded in alkaline conditions (1.0 M, 5 h, 60°C) and the degradation products were observed at retention times of 3.7 and 3.9 min (Figure 2A). However, when kept in alkaline conditions at room temperature for 24 h, 25.8% degradation was observed and the degradation products were observed at retention times of 3.4, 3.6, and 3.7 min (Figure 2B). These peaks of degradation products were not well resolved. Under oxidative conditions, the drug

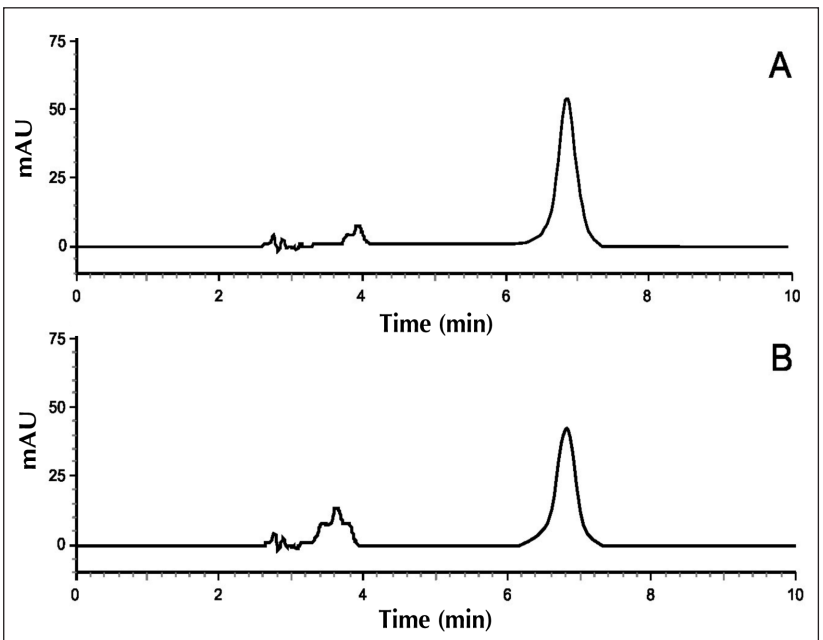

Figure 2. Chromatograms of lodenafil carbonate solution after alkaline hydrolysis (1.0 M NaOH) at 60°C, 5 h (A); at room temperature, 24 h (B).

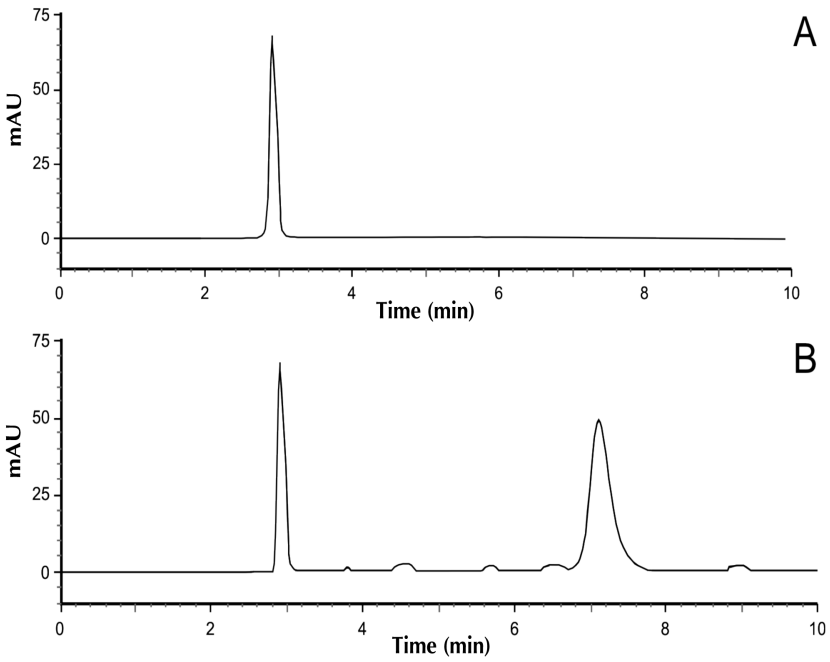

Figure 3. Chromatograms of blank solution after oxidation with 3.0% hydrogen peroxide (A) and lodenafil carbonate solution after oxidation with 3.0% hydrogen peroxide (B).

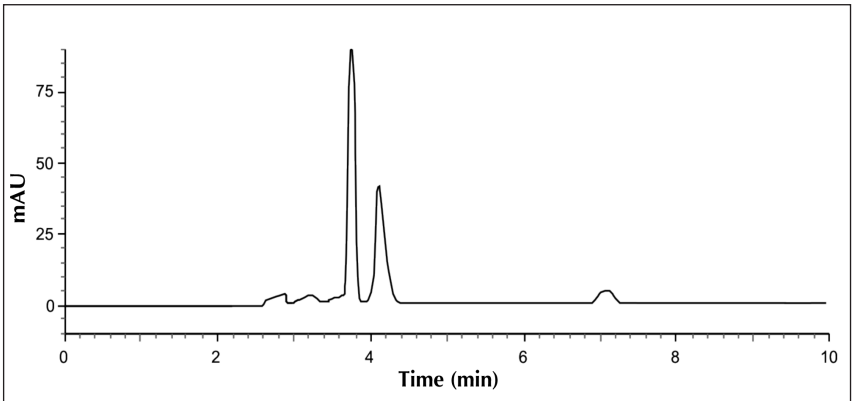

Figure 4. Chromatograms of lodenafil carbonate solution after exposition to UV light.

content decreased by about 27.2% and the degradation products were observed at retention times of 3.7, 4.5, 5.6, 6.5, and 8.9 min (Figure 3). Degradation of 94% was observed after UV radiation exposition. The major degradation product was detected at 3.7 min (Figure 4). Other peaks were observed at 2.7, 3.2, and 4.1 min. No degradation peaks were observed after exposure to dry heat at 50°C for 24 h but lodenafil peak area decreased by 4.9%.

Precision

To evaluate the precision of the proposed method, the RSD analysis (relative standard deviation) was developed, as recommended by ICH (12). It was found a RSD = 0.9 % (recommended value ≤ 2.0 %), as presented in Table I.

Accuracy

The results obtained for the accuracy study are presented in Table II. The mean recovery obtained for each level, as well as for all levels (100.3%) showed that the method was accurate. The statistical analysis of the accuracy results (ANOVA) showed no evidence of significative difference between the mean recoveries in the evaluated concentration levels (p = 0.5248).

Robustness

The robustness of the method was studied and no significant effect on assay data performance was observed (Table III) with change of pH (3.8 and 4.2), flow rate (0.8 and 1.2 mL) and mobile phase composition (70:30 and 60:40 v/v).

Stability of sample solution

The stability of the sample solutions was studied and the data obtained showed no change till 24 h at room temperature. The RSD values were within 0.2%.

Table I. Method Repeatability/Intermediate Precision for Lodenafil Carbonate

	% Label claim		
Sample	Day 1	Day 2	Day 3
1	105.1	106.0	106.1
2	106.5	105.3	105.1
3	104.6	106.8	105.6
4	103.6	106.5	104.9
5	104.3	106.0	104.6
6	105.8	106.2	104.1
Mean (6)	105.0	106.1	105.1
%RSD	0.9	0.5	0.7
Mean (18)	105.4		
%RSD	0.9		

Table II. Accuracy of Method Determined by Recovery of Lodenafil Carbonate from Tablets Solutions Spiked with Standard Solution

Sample	Level	% Recovery	Mean (3)	%RSD
1	70	100.34	99.9	0.4
2		99.50		
3		99.98		
1	100	99.68	100.6	0.8
2		100.84		
3		101.13		
1	130	99.09	100.4	1.2
2		101.41		
3		100.84		
Mean (9)	100.3			
% RSD	0.8			

Table III. Results of the Method Robustness Study

Variations	Retention time	Theoretical plates	Capacity factor	Tailing factor	Assay as % of label claim
Mobile phase proposed*,†	7.04	5412	3.60	1.47	105.3
Methanol content 60 %†,‡	8.75	3432	4.83	1.67	104.7
Methanol content 70 %†,‡	5.75	4507	2.83	1.61	104.6
Aqueous phase pH 3.8†,§	6.77	3554	3.51	1.75	104.4
Aqueous phase pH 4.2†,§	7.67	6289	4.12	1.54	105.3
Flow rate 0.8 mL/min*,†	8.54	4630	4.69	1.84	105.3
Flow rate 1.2 mL/min*,†	5.94	4254	2.96	1.60	106.4
Phenomenex Synergi C18 column (150 mm Å 4.6 mm)*	4.55	3334	2.04	1.06	106.5

* Methanol–acetic acid 0.1 % pH 4.0 (65:35, v/v).
† Phenomenex Synergi C18 column (250 mm x 4.6 mm).
‡ Value of pH 4.0.
§ Proportion of organic and aqueous phase 65:35.

Table IV. Results of Peak Purity of Forced Degradation Study Using Proposed Method

Stress condition	Peak purity
Control sample	0.9999
Acid hydrolysis (1.0 M HCl, 5 h, 60°C)	0.9999
Acid hydrolysis (1.0 M HCl, 24 h, room temp.)	0.9999
Alkaline hydrolysis (1.0 M NaOH, 5 h, 60°C)	0.9999
Alkaline hydrolysis (1.0 M NaOH, 24 h, room temp.)	0.9990
Photolytic degradation (15 min)	0.9999
Oxidation (3% H_2O_2, 2 min)	0.9990
Thermal degradation (24 h, 50°C)	0.9999

The sample stock solutions were kept at 2–8°C during 18 days and showed result of 105.6% of the labeled value of lodenafil carbonate (correspondent to 100.5% of the initial potency of the same solutions, peak purity of 0.9999), which demonstrate that they remain with acceptable stability.

System suitability test

The %RSD values calculated for the retention time, tailing factor, number of theoretical plates, peak area, and capacity factor were 0.14, 0.24, 0.12, and 0.18, respectively. The experimental results show that the parameters tested were within the acceptable range (RSD < 2.0 %), indicating that the system was suitable for the intended analysis.

Discussion

This isocratic-mode method with UV detection was developed for the determination of lodenafil carbonate in tablets. Preliminary trails using different compositions of mobile phases were conducted and the final mobile phase was chosen based in the chromatographic parameters (tailing factor, theoretical plates, resolution, and capacity factor). A mixture of methanol: acetic acid 0.1% pH 4.0 (65:35, v/v) was adopted. The column efficiency for lodenafil carbonate was about 5560 theoretical plates, the peak symmetry was 1.47 and the capacity factor was 3.61. For the selection of the best detection wavelength a PDA detector was used. No interference from the sample solvent, impurities and dosage form excipients were observed at the detection wavelength (290 nm) (Table IV).

Linearity of the assay method was checked during three consecutive days over the same concentration range. The results showed that an excellent correlation was obtained between the peak area and concentration of the analyte. The validity of the assay was verified by means of the analysis of variance (ANOVA) indicating that there was no significant deviation from linearity ($p < 0.05$).

The described LC method was specific. No interfering peaks of degradated products were observed at retention time of about 7.0 min. Slight degradation was observed in acid and basic hydrolysis conditions at room temperature and on heating at 60°C. In the same way slight degradation was found in oxidative stress, while significant degradation of the drug was observed under UV radiation exposition at room temperature. Photolytic degradation lead to the formation of one major degradation product at 3.7 min, at the same retention time as the one observed after alkaline and oxidative decomposition. No degradation products were detected, on dry heating at 50°C for 24 h and in acid condition indicating that lodenafil carbonate was degraded to non-chromophoric compounds or it decomposed to low molecular weight fractions, which could not be detected under the experimental conditions. The presence of potential degradation products in the solution did not interfere with the determination of lodenafil carbonate under the proposed LC conditions. All the degradation products eluted at different retention times and did not co-elute with the parent compound. The purities of the peaks (Table IV), verified by PDA, indicated that they were not contaminated with any of the degradation products. In all the degradation conditions, the tailing factor and the resolution followed the reference values (tailing factor ≤ 2.0 and resolution > 2.0). The isolation and identification of the degradation products will be the next step of this work. Thus, the developed method was shown to be selective by nature.

The relative standard deviations values for repeatability and intermediate precision were within the acceptance criteria of 2.0%. Also, the obtained accuracy values were within the range of 98.0–102.0%, satisfying the acceptance criteria for the study.

The result of the robustness study is demonstrated in Table III. Deliberate alterations of mobile phase, flow-rate and column showed that the changes of the operational parameters did not lead to essential changes of the performance of the chromatographic system. In short term stability study, the sample solution was also stable until 24 h at room temperature and the sample stock solution for 18 days at 2–8°C. System suitability tests were performed and chromatographic parameters such as capacity factor, tailing factor, theoretical plates, etc. were calculated from experimental. All the values for the system suitability parameters were within the acceptable range.

Conclusion

The isocratic LC method developed proved to be simple, linear, precise, accurate and specific for the determination of lodenafil carbonate in tablets, without any interference from the excipients. Therefore, the method may be useful for routine quality control assay of lodenafil carbonate in tablets and stability studies.

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