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Optimization and Validation of HPLC-UV Method for the Determination of Vardenafil, Sildenafil, and Tadalafil in Honey-Mixed Herbal Sachets Using a Design of Experiment

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ABSTRACT

A method was developed for the simultaneous determination and analysis of sildenafil, vardenafil, and tadalafil in honey-mixed herbal sachets using high-performance liquid chromatography with a UV detector (HPLC–UV). This method eliminates the employment of complex procedures and abolishes time-consuming and labour-intensive pre-treatment processes. In ten minutes, the separation process (at 25oC) of sildenafil, vardenafil, and tadalafil using a C18 150 mm × 4.6 mm x 5 µm column (Shim-pack GIST) was successful with high selectivity and sensitivity. The mobile phase was a 60:40 (v/v) mixture of 0.1 percent formic acid in water and 0.1 percent formic acid in acetonitrile. Using the mobile phase as an extraction mixture, it gave recoveries in the range of 93.0-103.3% at spike levels of 50–150 mg/kg with relative standard deviations (RSDs) lower than 10%. The intra-day and inter-day precision results were in the range of 0.4–0.8% and 1.0–1.7%. Furthermore, the retention times for sildenafil acid, vardenafil acid, and tadalafil were 1.93, 2.47, and 9.62 minutes, respectively, and the limits of detection (LOD) were 1.70, 2.16, and 1.03 mg/L, while the limits of quantification (LOQ) were 5.65, 7.21, and 3.42 mg/L.
Keywords: HPLC, experiment design, sildenafil, vardenafil, tadalafil, a PDE_5 inhibitor.

INTRODUCTION

Erectile dysfunction (ED) is the inability to sustainably achieve or maintain an erection sufficient for satisfactory sexual performance [1]. This prevalent condition is common in men over the age of 40 and can have a significant impact on quality of life and self-esteem.

In the past, due to limited understanding of the physiological mechanism of erection, treatment of ED was limited to vacuum contractors, prosthetic implants, intracavernosal injections, and intraurethral suppositories [2]. Since its emergence, a class of drugs known as type 5 phosphodiesterase (PDE_5) inhibitors has revolutionized the treatment of ED. PDE_5 inhibitors have become the first-line therapy for ED recommended by the American Urological Association (AUA) and the European Association of Urology (EAU) [2, 3].

In the mid-1980s, the relationship between nitric oxide (NO) and the PDE family increased drug innovation. Many physiological effects of NO have had dramatic effects on many illnesses. PDE enzymes are ubiquitous in the body, and 11 recognized isozymes are expressed at different levels in different tissues. The PDE5 enzyme is widespread

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but more common in penile tissue. Before discovering the association between NO and PDE, non-selective PDE inhibitors (such as theophylline) were used, but selective PDE inhibitors have not yet been developed. Since then, several selective PDE inhibitors have been approved to treat conditions ranging from ED to pulmonary hypertension [4].

Four oral PDE5 inhibitors commercially available in the United States are sildenafil® (Viagra, Pfizer), vardenafil® (Levitra and Staxin, Bayer / GlaxoSmithKline), tadalafil® (Cialis, Eli Lily), and recently approved. It is a drug, avanafil® (Stendra), Vivis). This class of extensions gives you more flexibility when prescribing based on individual responses. During sexual arousal, NO is released from the corpus cavernosum nerve endings and endothelial cells. NO activates guanylate cyclase to convert guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP), triggering cGMP-dependent events. Accumulation of cGMP leads to relaxation of smooth muscle in the corpus cavernosum and increased blood flow to the penis [5, 6].

The most frequently reported adverse events with oral PDE_5 inhibitors are headache, flushing, dyspepsia, dizziness, and rhinitis [5, 7-11]. All PDE_5 inhibitors currently available are highly selective for the type 5 gene family. However, sildenafil and vardenafil are less selective for PDE_6 expressed in the retina [12, 13]. Patients have reported vision-related side effects associated with PDE_6 inhibition, including dose-dependent disorders of color discrimination (blue/green) or cyanopsia (objects appear blue) [14-16]. In contrast, tadalafil is selective for PDE6 and PDE_{11}, which is concentrated in the prostate, testis, and skeletal muscle [12, 13]. Inhibition of type 11 PDE isozymes is associated with pain and myalgia [17]. Analytical techniques for analyzing PDE_5 inhibitors and their analogs have been developed for public health safety and regulation [18-20]. In this case, various spectroscopic and chromatographic methods are used to inspect illegally grown foods containing undeclared synthetic drugs in a complex matrix. The most common techniques for measuring PDE5 inhibitors and their analogs are UV-Vis detectors (UVs) [21-23], spectrophotometers [24], and gas chromatography combined with mass spectrometry (High-Performance Liquid Chromatography (HPLC) with GC-MS) [25]. Recently, liquid chromatography (LC-MS) combined with mass spectrometry and liquid chromatography (LC-MS / MS) combined with tandem mass spectrometry have been adopted [19, 26-28]. High-Performance Liquid Chromatography (HPLC-PDA) with a diode array detector can identify the analyte by comparing the UV spectra of the reference compound and the target compound. In this regard, MS has a great deal of interest in qualitative and quantitative analysis[29]. Especially in quantitative analysis, multiple reaction monitoring (MRM) of triple quadrupole mass spectrometry (QQ MS) shows high sensitivity and remarkable property selectivity[30].

This study aims to develop an easy and quick way to determine the presence or absence of sildenafil, vardenafil, and tadalafil in herbal sachets mixed with honey. It uses a simplified solvent extraction method followed by HPLC with a UV-Vis detector. The proposed method is internally validated for linearity, daytime and daytime accuracy, LOD, and LOQ.

EXPERIMENTAL SECTION

Materials

Certified standard solutions of sildenafil (98.0 %), vardenafil (98.0 %), and tadalafil (98.0 %) were procured from AmBeed, Illinois, United States. Daily working standard solutions were prepared by diluting the stock solutions in the mobile phase. Ammonium formate was purchased from Agilent Technologies (USA). Formic acid (99.8 %) was obtained from Fluka (Buchs, Switzerland). Water was purified using reverse osmosis technology and an Electrodeionization (EDI) system (Maxima Ultra-Pure Water, England). A non-sterile PTFE Syringe Filter with a disposable membrane filter (0.45 μm) was purchased from

Whatman GmbH (Dassel, Germany).

Instrumentation

The HPLC analysis was performed using a Shimadzu LC-2030C system (Kyoto, Japan) consisting of a degasser, four-solvent low-pressure gradient pump, autosampler, column oven, a UV-Visible detector, and an autosampler equipped with a 200-µL sample loop and controlled by the Lab Solution system (version 5.90, Shimadzu, Japan). The chromatographic separation was performed with Shim-pack GIST, C18, 5 µm, 4.6 x 150 mm chromatographic column purchased from Shimadzu Corporation (Kyoto, Japan). In the mobile phase, the sample extracts were analyzed using 0.1% formic acid in water and 0.1% formic acid in an acetonitrile 60:40 (v/v) mixture. The column was kept in a column oven at 25° C at a flow rate of 0.8 mL/min to achieve the optimum resolution between PDE_5 inhibitors. The injection volume was maintained at ten µL for both sample and standard solutions. The wavelength at 220 nm was applied to detect all PDE_5 inhibitors.

Procedure

Sample preparation

Honey samples were prepared using a solid-liquid extraction procedure described below [31].

Step I: A thoroughly homogenized honey sample (1.0 g) was weighed in a polypropylene centrifuge tube (15 mL).

Sample recovery was made with 1.0 g of the non-contaminated honey samples with three different fortification levels; 0.5 mL of PDE_5 inhibitors mixed standards were spiked at 5.0, 10.0, and 20.0 µg/kg of honey. The spiked samples were left overnight (14 h) in the dark at room temperature (humidity between 30% and 60%) to allow the solvent to evaporate and for PDE_5 inhibitors absorption into the matrix. Then they were extracted via the following steps (II to III).

Step II: 10.0 mL of 50:50 (v/v) acetonitrile/water mixture was added to the fortified samples, and the centrifuge tube was manually shaken vigorously for 1 min to ensure that the solvent had mixed thoroughly with the entire sample. Therefore, the extraction of the analyte was complete.

Step III: The extract was centrifuged for 5 min at 4000 rpm. 1.0 mL of the upper organic layer was filtered through a 0.45 µm nylon syringe filter before HPLC analysis.

Preparation of standard stock solution and calibration standard.

The standard stock solution of PDE_5 inhibitor (1000 mg/L) was prepared with the mobile phase. A series of standard solutions (12.50, 25.00, 50.00, 100.0, and 200.0 mg/L) was prepared by diluting adequate volumes of the PDE_5 inhibitors stock solution with the mobile phase.

Optimization procedure

The pH of the mobile phase and mobile phase ratio plays a significant role in chromatographic separations. The DOE and statistical data analysis were performed using Minitab® 20.0 software system. For the robustness study, a CCD with twenty-six experimental points (Table 1) was performed randomly at all points. Resolution (R_S), in addition to the maximum peak, was chosen as the response of the food PDE_5 inhibitors.

Table 1: The effects of the acetonitrile percentage, flow rate, and format amount on the chromatographic peak areas for PDE_5 inhibitors, on retention of tadalafil, and the resolution between vardenafil and sildenafil analysed by Zorbax Eclipse XDB-C18 150 mm × 4.6 mm column at 285 nm at room temperature.

Run Order	0.1% Formic Aid (v/v) Acetonitrile	Flowrate	Formate amount	Area VAR	Area SIL	Area TAD	TAD retention time (min)	Resolution between VAR & SIL
1	45	1.2	0	5596588	3716224	4203470	3.266	0.84
2	35	1	10 mM	7347353	4717276	5933050	7.619	2.30
3	25	0.8	0	11088708	11192518	7678229	11.657	5.00
4	45	1.2	10 mM	7163758	7475723	7475723	3.803	0.81
5	25	1.2	10 mM	7732072	5317255	10611312	46.991	2.90
6	45	0.8	10 mM	7117393	5175559	5834347	3.925	0.86
7	45	0.8	0	7078510	5173909	5810842	3.926	0.87
8	35	1	0	8794939	6003490	8779507	7.598	2.20
9	25	1.2	0	7732072	5317255	10611312	46.991	2.40
10	25	0.8	10 mM	7516271	4917832	5911307	7.621	4.80

Food samples

In the first quarter of 2022, 66 samples of honey mixed herbal, mostly Ginseng and Tongkat Ali, sachets of different brands were randomly obtained from various pharmacies and drug stores in Amman, Jordan. The samples were stored in the dark at room temperature (20–25^o C). The samples were mixed at room temperature until a homogeneous solution was obtained.

RESULTS AND DISCUSSION

Optimization of HPLC conditions

The chromatographic conditions were optimized by investigating various mobile phase compositions, flow rate, and addition of buffer to obtain the best chromatogram separation in the shortest analysis time. Variations in the ratio of 0.1% formic acid in acetonitrile at a proportion of 25–45 % in the mobile phase, variation of flow rate from 0.8 to 1.2 mL/min, and ten mM ammonium formate were also investigated. Data were analyzed using Minitab® 20.0 software to maximize the peak area of PDE_5 inhibitors, optimize the resolution between vardenafil and sildenafil and decrease the retention time of tadalafil.

The present work sought to determine the combined effects of buffer, flow rate, and mobile phase composition on the reverse-phase liquid chromatographic behaviour of PDE_5 inhibitors. The effect of a buffer was also tested by adding ten mM ammonium formate to provide a maximum peak area of PDE_5 inhibitors and to decrease the retention time of tadalafil without affecting good separation resolution between the vardenafil and sildenafil peaks. Moreover, a flow rate between 0.8 to 1.2 mL/min was used to examine its potential impact on the resolution between vardenafil and sildenafil and the retention time of tadalafil. The DOE was applied to unearth the best suitable percentage of 0.1% formic acid in water to 0.1% formic acid in acetonitrile. Furthermore, adding ammonium formate as a buffer modifier at a concentration of 10 mM and finding the optimum flow rate to get the best-resolved peak area of vardenafil and sildenafil and decrease the retention of tadalafil. CCD with ten experimental points (Table 1) was performed randomly at all points. A quadratic polynomial model was used to fit the experimental data. Figures 1 and 2 depict the prediction profilers available on the response surface (Figures 1 and 2).

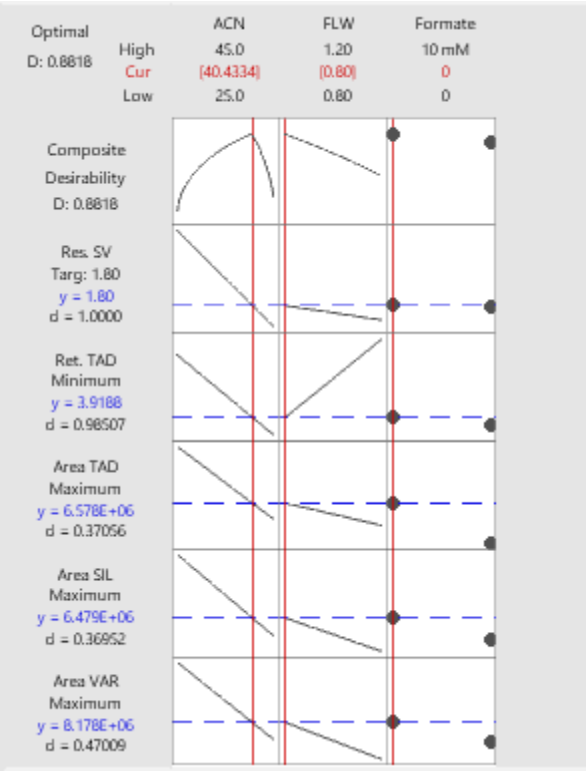
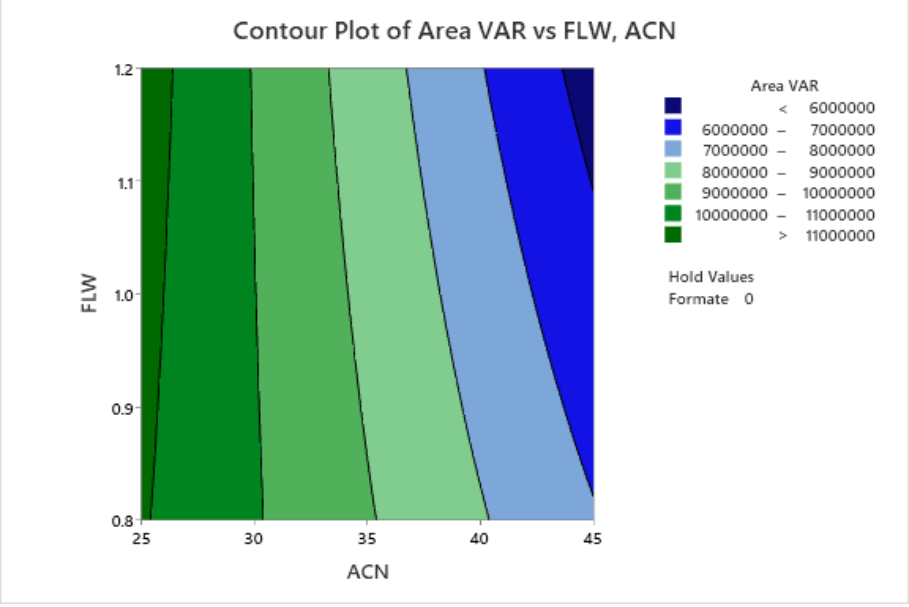


Figure 1: The Maximum Desirability Profiler displays optimal settings of flow-rate 0.8 and 0 mM ammonium formate using 60:40 (%, v/v) 0.1% formic acid in Water/ 0.1% formic acid in acetonitrile solution. It gave 0.88 composite desirabilities of peak area of PDE_5 inhibitors, resolution between vardenafil and sildenafil and the retention time of tadalafil.

a



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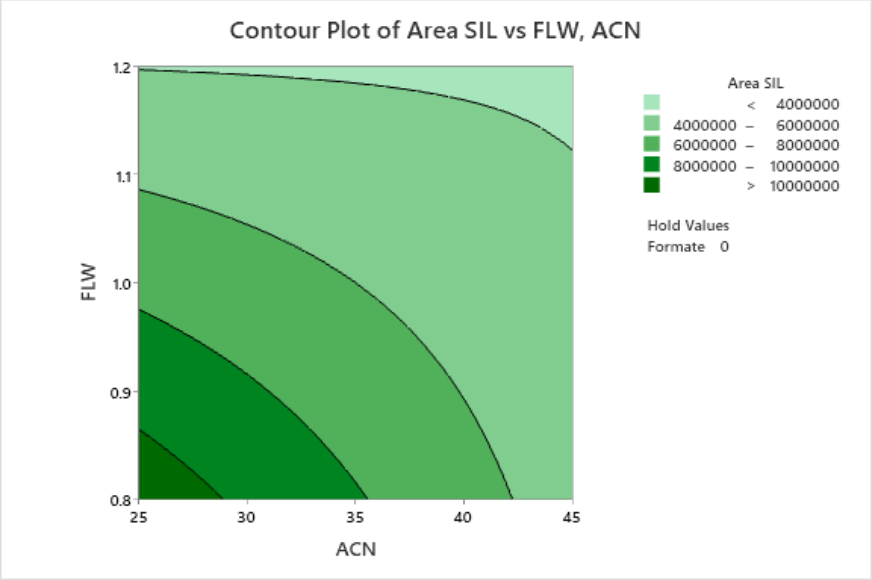

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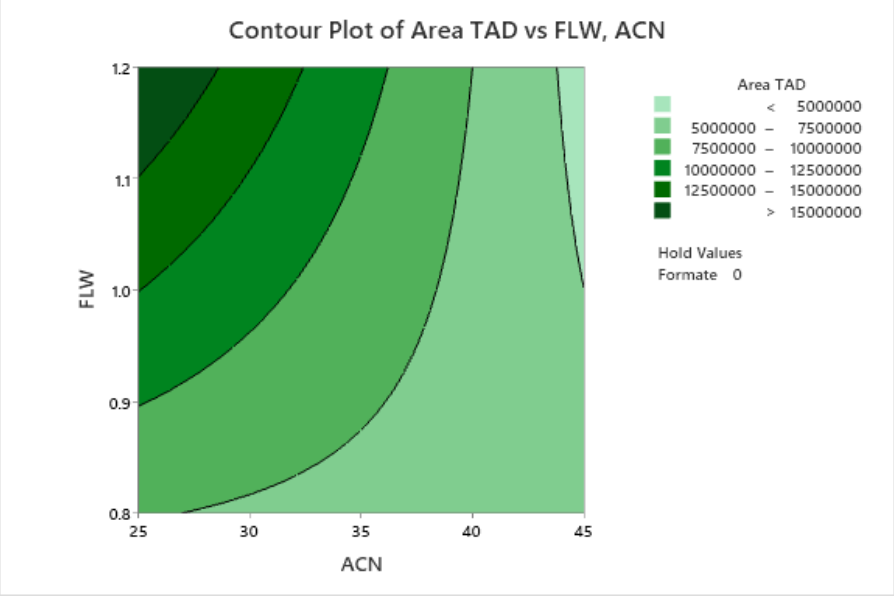

Figure 2: An overlay contour plot of both PDE5 inhibitors Vardenafil (a) and Sildenafil (b) peak area with 24 experimental points (the black dot point)

Figure 1 illustrates the Maximum Desirability Profiler and indicates optimal settings of flow-rate 0.8 and 0 mM ammonium formate using 60:40 (%, v/v) 0.1% formic acid in Water/ 0.1% formic acid in acetonitrile solution. It gave 0.88 composite desirabilities of peak area of PDE_5 inhibitors, resolution between vardenafil and sildenafil, and comparatively short retention time of tadalafil.

Figure 2 indicates that the maximum peak area under the

curve can be achieved for PDE_5 inhibitors using 75:25 (v/v) 0.1% formic acid in Water/ 0.1% formic acid in acetonitrile solution at different flow rates. However, this composition solution was avoided due to the broad peaks and comparatively long retention times when the water composition exceeded 70%. Additionally, using mobile phase composition of 55:45 (v/v) 0.1% formic acid in Water/ 0.1% formic acid in acetonitrile solution at 1.2 mL/ min, vardenafil, and sildenafil did not separate. Conversely, to the compositions above, a mobile phase composition of 60:40 (v/v) 0.1% formic acid in water/acetonitrile solution at a flow rate of 0.8 mL/min displayed optimal settings and offered adequate separation between vardenafil and sildenafil peaks with reasonable and acceptable retention times, as well as good peaks areas for all three PDE_5 inhibitors (Figure 3). The experimental data is in good agreement with the prediction of the Maximum Desirability Profiler.

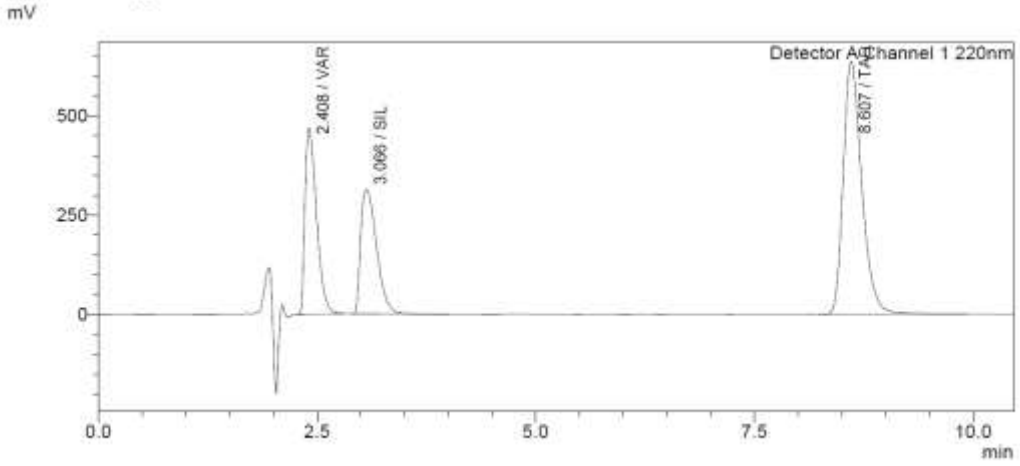

Figure 3: HPLC chromatogram of PDE5 inhibitors standard solutions containing 50 mg/L of vardenafil, sildenafil, and tadalafil with a retention time of 2.4, 3.0, and 8.6 minutes, respectively.

The bottleneck of any chromatographic system is the column, where the actual separation between analyte mixtures occurs. The column selection strongly relies upon the prior knowledge of the physical and chemical properties of the analyte and matrix [31]. The column type, length, and particle size were optimized by studying different HPLC columns under the optimized mobile phase conditions to obtain better chromatography in the shortest analysis time. The studied columns were:

A. Shimadzu Shim-pack GIST 5µm C8 150 x 4.6 (Japan),
B. Shimadzu Shim-pack GIST 5µm C18 150 x 4.6 (Japan),
C. Fortis Technologies 5µm Fortis C18 250 x 4.6 (Munich, Germany),
D. Agilent Technologies ZORBAX Eclipse Plus, C18, 3.5 µm, 4.6 x 150 mm (Santa Clara, USA).

Table 2 shows that the column significantly affects the retention time and sensitivity (Area %) of PDE_5 inhibitors. Column A (Shim-pack GIST 5µm C8) has a high sensitivity for sildenafil (97%) and tadalafil (96%) but suffers from less sensitivity for vardenafil (91%). On the other hand, column B (Shim-pack GIST 5µm C18) shows excellent sensitivity for vardenafil (100%) and high sensitivity for sildenafil (97%) and tadalafil (96%). Additionally, column B showed an optimal resolution (Rs ~ 1.93) between vardenafil and sildenafil and the second shortest retention time (less than 10 minutes) after column D.

Table 2: The HPLC column packing materials investigated (Vardenafil, Sildenafil, and Tadalafil at 260, 227 and 303 nm at mobile phases (60:40 (%, v/v)) flow rate mL/ min and column temperature 20°C.

Column		PDE_5 inhibitors	Retention Time (min)	Area %	Resolution
A	**Shimadzu Shim-pack GIST 5µm C8 150 x 4.6**	Vardenafil	2.466	91%	-
		Sildenafil	3.275	97%	3.106
		Tadalafil	10.307	96%	19.849
B	**Shimadzu Shim-pack GIST 5µm C18 150 x 4.6**	Vardenafil	1.934	100%	-
		Sildenafil	2.474	97%	2.586
		Tadalafil	9.615	96%	20.770
C	**Fortis Technologies 5µm Fortis C18 250 x 4.6**	Vardenafil	3.429	99%	-
		Sildenafil	4.667	100%	3.641
		Tadalafil	19.085	100%	19.666
D	**Agilent Technologies Zorbax Eclipse XDB-C18 150 mm × 4.6 mm**	Vardenafil	1.959	91%	-
		Sildenafil	2.510	93%	2.716
		Tadalafil	6.870	94%	16.315

The 25 cm columns (column C; 5µm Fortis C18 250 x 4.6) showed the highest sensitivity for all analytes; 99% for vardenafil and 100% for sildenafil and tadalafil, but was associated with a significant increase in retention times (~20 min).

The 3µm particle size column D showed good sensitivity for vardenafil (91%), sildenafil (93%), and tadalafil (94%). Furthermore, it had an optimum resolution (Rs ~ 1.96) between vardenafil and sildenafil, with the shortest retention time of fewer than 8 minutes. Ultimately, we chose column B as it had the highest sensitivity and the second shortest retention time. The optimized conditions resulted in an effective separation of the PDE_5 inhibitors in a run time of 10 min. The average retention time was 1.9 min for vardenafil, 2.5 min for sildenafil, and 9.6 min for tadalafil.

To assess whether the PDE_5 inhibitor mixed standards could be distinguished and well-separated from the interfering substances in the sample matrix, an adulterated honey-mixed herbal sample was pretreated using the modified sample preparation method and separated using a BDS 3µm C8 150 x 4.6. The chromatograms, which indicate the selectivity of the procedure, are shown in Figure 4. The chromatograms of the adulterated honey-mixed herbal sample showed that all peaks of the PDE_5 inhibitor standard are well separated from the interfering substances found in the matrix with reasonable retention times.

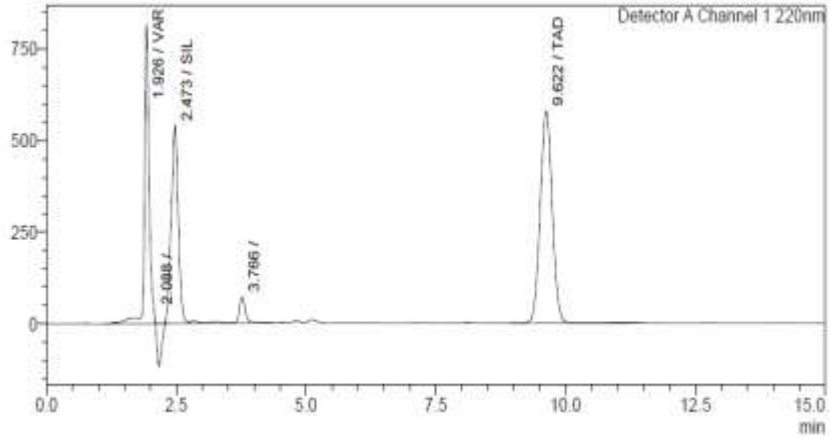

Figure 3: HPLC chromatogram of PDE_5 inhibitors standard solutions containing 50 mg/L of vardenafil, sildenafil, and tadalafil with a retention time of 2.4, 3.0, and 8.6 minutes, respectively.

Selection of detection λ-max

To choose the best wavelength, a spectrum of three PDE_5 inhibitors solution (50 mg/L) analyzed using the chosen HPLC mobile phase at a flow rate of 0.8 mL/min (Figure 3) was generated to optimize the detection of PDE_5 inhibitors. Based on a review of pertinent literature, we found that many articles analyzed sildenafil, vardenafil, and tadalafil at different wavelengths [24, 32]. Sildenafil was analyzed at λs of 225 [33], 230 [32, 34], and 292 [21, 33, 34], and vardenafil was analyzed at λs of 230 [32, 34], and 292 [21, 34], whereas tadalafil was analyzed at λs of 220 , 230 [32, 34], 283 [34] , 284 [24] and 292 [21, 34]. The optimization procedure for the wavelength selection was carried out by analyzing the targeted PDE_5 inhibitors at five different wavelengths (i.e., at λs of 220, 230, 245, 284, and 292) until the maximum sensitivity (peak area counts of sildenafil, vardenafil and tadalafil standards mixture) was obtained.

Table 3 shows substantial differences in the peak area counts for sildenafil, vardenafil, and tadalafil when applying different wavelengths. As the wavelength changed from 220 to 295 nm, the peak area for vardenafil, sildenafil, and tadalafil were substantially decreased, from 100% to 15%, 45%, and 24%, respectively. Therefore, a wavelength of 220 nm was selected to quantify sildenafil, vardenafil, and tadalafil in honey samples. Alternatively, the wavelength of 230 nm can be selected to avoid UV absorbance cut-off of the selected mobile phase for quantifying sildenafil, vardenafil, and tadalafil in honey samples as the peak area for sildenafil, and vardenafil, were slightly decreased, from 100% to 93%, 89%, and 72%, respectively (Table 3).

Table 3: The effects of the wavelength (nm) on the chromatographic peak areas percentage for PDE_5 inhibitors.

PDE_5 inhibitor	**Wavelength (nm)**								
	220	230	245	283	284	285	291	292	295
VAR	100%	89%	77%	22%	22%	21%	17%	21%	15%
SIL	100%	93%	66%	41%	41%	42%	45%	44%	45%
TAD	100%	72%	25%	29%	29%	35%	26%	26%	24%

Method validation

The method was validated internally regarding linearity, accuracy, intra-day and inter-day precision, the LOD, and the LOQ. The linearity was tested using a mixture of the three PDE_5 inhibitors standards in a concentration range from 12.5 to 200 mg/L for sildenafil, vardenafil, and tadalafil.

Table 4 shows good linear relationships between the concentration of the analyte and the peak response, with correlation coefficients more significant than 0.9999 for all analytes. Calibrations with ICH standard solutions were utilized for quantitation of PDE5 inhibitors, and Statistical analysis was performed using SPSS program version 19. Observed differences between groups were compared using analysis of variance (one-way ANOVA). Differences were regarded as significant, with $P \leq 0.05$.

Table 4: Linearity range, Equation, r^2 value, LOD and LOQ of sildenafil, vardenafil, and tadalafil.

PDE_5 inhibitor	**Linearity Range** (mg/L)	**Equation**	**r^2**	**LOD** (mg/L)	**LOQ** (mg/L)
Sildenafil	12.5 – 200.0	y = 33306x - 64947	0.9999	1.70	5.65
Vardenafil	12.5 – 200.0	y = 30981x - 17137	0.9999	2.16	7.21
Tadalafil	12.5 – 200.0	y = 70515x - 21690	0.9999	1.03	3.42

The accuracy was calculated by determining the recoveries of PDE_5 inhibitors from honey samples at concentrations of 50, 100, and 150 mg/L of sildenafil, vardenafil, and tadalafil standards; the spiked samples were analyzed in triplicates (Table 5) and calculated using the following formula: [35]:

$$\text{Recovery (\%)} = \frac{\text{Recovered Amount (mg/L)}}{\text{Added Amount (mg/L)}} \times 100$$

Table 5: Mean of recoveries and RSDs (n=5) of sildenafil, vardenafil, and tadalafil spiked into clean Honey-Mixed Herbal Sachets and Honey Sachets samples at three spiking levels using HPLC method (n = 3)[a]

PDE_5 inhibitor	Spiking Level (mg/L)	Honey-Mixed Herbal Sachets	Honey Sachets
		Mean of Recovery (%) ± RSD (%)	
Sildenafil	50	94.1±0.8	93.0±0.7
	100	103.3±0.3	98.6±0.8
	200	101.2±0.6	100.1±0.8
Vardenafil	50	91.4±1.2	96.0±0.4
	100	102.7±0.6	101.2±0.1
	200	101.2±0.7	100.5±0.3
Tadalafil	50	95.7±0.4	96.0±1.1
	100	103.2±0.7	100.2±0.5
	200	101.0±0.3	102.5±0.3

[a] n: is the number of replicates

The recovery percentages ranged from 93.0% to 103.3%, with an RSD of less than 2%.

The sensitivity was determined by assessing the LOD and LOQ. LODs and LOQs were determined experimentally as the lowest concentration giving a response of three- and six times to the baseline noise, respectively. The LOD of sildenafil acid, vardenafil acid, and tadalafil was 1.70, 2.16, and 1.03 mg/L, respectively. The LOQs were 5.65, 7.21, and 3.42 mg/L, respectively (Table 4). Or Table 5

Intra-day precision was calculated by assaying five replicates of the same sample at a spiked level of 50 mg/L of sildenafil acid, vardenafil acid, and tadalafil on the same day. For the inter-day precision, five replicates of the same sample at a spiked level of 50 mg/L of sildenafil acid, vardenafil acid, and tadalafil were analyzed on three consecutive days. The intra-day precision and inter-day precision were calculated and tabulated in Table 6. The intra-day precision (n= 5) values were between 0.4 and 1.2%, whereas the inter-day variation (n= 15) values were between 1.0-1.7%. The complete separation of the peaks, the low RSD, LOD, and LOQ data obtained from this work compared with reported values confirm this method's good reproducibility and repeatability.

Table 6: The intra-day precision and inter-day precision of sildenafil, vardenafil, and tadalafil expressed as RSD% values

PDE_5 inhibitor	**Spiking Level** (mg/L)	**Intra-Day Precision** (n = 5)[a]	**Inter-Day Precision** (n = 15)[a]
Sildenafil	50	0.8	1.1
Vardenafil	50	1.2	1.7
Tadalafil	50	0.4	1.0

[a] n: number of replicates

Considering the data obtained from the method validation, the current HPLC–UV analysis measured with the aid of response surface methodology, experimental design, and sample preparation procedures is considered a selective, precise, and robust method to determine sildenafil acid, vardenafil acid, and tadalafil PDE_5 inhibitors in honey samples.

Food samples analysis

The developed method was applied to analyze the targeted PDE_5 inhibitors in 66 samples of honey mixed herbal sachets declared as purely 'natural' erectile supporters, which were collected in the first quarter of 2022 from various community pharmacies in the capital city of Amman. The collection method was based on approved international methods, where the samples were preserved and transported to the laboratory in conditions commensurate with the storage methods. In addition, all information related to each sample was recorded in a sample record, including the type of sample, its source, and the date taken to interpret and evaluate the results. The samples were distributed as follows: 8 samples of local origin, 28 samples from the United States of America and Canada, 30 samples from European countries, and finally, 18 samples from Asia.

The analysis showed that eight samples turned out to be positive for non-declared PDE_5 inhibitors. Sildenafil was considered to be the primary active compound to be detected (1.1 mg/sachet [local samples] to 124 mg/sachet [mixed herbal sachets from Asia]). In contrast, tadalafil was only detected in five samples ranging from 0.67 mg/sachet [local samples] to 76.6 mg/sachet [mixed herbal sachets from Europe]. On the other hand, vardenafil was not detected in any of the tested samples from all sources. It is worth mentioning that samples of the same commercial brand tested positive for the PDE5 inhibitors with varying concentrations in each sachet. In contrast, others tested negative, indicating no systemic way of fortifying the PDE5 inhibitors and lack or absence of quality control and quality assurance procedures. This is a catastrophic and lethal error as the consumer might take a multi-sachet dose, mainly that all obtained honey-mixed herbal sachets samples lacked patient leaflet with some dare to claim it is safe for use by printing a legal Disclaimer as below:

The product does not cause any side effects and is safe for men of all ages; however, it is not to be used by people with kidney failure, heart disease, chronic hypertension, ischemia, and children under 18 years old.

Patients over 40 with chronic diseases such as hypertension, ischemic heart disease, diabetes, depression, and atherosclerosis generally have ED as a complication. In recent years, herbal products are becoming more popular than ever as an alternative to prescription drugs for numerous reasons, including a common belief that they are safe, obtaining herbal products without a prescription or seeing a doctor or even a pharmacist, and the myth belief of folklore tradition that these herbs work better than prescribed drugs in the market. However, our findings are in-line with all previous observations, which further emphasize that herbal remedies are frequently being adulterated with undeclared synthetic drugs to achieve the desired action, and this may inevitably be associated with life-threatening ramifications on its own or as a result of clinically significant drug-drug interactions [18, 19, 21, 24].

CONCLUSION

The importance of this work paves the foundations for quickly and effortlessly performing a simple analytical method to determine and quantify the presence of PDE_5 inhibitors in honey-mixed herbal sachets. This is paramount as commercial fraud in such products might lead to fatalities or severe side effects. The findings of this project revealed astonishing and alarming information about these products, such as the random fortification quantity of PDE5 inhibitors and the failure to pronounce the existence of such materials on the product's packaging. Moreover, a simple, rapid, inexpensive, and effective sample preparation method has been developed to determine vardenafil, sildenafil, and tadalafil in honey-mixed herbal sachets in the hope of encountering and combating commercial frauds and ultimately saving lives. The sensitivity of the HPLC–UV instrument could be significantly enhanced by optimizing the mobile phase composition and the type, length, and particle size of the HPLC column. The developed sample preparation procedure is based on a single extraction step without employing pre-treatment processes and thus can be recommended as an alternative to the time-consuming precipitating step, steam distillation multiple steps, or solid-phase extractions clean up a step for PDE_5 inhibitors determination in food. A C18 150 mm × 4.6 mm x 5 µm column at 20^{o}C performed separation of the vardenafil, sildenafil, and tadalafil with higher selectivity and sensitivity and within shortened retention time. Excellent linearity and repeatability, high recoveries, , and reproducibility values were obtained, indicating the developed method's suitability for determining PDE_5 inhibitors in Honey-Mixed Herbal Sachets. Compartively short retention times for sildenafil acid, vardenafil acid, and tadalafil were found 1.93, 2.47, and 9.62, respectively, while the limits of detection (LOD) were 1.70, 2.16, and 1.03 mg/L, and the limits of quantification (LOQ) were 5.65, 7.21, and 3.42 mg/L.

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تحسين والتحقق من طريقة HPLC-UV لتحديد Vardenafil وSildenafil وTadalafil في أكياس العشبية المصنوعة من العسل باستخدام تصميم التجربة

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ملخص

تم تطوير طريقة لتحديد وتحليل متزامن للسيلدينافيل، فاردنافيل، وتادالافيل في أكياس عشبية مخلوطة بالعسل باستخدام كروماتوجرافيا سائلة عالية الأداء مع كاشف الأشعة فوق البنفسجية (.HPLC-UVهذه الطريقة تلغي استخدام الإجراءات المعقدة وتلغي عمليات المعالجة المسبقة التي تستغرق وقتًا طويلاً وعملا كثيفا. في غضون عشر دقائق (عند 25 درجة مئوية) تمت عملية الفصل بنجاح لكل من سيلدينافيل و فاردينافيل و تادالافيل باستخدام عمود الفصل من نوع C18 GIST Shim-Pack الطول 150 ملم، القطر الداخلي 4.6 ملم، قطر الحبيبات 5 ميكروميتر) مع انتقائية وحساسية عالية. كانت تركيبات الطور المتحرك عبارة عن خليط بنسبة 60:40 (حجم لحجم) من حمض الفورميك 0.1 % المذاب في الماء وحمض الفورميك 0.1 % المذاب في الأسيتونيتريل. باستخدام الطور المتحرك كخليط استخراج، أعطت معدلات استرداد في نطاق 93.0–103.3٪ عند تراكيز من 50–150 ملغم / كغم مع الانحرافات المعيارية النسبية (RSDs) أقل من 10٪. وكانت النتائج الدقيقة داخل اليوم وبين اليوم في حدود 0.4–0.8 ٪ و1.0–1.7 ٪. علاوة على ذلك، كانت زمن انحباس ل السيلدينافيل، الفردنافيل، وتادالافيل 1.93، 2.47 و 9.62 دقيقة، على التوالي، و لقد كان الحد الأدنى للقياس النوعي 1.70 (LOD)، 2.16 و 1.03 ملغم/لتر، في حين أن الحد الأدنى للقياس الكمي (LOQ) كانت 5.65 و 7.21 و 3.42 ملغم/لتر.

الكلمات الدالة: HPLC، تصميم التجربة، فاردينافيل، سيلدينافيل، وتادالافيل، مثبط PDE5.

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