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Contributed Paper

Analytical Validation of Sildenafil Citrate Inhaler Preparation

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ABSTRACT

The aim of this study was to develop and validate a high performance liquid chromatographic (HPLC) system for the analysis of sildenafil citrate in pharmaceutical inhaler preparations. Sildenafil was partially degraded by acid and alkali and was completely degraded by oxidation. Direct exposure to heat and UV in solid state had no effect on the degradation of sildenafil. This method was able to resolve any degradation products from the sildenafil peak. Sildenafil citrate showed UV absorption maximum at 240 and 276 nm. A reversed phase HPLC system for the separation and quantitation of sildenafil citrate assay was successfully developed and validated. The chromatographic conditions employed a C18 column (4.6 × 250 mm) with a mobile phase of 0.2 M ammonium acetate buffer and acetonitrile (40:60 v/v, pH 7.0) at a flow rate of 1.0 mL/min with UV detection at 240 nm. The retention time of sildenafil citrate was about 5 min. The method was specific to the sildenafil at the presence of other common excipients in the inhaler preparation. The method was accurate (99.5% recovery) and precise for both intra-day (0.5-1.0%) and inter-day (1.1%) assays. A linear correlation was obtained over the concentration range of 0.5-500 μg/mL (r^2=1.0). The sample solution from metered dose inhalers (MDIs) was stable over 24 h. The LOD and LOQ were 1.30 and 6.10 ng/mL, respectively.

Keywords: sildenafil citrate, validation, liquid chromatography, degradation

1. INTRODUCTION

Sildenafil or sildenafil citrate (Figure 1) is chemically known as 1-[[3-(6,7-dihydro-1-methyl-7-oxo-3-propyl-1*H*-pyrazolo [4,3-*d*] pyrimidin-5-yl)-4-ethoxyphenyl] sulfonyl] -4-methyl-piperazine citrate [1]. The structural formula is shown in Figure 1 with a molecular weight of 666.7 for its citrate salt [2]. There are two pK_a values at 9.84 (NH-amide) and 7.10 (NH-piperazine) [3]. It is a highly selective phosphodiesterase-5 inhibitor for the treatment of erectile dysfunction. The dosage form is as a single oral dose tablet of 25 - 100 mg [4-6]. Sildenafil was subsequently shown to have effects on the pulmonary hypertension as an oral dose of 20 - 50 mg tid [6-10]. It has been confirmed that sildenafil has a greater efficacy and is safe when administered by inhalation. Such a product is

not yet available in the market. The delivery of sildenafil to the lung or target directly could have several advantages over conventional treatments from its onsite delivery, a more rapid onset of response, reduced side effects, selectivity of hemodynamic effects on lung vasculature. As the receptors of pulmonary hypertension are located on the smooth muscle cell in the lung, hence an increased bioavailability of the delivered dose is beneficial [9, 11-12]. In addition, inhaled sildenafil in the prophylaxis of pulmonary hypertension in the postoperative setting could be an additional tool to prevent an important clinical problem better than intravenous sildenafil [13]. Overall there is a need for extensive investigations and developments for an inhaler dosage form. There have been a few methods for determination of sildenafil reported in pharmaceutical products based on HPLC methods but the limit of detection and quantitation is still high [14-16]. In addition, the methods for determining sildenafil in biological fluids are more complicated when used as a quality control in routine work [17-20]. In general, the dose of sildenafil in an inhaler preparation is much lower than that for an oral preparation by approximately 10-100 times so any assay method needs to be more sensitive. As sildenafil inhaler preparation not available, we have now developed a new dosage form of sildenafil citrate as a pMDI for treatment of pulmonary hypertension. The aim of this study was to develop and validate an HPLC system for an analysis of sildenafil in the new dosage form as pMDI. The degradations of sildenafil citrate by acid/base hydrolysis, oxidative, thermal and UV degradation were carried out.

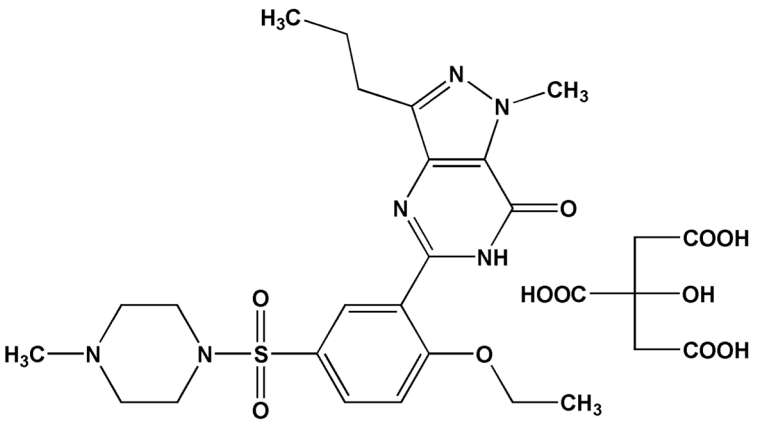

Figure 1. The chemical structure of sildenafil and its citrate salt.

2. MATERIALS AND METHODS

2.1 Materials and Reagents

Sildenafil citrate (Lot no. SCVIID 1209040) and reference standards of sildenafil citrate (potency 99.40% as is) were obtained from Smilax Laboratories Limited, India. Other chemicals were analytical grade from Merck, Germany. All solvents used were HPLC grade from RCI Labscan, Bangkok, Thailand.

2.2 Instrumentation and Chromatographic Conditions

Chromatography was performed with the HPLC instrument system (Ultimate 3000 SR-3000, USA). A high performance liquid chromatographic system consisting of a water solvent delivery pump, a water injector with an injection volume of a 20 µL sample loop (Ultimate 3000 WPS 3000SL), by an auto-sampler was used for analysis. The data

was recorded using LC 2010 solution software. The separations were performed on a reversed phase stainless steel column 250 mm long, 4.6 mm internal diameter filled with octadecylsilane (ACE 5 C-18-AR 4.6 × 250 mm, 5 μm) maintained at 25°C. The mobile phase contained a degassed mixture of 0.2 M ammonium acetate buffer and acetonitrile in the ratio of 40:60 by volume at the ambient temperature. The flow rate was maintained at 1.0 mL/min, and the separation was monitored by UV detection at a wavelength of 240 nm.

2.3 Preparation of the Mobile Phase

The mobile phase was prepared by dissolving 6.10 g of ammonium acetate in 400 mL of water, adjusted to a pH 7.0 with phosphoric acid followed by the addition of 600 mL of acetonitrile into the aqueous mixture. The mobile phase was sonicated for 15 minutes and then filtered through a polyamide 0.45 μm membrane filter paper. The mobile phase was adjusted to pH 7.0 with 0.1 N NaOH prior to use. The mobile phase was adjusted if and when necessary.

2.4 Maximum Absorption of Sildenafil Citrate

Sildenafil citrate in an aqueous solution at a concentration of 10 μg/mL (0.022 mM) was prepared. The UV absorption spectra at 190-790 nm were compared with absorption bands of other excipients (Genesys 6, Thermo electron corporation, USA). The molar absorptivity of sildenafil was also calculated.

2.5 Degradation Studies of Sildenafil Citrate

Acid / Base degradation in an aqueous solution

The degradation of sildenafil citrate in an aqueous solution was conducted at pH 2 and pH 12 at ambient temperature. Sildenafil citrate sample was dissolved and adjusted with the mobile phase to a concentration of 100 μg/mL. The sample preparation was adjusted to a pH of 2 with 1 N HCl or 1 N NaOH solution to a pH of 12, and left for 2 weeks. The sample solution at pH 7.0 (without treating with either acid or base) was prepared as a control. At a specified time, the resultant solution was analyzed to determine the sildenafil degradation products.

Oxidative degradation

Sildenafil citrate was accurately weighed and transferred into a volumetric flask and diluted with a mobile phase to a concentration of 1 mg/mL. The sample was then diluted with the mobile phase and a 50% H_2O_2 solution added to a final concentration of 0.1%, 1%, 2% and 30% of H_2O_2 in the sample solution. These solutions were left to react for 24 h. A treated sample (5 mL) was then pipetted into a 50 mL volumetric flask and adjusted the volume with the mobile phase before analysis.

Thermal degradation

Sildenafil citrate was kept in a hot air oven at 120°C for 48 h in order to activate the degradation of sildenafil in its solid state. The sample was then prepared to a final concentration of 100 μg/mL with the mobile phase before injection.

UV degradation

Sildenafil citrate was exposed to UV at the ambient temperature for 48 h in order to activate the UV degradation of the solid state sildenafil. After that the sildenafil citrate sample was diluted with the mobile phase, yielding a final concentration of 100 μg/mL.

2.6 Preparation of Placebo Solutions

A placebo of sildenafil MDI of about 265 mg was weighed and transferred into a

50 mL of volumetric flask and after being completely dissolved was then diluted with the mobile phase. Five mL of this solution was pipetted into a 50 mL volumetric flask, and diluted to volume with a mobile phase. The solution was filtered through a 0.45 μm membrane before injection. The components of the placebo consisting of dried ethanol, cyclodextrin, glycerin, propylene glycol, benzalkonium chloride. The all excipients components of the MDI were except the propellant because the propellant evaporated after actuation. The UV maximum absorption of inhalation excipients did not interfere with sildenafil citrate at 240 nm.

2.7 Preparation of Sample Solutions

The sample of sildenafil citrate MDI (prototype formula without propellant) was accurately weighed to obtain 5 mg of sildenafil into a 50 mL volumetric flask and diluted with a mobile phase. Five mL of the sample was pipetted into a 50 mL volumetric flask, dissolved and diluted with the mobile phase. The solution was filtered through a 0.45 μm membrane filter before injection.

2.8 Preparation of the Standard Solutions

The sildenafil citrate reference standard was accurately weighed (25 mg) and added to a clean 25 mL volumetric flask. This reference standard was then dissolved and diluted with the mobile phase. This final solution contained 1 mg/mL of sildenafil. Then, aliquots of 5 mL stock solution into a 50 mL volumetric flask and diluted with a mobile phase and used as a stock solution (0.1 mg/mL of sildenafil). The stock solutions were stable for at least one month (data not shown) but in this experimental it was used within 2 weeks.

2.9 Method Validation

The method was validated for 8 parameters: specificity, range, linearity, precision, accuracy, limit of detection (LOD), limit of quantitation (LOQ), and robustness. In addition the system suitability parameter was also calculated. To demonstrate specificity in the presence of the excipients used in the inhaler formulations, sildenafil citrate was spiked (approximately 25 μg/mL) into the drug product (prototype sildenafil inhaler formulation), a chromatogram was run and the result observed and compared with that of the standard solution to confirm the same retention time and also that the product used was capable of separating the analyte peak from its inhalation excipients. In addition, the sample solution, placebo solution and standard solution were injected separately to compare the retention times of the sildenafil peak from different samples. A standard solution of sildenafil was prepared from the stock solution at a concentration of 0.50 mg/mL and this was then further diluted to a final concentration of 50, 75, 100, 125 and 150 μg/mL to evaluate linearity. The samples were injected (20 μL) and the signals from the samples were recorded. A calibration curve for sildenafil citrate was constructed using the observed peak area versus the nominal concentration of the analyte. To determine the repeatability and intermediate precision, six replicate samples of sildenafil citrate obtained from the spike placebo method was analyzed (at the 100% concentration level). To determine the intermediate precision, sample solutions of sildenafil citrate at eight different concentrations were analyzed three times within the same day (intra-day) and over three other days (inter-day variation). To

determine the accuracy of the method, the spiked placebo method was chosen. A working standard of sildenafil citrate was added into the placebo solution in triplicate at the level of 75, 100 and 125% and analyzed. To determine if the interval between the upper and lower concentrations of sildenafil in the sample (including the above concentrations) had the appropriate precision, the results from the accuracy determination were used. The stability of the sildenafil in the test solutions was determined by assay of the same solution after storage for 0, 2, 4, 6, 8 and 24 h at room temperature. The LOD and LOQ values were calculated according to Thongchai *et al.* [21] and USP guidelines [22] from the standard deviation (SD) of response and slope of curve (S) as following equation:

$$LOD = 3.3\ (SD/S)$$
$$LOQ = 10\ (SD/S)$$

The robustness of the procedure was evaluated for the following parameters; column temperature (20, 25, 30°C), flow rate (0.5, 1.0, 1.5 mL/min), mobile phase ratio (54, 60, 66% of acetonitrile), wavelength (235, 240, 245 nm), sonication time (10, 15, 20 min) and the column type that was used in this study (old pre-used and new column).

2.10 Assay of Sildenafil Citrate Inhaler Formulations

Due to the sildenafil inhaler preparations not being available the inhaler products of sildenafil citrate were made in-house for the pMDI prototype. The formulations consisted of ethanol as a co-solvent, PEG 400 as a stabilizing agent, sorbitan monooleate as a surfactant, and tetrafluoroethane (HFA 134a) as a propellant. These formulations were developed in-house with adaptations from the results of previous work [23]. Briefly, the required quantity of sildenafil citrate and dried ethanol were mixed in a vessel and maintained the temperature in an ice-bath. The stabilizing agents (PEG 400 and sorbitan monooleate) were added and then mixed by Vortex-2-Genie (Scientific Industries, Inc., USA) to obtain the product concentrate. The product concentrate (1000 mg) was weighed into glass bottle canister. Then 50 μL metering valves were immediately crimp-sealed onto the canisters using an aerosol crimping machine (Pamasol Willi Mäder AG, Switzerland) with 20 mm neck diameter and the canisters were then filled with propellant at a specified amount with the same machine. That product met the requirements for an MDI and produced a physically stable pMDI. The sample preparation was prepared in a similar manner to the sample preparation using the mobile phase as a solvent. The sildenafil pMDI formulations were prepared without propellant and the amount of sildenafil citrate in the pMDI were varied over 5 concentrations. The samples were filtered through a 0.45 μm membrane and injected for HPLC analysis.

3. RESULS AND DISCUSSION

3.1 Method Development

Sildenafil citrate showed two UV absorption maximum wavelengths at 240 and 276 nm and this was similar to a previous report [15] (see contour plot wavelengths 190-790 nm from Figure 2). There were no interferences from other excipients at 240 nm. The molar absorptivity of sildenafil citrate at 240 nm is 15,100. Analytical methods of sildenafil citrate appeared in the literature with various wavelengths i.e. Dinesh *et al.* [1] used detection wavelength at 245 nm, Tripathi *et al.* [24] determined at 230 nm while Elshafeey *et al.* [25] determined sildenafil citrate in plasma at 220 nm. We adapted

the chromatographic conditions from Daraghmeh *et al.* [15] and chose detection wavelength at 240 nm. It was found that other inhalation excipients did not interfere with sildenafil citrate running with this condition. Better resolution between the chromatographic peaks of sildenafil and its impurities was obtained. The ratio of 0.2 M

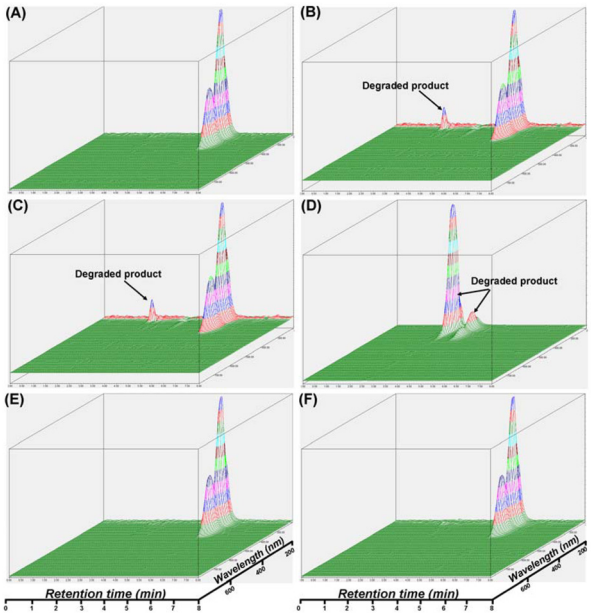

Figure 2. Contour plots of sildenafil citrate from HPLC at scanning wavelengths of 190-790 nm (A) standard sildenafil (B) forced degradation at pH 2 with 1 N HCl for 2 weeks (C) forced degradation at pH 12 with 1 N NaOH for 2 weeks (D) forced degradation with 0.1% H_2O_2 for 24 h (E) forced degradation by being kept at 120°C for 48 h (F) forced degradation by exposure to UV for 48 h.

ammonium acetate buffer and acetonitrile was modified from 50:50 v/v [15] to 40:60 v/v in order to reduce the retention time of sildenafil from 10 min to 5 min. The validation methods were performed according to the USP and US FDA guideline [22].

3.2 Determination of Degradation Products

Figure 2 shows the contour plot of sildenafil between the wavelengths of 190-790 nm. Figure 2 (A) is a standard sildenafil. Figure 2 (B) - (F) shows the peak of the degraded products of sildenafil after hydrolysis in an acidic condition (pH 2) (B) and basic condition at pH 12 (C), oxidized with H_2O_2 (D), exposure to heat (E) and UV (F), respectively. The retention time of standard sildenafil was about 5 min (Figure 2 (A)). Table 1 shows the degradation of sildenafil citrate after treating with acidic, basic, oxidizing agent, and exposure to heat and UV. Sildenafil was partly degraded by acid and alkali and was completely degraded by oxidation. At neutral pH, exposure to heat and UV exposure had no effect on the degradation of sildenafil. The acid and alkali catalyzed degradation of sildenafil as shown in Figure 2 (B, C) with only one small peak (39.4-33.4%) being detected during the assay for sildenafil using these chromatographic conditions. Figure 3 shows the content of sildenafil that remained

after storage at a pH of 2 and 12 during 2 weeks. The decrease of sildenafil contents in acidic and basic solution was in each case linear and time dependent. Treatment of sildenafil with heat and UV caused no degradation of sildenafil citrate, as there was no detectable change after these treatments. In addition, oxidation with H_2O_2 caused degradation of sildenafil citrate. The oxidizing agent (0.1% hydrogen peroxide) produced complete degradation of sildenafil citrate at a low concentration (Table 1 and see Figure 2 (D)). The degradation products of sildenafil were eluted at retention times of 2-4 min and the sildenafil peak disappeared from the chromatogram. Reddy and Reddy [17] and Badawy *et al.* [26] also demonstrated the chemical degradation of sildenafil and found that it was almost completely degraded with H_2O_2 to sildenafil sulfonate. Badawy *et al.* [26] studied the degradation of sildenafil by refluxing sildenafil with 30% H_2O_2 for 6 h and separated the degradation products using TLC. The R_f values of the degraded products were less than that of sildenafil citrate (0.11 and 0.62, respectively). The sildenafil citrate degraded product induced by oxidizing agents was sildenafil sulfonic acid. It is important to note that this oxidizing agent completely degraded sildenafil within 24 h. For the other treatments (heat and UV), no degraded products were observed as shown in Figure 2 E and F. This confirmed that sildenafil was stable after heat treatment (120°C) and UV irradiation. This method was able to detect the degradation of sildenafil. The degraded products were eluted first following by sildenafil. Sildenafil was partially degraded by acid and alkali and was completely degraded by oxidation.

Table 1. Forced degradation studies of sildenafil solution at concentration of 0.1 mg/mL in various conditions (mean ± SD, n = 3).

Stress conditions and time studies	Sildenafil (%)	Degradation (%)
Acidic (pH 2) / 2 weeks	66.7 ± 0.2	33.3 ± 0.2
Neutral (pH 7) / 2 weeks	101.5 ± 0.4*	0 ± 0.0
Basic (pH 12) / 2 weeks	70.6 ± 1.2	29.4 ± 0.7
Oxidizing with 0.1% H_2O_2 / 24 h	0 ± 0.0	100 ± 0.0
Oxidizing with 1% H_2O_2 / 24 h	0 ± 0.0	100 ± 0.0
Oxidizing with 2% H_2O_2 / 24 h	0 ± 0.0	100 ± 0.0
Thermal 120°C / 48 h	101.5 ± 0.4	0 ± 0.0
UV / 48 h	100.4 ± 0.2	0 ± 0.0

* n = 2

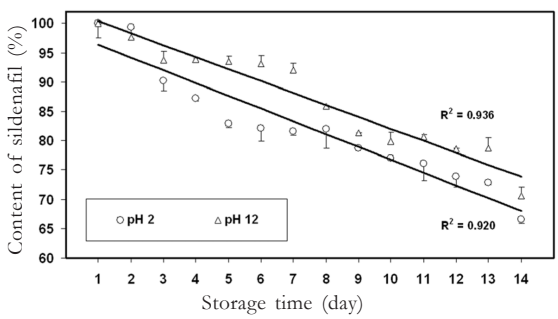

Figure 3. Degradation of sildenafil in aqueous solution pH 2 and 12 after storage at room temperature (mean ± SD, n = 3).

3.3 System Suitability

After the HPLC system was equilibrated with the standard mobile phase, the tailing factor of sildenafil peak was 1.37. The system therefore met the requirements of USP with a tailing factor of a standard solution of not more than 2.0. The percentage RSD standard response of 6 replicate injections was 0.17% (not more than 2.0%). All parameters were therefore satisfactory with system suitability.

3.4 Specificity

The specificity of the method was investigated by observing any interference encountered from the excipients present in the formulations. Using the chromatographic conditions previously described, the retention time observed during the validation assays were about 5 min for sildenafil. It was shown that none of other excipients interfered with the proposed method. The HPLC method presented in this study was selective for sildenafil citrate. The method was specific to sildenafil as it resolved sildenafil from any degraded products. The recovery of sildenafil was close to 100% after storage at 60°C for 24 h without protection from light.

3.5 Accuracy and Stability of the Sample

In our studies, sildenafil citrate was spiked into placebo solutions. Table 2 shows the validation results for accuracy. The method was accurate for all concentrations (recovery of sildenafil was 99.5%). The stability of the sample solutions was tested by HPLC over 24 h. The freshly prepared and stored samples at 2, 4, 6, 8, 24 h were analyzed by this method. The percentage recoveries for the assay of sildenafil were 100.7, 100.7, 100.6, 100.5, 100.5 and 101.0 after the initial, 2, 4, 6, 8 and 24 h storage, respectively. These results demonstrated that the sample solution of sildenafil can be accurately assayed within 24 h.

Table 2. Accuracy of the HPLC assay for sildenafil citrate (mean ± SD, n = 6).

Level	Concentration added (μg/mL)	Concentration found (μg/mL)	%Recovery
75%	75.2 ± 0.5	75.1 ± 0.8	99.9
100%	99.0 ± 0.4	98.4 ± 0.8	99.3
125%	126.8 ± 0.2	125.9 ± 0.2	99.3

3.6 Precision

The precision is the degree of reproducibility or repeatability of the analytical method under normal operating conditions. The intermediate precision is within laboratory variations. Precision was examined by injecting each of six test solutions three times. The results of intra- and inter-day variation are shown in Table 3. The first day repeatability gave the overall percentage recovery of 101.42 with a percentage RSD of 0.47. The second day gave the overall percentage recovery of 102.92 with the % RSD of 1.04. The third day gave the overall percentage recovery of 103.36 with the RSD of 0.59% and the inter-day precision of 3 different days was 1.07%. From these results it was concluded that this method was precise within the same and between days.

Table 3. Precision of the HPLC assay for sildenafil citrate (mean ± SD, n = 6).

Sample No.	% LA		
	1st Day	2nd Day	3rd Day
1	101.7	102.4	103.3
2	100.7	102.3	102.7
3	102.0	102.5	103.2
4	101.0	102.9	104.0
5	101.6	102.4	102.8
6	101.5	104.9	104.1
Average	101.4	102.9	103.4
RSD (%)	0.47	1.04	0.59
Inter-day precision (%)		1.07	

3.7 Linearity, Limit of Detection and Quantitation

The linearity was obtained over a concentration range of 0.5 - 500 μg/mL demonstrating its suitability for analysis. The correlation coefficient (r^2) was found to be 1.0. The LOD and LOQ for sildenafil citrate were 1.30 and 6.10 ng/mL, respectively. The LOD and LOQ reported by Daraghmeh *et al*. [15] were 0.413 and 1.38 μg/mL, respectively. Higher sensitivity (200 times) of this system was obtained even though the chromatographic condition was very similar because inhalation preparation was clean in comparison with the tablet. The proposed HPLC method was therefore suitable for assay low concentrations of sildenafil present in the MDI.

3.8 Robustness

The effects of the running conditions were determined for their robustness (Table 4). The changes of column temperature that differed from the standard method (within 5%) had only a slight effect on the recovery of sildenafil (0.8%). The flow rate changes of between 0.5 - 1.5 mL/min (±50% from the standard method) caused no changes of the percentage recovery of sildenafil. Other parameters including the mobile phase composition ratio (54, 60 and 66% of acetonitrile), detection wavelength (235, 240 and 245 nm), sonication time (10, 15 and 20 min) and the column conditions (used or new column) were evaluated. All the changes of parameters had no affect on the assay results for sildenafil and only a minor influence on the retention time, resolution, peak and area on the chromatogram. The percentage recoveries of sildenafil were good under these slightly modified methods as overall there was less than 2% variation (assay results between 98.6% - 102.1%) in all experiments (see Table 4). The tailing factor for sildenafil was always less than 2%.

Table 4. Effect of the HPLC condition on the percentage recoveries of sildenafil citrate (mean ± SD, n = 6).

Parameters	Conditions	Sildenafil recovery (%)
Column temperature (°C)	20	99.6 ± 0.25
	25	100.4 ± 0.47
	30	99.9 ± 0.83
Flow rate (mL/min)	0.5	101.2 ± 0.18
	1.0	100.4 ± 0.47
	1.5	98.6 ± 0.65
Mobile phase ratio (v/v)	Acetonitrile : Buffer	
	54 : 46	100.1 ± 0.33
	60 : 40	100.4 ± 0.47
	66 : 34	102.0 ± 0.58
Wavelength (nm)	235	100.1 ± 0.34
	240	100.4 ± 0.47
	245	102.1 ± 0.27
Sonication time (min)	10	100.0 ± 1.22
	15	100.4 ± 0.47
	20	99.7 ± 0.42
Column type	Used column	98.6 ± 1.26
	New column	100.4 ± 0.47

3.9 Application of the Analysis for Sildenafil Citrate in MDI Formulations

The validity of the method developed here was applied to the determination of sildenafil in the MDI formulations over a concentrations range of 0.5 - 500 μg/mL. The formulations were developed, in-house, for sildenafil citrate suspensions in the MDI prototype. The percentage recoveries of sildenafil citrate were between 97.2 to 101.4% with the % RSD of 0.44 (data not shown). The analytical data obtained from MDI formulation compared with the reference standard were not statistically different ($p > 0.05$). This method was acceptable for the assay of sildenafil and could be used as a reference method for the assay of sildenafil in sildenafil citrate inhaler preparation.

4. CONCLUSIONS

The proposed HPLC method for the assay of sildenafil citrate in pharmaceutical inhalers has been validated. The method employed a reversed phase C18 HPLC separation with UV detection at wavelength 240 nm. The mobile phase containing 0.2 M ammonium acetate buffer: acetonitrile (40:60 v/v) pH 7.0 at a flow rate of 1.0 mL/min was suitable as it was able to resolve any degradation products from sildenafil peak. The method can be used for determination of the low concentrations of sildenafil in pharmaceutical aerosols.

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