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Structure elucidation of a novel analogue of sildenafil detected as an adulterant in an herbal dietary supplement

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Abstract

A new analogue of sildenafil was detected in an herbal dietary supplement, which was sold over the internet and promoted as a product for the enhancement of sexual performance. The structure of the compound was established using LC–MS, UV spectroscopy, MS–MS, and NMR. In addition, the compound was cleaved at its sulfonamide S–N bond yielding a sulfonic acid and an amine, which were independently characterized using LC–MS, GC–MS, and derivatization. The compound, named methisosildenafil, is a novel synthetic analogue of sildenafil in which the *N*-methylpiperazine moiety has been replaced with 2,6-dimethylpiperazine.

Keywords: Dietary supplements; Sildenafil analogue; Methisosildenafil; Liquid chromatography–mass spectrometry (LC–MS); Nuclear magnetic resonance (NMR)

1. Introduction

Sildenafil was introduced onto the U. S. market in 1998, marketed by Pfizer under the trade name Viagra®, for the treatment of erectile dysfunction (ED). Subsequently, two other ED drugs have been approved by the U.S. Food and Drug Administration (FDA): vardenafil (Levitra®), manufactured by Bayer, and tadalafil (Cialis®), manufactured by Lilly. These ED drugs are synthetic compounds, which function by inhibiting the phosphodiesterase type 5 enzyme [1,2].

The advent of these highly successful drugs has spurred the marketing of herbal dietary supplements as natural alternatives for the enhancement of sexual performance. In recent years, there have been reports on the detection of synthetic ED drugs in herbal dietary supplements. Herbal products have been adulterated not only with the three FDA approved ED drugs, but also with synthetic analogues of these drugs [3–13]. The list of ED drug analogues found in herbal dietary supplements continues to grow and includes such compounds as homosildenafil [3–7], hydroxyhomosildenafil [4,5], acetildenafil, [4,5], hydroxyacetildenafil [8,9], piperadino acetildenafil [9], aminotadalafil [9,10], and piperidenafil [9,11].

In a previous study, the structure of piperidenafil, a vardenafil analogue, was first established in our laboratory using LC–UV–MS, direct infusion MS^n, and hydrolysis followed by LC–MS and GC–MS of the hydrolysis products [11]. In the current study, these same techniques and NMR analysis were applied to the detection and structure elucidation of a new sildenafil analogue. The compound was identified as 1-[[3-(6,7-dihydro-1-methyl-7-oxo-3-propyl-1H-pyrazolo[4,3-d]pyrimidin-5-yl)-4-ethoxyphenyl]-sulfonyl]-3-(*R*),5-(*S*)-dimethylpiperazine. The structure of this new analogue, named methisosildenafil, is given in Fig. 1.

2. Experimental

2.1. Materials

2,6-Dimethylpiperazine was purchased from Aldrich (Milwaukee, WI, USA). Sildenafil citrate was obtained from Pfizer, tadalafil from Lilly, and vardenafil hydrochloride trihydrate from Bayer. Reagent grade formic acid and hydrochloric acid and HPLC-grade Omni-Solve acetonitrile were purchased from EM Science (Gibbstown, NJ, USA). Water was purified to 18 MΩ cm using a Milli-Q Water System (Millipore, Bedford, MA, USA).

Two bottles containing five capsules each of the herbal dietary supplements were purchased over the internet. The product label

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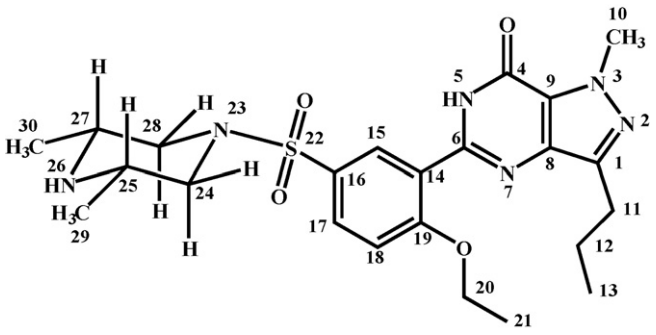

Fig. 1. Structure of methisosildenafil. The numbering system corresponds to the one in Ref. [4].

listed seven herbal substances, all of which are traditional Chinese herbal drugs. A capsule contained, on average, 487 mg of brown amorphous powder. A composite was prepared from the contents of three capsules.

2.2. LC–UV–MS

An amount of the capsule composite equivalent to one dosage unit was extracted into 25 ml MeCN–H_2O (1:1) in an ultrasonic bath for 20 min, centrifuged at 4500 rpm for 20 min, and the supernatant liquid was used for LC–UV–MS and collision-induced dissociation (CID) MS analysis. The LC–MS analytical procedure has been reported previously [11] and is based on the method of Gratz [6]. LC–MS was conducted on an Agilent 1100 system with a diode array detector and a single quadrapole mass spectrometer, model G1946A, operating in series. The UV signal was monitored at 230 nm and a spectrum was collected for each component recognized as a peak. An atmospheric pressure electrospray ionization source was operated in the positive ion mode and the analyzer scan range was 130–500 amu. The mass spectrometer has in-source fragmentation capabilities, and the fragmentor voltage was set to 115 V. The analytical column was a Zorbax SB-C_{18} stationary phase, 150 mm × 4.6 mm, 5 μm particle size (Agilent Technologies, Wilmington, DE, USA), and the guard column was a BrownLee NewGuard column, C_{18}, spherical, 300A, 7 μm particle size, 3.2 mm × 15 mm (Applied Biosystems, Foster City, CA, USA). The flow rate was 1 ml min^{-1}. Mobile solvent A was 0.1% formic acid in water and mobile solvent B was 0.08% formic acid in MeCN. A slightly lower concentration of formic acid in mobile phase B than in mobile phase A reduces the upward baseline drift during the gradient elution. A gradient was used for the mobile phase starting with 15% B for the first 5 min, changing linearly to 90% B over 5–15 min, and holding at 90% B for 5 min. The column was re-equilibrated for 5 min before the start of the next run.

2.3. Collision-induced dissociation (CID) MS

Positive ion electrospray ionization (ESI) CID MS was conducted on a Thermo-Finnigan LCQ Deca XP ion trap mass spectrometer by direct infusion of the acetonitrile-water extract of the capsule contents at 3 μl min^{-1}. The ion transfer capillary temperature was 250 °C, sheath gas 5 (arbitrary units), auxiliary gas 0 (arbitrary units), capillary voltage 7 V, and spray voltage 5 kV. The collision energy varied from 36 to 43%.

2.4. Hydrolysis

Sildenafil, vardenafil and the unknown substance were heated in acid media to cleave the sulfonamide bond, thus yielding a sulfonic acid and an amine. A composite of the herbal product (154 mg) was extracted with 25 ml MeOH for 20 min on an oscillating shaker, centrifuged, decanted and evaporated. The residue was dissolved in 3 ml MeOH and a 1 ml portion was used for the hydrolysis reaction. The 1 ml portion was evaporated to dryness and the residue was redissolved into 3 ml 6.1 M HCl. Two additional reaction solutions were prepared by dissolving 3 mg sildenafil citrate and 3 mg vardenafil hydrochloride separately in 3 ml 6.1 M HCl. The three solutions were placed in 15 ml glass test tubes, sealed tightly with PTFE-lined screw caps, and heated at 105 °C for 24 h in a Reacti-Therm™ heating module (Pierce, Rockford, Illinois, USA). The sample solution turned black, while the two standard solutions remained clear and colorless. The sildenafil and vardenafil hydrolysis reaction solutions were evaporated on a rotary evaporator. The herbal hydrolysis reaction mixture was centrifuged and the clear amber supernatant liquid was removed and evaporated to dryness on a rotary evaporator.

Each of the reaction residues was dissolved in 1 ml MeOH. A 30-μl portion was mixed with 150 μl 0.1% formic acid in MeOH–H_2O (1:1) and used for LC–MS analysis. The remaining MeOH solutions of sildenafil and vardenafil were mixed with 1 or more drops of concentrated ammonia–MeOH (1:9) until the solution was no longer acidic to wet pH paper and analyzed by GC–MS. The remaining MeOH solution of the herbal reaction product was evaporated, and the residue dissolved in 2 ml 1 M HCl, washed with two portions of 2 ml EtOAc, and mixed with 3 ml 1 M NaOH and 1 ml dichloromethane. The lower dichloromethane layer, which contained the amine, was analyzed by GC–MS.

2.4.1. Identification of the amine hydrolysis product

2.4.1.1. GC–MS analysis of the amine. The amines generated by acid hydrolysis of sildenafil, vardenafil and the unknown component were analyzed by GC–MS on a Hewlett-Packard gas chromatograph, model 5890 series II, with a Hewlett-Packard 5972 mass selective detector using a DB-5, 30 m × 0.25 mm × 0.25 μm capillary column under the following conditions: injector: 220 °C, 1 or 2 μl injection volume, split mode; carrier gas: helium at 1.3 ml min^{-1} constant flow; oven temperature: 60 °C for 1 min, increased to 130 °C at 12 °C min^{-1}, increased to 220 °C at 30 °C min^{-1}; detector temperature: 230 °C; MS scan range 25–550 amu.

2.4.1.2. Preparation of a benzoyl derivative of the amine and analysis by LC–MS. The residue from the hydrolysis reaction, obtained as described above, was reconstituted into 3 ml 0.5 M HCl and filtered through Whatman #1 filter paper. The resulting clear amber solution was washed twice with 3 ml EtOAc. Benzoyl chloride (50 μl) and 2 M NaOH (1.5 ml) were added

with stirring to the aqueous HCl solution. After 2 min, additional 50 μl benzoyl chloride and 0.5 ml 2 M NaOH were added. Additional aliquots of 2 M NaOH were added as needed to make the solution basic to pH paper. This process was repeated every 2 min until the addition of these two reagents was made six times. The solution was stirred for an additional hour, with continuous monitoring to ensure the solution remained strongly basic. The alkaline solution was extracted with 10 ml EtOAc. The upper EtOAc solution was back washed with 5 ml 1 M NaOH, dried over anhydrous K_2CO_3, and evaporated to dryness.

The benzoyl derivative of 2,6-dimethylpiperazine standard was synthesized under Schotten-Baumann conditions in a similar manner. Thus, 10 mg 2,6-dimethylpiperazine, 1 ml water, 50 μl benzoyl chloride (~0.35 mmol) and 0.5 ml 2 M NaOH (1 mmol) were mixed. Every 2 min another portion of benzoyl chloride and NaOH were added and the process continued as described above. White crystalline solid, which formed in suspension, was removed by centrifugation, washed twice with 1 ml water, resuspended in water, filtered and dried. Additional compound was collected by EtOAc extraction in a similar manner as above.

The LC–MS method described above for the analysis of the intact molecule was also used in the analysis of the benzoyl derivative of the amine generated by hydrolysis. To confirm identification of the amine portion of the herbal hydrolysis product to 2,6-dimethylpiperazine by retention time match, the benzoyl derivatives of each were chromatographed by two additional mobile phase systems: (1) a gradient of 20–80% B in 0–15 min, and (2) an isocratic mobile phase consisting of 40% B.

2.4.2. *Identification of the sulfonic acid hydrolysis product*

The LC–MS method described above for the analysis of the intact molecule was also applied to the analysis of the sulfonic acid hydrolysis products. Products generated from the hydrolysis of sildenafil, vardenafil and the component in the herbal dietary supplement were compared by retention time and mass spectra.

2.5. NMR

The residue of a methanol extract of the herbal composite, prepared as described under Section 2.4, was reconstituted in 1 ml MeOH and mixed with 1 ml 0.1% aqueous formic acid. The main component in the herbal product was isolated by repeated injection of 100 μl of the solution on the Zorbax-SB C_{18} analytical column using an isocratic mobile solvent of 53% MeOH-0.1% formic acid in water. The eluent was collected manually when a signal was observed from the UV detector at 7.8 min. The fractions corresponding to the compound were pooled, evaporated to dryness, and the residue was partitioned between 5 ml 0.1 M NaOH and 10 ml dichloromethane. The aqueous layer was extracted with two more 10 ml portions of dichloromethane. The combined dichloromethane extracts were dried over anhydrous sodium sulfate, filtered through a layer of anhydrous sodium sulfate, and evaporated to dryness. The residue was dissolved in $CHCl_3$ and evaporated again yielding 14.0 mg of a white solid. The sample was dissolved in $CDCl_3$ for NMR analysis.

^{1}H-, 2-D ^{1}H–^{1}H-correlation spectroscopy (COSY), 2-D ^{1}H-^{13}C correlation spectroscopy (HMQC), and 2-D nuclear overhauser enhancement spectroscopy (NOESY) NMR data were recorded on a Varian Inova 500 MHz instrument using $CDCl_3$ as solvent.

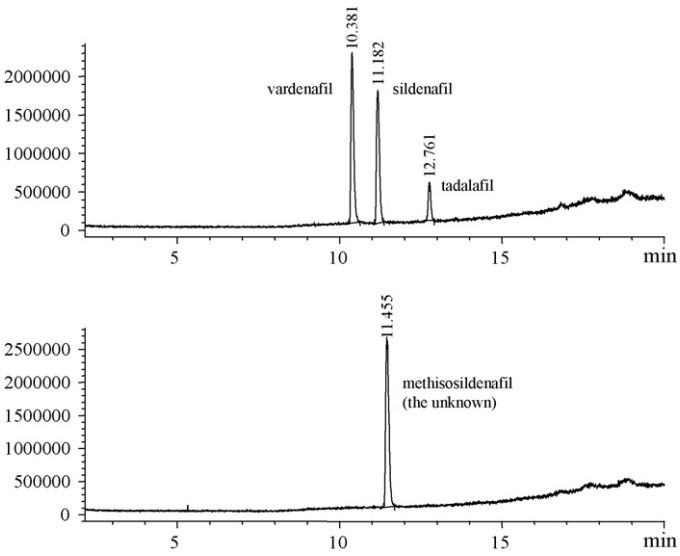

Fig. 2. Total ion chromatograms of a mixture of sildenafil, vardenafil, and tadalafil standards and the adulterated herbal dietary supplement.

3. Results and discussion

3.1. LC–UV–MS

The total ion chromatograms for the herbal product and a mixture of the three ED drugs are shown in Fig. 2. Tadalafil gives a weaker ESI-MS response than sildenafil or vardenafil. The three ED drugs are well resolved on the reversed-phase LC system and elute in the following order: vardenafil, sildenafil, tadalafil. The unknown component in the herbal product elutes slightly after sildenafil and generates a pseudo-molecular ion at *m/z* 489 (Fig. 3). This mass is the same as that for vardenafil and homosildenafil, and is 14 units higher than sildenafil.

The structures of vardenafil and sildenafil share many common features and differ in two aspects. First, the piperazine

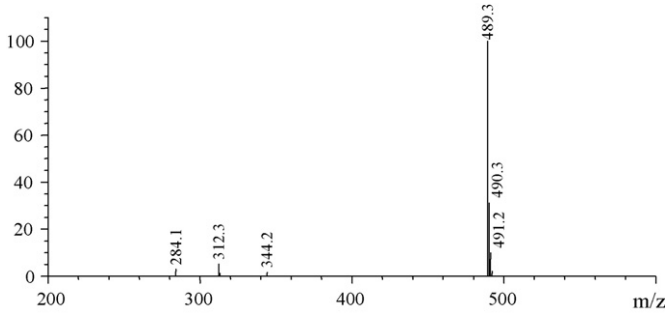

Fig. 3. Mass spectrum of the unknown component (methisosildenafil).

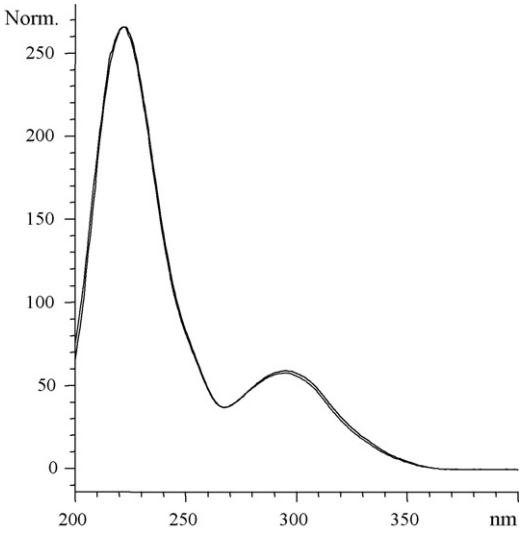

Fig. 4. Superimposed UV spectra of sildenafil (dashed line) and the unknown component, methisosildenafil (solid line).

ring of sildenafil has a methyl substituent, while vardenafil has an ethyl substituent. Second, there is a difference in the position of one nitrogen atom in the heterocyclic ring. The latter difference changes the chromophore of the molecule, thus easily differentiating sildenafil and vardenafil by their UV spectra. Furthermore, the UV spectrum of an analogue of sildenafil will be similar to that of sildenafil, and the UV spectrum of an analogue of vardenafil will be similar to that of vardenafil, providing no change was made in the chromophore when the analogue was made. As shown in Fig. 4, the UV spectra of the unknown component and sildenafil are practically superimposable, which supports a sildenafil type structure for the component.

Since this compound and sildenafil have the same chromophore, it is reasonable to assume that they have similar molar absorptivities. Using HPLC with UV detection at 230 nm and with sildenafil serving as an external reference standard, the amount of this sildenafil analogue found in the herbal dietary supplement was 54 mg per capsule. The therapeutic dose of sildenafil citrate (Viagra®) is typically 50 mg.

3.2. CID-MS

Using electrospray ionization MS in the positive ion mode, CID-MS of the pseudo-molecular ions of sildenafil [4,11], homosildenafil [4], hydroxyhomosildenafil [4], vardenafil [11], and piperidenafil [11] generate a common prominent fragment mass at *m/z* 377. This occurs even though the intact compounds have different molecular weights, and some compounds are based on the structure of sildenafil while others are based on the structure of vardenafil. The fragment ions at *m/z* 377 are due to cleavage of the S–N sulfonamide bond with a hydrogen transfer. Because different pseudo-molecular ions generate a fragment with the same mass, they must lose different groups, and this group must be associated with the nitrogen side of the sulfonamide bond in the molecule. In such a case, the analogue must result from a difference in the piperazine portion of the sildenafil or vardenafil molecule.

CID-MS of the pseudo-molecular ion (*m/z* 489) of the unknown component in this study also generates an *m/z* 377 fragment, corresponding to a neutral loss of 112. Since there is a hydrogen transfer in the fragmentation process, the mass of the amine molecule is expected to be 114. This amine molecule must have at least one nitrogen atom to form a sulfonamide bond, must have an even number of nitrogen atoms according to the nitrogen rule, and is likely to be related to the piperazine ring of sildenafil. Taken together, this data supports an empirical formula of $C_6H_{14}N_2$ for the amine portion of the unknown.

Since its UV spectrum is like that of sildenafil and unlike that of vardenafil, the unknown compound is likely to be a sildenafil analogue created by altering the piperazine ring group.

Many of the properties of the unknown component are similar to those reported for homosildenafil. Both compounds have a retention time in reversed-phase LC slightly longer than sildenafil, a UV spectrum similar to sildenafil, a mass spectrum with a pseudo-molecular ion at *m/z* 489, and a CID-MS fragment at *m/z* 377. While there are many common fragments in the CID-MS of both compounds, there are also critical differences. MS^2 of $[MH]^+$ of the unknown compound (Fig. 5) has peaks at *m/z* 472 and 432, which are absent in the reported spectrum of homosildenafil [4]. MS^2 of $[MH]^+$ of homosildenafil generates a base peak at *m/z* 461 (loss of $CH_2{=}CH_2$) [4], which is weak in the spectrum of the unknown component. From this information it can be deduced that the unknown is an isomer of homosildenafil differing in the piperazine ring moiety of the molecule. The MS^2 peak at *m/z* 472 is attributed to a loss of NH_3 and *m/z* 432 is attributed to a neutral loss of C_3H_7N. While the unknown and homosildenafil have practically the same retention time using the gradient LC–MS system described above, the two compounds were almost baseline resolved on the same column using an isocratic mobile phase consisting of 35% MeCN-0.1% formic acid in water, giving retention times of 4.48 and 4.13 min for the unknown and homosildenafil, respectively.

3.3. Hydrolysis

While mass spectrometry is a powerful technique in structure elucidation of ED drug analogues, it may not define the structure of the molecule or a simple fragment unambiguously even if the molecular formula is known because there may be more than one structural isomer with that molecular formula. We encountered this situation during the identification of piperidenafil [11], when piperidine, ultimately determined as the amine portion of the molecule, was one of four likely candidates with a molecular formula of $C_5H_{11}N$. Unequivocal structure assignment of piperidenafil was made by acid hydrolysis of the sulfonamide bond followed by independent analysis of the two hydrolysis products: an amine (piperidine in the case of piperidenafil) and a sulfonic acid [11].

Similarly, in the current study, mass spectral analysis alone was insufficient in establishing the structure of methisosildenafil, "the unknown". MS–MS analysis of the unknown indicates that the amine molecule has a probable molecular formula of

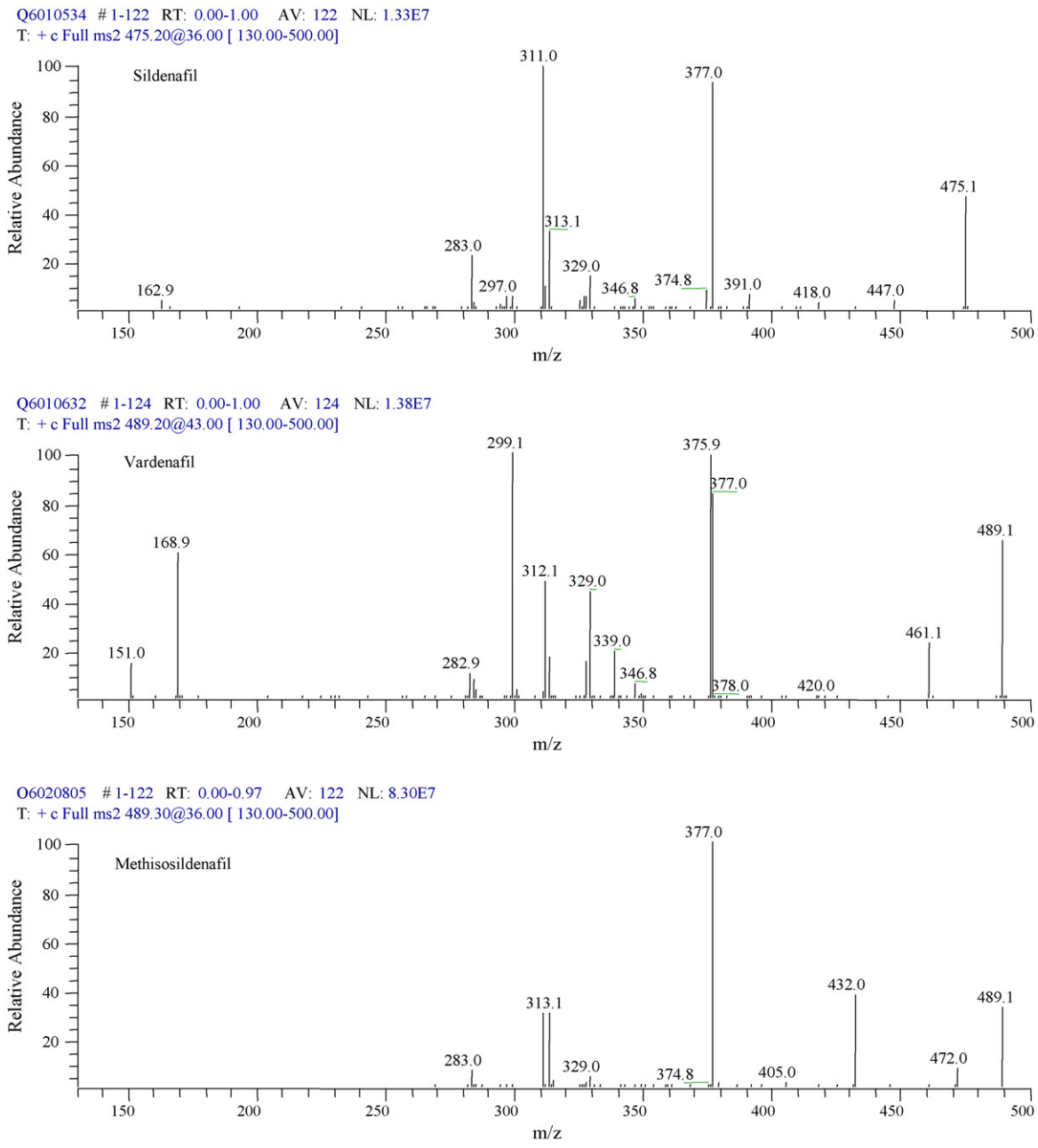

Fig. 5. CID mass spectra generated from the pseudo-molecular ions of sildenafil, vardenafil, and methisosildenafil.

$C_6H_{14}N_2$ (see discussion above). There are 13 commercially available compounds with that molecular formula which possesses a primary or secondary amine capable of forming a sulfonamide bond. Accordingly, the hydrolytic technique was applied as described previously [11] to establish the structure of the amine moiety and confirm the structure of the sulfonic acid moiety of the unknown herbal component.

3.3.1. Characterization of the sulfonic acid generated by hydrolysis

The sulfonic acid of the unknown was characterized by comparison to the sulfonic acids generated by hydrolysis of sildenafil and vardenafil. LC–MS of the hydrolysis products is shown in Fig. 6. The sulfonic acid resulting from hydrolysis of the sulfonamide S–N bond of sildenafil, vardenafil, or the analogue, is the initial and principle product detected. For all three compounds, the mass spectra have a prominent $[MH]^+$ at 393. The retention times for the sulfonic acids generated from vardenafil, sildenafil and methisosildenafil were 9.14, 10.13, and 10.19 min, respectively. The slight difference in retention times between the latter two compounds was found to be due to a difference in the concentration of the compounds. In the LC system, retention times are sensitive to sample concentration; an increase in concentration causes the retention time to shorten.

A secondary hydrolysis product common to both sildenafil and methisosildenafil has a retention time of 9.3 min with a pseudo-molecular ion at *m/z* 411. This compound is likely due to the hydrolysis of the initially formed sulfonic acid compound by

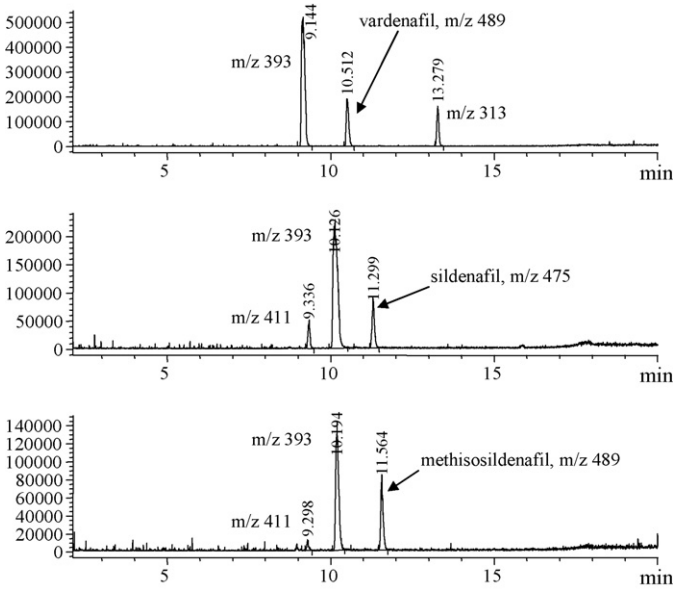

Fig. 6. Total ion chromatograms of the hydrolysis products of sildenafil, vardenafil, and methisosildenafil (the unknown).

addition of a molecule of water to the heterocyclic ring. While there are two hydrolysis products generated from methisosildenafil that match those from sildenafil by retention and mass spectra, none match the hydrolysis products of vardenafil.

3.3.2. Characterization of the amine generated by hydrolysis

A compound in the hydrolysis solution of the herbal extract was detected on the GC–MS system with a retention time of 7.06 min which matched the spectrum of 2,6-dimethylpiperazine in the Wiley 138 K Mass Spectral Database (94% quality index). This compound was purchased and used as a standard for direct comparison to the compound in the herbal product hydrolysis solution by GC–MS. When injected concurrently, the herbal hydrolysis component and 2,6-dimethylpiperazine had retention times of 6.84 min and 6.87 min, respectively, and their mass spectra were practically the same. The molecular ion was detected at *m/z* 114 (18% relative abundance) and other prominent ions were detected at *m/z* 99, 71, 70, 56, 44, and 42.

For additional conformation of 2,6-dimethylpiperazine, the compound was derivatized with benzoyl chloride and analyzed by LC–MS using various gradient and isocratic mobile phase conditions (see Section 2.4.1.2). The benzoyl derivative was shown by MS to be the disubstituted product with pseudo-molecular ions at *m/z* 345 $[M+Na]^+$ and 323 $[M+H]^+$ and a prominent fragment ion at *m/z* 105, due to the benzoyl ion. The retention times for the benzoyl derivative of 2,6-dimethylpiperazine standard and the benzoyl derivative of the herbal hydrolysis component were 5.86 min for both using 40% B isocratically, 12.17 and 12.18 min using the original gradient conditions, and 9.34 and 9.33 min using a gradient of 20–80% B in 15 min (data not shown). The hydrolysis experiment, supported with previous data, demonstrates that the structure of the unknown herbal component is a sildenafil analogue in which the *N*-methylpiperazine moiety is replaced with 2,6-dimethylpiperazine.

3.4. NMR

Detailed ^{13}C and ^{1}H NMR data have been reported for sildenafil and three sildenafil analogues using either $CDCl_3$, DMSO-d_6, or D_2O/acetone-d_6 as solvent [3,4,14,15]. The NMR data for methisosildenafil (Table 1), sildenafil [14] and homosildenafil [4], all in a solvent of $CDCl_3$, are consistent in their molecular assignments except for the piperazine portion of the molecule where the structures of the compounds differ. There are four possible configurations for the structure whereby 2,6-dimethylpiperazine is attached to the sulfonyl group: (1)

Table 1
NMR data for methisosildenafil

Group	Atom #	δ (^{1}H, ppm)	Multiplicity	^{1}H–^{1}H COSY	^{1}H–^{13}C COSY, δ (^{13}C, ppm)	NOESY
N–H	5	10.77	1H, br S			
N–CH_3	10	4.25	3H, S			
$CH_2CH_2CH_3$	11	2.89	2H, t, $J=7.6$	H-12	27.80	H-12, H-13
	12	1.82	2H, m	H-11, H-13	22.36	H-11, H-13
	13	0.98	3H, t, $J=7.6$	H-12	14.22	H-11, H-12
Benzene ring	15	8.76	1H, d, $J=2.3$	H-17	131.04	
	17	7.78	1H, dd, $J=9.2$, $J=2.3$	H-15, H-18	131.48	H-18, H-24 eq
	18	7.12	1H, d, $J=9.2$	H-17	113.27	H-17, H-20
OCH_2CH_3	20	4.35	2H, q, $J=7.0$	H-21	66.08	H-18, H-21
	21	1.61	3H, t, $J=7.0$	H-20	14.76	H-20
Dimethyl-piperazine[a]	24 (28) ax	2.07	2H, br m	H-24 eq	51.43	H-24 eq, H-29
	24 (28) eq	3.69	2H, d, $J=11.3$	H-24 ax	51.43	H-17, H-24 ax, H-25, H-29
	25 (27)	3.07	2H, br m	H-29	51.10	H-24 eq, H-29
	29 (30)	1.13	6H, br m	H-25	18.56	H-24 ax, H-24 eq, H-25

[a] Designations for protons on the piperazine ring: ax = axial proton, eq = equatorial proton. NMR phenomena that occur to a proton on one side of the piperazine ring also occur to the analogous proton on the other side of the ring; only protons on one side of the ring are cited in table.

cis-dimethyl configuration where the methyl groups are β to the sulfonamide nitrogen atom, (2) *cis*-dimethyl configuration where the methyl groups are α to the sulfonamide nitrogen atom, (3) trans-dimethyl configuration where the methyl groups are β to the sulfonamide nitrogen atom, and (4) *trans*-dimethyl configuration where the methyl groups are α to the sulfonamide nitrogen atom. The NMR data presented in Table 1 supports configuration (1) as the molecular structure of the unknown component. This interpretation is explained in the discussion that follows. Configuration (1) is shown in Fig. 1.

In the case of sildenafil [14] or homosildenafil [4], which have no C-methyl piperazine ring substituents, the ring can flip between two chair conformations, and the NMR spectrum displays two groups of protons: four equivalent methylene protons on the two ring carbon atoms β to the sulfonamide nitrogen atom and four equivalent methylene protons on the two ring carbon atoms α to the sulfonamide nitrogen atom. The latter protons are farther downfield due to an anisotropic effect of the sulfonyl π electrons.

trans-2,6-Dimethylpiperazine has one methyl in an axial position and one in an equatorial position. Energetically, the two chair conformations are equivalent and can flip back and forth, in which case, one would expect the four methylene protons to be equivalent and the two methine protons to be equivalent. This is not observed in the ^{1}H-NMR of methisosildenafil.

cis-2,6-Dimethylpiperazine can have the methyl groups either diaxial or diequatorial; however, steric interaction between two axial methyl groups would be so severe that the methyl groups would strongly favor the diequatorial position and flipping between two chair configurations would be severely restricted. This restriction translates into three distinct types of protons: an axial methine proton, an axial methylene proton and an equatorial methylene proton, with each proton on the two ring carbons on one side of the ring equivalent to a corresponding proton on the other side of the ring.

In accordance with this interpretation for the *cis*-dimethyl configuration, the ^{1}H-NMR of methisosildenafil shows three types of piperazine ring protons at δ 3.69, 3.07, and 2.07 ppm, each integrating for two protons. HMQC NMR shows a correlation between C25 (C27) at δ 51.10 and the proton at δ 3.07, which means that this proton is the ring methine proton. Correlations between C24 (C28) at δ 51.43 with the protons at δ 3.69 and 2.07 show that these are the ring methylene protons. When a double bond group, such as carbonyl or sulfonyl, is bonded to a rigid cyclohexane ring, the π-electrons of the double bond have a strong anisotropic effect on the equatorial proton on the α carbon. Therefore, the proton at δ 3.69 is assigned to the equatorial methylene ring proton, and the proton at δ 2.07 assigned to the axial methylene ring proton. The downfield position of a methylene ring proton supports a structure in which the methyl groups are β to the sulfonamide nitrogen atom.

2D-NMR NOESY experiments show a strong NOE between protons at δ 3.69 and 2.07, characteristic of geminal protons. There is also an NOE between the aromatic proton ortho to the sulfonyl group at δ 7.78 and the piperazine ring equatorial proton at δ 3.69. This observation of an association through space of an ortho aromatic proton with a piperazine ring methylene proton confirms that the methyl substituents on the ring are attached to the ring carbon β to the sulfonamide nitrogen atom as shown in Fig. 1.

There is evidence in the NMR spectra for the presence of a minor component, probably one or more of the other configurations.

4. Conclusions

An herbal dietary supplement sold over the internet and promoted to enhance sexual performance was found to contain a synthetic analogue of sildenafil in which the *N*-methylpiperazine moiety had been replaced with 2,6-dimethylpiperazine. In the past few years, there has been a trend toward the addition of designer drugs of the three approved PDE-5 inhibitors, sildenafil, vardenafil and tadalafil, in herbal aphrodisiacs, perhaps with the intent to avoid detection by routine procedures designed to screen specifically for the three approved prescription drugs. This practice presents a danger to the public, who may unwittingly consume a synthetic compound which has not been tested for efficacy or toxicity. Furthermore, the PDE-5 inhibitors are contraindicated for patients who take nitrate vasodilators and patients with various medical conditions.

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