Identification by Liquid Chromatography-Mass Spectrometry of Herbal Food Supplements Adulterated with PDE-5 Inhibitors

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The aim of this study is to develop a new, easy and efficient analitical method, in order to perform the screening of herbal food supplements for improving the quality of sexual life, suspected to be adulterated with phosphodiesterase-5 inhibitors (PDE-5 inhibitors). PDE-5 inhibitors are substances such as: sildenafil (Viagra®), tadalafil (Cialis®), vardenafil (Levitra®) and more recently, avanafil (Stendra™), used in the treatment of erectile disfunctions. In recent years, there have been many cases reported where food supplements for enhancing sexual performance, promoted as „100% natural", turned out to be adulterated with this type of substances or their analogues. On the label of this sort of products such substances are not mentioned, and thus, serious health problems can be caused to consumers, because PDE-5 inhibitors interact with nitrates based drugs. For this reason, a number of 26 herbal supplements found on the Romanian market were analysed using ion-spray liquid chromatography/tandem mass spectrometry (LC-MS/MS). These supplements have originated from various countries, such as: China, Canada, Japan, United Kingdom, USA, France, India and Romania. The identification of these pharmaceutically active substances was performed by selecting the specific molecular ions for each compound: sildenafil (parent ion 475.2, product ions: 311.2, 299.1, 255.1), tadalafil (parent ion 390.2, product ions: 268, 169, 135) and vardenafil (parent ion 489.2, product ions: 312.1, 299, 284). As a result of the screening of these products, 18 of them were identified having PDE-5 inhibitors in their composition, which means that 69% of analysed herbal food supplements, legally entered and marketed on the Romanian market, are adulterated.

Keywords: herbal food supplements, adulteration, PDE-5 inhibitors, LC-MS/MS

Food supplements represent a special category of products found at the borderline of medicines and food [1]. The consumption of this type of products has an alarming growth, due to the false impression that herbal supplements: are 100% natural, do not cause side effects, and are safe for human consumption. Together with this increasing consumption, herbal food supplements used to enhance sexual performance has also increased, being most frequently adulterated with PDE-5 inhibitors (sidenafil, tadalafil, vardenafil) and also their analogues. The manufacturers „spiked" herbal suplements by adding PDE-5 inhibitors and their analogues, in order to increase the efficacy of the products, thus bigger sales. They also continuously synthesize new analogues, modified from a structural point of view, in order to make the detection of their presence in the product formula more difficult [2]. According to literature data, in the year 2012, a number of 46 analogues were reported, whereas nowadays this number has incresed to approximately 50 PDE-5 inhibitors analogues detected in food supplements [3, 4].

Adulterated herbal supplements that are mislabeled can be the source of serious medical incidents, especially for consumers that suffer from atherosclerosis, hypertension or diabetes, for which PDE-5 inhibitors are forbidden. Even more dangerous is the adulteration of these supplements for increasing sexual performance, with PDE-5 inhibitors analogues, because there are no clinical studies on the effectiveness, toxicity, possible side effects or safety profiles [5]. The most common adverse reactions induced by the three PDE-5 inhibitors are: headache, flushing, dyspepsia, nasal congestion, dizziness, myalgia, and back pain, abnormal vision [6].

Food and Drug Administration (FDA) has defined adulterated foods as being food that „contains any poisonous or deleterious substances, such as chemical contaminants, which may ordinarily render it harmful to health" [7]. Herbal food supplements can thus be considered as adulterated if: a) contain ingredients that can lead to „a significant or unreasonable risk of illness or injury", when used according to the label instructions or in case that from the label proper reccomendations of usage are missing; b) contain ingredients that have not been evaluated „for offering reasonable assurement", that indicate they do not pose ilness or damage risks and do not represent an iminent danger for public health [8].

In recent years, the Internet has become the most complex supply chain at a worldwide level with: „lifestyle" drugs (Viagra, Cialis, Levitra) for the treatment of erectile disfunction; recreation use products that include dangerous combination of PDE-5 inhibitors with 3,4-methylenedioxymethamphetamine (MDMA/ecstasy); but also herbal food supplements (promoted as 100% natural), that are adulterated with PDE-5 inhibitors or their analogues. This type of illegal trade that takes place on a large scale causes serious problems on public health of potential consumers [9].

According to literature data, analysis methods used so far for determining undeclared substances in herbal food

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LC	Agilent 1200 Infinity Series
Column	Zorbax C18, 50 mm × 2.1 mm, 1.8 μm
Column temperature (°C)	45
Mobile phase	A – 4 mM of ammonium format in water + 0.05% formic acid B – 4 mM of ammonium format in methanol + 0.05% formic acid
Column flow	0.3 mL/min.
Gradient	0 min. 40% B, 0 – 4.1 min. 90% B, and held 3.9 min, then canged to 40% B at 8.1 min
Injection vol.	2 μL
MS	Agilent 6410 LC/MS Triple Quadrupol
Ionization	ESI(+)
Capillary	3500 V
Nebulizer pressure	35 psi
Drying gas	8 L/min
Gas temperature	350 °C
Nozzle voltage	500 V
Collision gas	Nitogen

Table 1
LC-MS/MS INSTRUMENT PARAMETERS

supplements used for improving sexual life are: TLC, HPLC and GC-MS [10], LC/MS/MS [11], NMR [12], LC-HR/MS [13], HPLC-DAD, HPLC-MS, RAMAN spectroscopy [14].

In this study, a number of 26 products aiming to enhance sexual performance were analysed by LC-MS/MS method, in order to identify the PDE-5 inhibitors adulterants. The samples originate from different countries, on almost all the continents, therefore: 8 products from Europe, 12 from Asia and 6 from North America.

Experimental part

Reference substances/ pure samples were: tablets of Viagra® (Pfizer Limited, for sildenafil 50 mg), tablets of Cialis® (Lilly, for tadalafil 10 mg) and Levitra® (Bayer, for vardenafil 10 mg). Methanol, acetonitrile, and formic acid used as solvents were provided by Merck. Ammonium formate was purchased from Sigma-Aldrich. Food supplement samples were supplied by the National Office of Medicinal, Aromatic Plants and Bee Products, or have been bought from the Romania market.

All the analyzed products were solid powder encapsulated (17 products) or compacted in tablets (9 products). Each capsule was emptied and each tablet was crushed. Every 200 mg of fine powder was mixed with 10 ml of 1:1 (v/v) acetonitrile/water. Samples were mixed thoroughly by vortexing, followed by 30 min of sonication and 5 minutes centrifugation at 4000 rpm. The supernatant was collected and filtered by 0.22 μm membrane filters.

The tablets of Viagra, Levitra and Cialis, used as reference substances were crushed and every 10 mg of fine powder extracted with 25 mL acetonitrile. The obtained standard solutions were mixed by vortexing followed by 30 min of sonication at room temperature and 5 min centrifugation at 4000 rpm. These solutions were serially diluted in acetonitrile/water 1:1 (v/v) to 25 ng/mL, 100 ng/mL, 250 ng/mL, 500 ng/mL and 1.0 μg/mL acetonitrile. The calibration curves for liquid chromatography tandem mass spectrometry were obtained by injecting the reference solutions and fitting the data by linear regression.

The measurement was performed on an Agilent 6410 LC/MS Triple Quadrupole with an electrospray source equipped with Agilent 1200 Infinity Series LC system. The operating conditions of the LC-MS/MS, including the chromatographic column and mobile phase gradient, are provided in table 1. This method used was adapted from F. Song et al. 2012 [15].

Results and discussions

All the 26 analysed products, and also the obtained results after applying the LC-MS/MS method are described in table 2. These herbal supplements aiming to improve

Sample	Product description	Country of origin	PDE-5 inhibitors
1	capsules	China	-
2	tablets	Canada	Tadalafil
3	capsules	China	-
4	capsules	China	-
5	tablets	Japan	-
6	tablets	United Kingdom	Sildenafil
7	tablets	China	-
8	tablets	SUA	Tadalafil, Sildenafil
9	capsules	China	Sildenafil, Tadalafil, Vardenafil
10	tablets	SUA	Sildenafil
11	tablets	SUA	Sildenafil
12	capsules	United Kindom	Sildenafil, Vardenafil
13	capsules	France	Sildenafil
14	tablets	Romania	Sildenafil
15	capsules	China	Sildenafil
16	capsules	India	Sildenafil
17	tablets	Japan	Tadalafil
18	capsules	SUA	Tadalafil, Sildenafil
19	capsules	Romania	Sildenafil
20	capsules	France	Sildenafil
21	capsules	United Kindom	Sildenafil
22	capsules	China	-
23	capsules	Canada	-
24	capsules	United Kindom	-
25	capsules	China	Vardenafil
26	capsules	China	Vardenafil

Table 2
IDENTIFICATION OF SILDENAFIL, TADALAFIL AND VARDENAFIL IN HERBAL FOOD SUPPLEMENTS BY LC-MS/MS

Compound name	Precursor ion	Product ion	Dwell time (ms)	Fragmentor Potential (V)	Collision Energy (V)
Sildenafil	475.2	311.2	20	110	28
Sildenafil	475.2	299.1	20	110	36
Sildenafil	475.2	255.1	20	110	40
Tadalafil	390.2	268	20	90	4
Tadalafil	390.2	169	20	90	36
Tadalafil	390.2	135	20	90	16
Vardenafil	489.2	312.1	20	162	40
Vardenafil	489.2	299	20	162	40
Vardenafil	489.2	284	20	162	50

Table 3
MASS SPECTROMETER PARAMETERS FOR ALL 3 COMPOUNDS OF INTEREST – PRODUCT IONS

sexual performance have been labelled as natural products that contain only plants. The majority of plants used in these products mainly originate from Asian areas. Therefore, regardless of the country of origin of the food supplement, in over 60% of cases, they contain specific Asian plants. The most frequent herbs that are found in the products used in order to enhance sexual life, analysed in this study, are: Epimedium leaf (*Epimedium grandiflorum*), Cuscuta seed (*Cuscuta sinensis*), Gingko leaf (*Ginkgo biloba*), Wolfberry fruit (*Lycium barbarum*), Reishi mushroom (*Ganoderma lucidum*), Amla (*Emblica officinalis*), Saw Palmetto berry (*Serenoa repens*), American and Asian Ginseng root (*Panax quinquefolius*/ *Panax ginseng*), *Mucuna pruriens* seeds, *Tribulus terrestris* fruit, *Ocimum sanctum* whole plant, *Polygonum multiflorum*, Glycyrrhiza rhizome (*Glycyrrhiza glabra*), *Angelica sinesis* root and others.

The identification of the 3 compounds of interest was made with the help of ESI system in positive mode. For the achievement of this screening, the selected parameters for the mass spectrometer are shown in table 3.

The 5 levels of concentrations, 25, 100, 250, 500 si 1000 ng/mL, were used in order to obtain the calibration curves specific to each reference substance; the curves were linear. The correlation coefficients for sildenafil, tadalafil and vardenafil are 0.9993, 0.9995 (fig. 1), and 0.9994.

The observed retention time for each compound during this investigation, and also during the scanning of mass spectrum used for identification, was of 4.8 min. for sildenafil, 5.6 min. for tadalafil and 4.9 min. for vardenafil.

After analysing the 26 samples, 18 products were found adulterated with sildenafil, tadalafil, vardenafil. Thus, in the composition of 10 food supplements was identified sildenafil (fig. 2), in 2 of them vardenafil, and 2 more were detected as being "enriched" with tadalafil (fig. 3).

The rest of 4 herbal supplements aimed to improve sexual performance were adulterated with combinations of PDE-5 inhibitors, such as: sildenafil and tadalafil (fig. 4) – substances detected in 2 products; sildenafil and vardenafil (fig. 5) – 1 product, and all of the 3 PDE-5 inhibitors (fig. 6) were identified in only one product of the samples analysed.

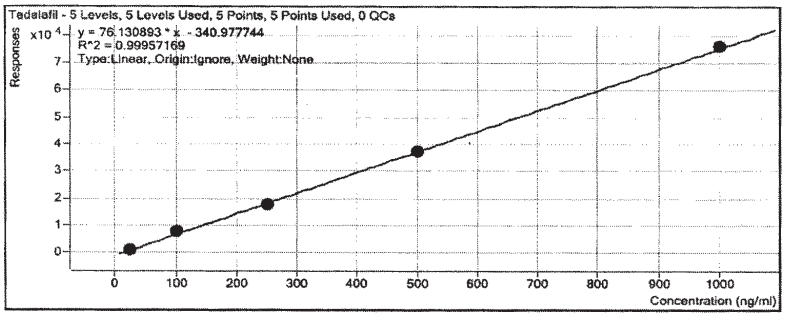

Fig. 1. Calibration curve of reference substances from liquid chromatograph tandem mass spectrometry: tadalafil

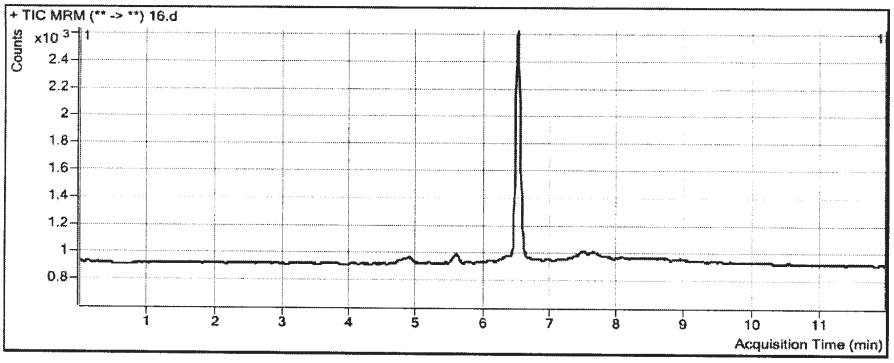

Fig. 2. Total ion chromatogram for sildenafil in sample 16 by LC-MS/MS

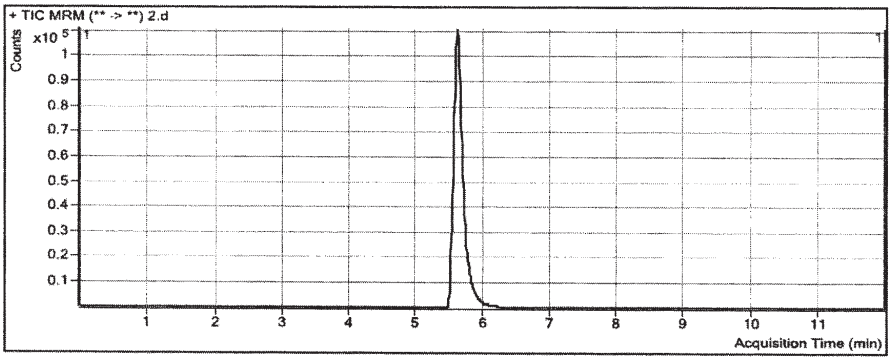

Fig. 3. Total ion chromatogram for tadalafil in sample 2 by LC-MS/MS

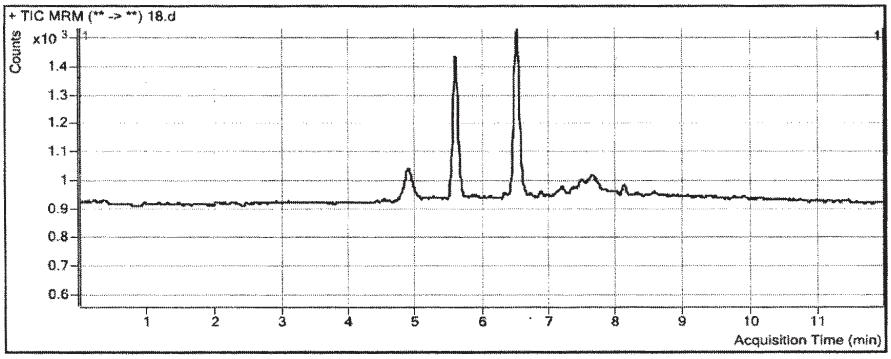

Fig. 4. Total ion chromatogram for sildenafil and tadalafil in sample 18 by LC-MS/MS

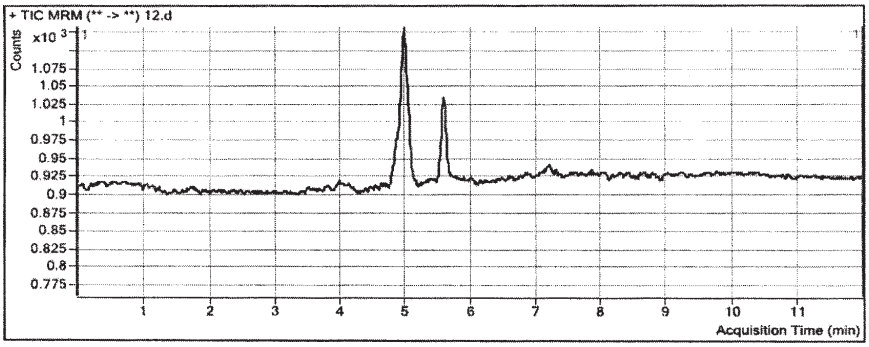

Fig. 5. Total ion chromatogram for sildenafil and vardenafil in sample 12 by LC-MS/MS

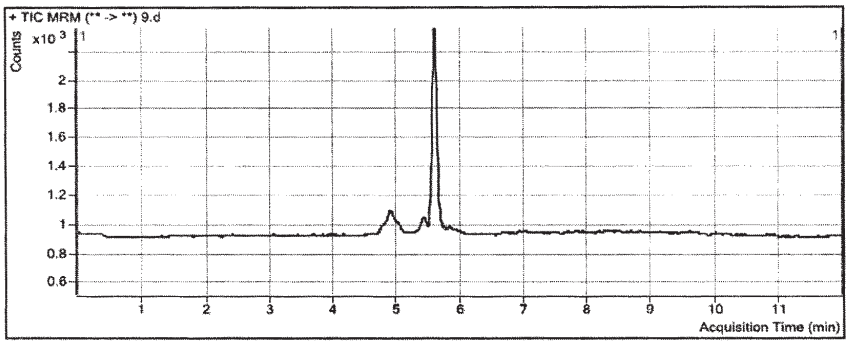

Fig. 6. Total ion chromatogram for sildenafil, tadalafil and vardenafil in sample 9 by LC-MS/MS

In the composition of the analysed herbal food supplements, the range of pharmaceutical active compounds is relatively small, from 25 to 665 ppb. Nevertheless, the risks to which consumers are exposed to are significant, because, under normal conditions, these types of products are recommended to be ingested during a longer period of time (3 months).

In these conditions, the effect of these products can be cumulative, but in the same time, they interact with consumers' diet, when simultaneously ingested with nitrates based medicines, leading to serious side effects. Another problem that appeared in this study is that the manufacturers of food supplements do not stop "spikinig" their products with only one substance, but, as we can see, they add two or even three pharmaceutical active substances, to get an almost immediate and efficient outcome.

Conclusions

LC-MS/MS is a sensitive and selective method that can be succesfully used for the screening of a large number of food supplements based on medicinal, aromatic plants, to detect adulterants, having a very short analysis time, of approximately 15 min, compared to the ones reported in literature, that range from 20 to 30 min , for just one sample. This method will be optimised and tested, aiming to a quantitative determination, but also the detection of novel analogues. In the future, this method can be applied in the monitoring process of PDE-5 inhibitors found on Romanian market of herbal supplements.

An alarming signal is the fact that, after this screening, 69% of the analysed products, that are found on the Romanian market, turned out to be adulterated.

Generally speaking, adulteration has serious consequences for the consumer health because the undeclared pharmaceutical substances from adulterated products could interact with some medications (PDE-5 inhibitors have potentially fatal interaction with drug nitrates). On the other hand, the consumer becomes a true victim because he is not warned by the product label, because that does not contain recommendations for use, or medical advice for special categories of vulnerable persons.

This screening of all herbal food supplements advertised as sexual performance enhancers proved to be necessary for the detection of PDE-5 inhibitors before the products' entries on the market.

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