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Research Article

Isocratic RP-HPLC Method for Detection of Sildenafil in Some Marketed Dietary Supplements

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ABSTRACT

Due to the widespread use of sildenafil, the market is full of different counterfeited preparations that contain either an unknown amount of sildenafil or mixed with one or more different varieties of unknown substances. These fake preparations are usually harmful to users. So, it is always necessary to develop specific as well as sensitive analytical procedures to detect sildenafil in such preparations. HPLC that equipped with modern sensitive detectors such as diode array detector (DAD) can play a great role in solving such problems. In this work, a new HPLC method was established for the detection of sildenafil in some marketed dietary supplements. Thermo scientific ODS HYPERSIL column (250 x 4.6 mm, 5µm) was used. The mobile phase was composed of methanol and potassium di-hydrogen phosphate buffer 0.05M (pH 3.5) at a ratio of 70:30 (v/v). Triethylamine (0.3%) was added to the mobile phase. The flow rate was 1mL/min. Detection was performed at 290 nm using a diode array detector. The method showed good linearity (5.0-80.0 µg/mL). The retention time was 5.3 min. The validation parameters were evaluated as per ICH guidelines. The findings given by the developed method were compared statistically to those achieved by a reported one. There was a good statistical agreement. The developed method is considered a sensitive and specific analytical method for the detection of sildenafil counterfeited dietary supplements.

Keywords: Sildenafil; dosage form; counterfeited dietary supplements; RP-HPLC.

1. INTRODUCTION

Several features help in the spreading of fake sildenafil, such as little authorized enforcement, great economic return, and an informal supply of illegal medications through the internet without supervision. Moreover, embarrassment about erection dysfunction leads patients to avoid looking for specialized medical visits. It was reported that 0.6 to 2.5 million men are using illegal sildenafil products[1]. Chemical analysis of 2,383 seized samples of counterfeit sildenafil showed that only 10% enclosed the identical concentrations of active sildenafil that were advertised on the drug packaging 2, Varying doses of active constituents (from 0% to > 200% of considered dose), impurities (comprising commercial paint, printer ink, and talcum powder) and alternate constituents that are dangerous[1]. Fake sildenafil from different republics was reported to comprise various adulterants such as amphetamine, clomiphene, quinine, chloramphenicol, gamma amino butyric acid, caffeine, yohimbine, paracetamol, metronidazole, and, and glyburide[1,3,4].

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Many products labeled as herbal or natural and claimed to treat erectile dysfunction and enhance sexual performance are marketed as over-the-counter (OTC) dietary supplements. However, analysis of these products proved that they contained phosphodiesterase type 5 (PDE5) inhibitors[5].

Sildenafil citrate **(Fig.1)** is used for the management of impotence, erectile dysfunction as well as pulmonary hypertension. It is PDE5 (phosphodiesterase-5) inhibitor[6].

Overdoses of sildenafil lead to serious side effects such as headache, gastroesophageal reflux, and facial flushing as well as nasal congestion, and pupil sparing in addition to cardiovascular side effects such as vasodilatation, hypotension, and tachycardia[7].

There are different UV spectrophotometric methods reported for the analysis of sildenafil alone and in combinations[8–13]. Also, different chromatographic methods were reported as HPLC[8,14–22], UPLC 23, UPLC with tandem mass spectrometry detection[8], LC-MS[24], GC-MS[8,25], LC-MS/MS[26–28], LC-MS/MS/MS[28] and LC-ESI-MSI[29].

Because sildenafil is considered an electroactive species **(Fig. 1)**, different electrochemical methods were also reported for its determination, such as potentiometry 30,31 and voltammetry using ion-selective membrane electrodes, glassy carbon electrode, as well as diamond paste electrode for square wave voltammetric analysis of sildenafil[8,32–34].

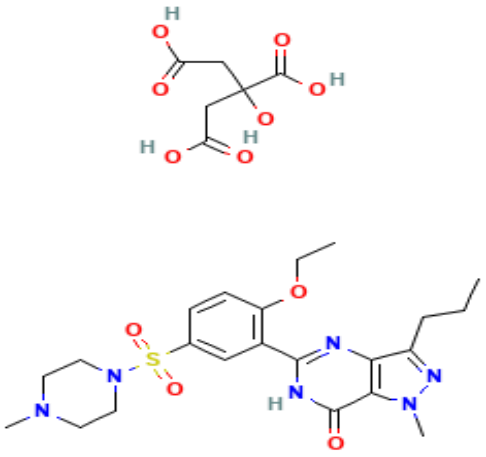

Fig.1. Chemical structure of sildenafil citrate.

HPLC (high performance liquid chromatography) is an analytical method that is used for separating molecules and ions in a mixture with higher selectivity and sensitivity than UV spectrophotometric and electrochemical analytical methods[35], so it was used in this work.

The current research is aimed at the development of a reversed-phase HPLC method for the detection and determination of sildenafil in its dosage form and adulterated dietary supplements. This is necessary because the market is full of dietary supplements that claim to achieve certain therapeutic effects based on herbal constituents only and are not labeled to contain sildenafil, which is already adulterating such supplements. The absence of such constituents from the preparation label constitutes a type of counterfeiting that may result in posing a danger to some people's lives. People, especially cardiac and hypotensive patients believe that these dietary supplements are safe for their health. People contraindicated from using sildenafil are exposed to cardiac problems and severe hypotension that may lead to death.

2. Experimental

2.1. Apparatus and software

Dionex Ultimate 3000 RS Thermo Scientific HPLC system (Sunnyvale, CA, USA) with diode array detector (DAD), Quaternary RS pump in addition to RS auto-sampler injector and Thermo stated RS column was used. The software Dionex Chromeleon version 7.1.2.1478 was used. HANA pH Meter (Rhode Island, USA) was used for the preparation of the mobile phase. The sonicator used was 3510 Bransonic® ultrasonic cleaner (Branson Ultrasonics Corporation, USA).

2.2. Materials and reagents

2.2.1. Active pharmaceutical ingredients (API) and dosage form

Sildenafil citrate (purity 99.80%) was given by Sigma Company for pharmaceutical industries (Quesna, Menuofia, Egypt). Five dietary supplement preparations for erectile dysfunction, claimed to contain only flavonoids and taurine, were purchased online. Three of them are in the form of sachets preparation (labelled: S®, T®, and X® preparations), and two are in the form of tablets formulation, which were labelled Y® and Z®.

Two illegal samples, which are labelled to contain either 125 mg (labelled B®) or 130 mg (labelled R®) of sildenafil citrate, were purchase also online. The true brand names for either dietary supplements or illegal samples were omitted from the study, and symbols were designed by the authors to refer to different preparations.

Erec® tablets (Adwia Company, 10th of Ramadan city, Egypt) were obtained from the local pharmacy. Each tablet is labeled to contain 140 mg of sildenafil citrate which is equivalent to 100 mg of sildenafil.

2.2.2. Solvent and reagents

Triethylamine (Merck Schuchardt, Germany) was used. Phosphoric acid (85%) and KH2PO4 (analytical grade) were obtained from Oxford Lab Fine Chem LLP (India). Methanol of HPLC grade (Sigma- Aldrich, Germany) was used.

2.2.3. Preparation of phosphate buffer

Phosphate buffer (0.05M) was prepared by weighing 3.4g of KH2PO4 and dissolving it in 100 mL distilled water, sonicated for 10 minutes then distilled water was added to 500 mL to obtain 0.05M buffer. pH was adjusted to 3.5 with phosphoric acid. The buffer was filtered using a filter membrane 0.45µm pore nylon membrane filter (Gelman, Germany) and then sonicated for 30 minutes for degassing.

2.3. Preparation of standard solutions

2.3.1. Standard stock solution

20 mg of sildenafil citrate was taken into a 100 mL-volumetric flask, dissolved in methanol, and sonicated for 10 minutes; then methanol was added to the mark to obtain a stock standard solution containing 200.0 µg/mL sildenafil citrate.

2.3.2. Standard working solution

Different volumes were taken from the standard stock solution of sildenafil citrate in 10 mL- volumetric flasks. Then methanol was added to the mark to obtain working standard solutions in the concentration range (5.0 – 80.0) µg/mL.

2.4. Optimum chromatographic conditions

Sildenafil citrate was determined by the RP-HPLC method. The mobile phase contained methanol and 0.05M KH2PO4 (pH 3.5) at a ratio of 70:30 (v/v). Triethylamine (0.3%) was added. The flow rate of the mobile phase was 1mL/min. DAD was used at 290 nm. Thermo scientific ODS HYPERSIL column (250 x 4.6 mm and particle size of 5µm) was used.

2.5. Construction of the calibration curve

Ten microliters of each prepared working standard solution were triplicate injected at the optimum chromatographic conditions, and the area under the curve was calculated. Then, the calibration curves were achieved by plotting the area under the curve versus the corresponding concentrations (in □g/mL). The regression parameters were calculated.

2.6. Assay of dosage forms

2.6.1. Preparation of assay solution of dietary supplements

2.6.1.1. For sachets, dietary supplements

500 mg of each of dietary supplements S®, T®, and X® (sachets) was weighed, taken into a 50-mL volumetric flask, dissolved in methanol, and sonicated for 15 minutes, and the volume was completed to the mark by methanol. 3 mL (solution A) and 5 mL (solution B) were taken from the previous solutions separately in 10 mL volumetric flasks. Then methanol was added to the mark. This was repeated three times. A membrane filter of 0.45µm was used to filter the solution. 10 µL of the diluted solutions were injected under the optimum chromatographic conditions. Under the same conditions, the same volume of standard solution of sildenafil citrate 50µg/mL was injected separately.

2.6.1.2. For tablet dietary supplements

Ten tablets of each dietary supplement Y®, Z® were weighed and grinded separately. The weight corresponding to one tablet content was taken, then dissolved in 20 mL methanol, sonicated for 20 minutes, then transferred into a 100-mL volumetric flask. Then methanol was added to the mark. 2mL (of tablet Y stock assay solution) and 1mL (of tablet Z stock assay solution) were transferred separately into a 10-mL volumetric flask, and then methanol was added to the mark. Filtration was carried out using a 0.45 µm membrane filter before injection. 10 µL of the diluted tablet assay solutions were injected under the optimum chromatographic conditions. Under the same conditions, the same volume of standard solution of sildenafil citrate 50 µg/mL was injected separately.

2.6.2. Preparation of assay solution of dosage form

Ten Erec® tablets (each tablet labeled to contain 140 mg sildenafil citrate) were weighed and grinded. The weight corresponding to one tablet was taken, then dissolved in

methanol, sonicated for 20 minutes, and then transferred into 100 mL volumetric flask. The volume was completed to the mark by methanol. 430 µL was transferred into a 10 mL volumetric flask. Then methanol was added to the mark. Filtration was carried out using a 0.45µm membrane filter before injection. 10 µL of the diluted tablet solution was injected under the optimum chromatographic conditions. Under the same conditions, a standard 50µg/mL sildenafil citrate solution was prepared. Ten µL was injected.

2.6.3. Preparation of fake tablet assay solution purchased through the internet

The same procedure as dosage form (section 2.6.2.), but the volume diluted was 400 µL and 500 µL for B® and R®, respectively.

The concentration of sildenafil citrate in the assay solution was calculated based on the peak area of the standard solution using this equation:

$$C_u = C_{st} * AUC_u / AUC_{st}$$

Where: C_u is the concentration of sildenafil citrate in its dosage form or dietary supplements, C_{st} is its concentration in standard solution, and AUC is the corresponding area under the curve for either the dosage form assay solution (AUC_u) or the standard solution (AUC_{st}).

In dietary supplements, the original concentration in the examined sample was obtained by multiplying in dilution factor.

3. RESULTS AND DISCUSSION

The Isocratic RP- HPLC method was developed for the determination of sildenafil citrate in its dosage form and in some commonly distributed dietary supplements suspected to be counterfeited by sildenafil citrate. The concentration of sildenafil citrate in dosage forms was calculated based on the peak area of the standard solution, as mentioned in the experimental section.

3.1 Method optimization

Several trials were made for the development of an analytical isocratic HPLC method for the analysis of sildenafil in its dosage form and dietary supplements counterfeited by sildenafil.

3.1.1. Effect of methanol ratio

Several ratios of organic solvent (methanol) were investigated. It was found that 70% methanol was the optimum one. Lower ratios of methanol were tried, but the tailing factor was more than 2, and retention time was longer. Using a higher ratio of methanol decreased retention time and tailing, but there was an overlap between the peaks of SDC and the solvent.

3.1.2. Effect of pH

Potassium dihydrogen phosphate (0.05M) at different pH values was investigated. It was found that pH 3.5 was optimum as at higher pH, retention time increased.

3.1.3. Effect of flow rate

Several flow rates were investigated as 0.7, 0.8, and 1 mL/min. It was found that at flow rates 0.7 and 0.8 mL/min, the retention time and tailing factor of SDC increased. The optimum flow rate was 1mL/min (retention time 5.3 min)

Detection was carried out using DAD at 290 nm. Sildenafil citrate was detected at a retention time of 5.3min as shown in **(Fig.2a)**. The developed method was applied for the determination of sildenafil citrate in its dosage form **(Fig.2b)** and in dietary supplements counterfeited by sildenafil **(Fig.3)** as well as in illegal tablets of sildenafil citrate (drug R® and drug B®) as shown in **(Fig.4)**.

3.2. Method validation

The developed method was validated according to ICH guidelines[36].

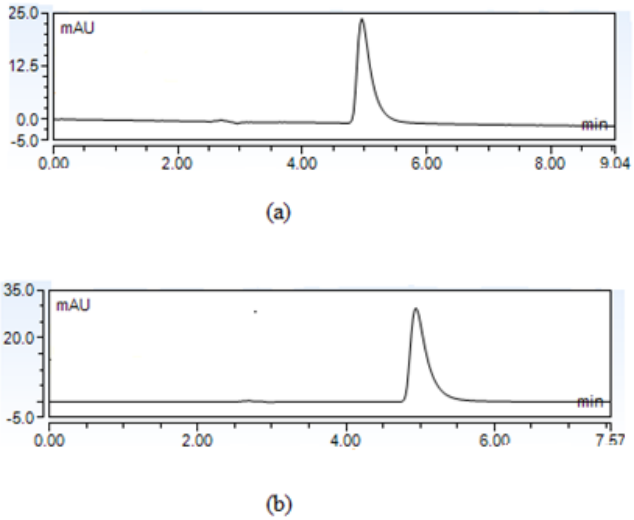

Fig. 2: Chromatogram of (a) Standard solution of sildenafil citrate (50µg/mL), (b) Assay solution of (Erec®) tablet (60µg/mL) obtained by the proposed isocratic RP-HPLC method.

3.2.1. System suitability

The factors of system suitability were calculated for the isocratic elution of sildenafil citrate by the proposed HPLC method **(Table 1)**. A good capacity factor (K`=3.79) was obtained. The asymmetry factor of the peak (A_{sym}) was less than 2.

3.2.2. Linearity

The linearity range for sildenafil citrate was 5-80 µg/mL. The regression parameters are shown in **(Table 2)**. A good correlation coefficient was obtained (0.9999).

3.2.3. Accuracy

Three concentrations of sildenafil citrate within the linearity range (20, 50, 70 µg/mL) were prepared and determined three times. Mean % recovery ± SD values indicated the accuracy of the developed RP-HPLC method **(Table 3)**.

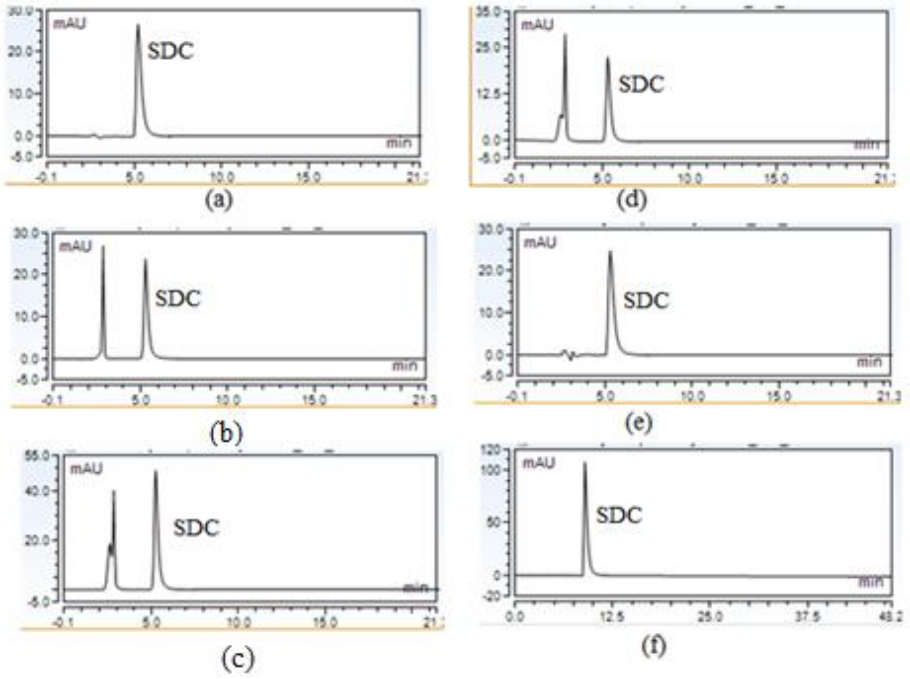

Fig. 3: Chromatogram of sildenafil citrate (SDC) in different dietary supplements (b to f) compared to (a) standard 40µg/mL, where (b) dietary supplement S ® sachet, (c) dietary supplement T ® sachet, (d) dietary supplement X ® sachet (e) dietary supplement Y ® tablet, (f) dietary supplement Z ® tablet using the proposed isocratic RP-HPLC method.

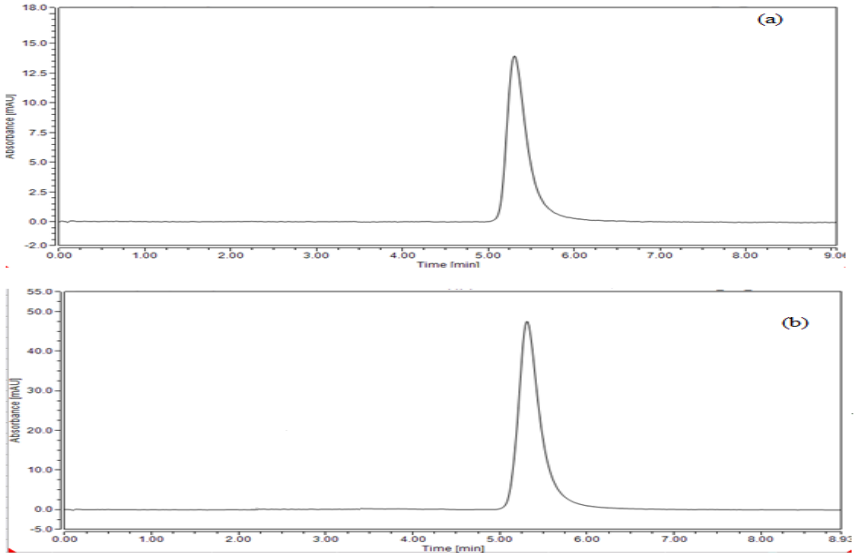

Fig. 4: Chromatogram of (a) Assay solution of (Drug B ®) tablet and (b) Assay solution of (Drug R®) tablet by the proposed isocratic RP-HPLC method.

Table 1: System suitability parameters for sildenafil citrate by the proposed isocratic RP-HPLC method.

Parameter	Sildenafil citrate	Reported values[38]
t_r (min)	5.3	-
A_{sym}	1.77	< 2
NTP	2375	> 2000
K`	3.79	2-10

K`: capacity factor, t_r: retention time
A_{sym}: asymmetry factor of the peak
NTP: number of theoretical plates

Table 2: Regression parameters for determination of sildenafil citrate by the proposed isocratic RP-HPLC method.

Regression parameters	sildenafil citrate
Linearity range (µg/mL)	5-80
R^2	0.9999
a	0.3851
b	0.1369
S_a	0.0330
S_b	0.0006
$S_{(y/x)}$	0.0069
LOD (µg/mL)	0.490
LOQ (µg/mL)	1.617

R^2: Correlation coefficient, a: intercept, b slope, S_a: standard deviation of intercept, S_b: standard deviation of slope, S(y/x): residual standard deviation, LOD: limit of detection, LOQ: limit of quantitation.

Table 3: Results of accuracy for determination of sildenafil citrate by the proposed isocratic RP-HPLC method.

Parameter									
Conc. taken (µg/mL)	**20**			**50**			**70**		
Conc. found (µg/mL)	19.83	19.76	20.12	49.93	49.49	49.78	70.31	69.43	69.72
%Recovery	99.16	98.79	100.62	99.85	98.98	99.56	100.44	99.19	99.60
Mean % Recovery±SD	99.52±0.19			99.46±0.22			99.74±0.45		

SD: standard deviation.

3.2.4. Limit of detection (LOD) & Limit of quantitation (LOQ)

The values of LOD, and LOQ for the determination of sildenafil citrate using the proposed isocratic HPLC method were mentioned in (Table 2) using the following equations:

LOD = (3.3x σ) / b **LOQ = (10x σ) / b**

Where σ: is the standard deviation of blank that was determined by triplicate measurement of the area under the curve of solvent (methanol) at the retention time of sildenafil citrate and b: is the slope obtained from the calibration curve.

The results shown in **(Table 2)** indicate the sensitivity of the developed method.

3.2.5. Precision

Three concentrations of sildenafil citrate (20, 50, 70 µg/mL) were prepared and then determined triple. The %RSD values were determined for intraday precision as given in **(Table 4)**. For inter-day precision, the same three concentrations were determined triple on three days, and %RSD was calculated as shown in **(Table 5)**.

3.2.6. Robustness

The isocratic method proved to be robust by changing the ratio of organic solvent (methanol) by ±2% and by changing pH by ±0.2, % RSD for three concentrations was less than two as shown in **(Table 6)**.

3.2.7. Specificity

The specificity of the method was confirmed by the accepted values of mean % recovery ± SD for sildenafil determination in the presence of excipients **(Table 7)**. By comparing the chromatograms (peak position and shape) of a standard solution of sildenafil citrate, and that of its tablet solution **(Fig. 2)**, it was found that there is no interference from excipients. This indicated that the developed isocratic RP-HPLC was specific for the determination of sildenafil in its tablet. Furthermore, the specificity was confirmed by statistical comparison of the obtained results with that of a reported spectrophotometric one[37]. There was no significant difference, as shown in **(Table 7)**. Also, when using the method for determination of sildenafil in dietary

supplements, other constituents were completely resolved (eluted earlier) from that of sildenafil citrate. Identical retention times for sildenafil in repetitive runs indicated the absence of matrix interference in the determination of the target analyte.

3.3. Assay of tablets

3.3.1. Assay of dosage form

Results of the assay of sildenafil citrate dosage form showed accepted recovery (98-102%) and tolerable standard deviation (not more than 2) for the assay sample measured. T-test and F-test were accepted as the calculated t-value was (1.58) lower than the tabulated one (2.77). Also the calculated F value (2.41) was lower than the tabulated one (19) by comparing the results of the developed RP-HPLC method to a reported UV spectrophotometric one[37] as shown in **(Table 7)**.

3.3.2. Assay of fake samples of sildenafil citrate (drug R® and drug B®)

When analyzing illegal tablets of sildenafil citrate, the first (drug R®) showed a higher recovery than the labeled, about 156.68 %, and other tablets (drug B®) showed a lower recovery of about 46.02% and tolerable standard deviation of not more than 2 for assay sample measured in triplicate. The student's t-test was accepted as the calculated t-value did not exceed the tabulated value (2.77). Also F-test was accepted as the calculated F value was lower than the tabulated value (19) by comparing the results of the developed RP-HPLC method to a reported spectrophotometric one[37,] as shown in **(Table 8)**. This indicates that these tablets (drugs R and B) are counterfeited.

Table 4: Results of intraday precision for determination of sildenafil citrate by the proposed isocratic RP-HPLC method.

Conc. taken (µg/mL)	Conc. found (µg/mL)			SD	%RSD
20	19.83	19.76	20.12	0.19	0.97
50	49.93	49.49	49.78	0.22	0.45
70	70.31	69.43	69.72	0.45	0.64

SD: standard deviation, %RSD: relative standard deviation.

Table 5: Results of inter-day precision for determination of sildenafil citrate by the proposed isocratic RP-HPLC method.

Parameters	Day 1			Day 2			Day 3		
Conc. taken (µg/mL)	**20**	**50**	**70**	**20**	**50**	**70**	**20**	**50**	**70**
Mean found Conc.* (µg/mL)	19.90	49.73	69.43	19.98	49.85	69.99	19.81	50.07	70.11
SD	0.09			0.17			0.36		
%RSD	0.43			0.35			0.52		

*Mean found concentration for three determinations (µg/mL), SD: standard deviation
%RSD: relative standard deviation.

Table 6: Results of the robustness of the proposed isocratic RP-HPLC method.

Parameters	Sildenafil citrate[a]					
	Conc. found (µg/mL)			Mean found Conc. (µg/mL)	SD	%RSD
MeOH 72%	71.24	71.25	68.85	70.44	1.39	1.97
***MeOH 70%**	70.31	69.43	69.72	69.82	0.45	0.64
MeOH 68%	70.16	70.96	70.94	70.69	0.46	0.65
pH 3.3	69.28	70.23	69.87	69.79	0.48	0.69
***pH 3.5**	70.23	70.38	69.72	70.11	0.35	0.49
pH 3.7	70.96	70.82	71.47	71.09	0.35	0.49

[a]: concentration taken of sildenafil citrate (70 µg/mL)
***:** optimum conditions

Table 7: Assay results for determination of sildenafil citrate in its tablet form by the developed isocratic HPLC method and comparison to a reported one.

Parameters	Developed method			Reported method[37]		
Conc. taken (µg/mL)	60			40		
Found conc.(µg/mL)	60.05	59.65	60.2	40	39.49	39.49
Mean % recovery ± SD	99.95±0.47			99.17±0.73		
F-test	2.41			1.58		
t-test	(19)*			(2.77)*		

Each (Erec®) tablet labeled to contain 140.48mg sildenafil citrate (100mg sildenafil)

* Tabulated values for t-test and F-test at 95% confidence interval.

Reported method [37]: UV direct spectrophotometry at the λmax of Sildenafil citrate (293.2nm).

Table 8: Assay results for determination of different illegal samples of sildenafil citrate purchased from the internet by isocratic HPLC method.

Parameters	Drug R®						Drug B®					
	Developed method			Reported method[31]			Developed method			Reported method[37]		
Conc. taken (µg/mL)	65			65			50			50		
Conc. found (µg/mL)	101.76	101.93	101.84	61.07	61.09	61.23	23.01	23.25	23.77	22.79	23.11	22.85
Mean % recovery ± SD	156.68 ± 0.13			156.73±0.86			46.02±0.48			45.84±0.34		
F-test	2.55			(19)*			1.96			(19)*		
t-test	2.35			(2.77)*			0.57			(2.77)*		

* Tabulated values for t-test and F- test at 95% confidence interval.

Tablets are labelled to contain 125mg and 130mg sildenafil citrate for drug B® and Drug R®, respectively.

Reported method[37]: UV direct spectrophotometry at the λmax of Sildenafil citrate (293.2nm)

3.4. Detection of sildenafil in dietary supplements

3.4.1. Sachet form

Different three types of sachet dietary supplements S, T, and X, were analyzed. Two samples (solution A and B) of assay preparation from each dietary supplement were prepared, and 10µL of each assay preparation of dietary supplement (solution A and B) was injected into HPLC.

The concentration of sildenafil in dietary supplements was calculated as following:

C_u final concentration of solution A or B was calculated from the following equation:

$$C_u = C_{st} * AUC_u / AUC_{st}$$

Where: Cu is the concentration of assay solution, C_{st} is the concentration of the standard solution. AUC_u and AUC_{st} represent the area under the curve (peak) for the assay and standard solutions, respectively.

Then, this concentration was multiplied by the dilution factor to obtain the original concentration in a sachet.

3.4.2. Tablet form

Two different types of tablets, Y and Z forms of dietary supplements, were analysed. One concentration in each tablet was calculated by injection of 10µL of each assay preparation; the final and the original concentrations were calculated in sachet form.

Sildenafil citrate was detected in dietary supplements S, T, and X sachets as shown in **(Figs. 2b, 2c, and 2d)**, respectively. It was also detected in dietary supplements tablets Y and Z, as shown in **(Figs. 2e and 2f)**, respectively. This, indicated that these dietary supplements were counterfeited by sildenafil citrate.

The advantages of the developed isocratic RP-HPLC method include having wider linearity range (5-80 µg/mL) compared to the reported method 37 (8-60 µg/mL) as well as providing higher sensitivity (LOD = 0.049 µg/mL) compared to that of reported one (LOD =1.012 µg/mL) in addition to

the ability of determination of sildenafil in its dosage form and in adulterated dietary supplements.

3 CONCLUSION

An accurate, precise, and simple isocratic RP-HPLC method was established and validated for the detection and determination of sildenafil in dietary supplements counterfeited by sildenafil. The advantages of the developed isocratic RP-HPLC method include having a wider linearity range (5-80 µg/mL) as well as providing higher sensitivity (LOD = 0.049 µg/mL) compared to that of the reported method in addition to the ability of determination of sildenafil in its dosage form and adulterated dietary supplements. The developed method was robust and can be used for routine analysis of sildenafil.

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