Simultaneous Analysis of Sexual Stimulants and Anabolic Steroids as Adulterants in Dietary Supplements by High Performance Liquid Chromatography with Photodiode Array Detection

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Abstract: Background: The illegal virtual market for food supplements facilitates fraud and adulteration. Worldwide concern for consumer safety is growing on the part regulatory agencies, healthcare professionals and consumers.

Objective: This work aimed to evaluate the presence of sexual stimulants and anabolic steroids commonly used in the adulteration of dietary supplements through the development of a high performance liquid chromatography with the photodiode array detection (HPLC-DAD) method for the identification and quantification of these compounds.

Methods: The mobile phase composed of an ammonium acetate solution, acetonitrile and methanol led to the efficient separation of vardenafil, testosterone base, testosterone propionate, tadalafil, sildenafil and yohimbine.

Results: The assay was linear (r² > 0.999), precise (RSD% < 0.5), accurate (99.1 to 105.2%), and the limits of detection and quantification were less than 0.05 and 0.15 μg/mL, respectively. Four samples of dietary supplements contained testosterone (n=1), tadalafil (n=2) and yohimbine (n=1) as adulterants. The adulterants found were in subtherapeutic doses, probably to reduce possible adverse effects and the action expected to appear natural. Since about 80% of adverse drug reactions are dose dependent, unpredictable adverse drug reactions are dose independent and based on idiosyncratic or allergic mechanisms or intolerance.

Conclusion: The developed method is convenient and easily applicable for adulteration detection of the analyzed drugs in the multicomponent supplements.

Keywords: Adulterants, anabolic steroids, dietary supplements, liquid chromatography with photodiode array, sexual stimulants, dysfunction.

1. INTRODUCTION

Dietary supplements are products that aim to complement the diet of healthy individuals, and may show nutritional, metabolic and physiological effects. Thus, they are generally composed of vitamins, minerals, fibers, proteins, amino acids, fatty acids, herbs and plant extracts, probiotics and other substances [1, 2]. The presence of stimulants, hormones and other substances is considered as doping in the list of substances prohibited by the World Anti-Doping Agency (WADA), as well as substances with therapeutic or medicinal purposes [3].

The large number of products available on the market, which are freely marketed on the internet without proper inspection, has facilitated the occurrence of fraud and adulteration. In addition, they are characterized as food or food supplements, and most of them are exempt from registration [4].

Adulteration is the practice of adding substances to a product, not declaring them as ingredients in order to intensify the desired effect and boost consumption. The registration as a foodstuff product turns the rules on production, quality control of the final product, trade and imports much more lenient than pharmaceutical guidelines. This situation makes the practice of adulteration increasingly easy and recurrent [5, 6].

Sexual stimulants are drugs used to treat erectile dysfunction, which is a medical condition for which the patient has
trouble getting or maintaining an erection. The first three approved inhibitors of the enzyme phosphodiesterase V (PDE-5) are sildenafil citrate (Viagra®, Pfizer), tadalaflil (Cialis®, Eli Lilly) and vardenafil hydrochloride (Levitra®, Bayer) [7]. Sildenafil citrate reached $411 million of sales in 2017 [9]. Anabolic androgenic steroids are hormones derived from testosterone, which is the main anabolic natural androgen [9]. Front to numerous undesirable adverse effects, the therapeutic use of these drugs is restricted to cases of male hypogonadism, Turner's syndrome, breast tumor, refractory anemias, severe catabolic states and in some cases osteoporosis [10]. The abuse of these substances has been reported by elite athletes, mainly those involved in sports of strength and speed. The main aim is to improve physical performance or to change body composition, such as increasing lean mass and reducing subcutaneous fat [11-12]. The use of anabolic androgenic steroids is associated with erectile dysfunction due to the reduction in the production of endogenous testosterone [12]. This justifies the track of the associated adulteration of supplements by sexual stimulants and anabolic steroids in the same product. Studies have pointed to the addition of PDE-5 inhibitors and Anabolic Androgenic Steroids (AAS) as adulterants in dietary supplements [13]. The fraudulent pretenses are to intensify the effect proposed by certain products to increase consumption.

In this scenario, the objective of this work was the simultaneous determination of sexual stimulants (sildenafil, tadalaflil, vardenafil and yohimbine) and anabolic steroids (testosterone base and testosterone propionate) as adulterants in samples of dietary supplements marketed via the internet using a high performance liquid chromatography with photodiode array detection (HPLC-DAD). This research aims to track the practice of adulteration of food supplements, considering the easy access by the illegal virtual market.

2. MATERIALS AND METHODS

2.1. Instrumentation and Apparatus

The chromatographic separations were carried out in an Agilent 1260 Infinity II (Berlin, Germany) HPLC system, which consisted of a G7111B quaternary pump with gradient separation (channels A, B, C and D); an auto-sampler model G7129A; and a multi-channel UV spectrophotometer detector based on diode array technology (WR G7115A Detector) equipped with Agilent OpenLAB® software (version A.04.07.28). The chromatographic runs were conducted at temperature (20 ±0.5 °C) using a reverse-phase C18 column (4.6 × 250 mm, 5 μm; Kinetex) with a C18 guard cartridge (Thermo Scientific® 4.6 × 10 mm, 5 μm). Samples were injected using an injector equipped with a 10 μL loop. The detection was performed at 245 nm for vardenafil, testosterone base and testosterone propionate; and at 290 nm for tadalaflil, sildenafil and yohimbine.

2.2. Reagents and Solutions

Standards of vardenafil, testosterone base, testosterone propionate, tadalaflil, sildenafil and yohimbine were of pharmaceutical grade and were obtained with certificates of analysis. The water was purified using a Milli Q Ultra-Pure Water System (Millipore Synergy® UV, Bedford, USA). HPLC-grade solvents ammonium acetate and methanol were obtained from the Tedla Company (Fairfield, CT, USA). Acetonitrile grade HPLC was also used (J.T. Baker, EUA). Stock solutions (1 mg/mL) of each adulterant standard were prepared using a mixture of acetonitrile and methanol (50:50, v/v) as a solvent. Work solutions (10 μg/mL) were prepared from these solutions in the same solvent.

2.3. Sample Preparation

Dietary supplements (n=50) were purchased online from randomly chosen Brazilian websites. Considering the scope of this work, the search was restricted to products that advertise and market food supplements with claims of "Muscle mass gain", "increased sexual performance", or "increased vasodilation".

The samples were supplied as powders in capsule, tablet or bulk forms. For encapsulated and tablet samples, a pool of 10 items was prepared in order to obtain homogeneous material for analysis. The average weight of each sample (or 0.5 g for samples in bulk) was weighed, dissolved in a 25 mL mixture of acetonitrile and methanol (50:50, v/v) as a solvent, and sonicated for 30 minutes. The sample was then filtered through cotton and regenerated cellulose acetate membrane (0.45 μm). Finally, the extract was diluted at least 10-fold in the mobile phase before injection into the chromatographic system.

2.4. HPLC Conditions

The chromatographic column (C18) was conditioned daily with the mobile phase for 60 min prior to use in experiments. Working solutions were injected using a 10-μL loop injection. At the end of the day, the chromatographic column was washed with a solution of acetonitrile containing ultrapure water (75:25, v/v) for 15 min at a flow rate of 1.0 mL/min; thereafter, the same mixture in the ratio of 90:10 (v/v) for 20 minutes. Besides, in order to avoid the accumulation of particles in the channels of the system, purging with isopropyl alcohol was performed. The analytes were separated by HPLC-DAD using an eluent composition of 30 mM ammonium acetate in water (solvent A) and a mixture of acetonitrile and methanol 50:50 v/v (solvent B). The mobile phase gradient was as follows: 15% A and 85% B (0-4.9 min); 10% A and 90% B (5.0-9 min).

3. RESULTS AND DISCUSSION

3.1. Development of Analytical Conditions

This work proposes to develop an analytical method for the simultaneous quantification of sex stimulants and anabolic steroids in dietary supplements by HPLC-DAD. Photodiode detection allows evaluating the purity of the chromatographic peak through the absorption spectrum generated for each compound, conferring a suitable selectivity to the method. The absorption spectrum scan between 200 and 400 nm was performed in order to determine the appropriate wavelength to each compound. The chosen wavelengths were 245 nm for vardenafil, testosterone base and testosterone propionate; and 290 nm for tadalaflil, sildenafil and yohimbine.
Fig. (1). Chromatogram of the studied adulterants using the proposed method under optimized conditions: Conditions: eluent composition of 30 mM ammonium acetate in water (solvent A) and a mixture of acetonitrile and methanol 50:50 v/v (solvent B); the mobile phase gradient was as follows: 15% A and 85% B (0–4.9 min); 10% A and 90% B (5.0–9 min); C18 column; flow rate 1.0 mL/min.

Table 1. Linearity data, detection and quantification limits for the determination of adulterants by the developed HPLC DAD method.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Linear Range (µg mL⁻¹)</th>
<th>Linear Regression Equation</th>
<th>r²</th>
<th>LOD (µg/mL)</th>
<th>LOQ (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tadalafil</td>
<td>0.08-5.0</td>
<td>y = 2.485.665,57x - 87.919,78</td>
<td>0.9999</td>
<td>0.03</td>
<td>0.08</td>
</tr>
<tr>
<td>Yohimbine</td>
<td>0.15-6.0</td>
<td>y = 1.162.591,70x - 11.322,66</td>
<td>0.9990</td>
<td>0.05</td>
<td>0.15</td>
</tr>
<tr>
<td>Sildenafil</td>
<td>0.11-5.0</td>
<td>y = 1.468.619,63x - 15.449,88</td>
<td>0.9990</td>
<td>0.04</td>
<td>0.11</td>
</tr>
<tr>
<td>Vardenafil</td>
<td>0.11-3.0</td>
<td>y = 2.951.922,30x + 40.633,89</td>
<td>0.9990</td>
<td>0.04</td>
<td>0.11</td>
</tr>
<tr>
<td>Testosterone</td>
<td>0.06-3.0</td>
<td>y = 4.235.450,93x - 7.747,50</td>
<td>0.9999</td>
<td>0.02</td>
<td>0.06</td>
</tr>
<tr>
<td>Testosterone Propionate</td>
<td>0.08-6.0</td>
<td>y = 3.660.678,62x - 47.547,79</td>
<td>0.9999</td>
<td>0.03</td>
<td>0.08</td>
</tr>
</tbody>
</table>

The reverse phase chromatography is ideal for analytes having a molecular weight below 2000, which dissolve only in weakly polar or apolar solvents, such as sildenafil, tadalafil, vardenafil, yohimbine, testosterone base and testosterone propionate [14]. In consequence, a chromatographic column attached to octadecyl groups (C18) with pre-column of the same nature coupled was chosen for the separation. Furthermore, acetonitrile and methanol combined with water or aqueous buffer provide sufficient dipole interactions or hydrogen bonds with solutes to separate a large number of compounds by reverse phase chromatography [14]. The initial conditions applied were a mobile phase "A" composed of a 10 mM potassium phosphate buffer with pH adjusted at 2.3, and a mobile phase "B" containing a mixture of acetonitrile and methanol (1:1). The flow rate was 1.0 mL/min, at a temperature of 24 °C.

The mobile phase gradient can be evaluated through the relationship between the retention times of the compounds and the variation in the composition of the mobile phase. The elution gradient is recommended when Δt/tg > 0.25, otherwise an isocratic elution can be applied if Δt / tg < 0.25. The Δt is the difference between the retention times of the first and the last peak of the chromatogram. The gradient time (tg) is the time that the solvent composition varies.

The first test conditions allowed to separate and identify all compounds. However, the Δt/tg ratio was 0.55, indicating that an elution gradient would be adequate to decrease the time of analysis and improve the efficiency of the method. Besides, the pressure of the equipment has quickly reached the limit supported by the equipment. This way, the ammonium acetate was tested as a mobile phase once that it is more apolar and has a greater affinity with the chosen organic compounds. The mixture containing ammonium acetate, acetonitrile and methanol provided satisfactory retention times for the analysis.

Different pH and ammonium acetate concentrations in the mobile phase, as well as column temperature, were test-
ed. The final separation conditions (as described in Section 2.4) were chosen in order to provide the most satisfactory results in terms of the criteria for proper separation. Fig. (1) shows the chromatogram obtained for the separation of the adulterants by the optimized HPLC-DAD method.

3.2. Validation of the HPLC-DAD Method

The optimized method was validated based on the primary analytical validation parameters: linearity, the limit of detection and quantification, precision, accuracy and robustness [15-16].

The linearity was evaluated from triplicate calibration curves on three different days. The levels of concentration of the standards were set from the sensitivity of the compounds. Analytical curves were constructed by evaluating the relationship between peak area and concentration by linear regression analysis. All analytes presented a correlation coefficient higher than 0.999, showing that there is a directly proportional relationship between the added concentration of each analyte and the area of the chromatographic signal. In all instances, a linear fit was found to be adequate for the purpose. Linearity data was validated using Analysis of Variance (ANOVA), which demonstrated a linear relationship and no significant deviation from linearity (P < 0.05). The sensitivity of the chromatographic system employed was assessed by determining the Limit of Detection (LOD) and the Limit of Quantification (LOQ). The limits of detection and quantification were calculated from the equations: LOD = 3.3 x Sa/b, and LOQ = 10 x Sa/b, where Sa = standard deviation of the intercept, b = slope. Linearity data, detection and quantification limits are summarized in Table 1.

The results for the precision of the proposed method determined as the repeatability (replicates obtained on the same day) and the intermediate precision performed on different days are shown in Table 2. The acceptance criterion of RSD <5 (n=6) for repeatability and RSD <5 (n=12) for intermediate precision was achieved for all compounds. In addition, the intermediate precision was evaluated statistically through the F-test. The data confirm that there was no significant difference between the results obtained for both tests according to the applied F test, where F values of 2.03, 0, 13, 0.44, 0.59, 0.86 and 0.05 for tadalafil, yohimbine, sildenafil, vardenafil, testosterone base and testosterone propionate, respectively, with 4.256 being the tabulated F value (P < 0.05).

The accuracy was assessed by the standard addition method (n = 3), which was applied in three randomly chosen samples free of the compounds evaluated by the method. Student's t-test was applied to verify the accuracy. The results show that there was no significant difference between the concentrations added and the concentrations recovered by the method., since the t-values found were lower than the tabulated value (2.776, P < 0.05).

Precision and accuracy results are in accordance with the AOAC (2013) requirements for validation experiments in botanicals and dietary supplements, considering the studied concentration levels [15].

The robustness test was performed in order to evaluate the ability of the method to resist small changes in the analytical parameters. The method changes were mobile phase pH, mobile phase composition, mobile phase flow, and column temperature. The same solution used for the intermediate precision test was submitted to the method variations, in triplicate. Each alteration was evaluated by the average of the chromatographic peaks retention time of the standards. The evaluated changes did not affect the performance of the method, confirming the robustness of the method in the range tested.

3.3. Interferents

Dietary supplements have infinite possibilities for combining ingredients, from their excipients to active compounds, and even undeclared ingredients. Its matrix complexity requires studies of selectivity of the analytical method. Thus, findings of different possible interferents were proposed. The anorexigens (sibutramine, amfepramone and fenproporex), antidepressants (fluoxetine and paroxetine), adrenergic amines (caffeine and synephrine), diuretics (furosemide, hydrochlorothiazide and chlorothalidone), laxatives (phenolphthalein and bisacodyl) and DHEA were tested as interfering in the method specificity test. The compounds were chosen based on reports of these adulterants in food supplements.

### Table 2. Experimental values obtained in the precision and the recovery test for the determination of adulterants by the developed HPLC-DAD method.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Repeatability RSD%* (n=6)</th>
<th>Intermediate Precision RSD%* (n=12)</th>
<th>Recovery (mean ±SD, %)**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tadalafil</td>
<td>0.14</td>
<td>0.11</td>
<td>103.0 ±0.4</td>
</tr>
<tr>
<td>Yohimbine</td>
<td>0.23</td>
<td>0.04</td>
<td>103.9 ±0.3</td>
</tr>
<tr>
<td>Sildenafil</td>
<td>0.18</td>
<td>0.06</td>
<td>102.7 ±0.1</td>
</tr>
<tr>
<td>Vardenafil</td>
<td>0.20</td>
<td>0.08</td>
<td>104.5 ±0.2</td>
</tr>
<tr>
<td>Testosterone</td>
<td>0.16</td>
<td>0.07</td>
<td>99.1 ±0.1</td>
</tr>
<tr>
<td>Testosterone Propionate</td>
<td>0.47</td>
<td>0.06</td>
<td>102.5 ±0.3</td>
</tr>
</tbody>
</table>

*RSD%* = relative standard deviation, **results are triplicates for three levels of concentration.
Table 3. The composition of adulterated formulations (as labelled by manufacturers) and information on the origin, claim, doses and adulterant content in products determined by the developed HPLC-DAD method.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Formulation Description</th>
<th>Claim</th>
<th>Origin</th>
<th>Adulterant</th>
<th>Drug Found (mg±RSD/capsule)</th>
<th>Calculated Ingested Doses (mg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Calcium alanine chelate, calcium arginine chelate, magnesium carbonate, and ascorbic acid.</td>
<td>Muscle builder</td>
<td>Brazil</td>
<td>Testosterone base</td>
<td>0.97 ±0.30</td>
<td>3.9</td>
</tr>
<tr>
<td>B</td>
<td>Tribulus terrestris</td>
<td>Increases testosterone level</td>
<td>Brazil</td>
<td>Tadalafil</td>
<td>0.05 ±3.54</td>
<td>0.20</td>
</tr>
<tr>
<td>C</td>
<td>Dehydroepiandrosterone (DHEA)</td>
<td>Promotes a balanced hormone level</td>
<td>USA</td>
<td>Tadalafil</td>
<td>0.10 ±0.56</td>
<td>0.10</td>
</tr>
<tr>
<td>D</td>
<td>Caffeine, theobromine, Citrus aurantium and yohimbine</td>
<td>Fat burner</td>
<td>USA</td>
<td>Yohimbine</td>
<td>0.07 ±1.30</td>
<td>0.15</td>
</tr>
</tbody>
</table>

Fig. (2). Chromatogram obtained for sample ‘A’ containing testosterone base as an adulterant. Sample composition is as described in Table 3. Other conditions are as described in Figure 1.

Each compound was injected individually to determine the retention time and the UV absorption spectrum. Subsequently, the interferents were injected simultaneously with the compounds of interest at 245 nm and 290 nm. Only the amfepramone peak partially overlaps the peak of the testosterone base at 245 nm. However, the selectivity of the method was not affected, since the peaks can be differentiated by the absorption spectrum.

3.4. Evaluation of Counterfeit Products

Worldwide, products sold for weight loss, gaining muscle mass and increasing libido are among the most fraudulent dietary supplements. Sexual stimulants and anabolic steroids are substances often reported in this type of adulteration [7-9, 13, 17-19]. Herein, fifty samples of dietary supplements were tracked by the developed method.

Table 3 shows the composition of the adulterated formulation (as labelled by manufacturers) and information of origin, claim, doses and the adulterant found by the developed method. The determination of the adulterants in the samples was evaluated according to the following analytical criteria: Retention Time (RT) and Ultraviolet (UV) absorption spectrum. Sample A presented RT (Fig. 2) and spectra compatible with a testosterone base, as well as samples B and C with tadalafil, and sample D with yohimbine. The quantification of the adulterants was based on the linear regression equation obtained by plotting the peak area responses versus concentrations.

Sample A presented testosterone in a dose used clinically. In women, the dosage applied in androgenic therapy with oral testosterone ranges from 1.25 mg to 2.5 mg per day. Adulterated dietary supplements can cause hepatotoxicity,
androgynic effects in women, including acne, increase the facial hair, changes in voice tone, amenorrhea, and aggravation of symptoms caused by polycystic ovarian syndrome, in case of gestation, virilization of the female fetus may occur. In addition, testosterone has several serious drug interactions, among them, it potentiates the effect of anticoagulants [20]. The risks are unpredictable, since the consumer does not know that they are ingesting this hormone. Sample A claims that it stimulates the nitric oxide production, promoting vasodilation. Besides, its website claims a fast gain of muscle mass, improvement of strength and increase of resistance to exercises.

Tadalafil was in subtherapeutic doses in analyzed dietary supplements (samples B and C), compared to the approved pharmaceutical dosage forms. Probably, deliberate contamination was malicious in order to increase the effectiveness of the product. Tribulus terrestris containing products, such as sample B, claim to promote the natural increase of testosterone, gain of strength and muscle mass, loss of body fat, increase in libido and sexual performance. Sample C contains dehydroepiandrosterone that is a precursor to testosterone hormone, which labels the same claims.

Common side effects of tadalafil include headache, low back pain, myalgia, dyspepsia, gastroesophageal reflux, facial flushing, and nasal congestion. Besides, hypotension may occur in patients taking antihypertensives. The tadalafil required caution to patients with cardiovascular problems, stroke, kidney problems or those requiring dialysis [21].

Sample D claims to increase hormone levels responsible for burning fat, control appetite, speed up metabolism and lose weight in a healthy way. It contained yohimbine at a daily dose of 0.15 mg, which is a drug with mandatory registration. The adverse drug reactions often are dose-dependent and related to known pharmacologic actions of the drug [23]. Probably this is the reason that most adulterants are added in subtherapeutic doses once that the effects appear in a mild and natural way. However, there are unpredictable adverse drug reactions based on idiosyncratic or allergic mechanisms or intolerance. These reactions are dose-independent and can cause harm to the consumer [24]. In this way, tampered products, even in subtherapeutic doses, can generate adverse reactions. Intolerance can occur if an individual presents a diminished threshold for the drug pharmacological action. Hypersensitivity reactions usually do not occur at the first exposure of the medicine once a previous sensitization period is required. The allergic re-exposure will result in a new reaction generally faster than the previous one [22-23]. Thus, the correct conduct should be the immediate suspension of the ingestion of the suspect product.

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The declared composition of analyzed food supplements can be divided into: 34% vitamin and mineral products, 24% protein based products, 10% food replacement products, 8% libido stimulants, 8% prehormonal, 6% hypercaloric, 6% thermogenic, and 4% middle chain triglycerides. Its most frequent ingredients were zinc \( (n = 18) \), vitamin B6 \( (n = 18) \), maltodextrin \( (n = 17) \), magnesium \( (n = 14) \), and concentrated whey protein \( (n = 12) \).

The market of food supplements consists of products with an asymmetry of information regarding their risks and benefits. Among the dietary supplements analyzed \( (n = 50) \), 42% presented regulatory irregularities. The main nonconformities were unregistered products, the presence of unauthorized ingredients and claims of unproven therapeutic properties. The adulteration process becomes facilitated and recurring because of this scenario of irregularities.

**CONCLUSION**

Liquid chromatography with DAD detection was applied for the simultaneous determination of sexual stimulants (sildenafil, tadalafil, vardenafil and yohimbine) and anabolic steroids (testosterone base and testosterone propionate) commonly used in the adulteration of dietary supplements. Liquid chromatography with DAD detection was applied for the simultaneous determination of sexual stimulants (sildenafil, tadalafil, vardenafil and yohimbine) and anabolic steroids (testosterone base and testosterone propionate) commonly used in the adulteration of dietary supplements. The developed HPLC-DAD method proved to be sensitive, linear, accurate, and reproducible for adulteration detection in dietary supplements. From fifty dietary supplements, four samples contained testosterone, tadalafil and yohimbine as adulterants. The adulteration of dietary supplements by synthetic drugs seems to be a growing trend. Regulatory agencies, healthcare professionals and mainly consumers must be aware of the potential risks from adulterated dietary supplements.

**ETHICS APPROVAL AND CONSENT TO PARTICIPATE**

Not applicable.

**HUMAN AND ANIMAL RIGHTS**

No animals/humans were used for studies that are basis of this research.

**CONSENT FOR PUBLICATION**

Not applicable.

**AVAILABILITY OF DATA AND MATERIALS**

Not applicable.

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**CONFLICT OF INTEREST**

The authors declare no conflict of interest, financial or otherwise.

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