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Identification of benzamidenafil, a new class of phosphodiesterase-5 inhibitor, as an adulterant in a dietary supplement

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Abstract

A sample labeled to be a natural herbal supplement for the enhancement of sexual function, was sent to Health Sciences Authority (HSA) of Singapore for testing. An unknown compound was detected and isolated from the product. The structure of the unknown compound was identified using LC-UV, high-resolution MS, ESI-MS/MS, IR, and NMR. The compound was characterized as a phosphodiesterase-5 (PDE-5) inhibitor, benzamidenafil. This is the first report of benzamidenafil, representing a new class of PDE-5 inhibitors, as an adulterant of a dietary supplement.

Keywords: Benzamidenafil; PDE-5 inhibitor; Adulterant; Dietary supplements; Liquid chromatography–mass spectrometry (LC–MS); Nuclear magnetic resonance (NMR)

1. Introduction

Synthetic phosphodiesterase-5 (PDE-5) inhibitors, e.g. sildenafil citrate, are used for the treatment of erectile dysfunction (ED). To date, there are three PDE-5 inhibitors that have been approved by the U.S. Food and Drug Administration for the treatment of ED: sildenafil citrate (Viagra®, manufactured by Pfizer), vardenafil hydrochloride (Levitra®, manufactured by Bayer), and tadalafil (Cialis®, manufactured by Lilly). It is important to note that Viagra®, Levitra® and Cialis® are prescription drugs and must be used under medical supervision. Adverse effects, such as headache, facial flushing, dyspepsia, visual disturbances and muscle aches have been reported [1]. Some patients may resort to herbal alternatives as herbal medicines are believed to be safer than synthetic ingredients. However, some herbal products advertised as "all natural" had been found to be adulterated with synthetic PDE-5 inhibitors [2–17]. Herbal products have been spiked not only with sildenafil, vardenafil and tadalafil [2–9], but also with analogues of these compounds. Analogues of sildenafil, including homosildenafil [9–12], hydroxyhomosildenafil [11,12], acetildenafil [11–14], hydroxyacetildenafil [15] and methisosildenafil [16], have been detected in dietary supplements. Our group previously reported an analogue of tadalafil [17] while other researchers detected a synthetic analogue of vardenafil (piperidenafil) in a natural herbal supplement [18–20]. It is dangerous for patients to unknowingly consume these analogues because of the lack of information regarding their safety and efficacy. Hence, it is important to determine the presence of synthetic PDE-5 inhibitors, especially those unknown analogues, in dietary supplements. All analogues described thus far can be seen as structural modifications of existing commercial PDE-5 inhibitors. In this paper, a PDE-5 inhibitor, benzamidenafil (Fig. 1), structurally unrelated to sildenafil, vardenafil and tadalafil, has been detected in a dietary supplement for the first time. It is so named because of the benzamide moiety in the structure. This molecule represents a new class of PDE-5 inhibitors detected as an adulterant.

2. Experimental

2.1. Chemicals and sample

Silica gel 60 (40–63 μm) for normal phase column chromatography was supplied by Merck (Darmstadt, Germany).

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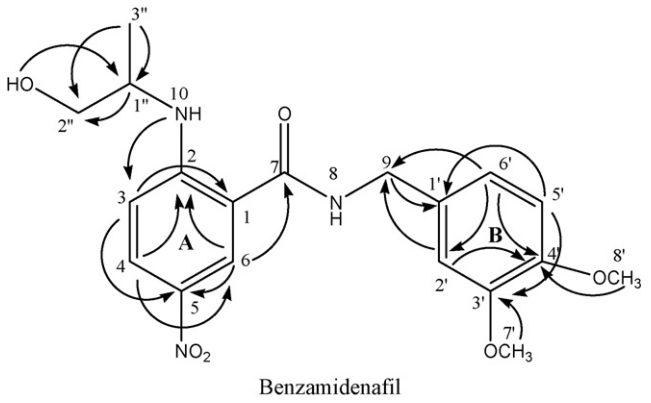

Benzamidenafil

Fig. 1. Chemical structure of benzamidenafil. Arrows indicate important HMBC interactions.

Chloroform (AR grade) and methanol (AR grade) were supplied by Labscan Asia Co., Ltd. (Bangkok, Thailand). $CDCl_3$-d_1 and tetramethylsilane used for NMR analysis and potassium bromide (KBr) powder used for IR analysis were purchased from Sigma–Aldrich (Steinheim, Germany). The dietary supplement sample was provided by the Health Sciences Authority (HSA) of Singapore.

2.2. Extraction and purification of the unknown compound

The contents of three capsules (1.02 g) were ultrasonically extracted in 50 mL methanol for 30 min. The extract was filtered and the solvent was evaporated under *vacuo*. The residue (150 mg) was dissolved in 2 mL methanol and the solution was adsorbed to 2 g of silica gel by evaporating off the solvent. The silica gel-bound sample was applied onto the top of the column which was prepared with 50 g silica gel for column chromatography. Then, the column was eluted with a mixture of chloroform and methanol (20:1). Fractions of 50 mL were collected and samples of each fraction were analyzed by TLC (Rf = 0.4) using chloroform and methanol (20:1). Silica gel 60 F254 coated TLC plates (Merck) were used with UV detection. All of the fractions containing the target compound were pooled and the solvent was evaporated.

2.3. Melting point

The melting point (uncorrected) of the unknown compound was measured on a Gallenkamp melting point apparatus (Loughborough, UK).

2.4. ESI-MS/MS and high-resolution MS analysis

The unknown compound was dissolved in MeOH/H_2O (1:1) at a concentration of 5 μg/mL. Sample was injected into the spectrometer at a flow rate of 5 μL/min using an external syringe pump. ESI-MS and MS/MS analysis were performed in positive ion mode using a QTRAP LC/MS/MS system from Applied Biosystems (Foster City, CA, USA). The $[M+H]^+$ was selected as the precursor ion to generate the ESI-MS/MS spectrum. The collision offset voltage was 10 V. Data acquisition and processing were performed using *Analyst* software (version 1.4.1) from Applied Biosystems (Foster City, CA, USA). The high-resolution MS spectrum was acquired in positive ion mode on a Finnigan/MAT 95XL-T mass spectrometer coupled with an electrospray ionization source.

2.5. NMR and IR analysis

The unknown compound was dissolved in $CDCl_3$-d_1 for NMR analysis. ^1H, ^{13}C, DEPT, COSY, and HMQC spectra were recorded on a Bruker AVANCE300 spectrometer (300 MHz). HMBC spectrum was recorded on a Bruker AMX 500 spectrometer (500 MHz). Chemical shifts are reported in ppm using TMS as an internal standard. IR spectrum was recorded over the spectral range 4000–400 cm^{-1} on a Shimadzu IR Prestige-21 FTIR spectrometer (Nakagyo-ku, Japan).

3. Results and discussion

About 74 mg of a yellow amorphous powder (melting point 125–126 °C, uncorrected) was isolated from the dietary supplement sample. As shown in Fig. 2, the UV spectrum of the compound in methanol shows maximum absorbances at 206 nm and 377 nm, different from the UV spectra of currently approved PDE-5 inhibitors, namely, sildenafil, vardenafil and tadalafil. High-resolution ESI-MS spectrum (Fig. 3) of the unknown compound reveals $[M+H]^+$ at *m/z* 390.1673 $[M+Na]^+$ at *m/z* 412.1496 and $[M+K]^+$ at *m/z* 428.1218 respectively. The accurate mass spectrum was recorded under full scan mode. The peaks at *m/z* 437 and 447 were background noise. The ^1H and ^{13}C NMR spectral data (Table 1) show that the unknown compound contains 19 carbons and 23 protons. Hence, the molecular formula of the unknown compound is determined as $C_{19}H_{23}N_3O_6$.

The ^1H NMR and COSY spectral data (Table 1) reveal 6 protons of two benzene rings (ring A: δ 6.74 ppm, 8.09 ppm, 8.32 ppm and ring B: δ 6.86 ppm, 6.90 ppm, 6.92 ppm) in the compound. A broad singlet (1H) at 1.66 ppm is the characteristic signal of a hydroxyl group. The protons with signals at 6.71 ppm and 8.88 ppm are not coupled with any carbon in the HMQC spectrum. Their chemical shifts suggest that they are not due to

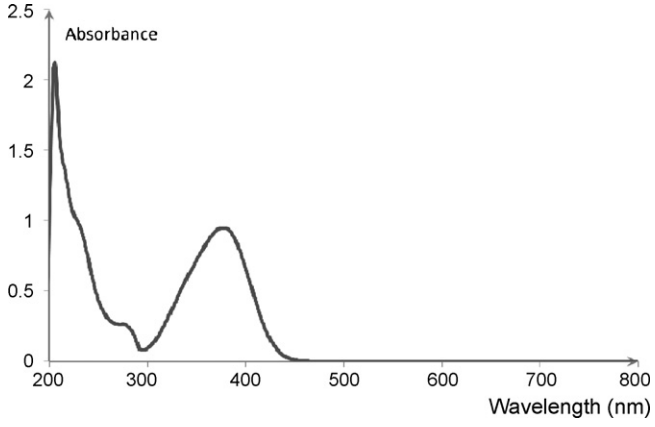

Fig. 2. UV–vis spectrum of the unknown compound in methanol, scanned from 200 nm to 800 nm, showing the maximum absorbances at 206 nm and 377 nm.

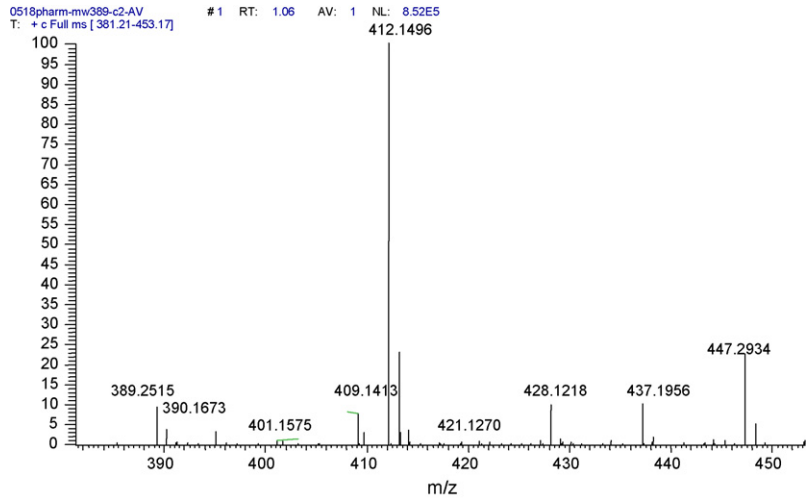

Fig. 3. High-resolution mass spectrum of the unknown compound.

the protons of hydroxyl groups. Hence, the signals at δ 6.71 ppm and δ 8.88 ppm are speculated to be caused by two –NH– groups. Two singlets at 3.89 ppm (3H) and 3.87 ppm (3H) suggest the existence of two methoxy groups in the compound. ^{13}C NMR and DEPT spectral data (Table 1) display the signals of three methyl (two methoxy), two methylene, seven methine (six are from benzene rings), and seven quaternary carbon atoms (six are from benzene rings). A quaternary carbon at 167.92 ppm suggests a carbonyl group.

The methylene carbon at 43.92 ppm is long-ranged coupled with the protons of benzene ring B at 6.90 ppm and 6.92 ppm in the HMBC spectrum, suggesting its connection with benzene ring B. The coupling between two protons of this methylene at 4.52 ppm and the proton of –NH– at 6.71 ppm in the COSY spectrum suggests the connection of the –NH– and the methylene. Furthermore, the carbonyl carbon at 167.92 ppm is long-ranged coupled with the methylene protons at 4.52 ppm and the proton of benzene ring A at 8.32 ppm in the HMBC spectrum. Hence,

Table 1
NMR data of the unknown compound

No.	Benzamidenafil				
	^{1}H(δ_H)	^{13}C(δ_C)	DEPT[a]	COSY	HMBC
1	–	113.25	0	–	H-3/H-8
2	–	153.90	0	–	H-4/H-6
3	6.74 (1H, d, J = 9.4)	111.17	1	H-4	H-10
4	8.09 (1H, dd, J = 9.4, 2.2)	128.70	1	H-3/H-6	H-6
5	–	135.39	0	–	H-3/H-6
6	8.32(1H, d, J = 2.2)	124.80	1	H-4	H-4
7	–	167.92	0	–	H-6/H-9
8	6.71(1H, *t*, J = 5.6)	–	–	H-9	–
9	4.55 (1H, dd, J = 5.6, 14.3)	43.92	2	H-8	H-2′/H-6′
	4.49 (1H, dd, J = 5.6, 14.3)				
10	8.88 (1H, d, J = 7.6)	–	–	H-1″	–
1′	–	129.98	0	–	H-9/H-5′
2′	6.90 (1H, s)	111.44	1	H-6′	H-9/H-6′/H-5′
3′	–	149.30	0	–	H-5′/H-7′
4′	–	148.80	0	–	H-2′/H-6′/H-8′
5′	6.86(1H, d, J = 7.9)	111.44	1	H-6′	H-2′
6′	6.92(1H, d, J = 7.9)	120.42	1	H-2′/H-5′	H-9/H-2′
7′,8′	3.87(3H, s)	55.96	3	–	–
	3.89 (3H, s)	55.99	3	–	
1″	3.82 (1H, m)	50.04	1	H-10/H-2″/H-3″	H-3″/OH
2″	3.65(1H, m)	66.14	2		
	3.76(1H, m)			H-1″	H-1″/H-3″
3″	1.29 (3H, d, J = 7.6)	17.07	3	H-1″	H-2″
OH	1.66 (1H, br, s)	–	–	–	–

δppm in $CDCl_3$, J in Hz.
[a] Number in DEPT is the number of attached protons.

it can be inferred that the two benzene rings are connected by an *N*-methylacetamide group.

The quaternary carbon at 129.98 ppm is coupled with the proton of ring B at 6.86 ppm and the methylene protons at 4.52 ppm in the HMBC spectrum, suggesting that the *N*-methylacetamide group is connected to the quaternary carbon at 129.98 ppm of ring B (numbered 1′). The quaternary carbons of ring B at 149.30 ppm and 148.80 ppm are coupled with methoxy protons at 3.87 ppm and 3.89 ppm, respectively. Meanwhile, the quaternary carbon at 148.80 ppm is coupled with the protons at 6.90 ppm and 6.92 ppm. The quaternary carbon at 149.30 ppm is coupled with the proton at 6.86 ppm. Hence, it is inferred that the two methoxy groups are connected to carbon 4′ and 3′ of ring B, respectively.

The quaternary carbon at 113.25 ppm is coupled with the proton of –NH– at 6.71 ppm, suggesting that the *N*-methylacetamide group is connected to the carbon at 113.25 ppm of ring A (numbered 1). The proton of the other –NH– at 8.88 ppm is coupled with the carbon of ring A at 111.17 ppm in the HMBC spectrum, suggesting that the –NH– group was connected to ring A. The methine proton at 3.82 ppm was coupled with the proton of –NH– at 8.88 ppm, the methyl protons at 1.29 ppm and methylene protons at 3.65 ppm in the COSY spectrum. Meanwhile, the methine carbon at 50.04 ppm was coupled with hydroxyl proton at 1.66 ppm in the HMBC spectrum. Hence, it can be inferred that a 1-propanol group was connected to the –NH– group. One nitrogen atom and two oxygen atoms in the compound have not yet been assigned. Based on the COSY, HMQC and HMBC spectra and a quaternary carbon of ring A at 135.39 ppm, a nitro group was assigned to be connected to the carbon atom at position 5 of ring A.

Hence, the structure of the unknown compound is elucidated as *N*-(3,4-dimethoxy benzyl)-2-{[(*1R,S*)-2-hydroxy-1-methylethyl]amino}-5-nitrobenzamide. This structure is further confirmed by the ESI-MS/MS and IR data. As shown in Fig. 4, the product ion at *m/z* 151 is derived from the $[M+H]^+$ by the cleavage of the bond between N8 and C9. The product ion at *m/z* 223 is formed by the cleavage of the bond between N8 and C7. The product ion at *m/z* 252 is produced by the cleavage of the bond between C9 and C1'. The IR spectrum shows absorption bands characteristic of hydroxyl (υ_{OH} 3520 cm^{-1}), amine (ν_{N-H} 3410 cm^{-1} and δ_{N-H}1600 cm^{-1}), carbonyl ($\nu_{C=O}$ 1650 cm^{-1}), aromatic ring (ν_{Ar-H} 3200 cm^{-1} and ν_{C-C} 1475 cm^{-1}) and other bands at 2920 cm^{-1}, 1335 cm^{-1}, 1265 cm^{-1}, and 1140 cm^{-1}.

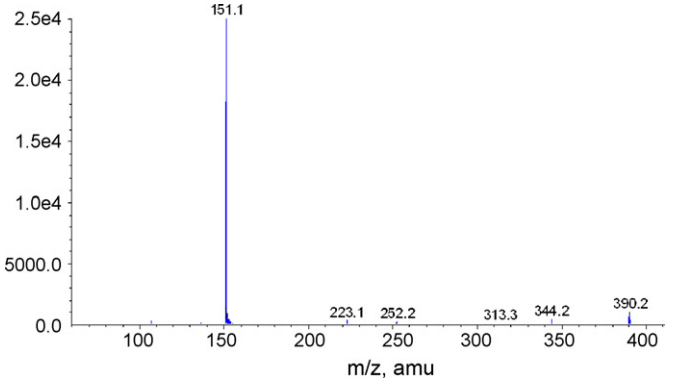

Fig. 4. ESI-MS/MS spectrum of the unknown compound.

This compound is named benzamidenafil because of the benzamide moiety in the structure. This compound was first synthesized and patented by Fujisawa Pharmaceutical Co., Ltd. of Japan [21]. Pharmacological study shows that this compound is a potent and specific inhibitor of PDE-5 [22], with an IC_{50} of 1.1 nM, fourfold lower than that of sildenafil (IC_{50} of 4.3 nM). It showed weak inhibitory activities for phosphodiesterase types 1, 2, 3 and 4 but inhibited phosphodiesterase type 6 with an IC_{50} of 20 nM, compared to IC_{50} of 15 nM for sildenafil. Hence, benzamidenafil may potentially have effects on the visual system due to inhibition of phosphodiesterase type 6 as reported for sildenafil [23,24]. To the best of our knowledge, no clinical trials have been reported, hence we do not know whether the amount of benzamidenafil (∼74 mg) isolated from the contents of 3 capsules is clinically significant for the treatment of erectile dysfunction. It is dangerous to unknowingly consume this compound in a herbal supplement because of the unknown safety and toxicity profile.

4. Conclusion

In the present study, a PDE-5 inhibitor, benzamidenafil was isolated from a dietary supplement and its structure was elucidated using NMR, IR, high-resolution MS and ESI-MS/MS. This is the first report of benzamidenafil detected as an adulterant in a dietary supplement and it represents a new class of PDE-5 inhibitors structurally unrelated to currently approved PDE-5 inhibitors. The ^1H and ^{13}C NMR signals are completely assigned based on the 1D and 2D NMR spectra. The reported NMR, UV, IR and MS data are helpful for future identification of related compounds. Benzamidenafil should be included as a target compound when screening for adulterants in herbal products. This molecule represents a new class of PDE-5 inhibitors detected as an adulterant.

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