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Analysis of undeclared synthetic phosphodiesterase-5 inhibitors in dietary supplements and herbal matrices by LC–ESI–MS and LC–UV

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Abstract

A liquid chromatography–electrospray ionisation–mass spectrometry (LC–ESI–MS) method was developed to screen for the presence of synthetic phosphodiesterase type 5 (PDE-5) inhibitors including sildenafil, tadalafil and vardenafil. The method was applied to the analysis of dietary supplements and bulk herbal materials. Bulk powders or composites of tablets, capsules or liquids were prepared and an extraction of PDE-5 inhibitors was performed using a mixture of acetonitrile and water with sonication. Identification of sildenafil, vardenafil or tadalafil was accomplished using a single quadrupole mass spectrometer coupled to a liquid chromatograph with an electrospray interface. Positive ion detection in the full scan mode was used while in-source collision induced dissociation (CID) provided several structurally significant fragment ions to aid in the mass spectral identification. Approximately half of the 40 botanical products analyzed were found to contain undeclared synthetic PDE-5 inhibitors. For products found to contain one of these three compounds by LC–MS, HPLC with UV detection was used for quantitation.
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Keywords: Dietary supplements; Phosphodiesterase-5; Sildenafil; Vardenafil; Tadalafil; Electrospray; Mass spectrometry; Reversed-phase liquid chromatography

1. Introduction

During the early 1990s, a drug designed to inhibit the enzyme phosphodiesterase-5 (PDE-5) was undergoing phase I and II studies as a potential anti-anginal agent. Investigators were surprised that many of the trial subjects refused to return their unused tablets. It turned out that many of the men in the study found that their previous erectile dysfunction was resolved. The drug was later given the name sildenafil citrate (Viagra®), and until August 2003, it was the only phosphodiesterase-5 inhibitor licensed for the treatment of erectile dysfunction [1]. Recently, two other PDE-5 inhibitors have gained notoriety. Vardenafil hydrochloride (Levitra®) and tadalafil (Cialis®) have been approved for use in both Europe and the United States. In addition, these drugs can be legally obtained only with a doctor's prescription.

The use of herbal remedies and dietary supplements has increased greatly during the past 40 years as an alternative to conventional medicines. There is widespread belief that natural ingredients are inherently safer and healthier than are synthetic ingredients. It should be noted that over 30% of modern pharmaceuticals are derived from natural botanical sources [2]. While the debate concerning the potential health benefits versus the potential risks associated with taking dietary supplements is seemingly endless, it is clear that the adulteration of these products with undeclared, synthetic pharmaceuticals poses a serious health risk. In this study alone, approximately 20 different "all natural" dietary supplements were found to contain synthetic PDE-5 inhibitors.

The structures of sildenafil and vardenafil exhibit only minor differences, while tadalafil differs markedly in terms of its molecular structure. Pharmacological studies indicate that interactions between PDE-5 inhibitors and certain prescription drugs containing nitrates (such as nitroglycerin) may drastically lower blood pressure. Nitrate medications are commonly used by people with diabetes, hypertension,

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hyperlipidemia and ischemic heart disease. Furthermore, sexual dysfunction is often associated with these conditions. Since the use of PDE-5 inhibitors is contraindicated in these patients, some may look to alternative medicines such as dietary supplements for treatment. The possibility of an individual taking nitrates in combination with supplements that have been adulterated with synthetic PDE-5 inhibitors may have serious health consequences [3]. Therefore, it is necessary to develop analytical methods that can selectively and simultaneously screen for the presence of these drugs in complex matrices such as those associated with herbal products.

Several authors have recently reported the adulteration of dietary supplements or functional foods with synthetic PDE-5 inhibitors. Tseng and Lin identified the presence of sildenafil in a dietary supplement capsule using electrospray LC–MS [4]. Shin et al. identified a sildenafil analog (homosildenafil) added illegally to a functional food marketed for erectile dysfunction [5]. This was accomplished using a combination of NMR, infrared spectroscopy and mass spectrometry. Mikami et al. developed an LC–MS method for the determination of sildenafil and phentolamine in adulterated soft drinks [6]. In addition, several authors have published methods for the detection and/or determination of individual PDE-5 inhibitors [7–11]. The present paper describes an LC–MS method for the simultaneous identification of sildenafil, vardenafil and tadalafil and discusses the LC–UV methods used for their quantitative determinations.

2. Experimental

2.1. Materials

Standard reference material of sildenafil citrate was received from Pfizer Inc. (New York, NY). Vardenafil hydrochloride reference material was received from Bayer Corporation (West Haven, CT). Tadalafil reference material was received from Eli Lilly and Company (Indianapolis, IN). Acetonitrile (HPLC grade) was purchased from Fisher Scientific (St. Louis, MO). Formic acid (FA) (88%) was purchased from Sigma–Aldrich (St. Louis, MO). All other reagents used were analytical grade. The water used was 18 MΩ deionized water (Millipore, Billerica, MA). Syringe filters were 25 mm nylon with a pore size of 0.2 μm and were purchased from Fisher Scientific.

2.2. LC–MS sample and standard preparation

Stock solutions of standards were prepared at concentrations of approximately 2 mg/mL in 50:50 acetonitrile:water and stored in the refrigerator. Working standards (5–25 μg/mL) were prepared fresh daily by diluting stock standards with acetonitrile. Sample preparation of capsules or tablets typically consisted of compositing 5–10 dosage units. Tablets were ground using a mortar and pestle and capsule contents were emptied and mixed using a vortex mixer. Approximately one dosage unit of the composite material was weighed and transferred to a glass scintillation vial. The sample was extracted in 5–10 mL of 50:50 acetonitrile:water with sonication for 15 min. A portion of the extract was filtered through a 0.2 μm nylon syringe filter and further diluted with acetonitrile prior to analysis. For bulk materials, between 0.1 and 0.2 g were generally weighed and extracted as described above. In the case of liquid samples, a sample volume equivalent to one dose was diluted with acetonitrile and analyzed. The use of acetonitrile as a diluent and extraction solvent was necessary due to poor solubility of tadalafil in methanol.

2.3. LC–MS instrument parameters

The operating conditions of the LC–MS, including the chromatographic column and mobile phase gradient, are provided in Table 1. The run time for the total analysis was 25 min, including a column equilibration step between samples.

2.4. LC–UV analyses of sildenafil citrate

The method used was adapted from the monographs "Sildenafil Citrate" and "Sildenafil Tablets" [12–13]. The separation was done on a Kromasil C4 column, 4.6 mm × 25 cm (Phenomenex). The mobile phase consisted of 68:28:4 H_2O:acetonitrile:buffer, where the buffer was composed of 0.5 M KH_2PO_4/0.01 M diethylamine in water, pH 4.5. The flow rate was 1.5 mL/min with detection at 230 nm. Typically, a 0.25 mg/mL stock solution of sildenafil citrate was prepared in mobile phase, and was serially diluted to produce solutions of 0.125 and 0.050 mg/mL. The calibration plot was obtained from these solutions and typically gave a correlation coefficient of 0.9999.

The results of the LC–MS experiments for each sample provided an estimated level of sildenafil citrate in terms of mg per g sample. A portion of sample, equivalent to 10 mg sildenafil citrate, was placed in a 100.0 mL volumetric flask, and approximately 50 mL mobile phase were added. The solution was shaken for 15 min, diluted to volume, and mixed. An aliquot was filtered through a 0.2 μm Nylon syringe filter prior to injection.

To prepare spiked solutions, approximately 10 mg of sildenafil citrate reference standard were added to the portion of sample composite. This spiked sample portion was prepared as above, and was diluted prior to injection, if necessary.

2.5. LC–UV analyses of tadalafil

The method used for the quantitation of tadalafil was adapted from a proprietary method, provided by Eli Lilly, which was developed for the analysis of Cialis®. Because the method is proprietary, the specific conditions used for the extraction and separation of tadalafil cannot be provided here.

Table 1
LC–MS instrument parameters

LC	Agilent 1100 series
Column	Zorbax stable bond C-18, 150 mm × 2.1 mm, 5 μm
Solvent A	18 MΩ deionized water (0.1% formic acid)
Solvent B	Acetonitrile
Gradient	0–5 min, 15% B; 5–15 min linear to 90% B; 5 min hold at 90% B
Column temperature (°C)	30
Flow (μL/min)	400
Injection volume (μL)	1
MS	Agilent 1100 MSD, VL
Ionization	Electrospray (positive ion)
Scan range	130–500 amu
Drying gas	Nitrogen, 10 L/min at 300 °C
Nebulizer gas	Nitrogen, 20 psi
Fragmentor (V)	115
V_{cap} (V)	2000

The tadalafil reference standard was dissolved in sample solvent to produce standard solutions of 0.5, 0.25, 0.1 and/or 0.05 mg/mL tadalafil. Depending on the estimated level of tadalafil obtained from the LC–MS experiments, the calibration plot used three of these standard solutions. The correlation coefficient for the calibration plot was typically 0.9999.

The equivalent of one unit dose of product was placed in a 50.0 mL volumetric flask, and approximately 30 mL sample solvent was added. The solution was shaken for 15 min, diluted to volume and mixed. The mixture was either centrifuged at 2000 × *g* for 10 min (Marathon 2100K/R; Fisher Scientific), or was filtered using 0.2 μm syringe filters.

To determine analyte recovery, tadalafil reference standard was added to another portion of the sample composite, at a level between 50% and 150% of the unit dose. For example, a product with an average tablet weight of approximately 0.5 g was assayed to contain tadalafil at a level of 8 mg/tablet. Analyte recovery was determined by weighing approximately 0.5 g of tablet composite followed by the addition of 6.3–9.3 mg of tadalafil reference standard, then treated as described above.

2.6. LC–UV analyses of vardenafil hydrochloride

Because no vardenafil has been detected in any dietary supplements to date, no separate method has been developed for the quantitation of vardenafil. However, the reference standard is observed in the LC–UV methods for sildenafil and tadalafil, and demonstrates retention times that differ from the other two PDE-5 inhibitors.

3. Results and discussion

3.1. LC–MS method optimization

Prior to this study, the analysis of sildenafil in finished pharmaceuticals in our laboratory was accomplished using LC–UV for initial identification and quantitation, while FTIR was generally used as a confirmatory technique. The appearance of other PDE-5 inhibitors in the marketplace, and the potential for them to be present in more complicated matrices like those associated with herbal supplements, made LC–MS a more suitable identification technique.

Initially, separations were attempted under isocratic conditions using acetonitrile or methanol with water containing 0.1% formic acid. Although the three compounds of interest were resolved, many of the herbal matrices evaluated required a gradient to force elution of the more hydrophobic sample components. It was also observed that the presence of methanol in the mobile phase generated significantly more background noise compared to acetonitrile. A linear solvent gradient using acetonitrile and water (0.1% FA) provided the most suitable mobile phase in terms of analysis time, resolution and sensitivity.

Using the flow injection analysis mode, MS parameters were optimized for sildenafil, vardenafil and tadalafil by adjusting the four major ESI parameters: the capillary voltage, the nebulizer gas pressure, the drying gas flow rate and the fragmentor voltage. Significant variation in the intensity of analytes was not observed when the capillary voltage, nebulizer gas pressure and drying gas flow rate were varied from 1500 to 4000 V, from 20 to 50 psi and from 4 to 12 L/min, respectively. In-source collision induced dissociation (CID) was used to generate useful fragment ions by varying the instrument's fragmentor voltage. Optimum CID conditions were obtained by injecting each PDE-5 inhibitor standard individually at several fragmentor voltages followed by careful review of the resulting mass spectra. In general, higher fragmentor voltage helps the transmission of ions through the relatively high-pressure region between the exit of the capillary and the entrance of the skimmer [14]. In this study, at fragmentor voltages less than 100 V, ions corresponding to a dimer and a doubly charged species were observed for both sildenafil and vardenafil and no fragmentation was observed. At fragmentor voltages above 130 V, the mass spectra exhibited extensive fragmentation and the molecular ion ($[M + H]^+$) was no longer observed for any of the three compounds

studied. An optimum fragmentor setting of 115 V was chosen for this analysis because it allowed for the formation of structurally useful fragment ions while maintaining sufficient $[M + H]^+$ response for all three of the compounds studied.

3.2. Analytical performance of LC–MS method

Retention time reproducibility was evaluated over a 90-day-period with a minimum of 30 injections for each of the three compounds. R.S.D. values for the retention of sildenafil, tadalafil and vardenafil were less than 0.5%, indicating stability of both the standard stock solutions and the chromatographic system.

The method limit of detection (based on signal:noise of 10:1) was determined for each compound by spiking low level standards into an herbal sample matrix. Signal to noise was evaluated using the peak area of extracted ion chromatograms corresponding to $[M + H]^+$ for each standard. The average noise level was calculated from a series of blank injections in the retention window corresponding to each compound. The method detection limits for sildenafil, tadalafil and vardenafil, on column, were 0.1, 0.3 and 1.4 ng, respectively.

3.3. LC–MS identification of sildenafil, tadalafil and vardenafil

Fig. 1 presents a total ion chromatogram (TIC) and corresponding mass spectra for a mixture of sildenafil, tadalafil and vardenafil standards. Despite their structural similarities, sildenafil and vardenafil were well resolved. Fig. 2 summarizes the average retention times observed for each compound during the course of this investigation and also presents the full scan mass spectral data used for identification. Also presented in Fig. 2, are structures of sildenafil, vardenafil and tadalafil along with the proposed fragmentation pathways for the ions observed in the CID mass spectra. The combination of chromatographic retention data along with mass spectral data that exhibits both a protonated molecular ion ($[M + H]^+$) and structurally significant fragment ions provides a relatively simple method for identification of these compounds.

3.4. LC–MS sample analysis

The samples evaluated in this study consisted primarily of dietary supplements marketed as sexual performance formulas. Although the samples were acquired from many different sources with different manufacturers, most were labeled to contain a common herb or group of herbs claimed to have sexual enhancement properties. Some of the more common herbs in this class include yohimbe bark, epimedium, cnidium monnier, muira puama, ginkgo biloba and xanthoparmelia scabrosa. In total, approximately 40 different herbal matrices were screened by this method. The results of the analyses of samples in which a PDE-5 inhibitor was detected are summarized in Table 2. Nearly 50% of the products analyzed were found to contain a synthetic PDE-5 inhibitor. For samples found to contain a PDE-5 inhibitor, the LC–MS data was used to provide a rough estimate of the level as guidance for the LC–UV assay. This was accomplished using the peak area from the extracted ion chromatogram for $[M + H]^+$ of the sample peak relative to that of the corresponding standard.

It is noteworthy that tadalafil, which was approved in Europe in November 2002, but only recently (November 2003) in the United States, was detected in more products than was sildenafil, which has been on the US market for nearly a

Table 2
Summary of chromatographic analysis of PDE-5 containing dietary supplements

Product	Dosage form	Compound detected	Number of determinations (n)	Range of levels determined for the product (mg per dosage unit)	Range of recovery, % (range of levels spiked, mg)
1	Capsule	Sildenafil	–	60	–
2	Capsule	Tadalafil	4	15.5–16.9	99–105 (11–14)
3	Tablet	Tadalafil	4	8.2–9.3	100–102 (6.1–7.6)
4	Capsule	Sildenafil	5	18.4–23.4	101–103 (12.4–20.6)
5	Capsule	Sildenafil	4	18.9–21.4	98.2–98.4 (20.4–20.7)
6	Tablet	Tadalafil	20	6.9–8.0	99–102 (6.3–9.3)
7	Tablet	Tadalafil	7	6.8–7.8	93–100 (4.8–7.0)
8	Tablet	Tadalafil	20	7.6–9.4	99–102 (6.3–7.2)
9	Tablet	Tadalafil	9	4.6–4.9	100–101 (4.0–6.8)
10	Capsule	Sildenafil	2	23.1–23.3	97 (23)
11	Tablet	Tadalafil	6	9.8–10.1	96–100 (7.0–11.3)
12	Capsule	Tadalafil	8	9.4–9.7	99 (7.0)
13	Capsule	Sildenafil	2	29.9–30.1	98 (25)
14	Tablet	Sildenafil	12	44.8–50.9	96–103 (34–42)
15	Capsule	Sildenafil	4	14.3–18.5	96–103 (14.8–15.3)
16	Capsule	Homosildenafil	–	9[a]	–
17	Tablet	Sildenafil	2	52.7–53.2	100 (42)
18	Liquid	Homosildenafil	–	140 mg/vial[a]	–
19	Bulk powder	Tadalafil	–	310 mg/g	–

[a] Estimated using sildenafil standard.

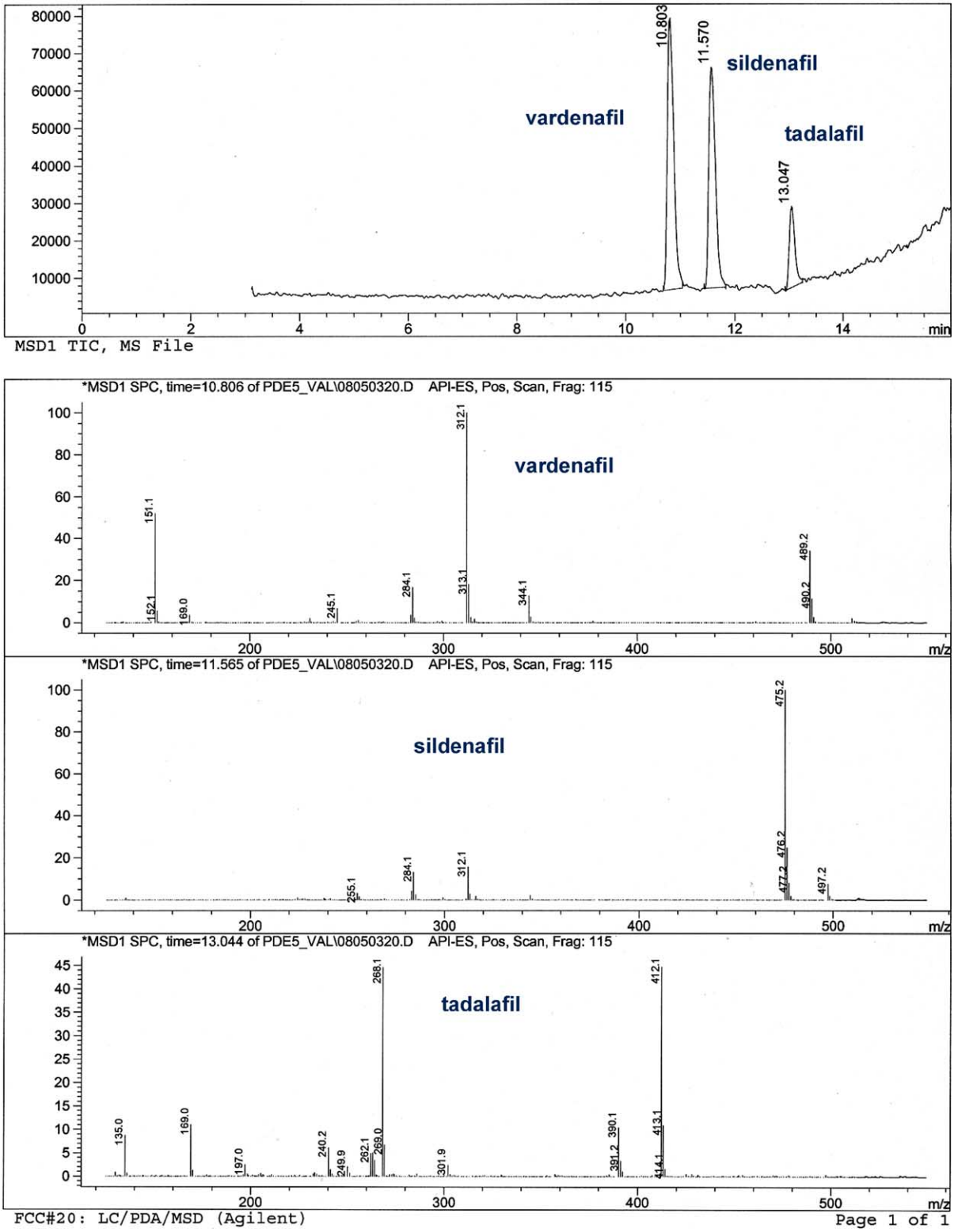

Fig. 1. LC–ESI–MS total ion chromatogram and corresponding mass spectra of synthetic PDE-5 inhibitors sildenafil, vardenafil and tadalafil on a Zorbax SB C-18 column, with an acetonitrile:water mobile phase containing 0.1% formic acid.

Name	FW	[M+H]+	Retention Time	[M-R]+	Other ions
Sildenafil Standard	474.6	475	11.6	312	284
Vardenafil Standard	488.6	489	10.9	312	151, 284
Tadalafil Standard	389.4	390	13.1	268	169, 412

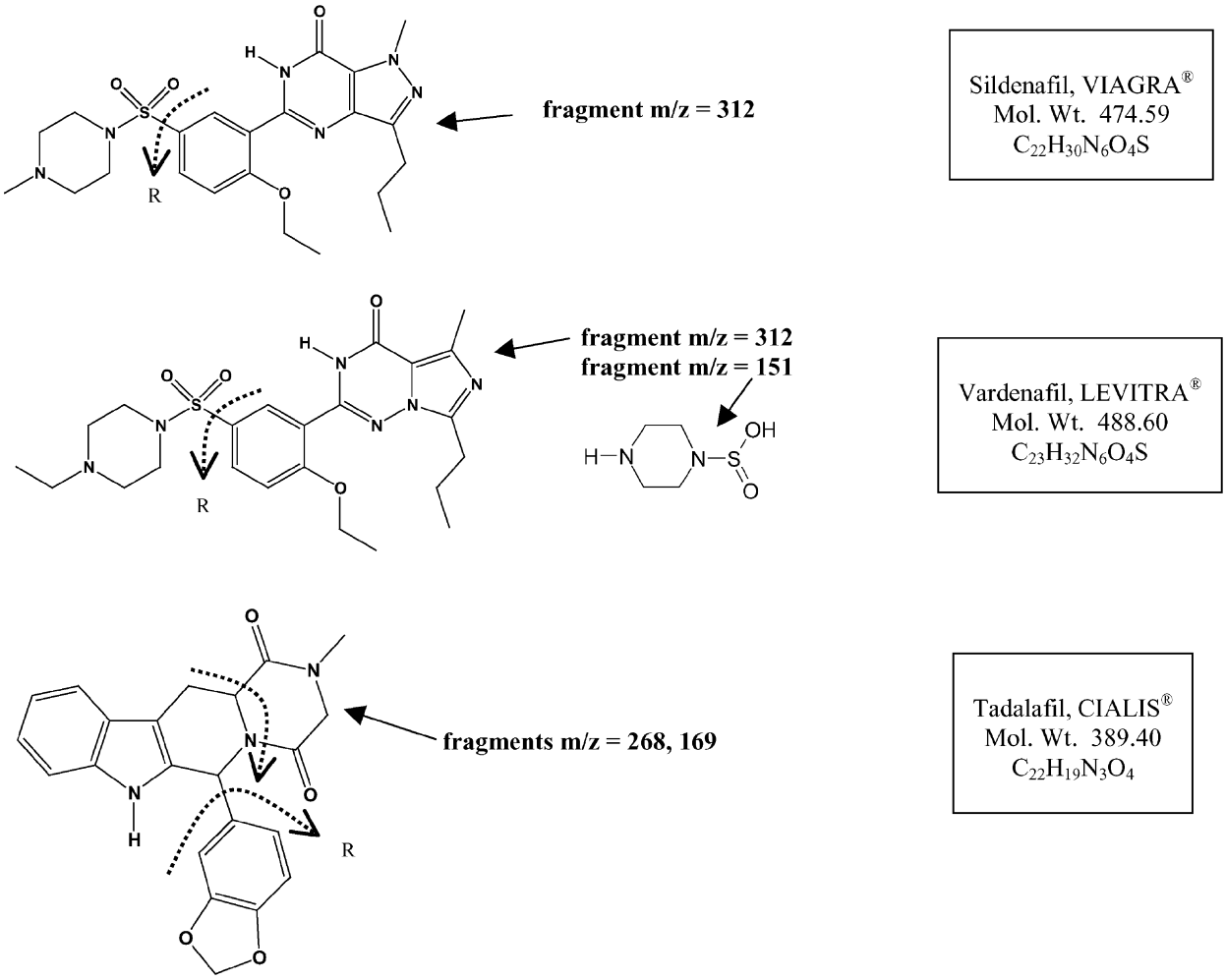

Fig. 2. Proposed LC–ESI–MS fragmentation pathways and chromatographic retention times of sildenafil, vardenafil, and tadalafil.

decade. Vardenafil, which gained FDA approval in the US in August 2003, was not detected in any of the products in this study.

A representative analysis of one of the products is shown in Fig. 3. This product (product 3) was labeled to contain a mixture of several herbal constituents including yohimbine, xanthoparmelia and epimedium. The peak with a retention time of 13.06 min in the chromatogram corresponds to tadalafil as indicated by the mass spectral data. However, two other peaks in the sample exhibit $[M + H]^+$ ions that likely correspond to yohimbine and icariin, which is a known component of epimedium. This example illustrates the selectivity of the method as neither of these compounds that are present naturally interfered with the detection of the synthetic PDE-5 inhibitors.

During the early stages of this study, analysis of a liquid sample (Fig. 4) revealed a peak that eluted immediately after the established retention time for sildenafil. This peak exhibited a mass spectrum that was also similar to that of sildenafil ($[M + H]^+$ of 489 with fragments at *m/z* 312 and 284). The only difference was that the unknown compound had a molecular ion 14 mass units greater than that of sildenafil. The difference of 14 amu was believed to arise from the presence of an additional methylene unit. Although vardenafil differs in mass by 14 amu compared to sildenafil, neither the retention time nor the mass spectrum of the unknown

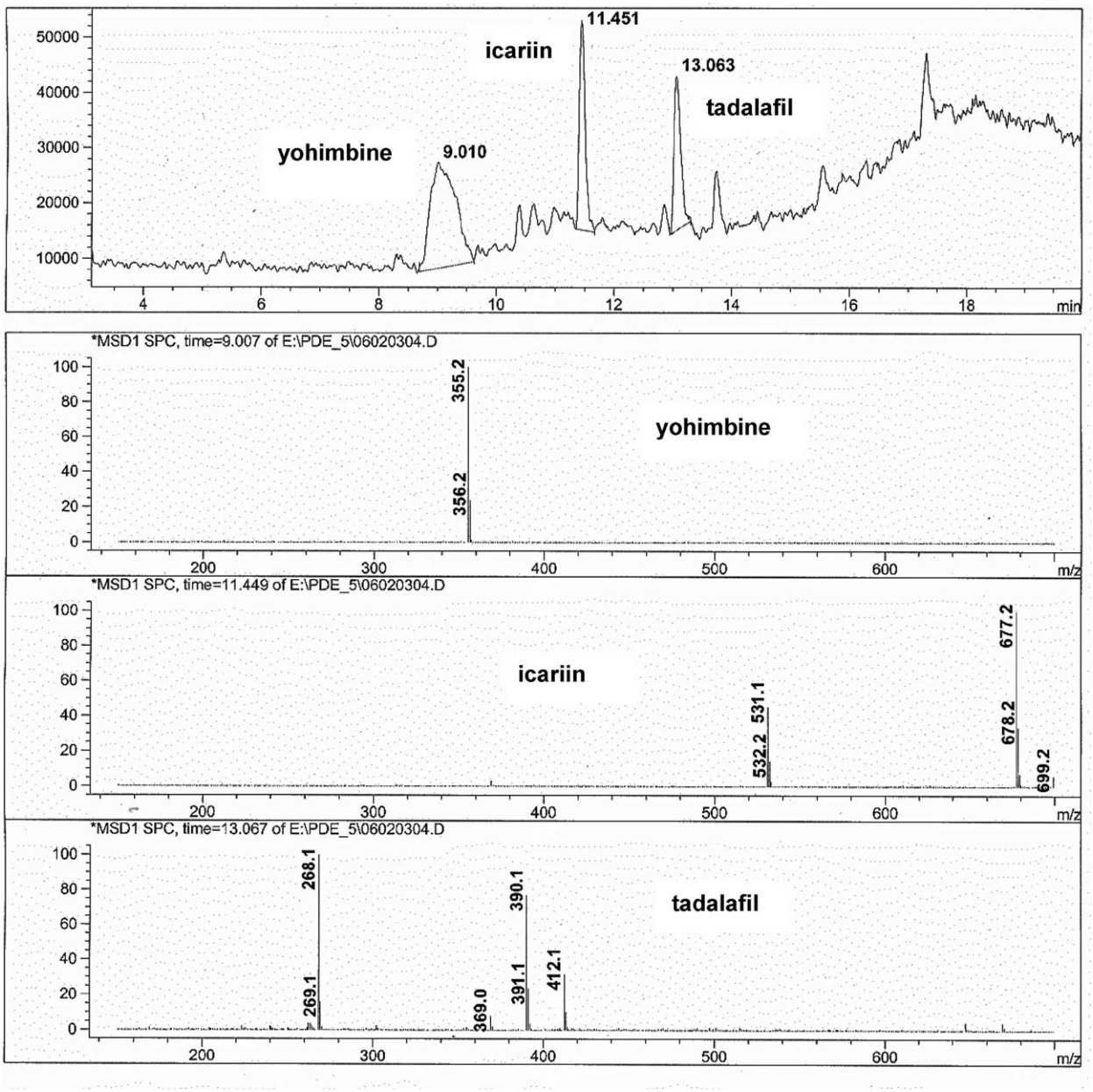

Fig. 3. Representative total ion chromatogram and mass spectra of dietary supplement containing tadalafil. Experimental conditions are provided in Table 1.

compound was consistent with vardenafil. In addition, the fragmentation pathway (ions at *m*/*z* 312 and 284) was identical to that of sildenafil. Recently, this compound was identified as homosildenafil, which is not an approved drug. Based on its structural resemblance to sildenafil, homosildenafil likely exhibits phosphodiesterase-5 inhibitory activity [5].

3.5. Quantitative determinations using LC–UV

Each of the LC–UV methods permits the quantitation of the relevant synthetic PDE-5 inhibitor without interference from other components that are extracted and separated under these conditions. For the samples listed in Table 2, spike recoveries for tadalafil ranged from 93% to 105%. The spike recoveries for sildenafil ranged between 96% and 103%. The level of active ingredient per dose for many of the products is provided as a range of values. For example, a total of 10 samples of product 6 were analyzed in duplicate (i.e. 20 determinations). Results ranged from 6.9 to 8.0 mg tadalafil per tablet. Six samples of product 14 were analyzed in duplicate, with results ranging from 44.8 to 50.9 mg sildenafil per tablet.

In terms of levels of active pharmaceutical ingredient, these dietary supplements can be compared to the approved pharmaceutical dosage forms. Viagra® is marketed in 25, 50 and 100 mg tablets, while Cialis® and Levitra® are marketed in 5, 10 and 20 mg dose tablets. For many of the dietary supplements analyzed, the recommended serving size is two units. Based on the levels per unit dose presented in

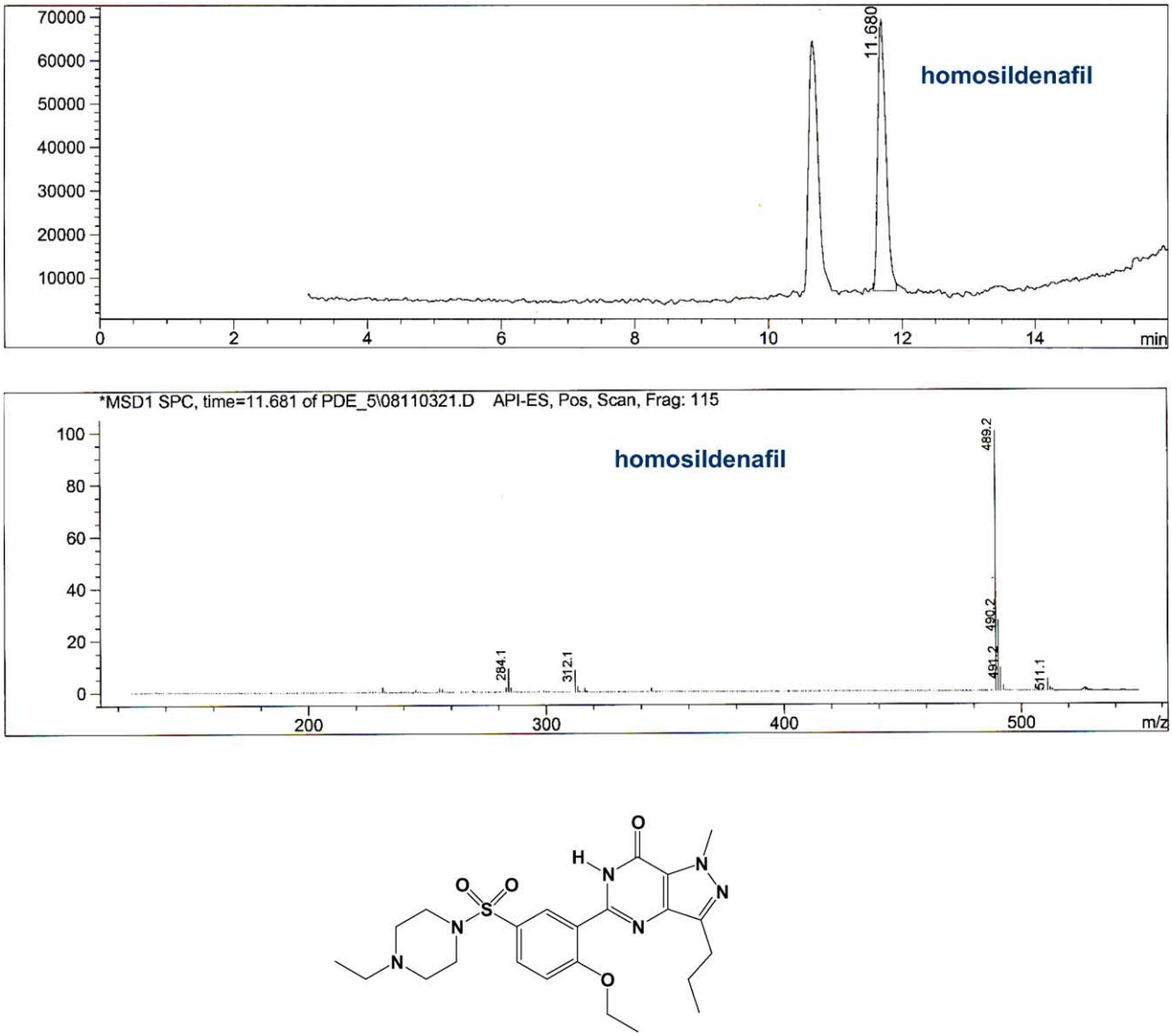

Fig. 4. Representative total ion chromatogram and mass spectra of dietary supplement containing homosildenafil. Conditions are provided in Table 1.

Table 2, the serving size ensures that these products deliver therapeutic dosage levels of the synthetic PDE-5 inhibitor detected.

For the supplements that contained homosildenafil, the sildenafil citrate reference standard was used to approximate the quantity of homosildenafil present.

4. Conclusions

In this study, liquid chromatography was used with both mass spectral and UV detection for the separation, identification and quantitation of synthetic PDE-5 inhibitors in dietary supplements and herbal matrices. Out of approximately 40 different products analyzed, 19 products were found to contain therapeutic levels of sildenafil, tadalafil or homosildenafil. The use of any prescription medication while not under the care of a physician can be dangerous. The possibility that patients who have health conditions that prevent them from taking PDE-5 inhibitors could, unknowingly, consume “all natural” supplements that contain undeclared synthetic PDE-5 inhibitors, poses a serious health threat. The method presented in this study is an important step in detecting the presence of pharmaceuticals that are illegally added to dietary supplements and herbal medicines.

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