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Original Article

METHOD VALIDATION FOR DETERMINATION OF SILDENAFIL CITRATE IN EXTEMPORANEOUS ORAL SUSPENSION

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ABSTRACT

Objective: The aim of this study was to validate a high-performance liquid chromatographic (HPLC) method for analysis of sildenafil citrate in an extemporaneous preparation, according to ASEAN guideline for the validation of analytical procedure.

Methods: The chromatographic condition used a C18 column with a mobile phase consisted of 50% 0.2 M ammonium acetate buffer pH 7.0 and 50% acetonitrile. The flow rate was performed at 1.0 mL/min, and UV detection was monitored at 245 nm.

Results: It was shown that the retention time of sildenafil was about 8.80 min. The analytical method used was specific to sildenafil in the presence of other common excipients in the preparation. The method was accurate (100.18% recovery), precise (< 2% RSD), and robust. Linear correlation was obtained over a concentration range of 0.01 - 60.00 μg/mL (r^2 = 0.9999). Limit of detection (LOD) and limit of quantification (LOQ) were 3.82 and 11.57 ng/mL. respectively. Forced degradation showed that the method would serve as a stability-indicating procedure that applied for the analysis of the drug in the stability studies.

Conclusion: The proposed method met the general requirements with an acceptable performance for validation. It was accurate and reliable, and served as a stability-indicating method. Therefore, it can be used to determine sildenafil citrate in the extemporaneous suspension preparation.

Keywords: Sildenafil Citrate, HPLC, Validation, Extemporaneous, Suspension.

INTRODUCTION

Sildenafil citrate (Fig. 1) is a potent and selective inhibitor of phosphodiesterase type 5 [1,2]. It is chemically known as 1-[[3-(6,7-dihydro-1-methyl-7-oxo-3-propyl-1H-pyrazolo- [4,3-d]-pyrimidin-5-yl)-4-ethoxyphenyl]-sulfonyl]-4-methyl-piperazine citrate with a molecular weight of 666.7 for citrate salt and 474.6 for base form. It is an amphoteric molecule with two pKa values at 9.84 (NH-piperazine ring) and 7.10 (NH-amide at pyrazolopyrimidine ring) [3].

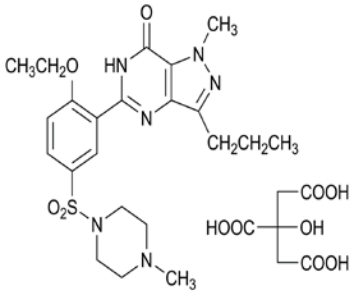

Fig. 1: Chemical structure of sildenafil citrate.

Sildenafil modulates nitric oxide and cyclic GMP signaling, and manages diverse medical conditions in cardiovascular diseases. Recently, it has been approved for treatment of pulmonary arterial hypertension by which an oral dose of 20 mg or 1 - 5 mg per kg three times a day showed an effective response in adult and pediatric patients, respectively [4,5]. Presently, sildenafil citrate is available in Thailand as a tablet dosage form. Extemporaneous preparation of the drug in an oral liquid form, such as suspension, is suitable for pediatric use in the hospitals [6]. Several HPLC-based procedures were used for the determination of sildenafil in pharmaceutical formulations [7-13]. A reversed phase C18 chromatography was used in conjunction with a mobile phase of water and acetonitrile (48:52 v/v) at a flow rate of 1.0 mL/min and UV detection at 245 nm [7]. This study employed piroxicam as an internal standard, and the result showed that the retention time of sildenafil was found to be 7.17 min. It was an accurate and precise method for quality control of the drug in bulk and tablet formulations. Another study was carried out a chromatographic method, using a C8 column and a mobile phase of methanol, water and triethylamine (630:370:2 v/v/v) adjusted to pH 4.0 [8]. The method used a flow rate of 1.0 mL/min, and the absorbance was measured at 220 nm. The result showed that the method achieved a good performance with simple, rapid and accurate characteristics for quantification of sildenafil in pharmaceutical preparations and industrial effluent samples. In addition, there was a reversed phase chromatography applicable to routine work on the analysis of sildenafil in formulations and human plasma samples [9]. They employed a C18 column with a mobile phase consisted of acetonitrile and phosphate buffer pH 7.0 (70:30 v/v) at a flow rate of 0.8 mL/min and UV detection at 228 nm. It was a stability-indicating method capable of separating sildenafil from degradation products. These mentioned methods were solely developed and validated for analysis of sildenafil in raw material and tablet formulations [7-13]. However, there were a limited number of methods applied to other pharmaceutical dosage forms such as inhaler, microcapsule, and microemulsion [14-16]. The present study was aimed to validate a HPLC method to exploit for quantitative analysis of sildenafil citrate in the drug preparation of extemporaneous suspension and its stability testing. It was followed on ASEAN guideline for the validation of analytical procedure, including specificity, range, linearity, accuracy, precision, LOD and LOQ, robustness, and forced degradation study [17].

MATERIALS AND METHODS

Chemicals and reagents

Sildegra® was purchased from GPO, Thailand. Methylcellulose-4000, methylparaben and propylparaben were obtained from P.C. drug center Co. Ltd, Thailand. Sildenafil citrate working standard (potency 99.40% as is) was obtained from Smilax Laboratories

Limited, India. Other chemicals were analytical grade from Merck, Germany. All solvents used were HPLC grade from RCI Labscan, Thailand.

HPLC instrument

Chromatographic separation was performed with Ultimate 3000 instrument system (Dionex Corporation, USA). A reversed phase C18 column (Inertsil® ODS-3; 4.6 x 250 mm; GL Sciences) was maintained at 25°C. The mobile phase was a degassed mixture of 50% 0.2 M ammonium acetate buffer pH 7.0 and 50% acetonitrile. Flow rate was used at 1.0 mL/min, and UV detector was set at 245 nm. The injection volume was 10 µL, and each sample was analyzed in duplicate. The data was recorded and interpreted using Chromeleon 7 software. Measurement of system suitability was determined by collecting data from 5 replicate injections of sildenafil standard to verify an adequate performance of the chromatographic system.

Extemporaneous preparation

Ten 100-mg sildenafil tablets (Sildegra®) were reduced to a fine powder by mortar and pestle. The vehicle was a mixture of 200 mL of 1% methylcellulose and 2% paraben concentrate and 200 mL of simple syrup [18]. An aliquot of the vehicle was added to the fine powder and mixed until yielding a uniform paste. The remaining vehicle was mixed and adjusted to 400 mL using a graduated cylinder to obtain an extemporaneous suspension with the nominal strength of 2.5 mg/mL. This extemporaneous sildenafil suspension was then transferred and stored in amber glass bottles.

Preparation of standard solutions

Sildenafil citrate working standard was accurately weighed (equivalent to sildenafil 25 mg) and added to a 100 mL volumetric flask. The standard was dissolved in the mobile phase, and used as a stock solution of sildenafil (250 µg/mL). It was diluted quantitatively with the mobile phase to obtain standard solutions of known concentration at a range of 0.01 - 60.00 µg/mL.

Preparation of sample solutions

Extemporaneous sildenafil citrate suspension was shaken well for 1 min. Five mL of the preparation was withdrawn from the center of the container and placed into a 50 mL volumetric flask. It was mixed with the mobile phase and sonicated for 10 min. Sample aliquot of 5 mL was then transferred into a 50 mL volumetric flask, and diluted with the mobile phase. The sample solution was filtered through a 0.45 µm nylon-66 membrane filter before analysis.

Method Validation

The method was validated, according to ASEAN guideline for the validation of analytical procedure [17]. Analytical performance was characterized on specificity, range, linearity, accuracy, precision, LOD and LOQ, and robustness. Specificity was assessed by spiking sildenafil standard (25 µg/mL) into the sample solution. The chromatogram was collected and compared with the result obtained from unspiked sample solution, standard solution, and vehicle. Linearity was evaluated across a range of 9 different concentrations between 0.01 - 60.00 µg/mL. It was done by individually weighing sildenafil standard to prepare three stock solutions. Data from three determinations of each concentration were analyzed by linear regression of a plot of peak area as a function of drug concentration.

The accuracy of the method was determined by the standard addition method. Sildenafil standard was spiked to the sample solution in triplicate at the level of 80, 100 and 120% of test concentration (20, 25, and 30 µg/mL, respectively). Precision parameter was reflected as repeatability (intra-day) and intermediate precision (inter-day). Repeatability was studied on the analytical variation in a single day, using 6 determinations of the sample solutions (at 100% test concentration) whereas the intermediate precision was assessed by analyzing the sample solutions three times on different days. LOD and LOQ values were obtained from calculation based on calibration curves by which factors of 3.3 and 10 were multiplied by a ratio of standard deviation of the response and slope of the curve, respectively.

Robustness was evaluated with respect to deliberate variations in the chromatographic conditions. It included changes in the following parameters; column temperature (20, 25, and 30°C), flow rate (0.5, 1.0, and 1.5 mL/min), mobile phase ratio (45, 50, and 55% of acetonitrile), wavelength (240, 245, and 250 nm), sonication time (5, 10, and 15 min), and column type (new Inertsil® ODS-3, and old Sepex® HP-18 columns).

Forced degradation studies

Degradation of sildenafil citrate in the extemporaneous preparation was conducted in various stress conditions. Acidic and basic hydrolysis were performed by which an individually 5 mL of the sample solution (about 250 µg/mL) was delivered to a 50 mL volumetric flask. The samples were refluxed with 5 mL of 1.0 N HCl as acid hydrolysis, or 1.0 N NaOH as basic hydrolysis, or distilled water as neutral hydrolysis at 80°C for 1 h. They were then neutralized with basic or acid solution before the analysis. For oxidative degradation, 5 mL of the sample solution in a 50 mL flask was mixed with aliquots of 30% H_2O_2 solution to make a final concentration of 0.1% and 1.0% H_2O_2. The samples were adjusted to the volume with the mobile phase, and the reactions were incubated at room temperature for 1 h in the dark. Thermal degradation was studied by placing the drug preparations in a hot air oven at 80°C for 1 week. A 100-fold dilution of sample with the mobile phase was made before injection. Photolysis of sildenafil in drug product was evaluated by exposing the samples to a fluorescent lamp at ambient temperature for 1 week. The samples were diluted with the mobile phase, yielding a final concentration of about 25 µg/mL.

RESULTS AND DISCUSSION

System suitability

After the HPLC system was equilibrated with the mobile phase, 5 replicate injections of the standard solutions were used to assess system suitability on each day of the experiment. The proposed method showed a sildenafil peak with tailing factor of 1.10. Variation on the drug response (%RSD) was 0.28%, and a theoretical plate of the column was found to be 10122.8. Therefore, the chromatographic system met the general requirements with an acceptable performance for drug analysis.

Specificity

There were several HPLC-based methods to determine sildenafil citrate in raw material and tablet dosage forms [7-13]. UV absorbance in a range of 220 -290 nm was used to quantitatively measure the drug content with high sensitivity and accuracy. The present study modified a chromatographic condition from Daraghmeh *et al*. that showed a good separation of sildenafil and its related substances [19]. In addition, the detection at 245 nm was chosen as an optimal wavelength [7]. The result showed that sildenafil had the retention time at 8.82 min (Fig. 2). It was eluted as a narrow peak with the resolution of 3.58 and tailing factor of 1.07. Methylparaben and propylparaben were also simultaneously found at 5.58 and 10.02 min, respectively. Increasing the composition of organic solvent up to 60% gave rapid elution but poor resolution between sildenafil and propylparaben (data not shown). Therefore, the proposed method was suitable because it was selective to sildenafil as judged by an apparent separation of the drug from other pharmaceutical excipients in the extemporaneous preparation. The specificity of the method was also demonstrated by forced degradation of sildenafil citrate in drug products. Treatments of sildenafil samples by refluxing with 0.1 N HCl or 0.1 N NaOH caused a partial degradation of sildenafil by which 95.99% of the drug content was remained (Table 1). Elution of hydrolytic products was detected at less than 4 min (Fig. 3A and 3B). A similar result was also obtained from hydrolysis of sildenafil standard with percent recovery of 93.35 ± 0.13 in acid and 92.40 ± 0.37 in basic catalysis (data not shown). This might imply that excipients in the formulation improved drug stability [20]. A previous study revealed that sildenafil was stable in various pH ranges from 3 to 12 at room temperature for 24 h [21]. Furthermore, drug degradation in acidic and basic solution was individually linear and time dependent after storage at ambient temperature for 2 weeks in a pH condition of 2

and 12 [14]. Cyclic amide and the sulphonamide functional group might be susceptible to hydrolytic reactions [22]. The significant decrease of sildenafil was found in the studies with chemical oxidation. There were 82.13% and 8.19% of the drug content that remained from a condition with 0.1% and 1.0% H_2O_2, respectively (Fig. 3D and 3E).The extent of drug degradation was directly dependent on an incubation time and H_2O_2 concentration. The degradation products were found to be eluted at the retention time of 2-4 min, suggesting that they became more polarity. It was consistent with previous studies which showed a complete decomposition when sildenafil was incubated with 0.1% H_2O_2 for a prolong time [9, 14]. Spectrometric and electrochemical analyses revealed that the oxidative reaction was taken place at the citrate anion, alkoxybenzenesulphonamide group, and piperazine ring [23]. Sildenafil sulphonate was shown to be a major degradation product by which its mobility on TLC plate was less than that of sildenafil citrate (Rf value 0.11 and 0.62, respectively) [24]. Furthermore, exposure of the sample to a dry heat and light unaffected the drug content (Fig. 3F and 3G). It confirmed that sildenafil preparation was stable to photolysis and high temperature (80ºC for 1 week). These result emphasized the specificity of the method, allowing the separation of sildenafil from other excipients and degradation products. Therefore, the method can be considered as a stability-indicating assay useful for quantitative determination of sildenafil in the preparation during pharmaceutical development and stability evaluation [25, 26].

Linearity, Limit of detection (LOD), and Limit of quantification (LOQ)

A linear relationship was observed from calibration curves of sildenafil standard over a concentration range of 0.01 - 60.00 µg/mL. Regression analysis showed that the method had a correlation coefficient (r^2) of 0.9999 and a linear equation Y = 0.7982X + 0.089, where Y was the peak area and X was the drug concentration in µg/mL (Fig. 4). LOD and LOQ of the method were determined based on the standard deviation of the response and the slope, and they were found to be 3.82 and 11.57 ng/mL, respectively (Table 2).

The result was comparable to that of the original method (LOD = 0.413 µg/mL and LOQ = 1.38 µg/mL) by which greater sensitivity of the proposed method (100 times) was gained attention, demonstrating its suitability for drug analysis in the extemporaneous oral suspension [19].

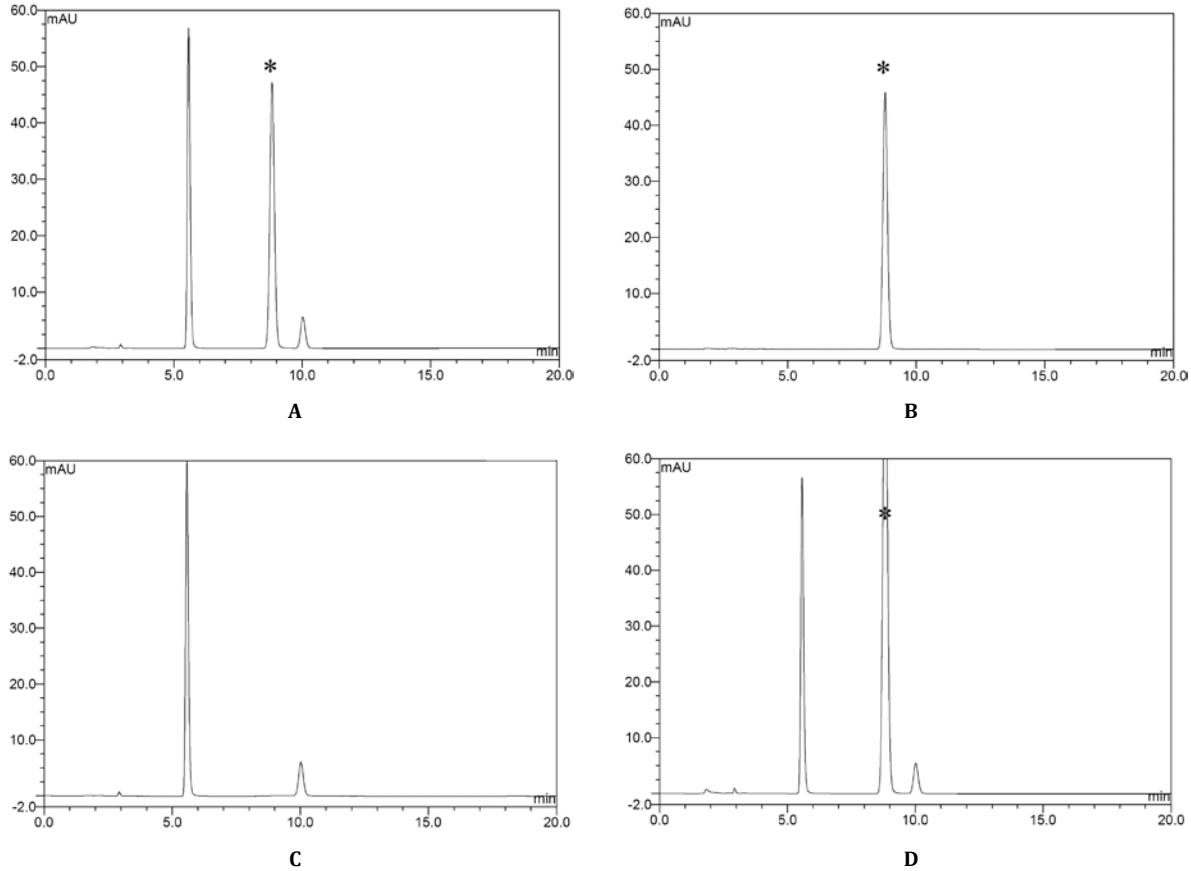

Fig. 2: Chromatrographic separation of (A) extemporaneous sildenafil suspension, (B) sildenafil standard, (C) vehicle, and (D) standard-spiked extemporaneous suspension. * indicated sildenafil peak.

Table 1: Forced degradation studies of extemporaneous sildenafil suspension in various conditions (mean±SD, *n* = 3).

Conditions	% Drug content
Acid hydrolysis/ 0.1 N HCl, 80°C, 1 h	95.99 ± 0.33
Basic hydrolysis/ 0.1 N NaOH, 80°C, 1 h	95.99 ± 0.65
Neutral hydrolysis/ Water, 80°C, 1 h	99.35 ± 0.71
Oxidation/ 0.1% H_2O_2, 1 h	82.13 ± 3.30
Oxidation/ 1.0% H_2O_2, 1 h	8.19 ± 4.10
Dry heat/ 80°C, 1 week	102.79 ± 0.21
Light, 1 week	101.40 ± 2.65

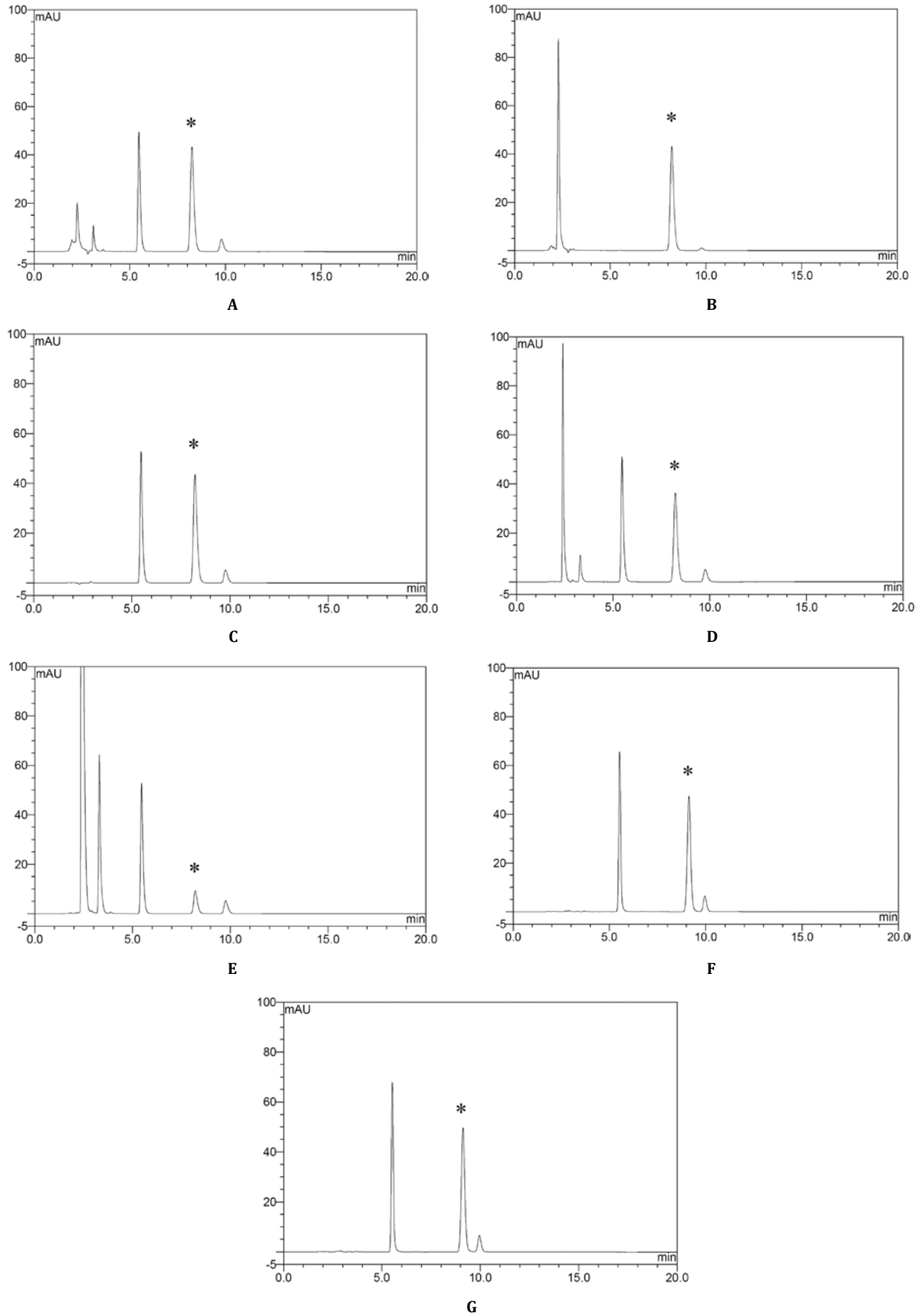

Fig. 3: Forced degradation studies of extemporaneous sildenafil suspension. (A) acid hydrolysis with 0.1 N HCl, (B) basic hydrolysis with 0.1 N NaOH, (C) neutral hydrolysis, (D and E) oxidation with 0.1% and 1.0% H_2O_2, respectively, (F) dry heat at 80°C, and (G) exposure to fluorescent lamp. * indicated sildenafil peak.

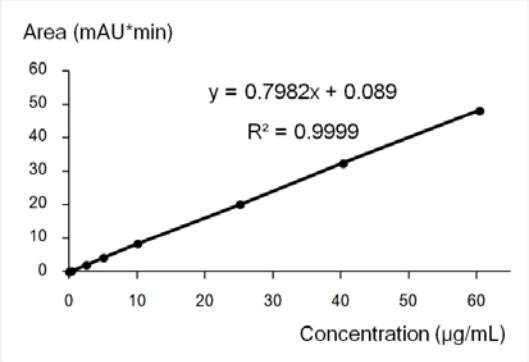

Fig. 4: Calibration curve of sildenafil standard over a concentration range of 0.01 - 60.00 µg/mL.

Table 2: Regression analysis of calibration curves for determining LOD and LOQ values.

Parameters	Results
Linearity range	0.01 – 60.00 µg/ml
Slope	0.7982
y-intercept	0.0890
Correlation coefficient	0.9999
Standard error of slope	0.0025
Standard error of y-intercept	0.0643
LOD	3.82 ng/mL
LOQ	11.57 ng/mL

Accuracy

To determine the degree of accuracy of the method, the standard addition method was used by which known quantity of sildenafil standard was spiked into the sample solutions in triplicate at three different levels of test concentration (80%, 100%, and 120%). The overall recovery across the specified range was 100.18% as shown in Table 3. The study revealed that the method was accurate for routine application. In addition, the stability of the sample solution was determined by HPLC. Analysis of the freshly prepared sildenafil samples at different time intervals (0, 2, 4, 6, 8, 10, 12, and 30 h) gave the percent recovery of 100.0, 100.0, 99.7, 99.8, 100.3, 100.2, 100.0, and 100.2, respectively. The result displayed a complete recovery without any degradations observed in the chromatogram, implying that the drug sample was stable in the mobile phase used. Therefore, the sample solution of sildenafil can be used within 30 h after its preparation.

Table 3: Accuracy of the proposed method for determination of sildenafil citrate in drug product (Mean ± SD, *n* = 3).

Level	Concentration added (µg/mL)	Concentration found (µg/mL)	% Recovery
80%	20.05	19.98 ± 0.67	99.66 ± 3.33
100%	25.06	24.99 ± 0.51	99.73 ± 2.04
120%	30.07	30.41 ± 0.11	101.14 ± 0.37
Overall (% recovery):		100.18 ± 2.09	

Precision

The degree of scatter between a series of measurement can be expressed as precision in the term of repeatability and intermediate precision. Six determinations of the sample solutions were used to describe the analytical variability under the proposed operating conditions on a single day and various days. The studies of repeatability showed the mean percentage of labelled amount and RSD of 102.95±1.46, 103.01±1.40, and 103.33±1.30 in the first, second, and third day, respectively (Table 4). Intermediate precision could be observed from sample examinations on three different days, and the result of overall %RSD was 1.30. Slight variation of the peak response reflected that the proposed method was precise within the same and between days of analysis.

Table 4: Precision of the HPLC method for determination of sildenafil citrate in drug product

Sample number	% LA		
	1st Day	2nd Day	3rd Day
1	103.25	103.21	103.59
2	102.18	102.61	102.66
3	101.84	102.23	102.39
4	101.73	101.46	102.44
5	102.98	102.89	103.06
6	105.76	105.68	105.81
Average	102.95	103.01	103.33
Repeatability (%RSD)	1.46	1.40	1.26
Overall average	103.10		
Intermediate precision (%RSD)	1.30		

Robustness

The robustness is a measure of reliability of the method during usage. The small change with respect to variations in the standard method parameters included column temperature (within ±5ºC), flow rate (±0.5 mL/min), mobile phase composition (±5% of acetonitrile), wavelength (±5 nm), sonication time (±5 min), and column condition (used and new columns). Examination of the percent recovery of sildenafil revealed an almost complete response, and one-way ANOVA statistics showed that all modifications of the critical parameters did not cause different results significantly (*p*-value > 0.05) (Table 5). A minor consequence in the chromatograms was only observed on the retention time, resolution, and peak area. The tailing factor for sildenafil was found to be less than 2.0, and the excipients were well separated under all the operational changes. These results demonstrated that the method was robust and reliable for the determination of sildenafil citrate in the drug product of extemporaneous suspension.

Table 5: Effects on method parameters on the percent recovery of sildenafil citrate in drug product (Mean ± SD, *n* = 6).

Parameters	Conditions	% Recovery	*p*-value
Column temperature (°C)	20	101.38 ± 1.61	0.479
	25	103.38 ± 1.68	
	30	101.34 ± 1.79	
Flow rate (ml/min)	0.5	101.37 ± 1.72	
	1.0	103.38 ± 1.68	0.498
	1.5	101.49 ± 1.68	
Flow rate (mL/min) (ACN: Buffer)	55:45	101.49 ± 1.68	0.528
	50:50	103.38 ± 1.68	
	45:55	103.42 ± 1.88	
Wavelength (nm)	240	101.37 ± 1.71	0.492
	245	103.38 ± 1.68	
	250	101.34 ± 1.81	
Sonication time (min)	5	100.01 ± 2.79	0.243
	10	103.38 ± 1.68	
	15	99.53 ± 0.62	
Column type	Used	101.31 ± 1.65	0.821
	New	100.85 ± 1.89	

Application of the method on sildenafil citrate analysis in the extemporaneous preparation

The proposed method was successfully used for the determination of sildenafil content in the extemporaneous oral suspension. The result showed that the percent content of the drug from 6 different samples with respect to their labeled claim was between 98.97 to 103.51% with %RSD of 1.99. Statistics analyses did not reveal a significant difference between the data (*p*-value > 0.05) (98.97%). It

indicated that the proposed method was sensitive, accurate and reliable for quantification of sildenafil, and could be served as a reference method for assay of sildenafil in the suspension preparation.

CONCLUSION

The HPLC method was validated, according to ASEAN guideline for the validation of analytical procedure for the determination of sildenafil citrate in an extemporaneously prepared suspension. It provided an acceptable performance with excellent accuracy, precision, and sensitivity. The method could resolve any degradation product peaks from sildenafil response that showed its selectivity, and served as a stability-indicating procedure. Slight variations in the analytical conditions did not cause significant changes on the resolution of sildenafil and their percent recovery, demonstrating the robustness of the analysis. Therefore, the method can be used for routine work on quality control purpose for the determination of sildenafil in this pharmaceutical dosage form.

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ABBREVIATIONS

HPLC, high-performance liquid chromatography; ACN, acetonitrile; LOD, limit of detection; LOQ, limit of quantification; SD, standard deviation; %RSD, percent relative standard deviation.

CONFLICT OF INTEREST STATEMENT

The authors declared that there were no conflicts of interest.

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