Development and Validation of HPLC/DAD Method for Simultaneously Determination of Six Prohibited Substances in Model Matrices

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The authorities have identified an emerging trend where over-the-counter products, represented as dietary supplements, contain hidden active ingredients that could be harmful. Consumers may unknowingly take products laced with varying quantities of approved prescription drug ingredients, controlled substances, and untested and unstudied pharmaceutically active ingredients. Hidden ingredients are increasingly becoming a problem in products promoted for sexual enhancement, weight loss, or bodybuilding. The tests have revealed the presence of some undesired substances like sildenafil, tadalafil, vardenafil, and their analogues in tainted sexual enhancement products. The content of these substances is usually around the daily curative dose. A simple high-performance liquid chromatography (HPLC) method for simultaneous determination of sildenafil, vardenafil, tadalafil, dapoxetine, yohimbine, and sibutramine was developed and validated. InfinityLab Poroshell 120 EC-C18 (150 '4.6 mm '4 µm particles) was used, as well as a diode-array detector (DAD) at 230 nm, and a gradient flow with 0.030 M ammonium acetate buffer and acetonitrile. The method is linear in the following range: 2.5–37.5 µg/mL for yohimbine, 2.06–30.9 µg/mL for vardenafil, 2.0–30.0 µg/mL for sildenafil, 3.1–46.5 µg/mL for tadalafil, 1.98–29.7 µg/mL for dapoxetine, and 2.2–66.0 µg/mL for sibutramine. The linearity coefficient is $R^2 = 1$ for all substances. Model matrices were spiked, and the analytical recoveries for all substances are in the range 97.5%–99.5%. The method exhibited an upper hand compared with previously reported methods in terms of speed and simplicity. Additionally, the mobile phase (also used as extracting, column washing, and diluting solvent) was composed of only buffer and acetonitrile, which rendered the method much cheaper than others.

Keywords: sildenafil, vardenafil, tadalafil, dapoxetine, yohimbine, sibutramine

1. Introduction

Food supplements contain certain substances in concentrated form with a nutritional or physiological effect. They are designed to correct nutritional deficiencies, maintain adequate intake of certain nutrients, or maintain certain physiological functions [1]. In recent years, the popularity of herbal medicines and nutritional supplements has increased worldwide owing to the high number of synthetic drugs associated with allergies and other side effects. The market for medicinal products and food supplements based on plant material is increasing rapidly. Food supplements are available in herbal stores, pharmacies, and on the internet with direct delivery. The marketing and sale of these supplements is mainly by internet and television, and they are not strictly controlled, so often they do not pass any safety and control tests [2, 3]. The authorities have identified an emerging trend where over-the-counter products, represented as dietary supplements, contain hidden active ingredients that could be harmful. Consumers may unknowingly take products laced with varying quantities of approved prescription drug ingredients, controlled substances, and untested and unstudied pharmaceutically active ingredients. Hidden ingredients are increasingly becoming a problem in products promoted for sexual enhancement, weight loss, or bodybuilding [4].

Presumably, herbal medicines should be considered safe and effective, with no side effects, but this huge market provokes deliberate tampering for higher profits. Undeclared sub-

stances or synthetic compounds in botanical products can be not only toxic but also often interact with other prescript medical drugs, and as a result, they may cause adverse effects that could even be life-threatening [5]. Counterfeiting consists of replacing the original herb partially or completely with another material based on similarity in morphological characteristics. It also can be done with synthetic chemical drugs used to increase the effectiveness of the food supplement, most often in anti-obesity and antidiabetic medical drugs, as well as those for potency. This type of counterfeiting can lead to serious health problems due to the long-term acceptance of the nutritional supplement [6]. In industrialized countries, one of the most falsified classes of food supplements is that of men's potency. Often, they include phosphodiesterase type 5 inhibitors (PDE5-i), such as sildenafil (trade name Viagra), vardenafil, (trade name Levitra), and tadalafil (trade name Cialis) [7, 8]. Another class of substances frequently subjected to counterfeiting is herbal dietary supplements for weight loss. The use of herbal products for these two types of adjustments has increased significantly. Because these plant products are classified as food additives and are falsified illegally, their safety, efficacy, and quality control are not subject of control. However, the presence of synthetic substances and analogues of prescription-only medicines can lead to a variety of health risks that present a serious risk, such as cardiovascular disease, liver disease, kidney failure, agitation, anxiety, confusion, depression, etc. For this reason, the World Health Organization (WHO), Food and Drug Administration (FDA), and the European Medicines Agency have issued guidelines and rules for the safe and appropriate use of herbal products.

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It has been found that one of the most likely impurities in the weight loss agents is sibutramine, a substance which can provoke anorexia. Yohimbine is also a drug commonly present in dietary supplements to improve athletic abilities, as well as weight loss. It is an indole alkaloid originally extracted from the bark of the *Pausinystalia yohimbe* tree, commonly used in the treatment of erectile dysfunction in men [9, 10]. Dapoxetine (trade name Priligy) is a short-acting medicine for the treatment of premature ejaculation in men. [11]. It is a selective serotonin reuptake inhibitor used to treat both premature ejaculation and anxiety [12]. Sibutramine is an oral drug that inhibits central reuptake of serotonin, norepinephrine, and, to a lesser extent, dopamine. It is prescribed for the treatment of obesity, along with diet and some specific practical exercise [13].

Analytical methods for simultaneous quantification of these compounds are essential, considering their nature and importance. Many authors have reported the separate determination of the aforementioned substances, either in products for the treatment of erectile dysfunction or in dietary supplements for weight loss by an ultra-performance liquid chromatography (UPLC) technique [14, 15], liquid chromatography–mass spectrometry [16], and high-performance liquid chromatography (HPLC) [17–20].

Taking into account all above mentioned herein, we report the development and validation of a simple HPLC method for simultaneously determination of the most used undeclared substances in weight-loss products and in products for sexual enhancement: sildenafil, vardenafil, tadalafil, dapoxetine, yohimbine, and sibutramine (Figure 1). Sildenafil and vardenafil differ in the heterocyclic ring system used to mimic the purine ring of cyclic GMP-specific phosphodiesterase type 5, as well as according to the substituent (ethyl/methyl) of a piperazine side chain. All target compounds have aromatic rings bonded to non-polar aliphatic chain. Due to their similar solubility and properties, their separation is quite difficult. In addition, sample preparation is also described for spiked model placebo matrix in this work.

2. Materials and Method

2.1. Chemicals and Reagents. Acetonitrile and methanol are purchased by Honeywell Research Chemicals (Seelze,

Germany), and ammonium acetate and formic acid are from Sigma-Aldrich (Deisenhofen, Germany). Deionized water used for preparation of all stock solution, as well as for HPLC system, was prepared using a Milli-Q system (Millipore, Bedford, MA, USA). All used solvents were of analytical or HPLC grade, and they are used without any additional treatment.

The European Directorate for the Quality of Medicines and HealthCare (EDQM, Strasbourg, France) supplies chemical reference substances (CRS), herbal reference standards (HRS), and biological reference preparations (BRP), as well as reference spectra for the tests and assays to be carried out in accordance with the official methods prescribed in the European Pharmacopeia. Specific batches of candidate material are selected. These are characterized at the EDQM laboratory according to the principles described in Chapter 5.12. (Reference Standards) of the European Pharmacopeia and 'ISO 17034 General requirements for the competence of reference material producers'.

Candidate materials can also be the subject of international collaborative studies. Once adopted by the European Pharmacopeia Commission, official reference standards become available for distribution. They are then used in pharmaceutical analysis, for example, for identification, purity tests, or assays according to the corresponding monograph of general chapter of the European Pharmacopeia.

All used standards in the current investigation are the following: sildenafil citrate – CRS purchased from European Directorate for the Quality of Medicines and HealthCare (Strasbourg, France) Batch 1.1; vardenafil hydrochloride – CRS purchased from European Directorate for the Quality of Medicines and HealthCare (Strasbourg, France) batch 1.0; tadalafil – CRS purchased from European Directorate for the Quality of Medicines and HealthCare (Strasbourg, France) batch 2.0; yohimbine hydrochloride – CRS purchased from

Table 1. Mobile phase gradient

t (min)	Mobile phase A (%)	Mobile phase B (%)	
0–5	70	30	Isocratic
5–15	70 → 20	30 → 80	Linear gradient
15–16	20 → 70	80 → 30	Linear gradient
16–20	70	30	Isocratic

Figure 1. Chemical structures of investigated compounds: (A) sildenafil, (B) vardenafil, (C) dapoxetine, (D) tadalafil, (E) yohimbine, and (F) sibutramine

Table 2. Concentration levels of the standard solutions used

Name	Weight (mg)	Stock solution volume (mL)	100%	10%	25%	50%	75%	150% (from stock solution)
Sildenafil	20.0	20	1 mL to 50 mL	1 mL to 10 mL	5 mL to 20 mL	5 mL to 10 mL	15 mL to 20 mL	3 mL to 100 mL
Vardenafil	20.6	20	1 mL to 50 mL	1 mL to 10 mL	5 mL to 20 mL	5 mL to 10 mL	15 mL to 20 mL	3 mL to 100 mL
Dapoxetine	9.9	10	1 mL to 50 mL	1 mL to 10 mL	5 mL to 20 mL	5 mL to 10 mL	15 mL to 20 mL	3 mL to 100 mL
Tadalafil	15.5	10	1 mL to 50 mL	1 mL to 10 mL	5 mL to 20 mL	5 mL to 10 mL	15 mL to 20 mL	3 mL to 100 mL
Yohimbine	12.5	50	5 mL to 50 mL	1 mL to 10 mL	5 mL to 20 mL	5 mL to 10 mL	15 mL to 20 mL	15 mL to 100 mL
Sibutramine	11.0	10	2 mL to 50 mL	1 mL to 10 mL	5 mL to 20 mL	5 mL to 10 mL	15 mL to 20 mL	6 mL to 100 mL

European Directorate for the Quality of Medicines and HealthCare (Strasbourg, France) batch 1.0; dapoxetine hydrochloride – Toronto Research Chemicals Inc. (Ontario, Canada) batch: 4-BLL-111-1; and sibutramine hydrochloride – Toronto Research Chemicals Inc. (Ontario, Canada) batch: 1-DXX-80-1.

2.2. Analytical Equipment and HPLC Conditions. The used HPLC system is HPLC Shimadzu LC20AD (Kyoto, Japan) equipped with a column InfinityLab Poroshell 120 EC-C18 (150 ‘4.6 mm ‘4 µm particles) from Agilent (Santa Clara, USA), a diode-array detector (DAD) scanned at 230 nm, and gradient flow as follows: mobile phase A: 0.030 M ammonium acetate buffer with pH 3.0 adjusted with formic acid; mobile phase B: acetonitrile; column temperature: 40 °C; flow rate: 1 mL/min; and injection volume: 10 µL; mobile phase gradient is shown in Table 1.

2.3. Preparation of Standard Calibration Solutions. All standard solutions are prepared by dilution in mixture water–acetonitrile = 50:50 v/v. The preparation of the standard

solutions in the dilution levels required for this study is presented in Table 2.

3. Results and Discussion

3.1. Optimization of Chromatographic Conditions. Initially, a conventional C18 column (Agilent Zorbax Eclipse, (150 ‘4.6 mm ‘5 µm)) and isocratic elution with acetonitrile–buffer (pH 3.0) at 30:70 v/v were tested for the separation of target compounds. The resolution between sildenafil and vardenafil and peak shapes for all substances, as well as a retention time of more than 30 min, were not acceptable. Some asymmetry and peak overlap were monitored. Taking into account the specific characteristics described by manufacturer, an Agilent InfinityLab Poroshell column (150 ‘4.6 mm ‘4 µm) based on superficially porous particle technology, which features a solid silica core and a porous outer layer, was subjected to experiment for the degree of separation of target compounds with the same mobile phase. The column shows

<Chromatogram>

Quantitative Results				
PDA				
ID#	Name	Ret. Time	Area	Height
1	Yohimbine	2.607	490757	68016
2	Vardenafil	4.897	693137	67404
3	Sildenafil	6.070	526377	45617
4	Tadalafil	11.021	1167635	165361
5	Dapoxetine	11.716	1005188	165642
6	Sibutramine	12.242	453375	68223

Figure 2. Obtained chromatogram for separation of 6 investigated compounds: (A) sildenafil, (B) vardenafil, (C) dapoxetine, (D) tadalafil, (E) yohimbine, and (F) sibutramine

Table 3. Retention times of investigated compounds

Name	Retention times t_R (min)
Yohimbine	2.61
Vardenafil	4.90
Sildenafil	6.08
Tadalafil	11.02
Dapoxetine	11.73
Sibutramine	12.25

perfect separation of sildenafil and vardenafil, which have very similar structure. In addition, excellent peak properties for sildenafil, vardenafil, tadalafil, and yohimbine peaks were registered, but peaks of sibutramine and dapoxetine still had been with poor symmetry. Further, various mobile phases were tested for better peak properties and shorter run time in order to optimized the analytical procedure. The following mobile phases were investigated: acetonitrile–buffer (pH 3–7) at different organic-to-aqueous solvent ratios (20–80%). The addition of triethylamine to the buffer solution lead to poor peak shapes for all peaks. Sibutramine was eluted with higher concentration of acetonitrile, and pH was very important. Thus, finally, a gradient method was chosen in order to complete all requirements of analytical method. It was optimized for good peak resolution and shorter run time. It was also monitored that fast increase of acetonitrile concentration leads to bad resolution between the peaks of tadalafil and dapoxetine.

Finally, all peaks were optimally separated with good symmetry of peaks and acceptable for the aim of analysis retention time with that described in Analytical equipment and HPLC conditions section gradient program. The effect of flow rate (0.5–1.2 mL/min), UV detection wavelength (220–254 nm), injection volume (10–20 μ L), and column temperature (25–45 $^{\circ}$ C) was also studied and adjusted accordingly. The optimal flow rate was found to be 1.0 mL/min. For UV wavelength, 230 nm was chosen, while 40 $^{\circ}$ C was found to be the optimal column temperature, and 10 μ L was the selected injection volume. The determining parameters used for choosing the optimal HPLC conditions were the ability of the mobile phase and the solvents to be used with liquid chromatography–mass spectrometry (LC–MS) equipment, total run time, reproducibility of retention times, separation of all peaks, and peak's shape, resolution, and symmetry.

The identification of the analyzed compounds was done by determining of the retention times (t_R) of the standard solutions for the chromatographic system presented in the materials and methods section. For this purpose, aliquots of each of the prepared standard solutions were injected into the chromatographic system. The obtained separation is shown on Figure 2, and assigned t_R values are presented in Table 3.

3.2. Method Validation. In order to demonstrate the linearity of the method for all test substances, solutions of the standards were prepared with 6 different concentrations (at level 10%, 25%, 50%, 75%, 100%, and 150% of the curative dose of each substance, Table 2). Each solution is injected into the chromatographic system three times. A measure of linearity is the coefficient of determination R^2 , and the acceptance limit in the case of quantity determinations is $R^2 > 0.99$. The obtained analytical results show that the method is linear in the following range: 2.5–37.5 μ g/mL for yohimbine, 2.06–30.9 μ g/mL for vardenafil, 2.0–30.0 μ g/mL for sildenafil, 3.1–46.5 μ g/mL for tadalafil, 1.98–29.7 μ g/mL for dapoxetine, and 2.2–66.0 μ g/mL for sibutramine. The characteristics of the obtained linearity for all 6 investigated substances are summarized in Table 4.

According to the International Conference on Harmonization (ICH) Harmonized Tripartite Guideline Validation of Analytical Procedures: text and methodology Q2 (R1), repeatability was assessed using 9 samples, e.g., 3 concentrations (70%, 100%, and 130% of the curative dose) per 3 replicates. For this purpose, 9 samples were prepared according to the following procedure: 1 g of placebo (taken from pre-powdered tablet of food supplement) is contaminated with a certain amount of standard solution of each substance. A small quantity of solvent (acetonitrile–H₂O = 50:50 v/v) is added and sonicated for 15 min, and the obtained solution is made up to 50 mL in a volumetric flask. The obtained sample is injected into the chromatographic system.

All spiked samples are prepared using standard solutions of each substance and placebo. A food supplement containing the active ingredients spirulina protein extract and humic acid and the excipients calcium hydrogen phosphate, magnesium stearate, maize starch, and povidone, is chosen as placebo. The tablets are previously finely powdered in the mortar. The blank solution of the placebo is prepared and injected. Any

Table 4. The characteristics of linearity for 6 investigated substances

Name	Ranges	Regression equation	Slope	y-Intercept	R^2
Yohimbine	2.5–37.5 μ g/mL	$y = 2E+07x + 2158.3$	19633841.83	2158.3131	1
Vardenafil	2.06–30.9 μ g/mL	$y = 3E+07x - 563.14$	33702693.97	–563.1444	1
Sildenafil	2.0–30.0 μ g/mL	$y = 3E+07x - 403.77$	26378612.92	–403.7654	1
Tadalafil	3.1–46.5 μ g/mL	$y = 8E+07x + 313.9$	75269880.91	313.9002	1
Dapoxetine	1.98–29.7 μ g/mL	$y = 1E+08x + 5852.8$	100675723.1	5852.8446	1
Sibutramine	2.2–66.0 μ g/mL	$y = 2E+07x + 354.01$	20585897.16	354.0127	1

Table 5. Recovery for investigated compounds

Conc. level %	Sample no.	Yohimbine (%)	Vardenafil (%)	Sildenafil (%)	Tadalafil (%)	Dapoxetine (%)	Sibutramine (%)
70	1	100.59	96.99	100.94	100.62	97.34	100.77
	2	98.27	101.95	99.28	97.70	98.88	96.84
	3	99.97	97.14	96.49	96.05	101.52	100.74
100	1	99.33	99.89	98.65	97.67	99.06	97.14
	2	101.10	97.83	98.08	96.67	99.31	98.35
	3	99.94	97.29	98.99	98.80	98.66	100.15
130	1	100.77	96.94	100.97	96.45	95.24	98.50
	2	98.66	101.23	100.75	96.95	98.96	100.29
	3	97.10	97.91	99.12	97.05	100.71	102.14
Average		99.53	98.58	99.25	97.55	98.85	99.44
SD		1.31	1.94	1.48	1.41	1.81	1.81
%RSD		1.32	1.97	1.49	1.45	1.83	1.82
CI (95%)	Mean	± 0.86	± 1.27	± 0.97	± 0.92	± 1.18	± 1.18

peaks are detected at the retention times of the analyzed substances.

According to the guidelines, the %RSD value for quantitative determination methods has to be less than 2.0% (Table 3). The obtained %RSD values for all compounds fit the limit.

The analytical recovery was also investigated. For this purpose, the same solutions mentioned above for repeatability were used. All results are summarized in Table 5.

According to the ICH guideline, the recovery has to be in the interval 95% to 105%. As it can be seen from the results in Table 5, all tested compounds fit in the range of this interval.

4. Conclusions

A simple and fast HPLC method for simultaneously determination of prohibited substances was successfully developed and validated. UV detection makes the method inexpensive and widely applicable. The selected mobile phase also makes the method applicable to LC–MS analysis if additional substance identification and confirmation are required. The selected chromatographic column achieved good separation and superior peak shapes even on conventional HPLC systems without the need for small particle size columns and high pressure operation with the UPLC system. The gradient program is chosen to achieve a good separation of all 6 substances in a relatively short run time. Additionally, the mobile phase (also used as extracting, column washing, and diluting solvent) was composed of only buffer and acetonitrile, which rendered the method much cheaper than others. The method is suitable for quantitative and qualitative routine control of sildenafil, vardenafil, tadalafil, dapoxetine, yohimbine, and sibutramine in different food supplements and pharmaceuticals.

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