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Isolation and identification of thiohomosildenafil and thiosildenafil in health supplements

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Abstract

Two unknown compounds are detected and isolated from health supplements for the enhancement of sexual function. The structures of the unknown compounds are elucidated using high-resolution MS, ESI-MS/MS, NMR, UV and IR. One compound is identified as an analogue of sildenafil in which the oxygen atom is substituted with a sulfur atom in the pyrazolopyrimidine moiety, and an ethyl group instead of a methyl group is attached to the piperazinyl nitrogen. Hence, this compound is named thiohomosildenafil. Another compound is also a sildenafil analogue in which the oxygen atom is substituted with a sulfur atom in the pyrazolopyrimidine moiety. This compound is named thiosildenafil. Both the two compounds are first detected in health supplements. The UV, IR and completely assigned NMR data of thiohomosildenafil and thiosildenafil are first reported.

Keywords: Thiohomosildenafil; Thiosildenafil; PDE-5 inhibitor; Adulterant; Health supplement

1. Introduction

Synthetic phosphodiesterase-5 (PDE-5) inhibitors, such as sildenafil citrate have been widely used for the treatment of erectile dysfunction (ED). Today, 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 contain synthetic PDE-5 inhibitors [2–20]. 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 [9–13], hydroxyhomosildenafil [11–13], acetildenafil [11–15], hydroxyacetildenafil [16,17] and piperidino acetildenafil [17] have been detected in health supplements. An analogue of tadalafil and an analogue of vardenafil, namely piperidenafil, were detected in herbal supplements [17–20]. It is dangerous for patients to consume these analogues because of the unknown safety and toxicity profile. Hence, it is important to determine the presence of synthetic PDE-5 inhibitors, especially those unknown analogues, in health supplements. In this paper, we report the isolation of two additional analogues, namely, thiohomosildenafil and thiosildenafil, from two health supplements and unambiguous structural elucidation using MS, NMR, UV and IR.

2. Materials and methods

2.1. Chemicals and samples

Homosildenafil was isolated from a herbal sample and its structure (Fig. 1) was confirmed by comparing its ^{1}H NMR

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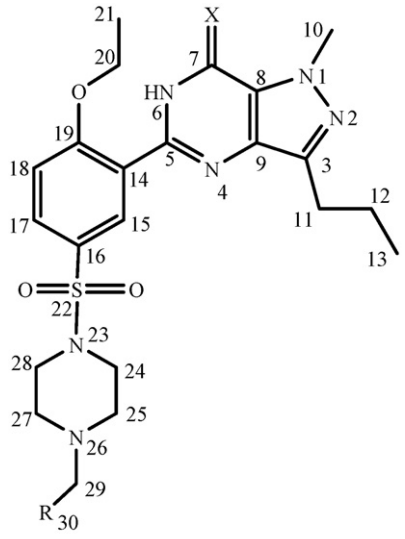

Fig. 1. Chemical structures of homosildenafil, thiohomosildenafil (compound 1) and thiosildenafil (compound 2).

and ^{13}C NMR spectra with reported data [11]. Silica gel 60 (40–63 μm) for normal phase column chromatography was supplied by Merck (Darmstadt, Germany). Chloroform (AR grade) and methanol (AR grade and HPLC grade) were supplied by Labscan Asia Co. Ltd. (Bangkok, Thailand). 0.45 μm nylon membrane filters were supplied by Whatman International Ltd. (Maidstone, UK). MilliQ water was obtained using a Synergy Purification System (Molsheim, France). $CDCl_3$ and tetramethylsilane used for NMR analysis and potassium bromide (KBr) powder used for IR analysis were purchased from Sigma–Aldrich (Steinheim, Germany). The health supplement samples A and B were submitted by a client to the Health Sciences Authority (HSA) of Singapore for analysis.

2.2. Extraction and purification of the compounds 1 and 2

The contents (1.23 g) of three capsules of sample A were ultrasonically extracted in 50 mL methanol for 30 min. The extract was filtered and the solvent was evaporated under vacuo. The residue was dissolved in 5 mL chloroform. Two grams of silica gel was added and mixed well. The solvent was evaporated. 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 (40:1, v/v). Fractions of 50 mL were collected and analyzed by TLC using chloroform and methanol (30:1, v/v). All of the fractions containing the target compound were pooled and the solvent was evaporated and compound 1 was obtained. Compound 2 was isolated with the same procedures. The contents (2.10 g) of 10 capsules of sample B were ultrasonically extracted to obtain compound 2.

2.3. Melting point

The melting points (uncorrected) of the unknown compounds were measured on a Gallenkamp melting point apparatus (Loughborough, UK).

2.4. LC-DAD

An Agilent 1100 series HPLC chromatograph with diode-array detector (Palo Alto, CA, USA) was employed. A Hypersil BDS C18 column (200 mm × 4.6 mm i.d., 5 μm) from Thermo Fisher Scientific Inc. (Waltham, MA, USA) was used. The mobile phase was 0.025 M NaH_2PO_4 (pH 3.2) and acetonitrile. The gradient elution profile was as follows: 0–30 min, acetonitrile rose from 10% to 70% (v/v), maintained for 5 min. The flow rate of mobile phase was 1 mL/min. The injection volume was 10 μL. The UV spectra from 200 to 400 nm were recorded on-line during the chromatographic run.

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

Homosildenafil and compounds 1 and 2 were dissolved in MeOH/H_2O (1:1, v/v) at a concentration of 1 μg/mL, respectively. Samples were 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 on an API 2000 mass spectrometer from Applied Biosystems (Foster City, CA, US). The $[M+H]^+$ was selected as a precursor ion and the ESI-MS/MS spectra were acquired. Collision energy (CE) was set at 50 V. Data acquisition and processing were performed using *Analyst* software (Version 1.4.1) from Applied Biosystems (Foster City, CA, US). The high-resolution MS spectrum was acquired in positive mode on a Finnigan/MAT 95XL-T mass spectrometer coupled with an electrospray ionization source.

2.6. NMR and IR analysis

Homosildenafil, compounds 1 and 2 were dissolved in $CDCl_3$ separately for NMR analysis. ^1H, ^{13}C, DEPT, COSY and HMQC spectra were recorded on a Bruker AVANCE300 spectrometer (300 MHz). Chemical shifts are reported in ppm using TMS as an internal standard. IR spectra were recorded on a Shimadzu IR Prestige-21 FTIR spectrometer (Nakagyo-ku, Japan) and recorded over the spectral range 4000–400 cm^{-1}.

3. Results and discussion

3.1. Compound 1

Approximately 200 mg of yellow amorphous powder (melting point 183–185 °C, uncorrected) was isolated from sample A. High-resolution ESI-MS spectrum of the compound 1 revealed $[M+H]^+$ at *m/z* 505.2049 and $[M+Na]^+$ at *m/z* 527.1868, suggesting a molecular formula of $C_{23}H_{32}N_6O_3S_2$. The errors between observed mass and theoretical mass of $[M+H]^+$ and $[M+Na]^+$ are −1.39 and −1.33 ppm, respectively. Compared to homosilde-

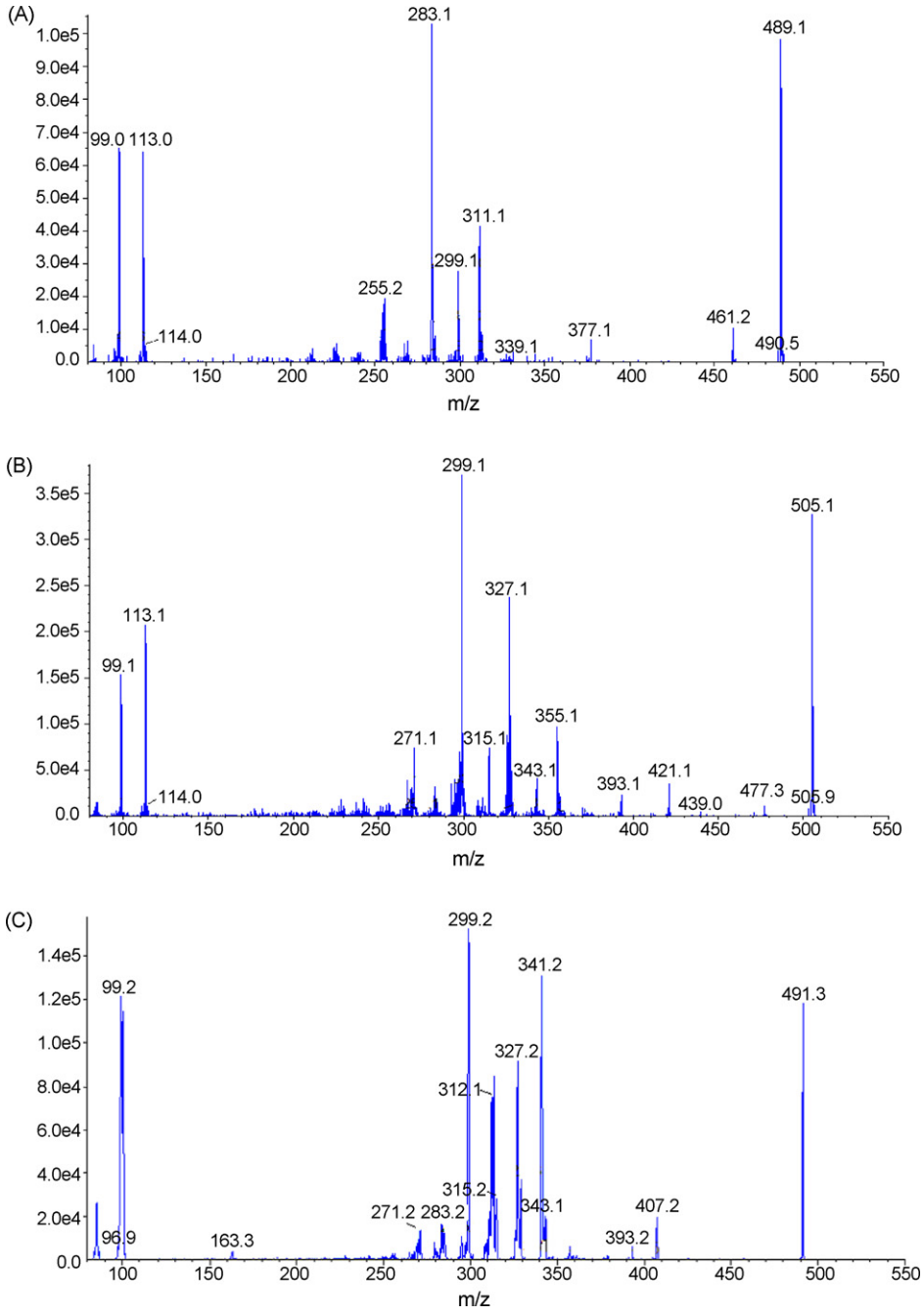

Fig. 2. ESI product ion spectra of (A) homosildenafil, (B) thiohomosildenafil (compound 1) and (C) thiosildenafil (compound 2).

nafil ($C_{23}H_{32}N_6O_4S$), compound 1 contains one more sulfur atom but one less oxygen atom.

As shown in Fig. 2(A) and (B), the protonated homosildenafil and the protonated compound 1 showed similar fragmentation pattern except that the product ions of compound 1 at *m*/*z* 477, 393, 355, 327, 315, 299 and 271 have a mass shift of 16 *m*/*z* units compared with the corresponding product ions of homosildenafil. The mass shift suggests that one oxygen atom is substituted with one sulfur atom. Hence, the structure of compound 1 is expected to be similar to that of homosildenafil.

As shown in Table 1, the ^{1}H and ^{13}C signals of homosildenafil are assigned according to the published NMR data [10,11]. It is obvious that the ^{1}H and ^{13}C signals of the compound 1 are quite similar to those of homosildenafil except the signals caused by the protons and carbons of the pyrazolopyrimidine moiety, suggesting that the sulfur atom is connected to the pyrazolopyrimidine moiety. A singlet at 4.53 ppm integrating for three protons and a singlet at 12.40 ppm integrating for one proton were observed in the ^{1}H spectrum of compound 1 and they were not found in the ^{1}H spectrum of homosildenafil. The signal at 4.53 ppm was assigned to the methyl at position 10 and signal at 12.40 ppm was assigned to the proton at position 6. Meanwhile, the ^{13}C and DEPT spectra of compound 1 revealed three quaternary carbon signals at 171.91, 132.41 and

Table 1
NMR data of homosildenafil, compounds 1 and 2

Carbon no.	Homosildenafil		Compound 1					Compound 2	
	δ^1H	δ^{13}C	δ^1H	δ^{13}C	DEPT[a]	COSY	HMQC	δ^1H	δ^{13}C
3	–	146.44	–	146.12	0	–	–	–	146.09
5	–	147.01	–	146.58	0	–	–	–	146.52
6	10.86	–	12.40 (1H, s)	–	–	–	–	12 .41 (1H, s)	–
7	–	153.66	–	171.91	0	–	–	–	171.82
8	–	124.52	–	132.41	0	–	–	–	132.34
9	–	138.40	–	133.98	0	–	–	–	133.94
10	4.27	38.23	4.53 (3H, s)	39.41	3	–	H-10	4 .52 (3H, s)	39.38
11	2.93	27.77	2.95 (2H, t, J = 7.5, 7.2)	27.61	2	H-11/H-12	H-11	2 .95 (2H, t, J = 7.5, 7.2)	27.59
12	1.86	22.27	1.85 (2H, m, J = 7.5, 7.2)	22.20	2	H-12/H-11, H-13	H-12	1 .86 (2H, m, J = 7.5, 7.2)	22.16
13	1.02	14.05	1.02 (3H, t, J = 7.2)	14.01	3	H-13/H-12	H-13	1 .02 (3H, t, J = 7.2)	14.01
14	–	121.13	–	120.01	0	–	–	–	119.86
15	8.80	131.24	8.85 (1H, s)	130.94	1	–	H-15	8 .84 (1H, s)	130.79
16	–	128.85	–	129.21	0	–	–	–	129.22
17	7.82	131.74	7.86 (1H, d, J = 8.3)	132.05	1	H-17/H-18	H-17	7 .84 (1H, d, J = 8.3)	131.98
18	7.15	113.05	7.17 (1H, d, J = 8.7)	113.11	1	H-18/H-17	H-18	7 .18 (1H, d, J = 8.7)	113.13
19	–	159.32	–	159.49	0	–	–	–	159.44
20	4.37	66.12	4.40 (2H, q, J = 7.2, 6.8)	66.44	2	H-20/H-21	H-20	4 .40 (2H, q, J = 7.2, 6.8)	66.44
21	1.64	14.54	1.73 (3H, t, J = 7.2, 6.8)	14.69	3	H-21/H-20	H-21	1 .72 (3H, t, J = 7.2, 6.8)	14.67
24, 28	3.11	46.12	3.13 (4H, bs)	45.94	2	H-24, 28/H-25, 27	H-24, 28	3 .11 (4H, bs)	45.95
25, 27	2.55	51.92	2.56 (4H, bs)	51.77	2	H-25.27/H-24, 28	H-25, 27	2 .50 (4H, bs)	54.05
29	2.41	51.83	2.43 (2H, q, J = 7.5, 7.2)	51.95	2	H-29/H-30	H-29	2 .27 (3H, s)	45.72
30	1.02	11.94	1.03 (3H, t, J = 7.2, 6.8)	11.75	3	H-30/H-29	H-30	–	–

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

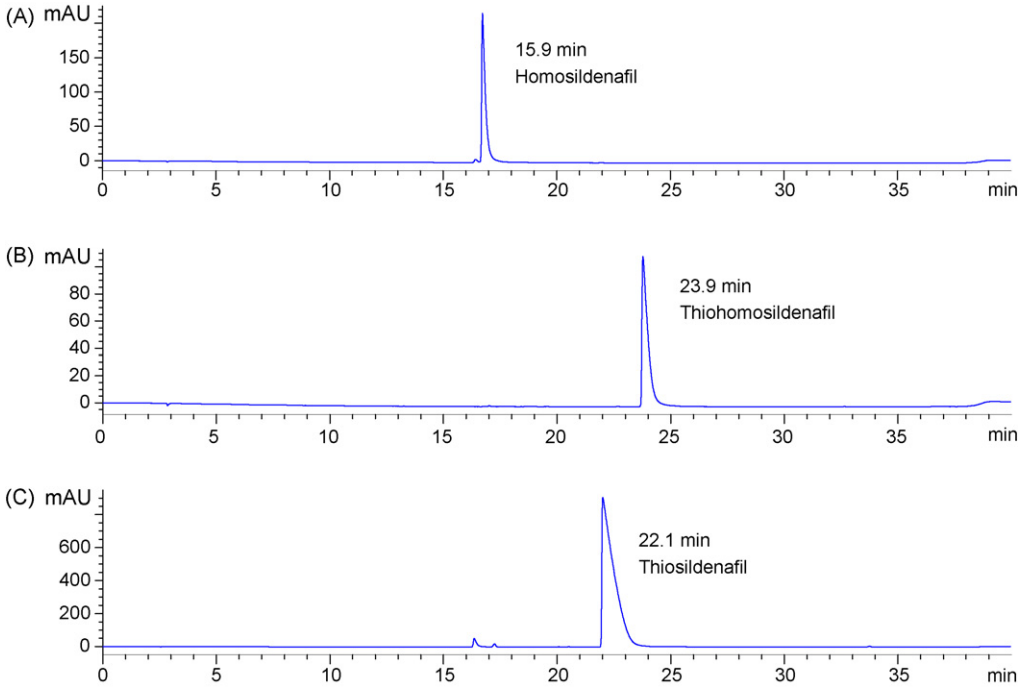

Fig. 3. HPLC chromatograms of (A) homosildenafil, (B) thiohomosildenafil and (C) thiosildenafil (wavelength 290 nm).

133.98 ppm, which were assigned to the carbons at positions 7, 8 and 9, respectively. Hence, it is speculated that compound 1 is an analogue of homosildenafil and the carbonyl group at position 7 of homosildenafil is replaced with a thiocarbonyl group (Fig. 1). This speculation is also supported by the COSY and HMQC results. The chemical name of the compound is 5-(2-ethoxy-5-(4-ethylpiperazin-1-ylsulfonyl)phenyl)-1-methyl-3-propyl-1*H*-pyrazolo[4,3-*d*]pyrimidine-7(6*H*)-thione, which was reported to be prepared as a PDE-5 inhibitor [21]. We name it thiohomosildenafil because of the substitution with a sulfur atom. All the other proton and carbon signals of thiohomosildenafil are assigned by comparing with those of homosildenafil.

3.2. Compound 2

Approximately 350 mg of yellow amorphous powder (melting point 172–174 °C, uncorrected) was isolated from sample B. High-resolution ESI-MS spectrum of the compound 1 revealed $[M+H]^+$ at *m/z* 491.1890 and $[M+Na]^+$ at *m/z* 513.1715, suggesting a molecular formula of $C_{22}H_{30}N_6O_3S_2$. Compound 2 contains one less CH_2 group than thiohomosildenafil.

As shown in Fig. 2(B) and (C), the protonated thiohomosildenafil and the protonated compound 2 showed similar fragmentation pattern except that the ion at *m/z* 113 was not detected in the product ion spectrum of compound 2. Product ion at *m/z* 113 is caused by the ethylpiperazine group of thiohomosildenafil and produce ion at *m/z* 99 by the loss of CH_2 [22]. The absence of ion at *m/z* 113 and detection of ion at *m/z* 99 in the product ion spectrum of protonated compound 2 suggest the structural modification on the piperazine ring. The product ions of compound 2 at *m/z* 407 and 341 have a mass shift of 14 units compared with the corresponding product ions of thiohomosildenafil, which is due to the intramolecular rearrangement [23].

As shown in Table 1, some of the ^1H and ^{13}C signals of the compound 2 were slightly different from those of thiohomosildenafil. The differences were attributed to the replacement of the *N*-ethyl group of thiohomosildenafil with an *N*-methyl group (Fig. 1). The chemical name of compound 2 is 5-(2-ethoxy-5-(4-methylpiperazin-1-ylsulfonyl)phenyl)-1-methyl-3-propyl-1*H*-pyrazolo[4,3-*d*]pyrimidine-7(6*H*)-thione and it was reported to be a PDE-5 inhibitor [21]. We name it thiosildenafil because of its structural similarity to sildenafil.

3.3. UV and IR spectra of thiohomosildenafil and thiosildenafil

Fig. 3 shows the HPLC chromatograms of (A) homosildenafil, (B) thiohomosildenafil (compound 1) and (C) thiosildenafil (compound 2). Thiohomosildenafil is more nonpolar than thiosildenafil and elutes after thiosildenafil.

As shown in Fig. 4, different from the UV spectrum of homosildenafil, the UV spectra of thiohomosildenafil and thiosildenafil are similar and show an absorption band at 350–370 nm, which should be ascribed to the conjugated heterocyclic thiones [24].

The IR spectra of both thiohomosildenafil and thiosildenafil show characteristic absorption bands of amine (υ_{N-H} 3285 cm^{-1}), aromatic ring (υ_{Ar-H} 2944 cm^{-1} and υ_{C-C} 1570, 1540 cm^{-1}), thione ($\upsilon_{C=S}$ 1745 cm^{-1}), sulfonamide ($\upsilon_{O=S=O}$

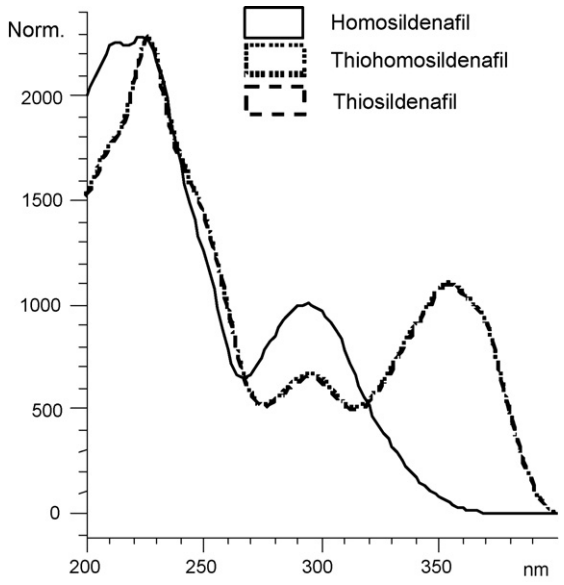

Fig. 4. UV spectra of homosildenafil, thiohomosildenafil and thiosildenafil.

1360, 1176 cm^{-1}) and other bands at 2860, 2810, 2365, 1261, and 954 cm^{-1}.

4. Conclusion

This work reports the first detection of two analogues of sildenafil and homosildenafil in two health supplements. The structures of the analogues are unambiguously elucidated using NMR, high-resolution MS, ESI-MS/MS, UV and IR. The adulteration with synthetic PDE-5 inhibitors and their analogues puts the consumers' health at risk. It is important and urgent to determine the presence of these synthetic PDE-5 inhibitors and their analogues in health supplements and herbal products. The NMR, MS, UV and IR data reported in this paper should be helpful for identification of sildenafil related adulterants in future.

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