Turk J Chem
34 (2010) , 157 – 163.

doi:10.3906/kim-1002-25

Isolation and identification of a new acetildenafil analogue used to adulterate a dietary supplement: dimethylacetildenafil

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Received 05.02.2010

A new sildenafil analogue was found to have been added illegally to a dietary supplement marketed for the enhancement of sexual function. The structure was determined as 5-(2-ethoxy-5-{[4-(3,5-dimethyl)piperazin-1-yl]-acetyl}phenyl)-1,6-dihydro-1-methyl-3-propyl-7$H-$pyrazolo [4,3-d]pyrimidin-7-one. Owing to the inclusion of a dimethyl group in acetildenafil, the detected compound was called dimethylacetildenafil. The sample was purified with column chromatography. The IR, LC/MS (ESI), and completely assigned NMR data of dimethylacetildenafil are reported for the first time. The structure was compared with that of acetildenafil and the results showed that the ethyl substitution at the 4-position of the piperazine ring had been replaced by a 3,5-dimethyl substitution. This new acetildenafil analogue was isolated and identified for the first time.

Key Words: Acetildenafil analogue, dimethylacetildenafil, adulteration, NMR, electrospray ionization (ESI), herbal aphrodisiac.

Introduction

Synthetic phosphodiesterase-5 (PDE-5) inhibitors, such as sildenafil citrate (Viagra®, Pfizer), vardenafil hydrochloride (Levitra®, Bayer), and tadalafil (Cialis®, Lilly) have been widely used for the treatment of erectile dysfunction (ED).[1] It is important to ensure that these are prescription drugs and they must be used under medical control. A number of adverse effects of these approved drugs have been reported. Recently,

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consumption of dietary supplements has been becoming more popular around the world. Unfortunately, the adulteration of dietary supplements with undeclared synthetic chemical compounds is steadily increasing according to the literature. Some herbal products advertised as "all natural" have in contrast been found to contain synthetic PDE-5 inhibitors. These kinds of commercially available herbal aphrodisiac products have been spiked with the above-mentioned legal drugs, but also with their analogues, which have not been subjected to formal pharmacokinetic or other pharmacological testing in either humans or animals. Over 20 sildenafil analogues[2] have been detected in adulterated dietary supplements, and this number is steadily increasing. As reported many times, consumption of these analogues represents a significant health risk to patients due to their unknown safety and toxicity profile. Therefore, it is very important to discover the presence of known or unknown synthetic analogues of PDE-5 inhibitors in health supplements. In our central research laboratory, we are analyzing these commercially available supplements (sent by the Ministry of Agriculture before it grants a license for import to Turkey) to determine whether they contain these kinds of analogues; unfortunately we have determined these analogues with certainty almost every time. In this paper we report the isolation of a new acetildenafil[3,4] analogue, namely, dimethyl acetildenafil, and unambiguous structural elucidation using several instrumental analyses (Figure 1).

Materials and methods

Equipment

Uncorrected melting points were measured on a Büchi B-540 capillary melting point apparatus. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectra were recorded employing a VARIAN MERCURY 400 MHz FT spectrometer, with $CDCl_3$ as solvent. Chemical shifts (δ) are in ppm relative to TMS. The LC/MS were taken on a Waters Micromass ZQ connected with Waters Alliance HPLC, using the ESI(+) method, with a C-18 column. Elemental analyses were performed using a Leco CHNS-932. The infrared spectrum was recorded in the 600-3600 cm^{-1} range using a Jacso FT-IR-420 spectrometer and KBr pellets.

Extraction and isolation

The contents of 2 capsules of sample were mixed with a little silica gel and loaded onto an open silica gel 60 column (0.04-0.063 mm) and eluted with ethylacetate-methanol-ammonium hydroxide (25%) (100:15:1.5). Fractions were collected and analyzed by TLC. All of the fractions having the target compound were collected and the solvent was evaporated and 0.021 g of white powder compound was obtained.

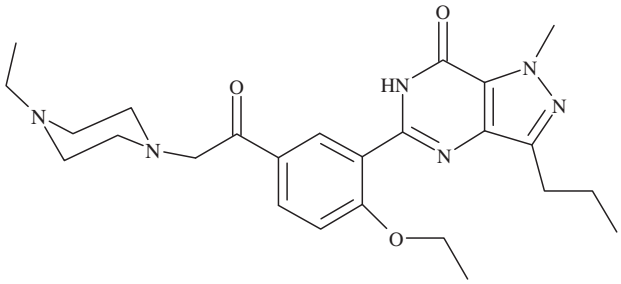
Structure identification

NMR correlation data of compound 1

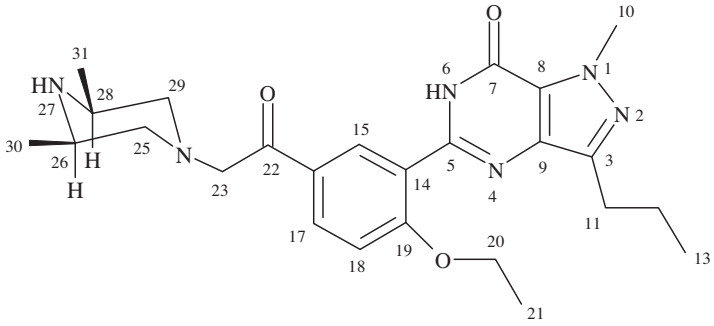
Compound **1** was dissolved in $CDCl_3$ and subjected to 1D and 2D NMR spectroscopic analysis (^{1}H, ^{13}C, DEPT, homo-COSY, NOESY, HSQC, and HMBC). The data are shown in the Table.

Analysis condition of LC/MS

LC-MS coupled with the positive (ESI+) Electro Spray method was used to determine its molecular weight. The HPLC of LC/MS was carried out on a column XTerra® MS C-18 (4.6 × 250 mm, 5 μm) with methanol:0.01 M ammonium acetate in water (65:35) as mobile phase. The flow rate was 0.7 mL/min, the injection volume was 5 μL, and the running time 20 min. The eluate was monitored by a photo-diode array detector at 273 nm. The analytical condition of mass was as follows: capillary voltage: 3.41 kV, cone voltage: 26 V, source temperature: 100 °C, desolvation temperature: 350 °C.



Acetildenafil



Compound 1

Figure 1. Chemical structure of the unknown compound (named dimethylacetildenafil).

Results and discussion

An unknown compound (referred to as compound **1**, Figure 1) was isolated from a dietary herbal aphrodisiac supplement by using column chromatographic techniques, as white powder; melting point: 136-139 °C, UV spectrum: λ max 233 and 276.4 nm (Figure 2). The IR spectrum showed absorption bands with the characteristics of an amine at 3312 cm^{-1}, an α,β-unsaturated lactam at 1689 cm^{-1}, an acetyl at 1698 cm^{-1}, an aromatic ring at 1558 cm^{-1}, and an ether group at 1244 cm^{-1} (Figure 3).

Compound **1** showed its $[M+H]^+$ ion at m/z 467.6, which corresponded to the molecular formula $C_{25}H_{34}O_3N_6$ (466.2771) (Figure 4).

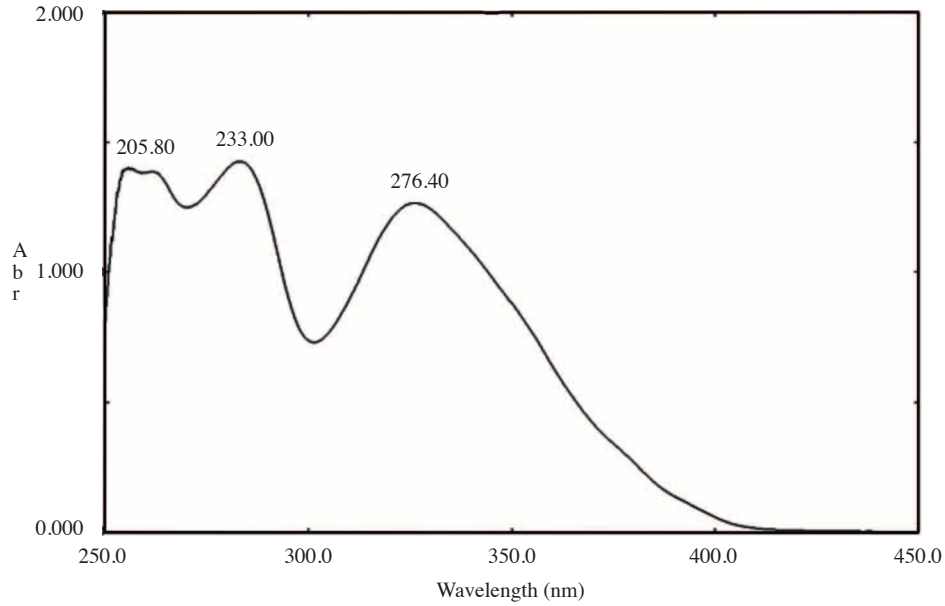


Figure 2. UV spectrum of compound **1**.

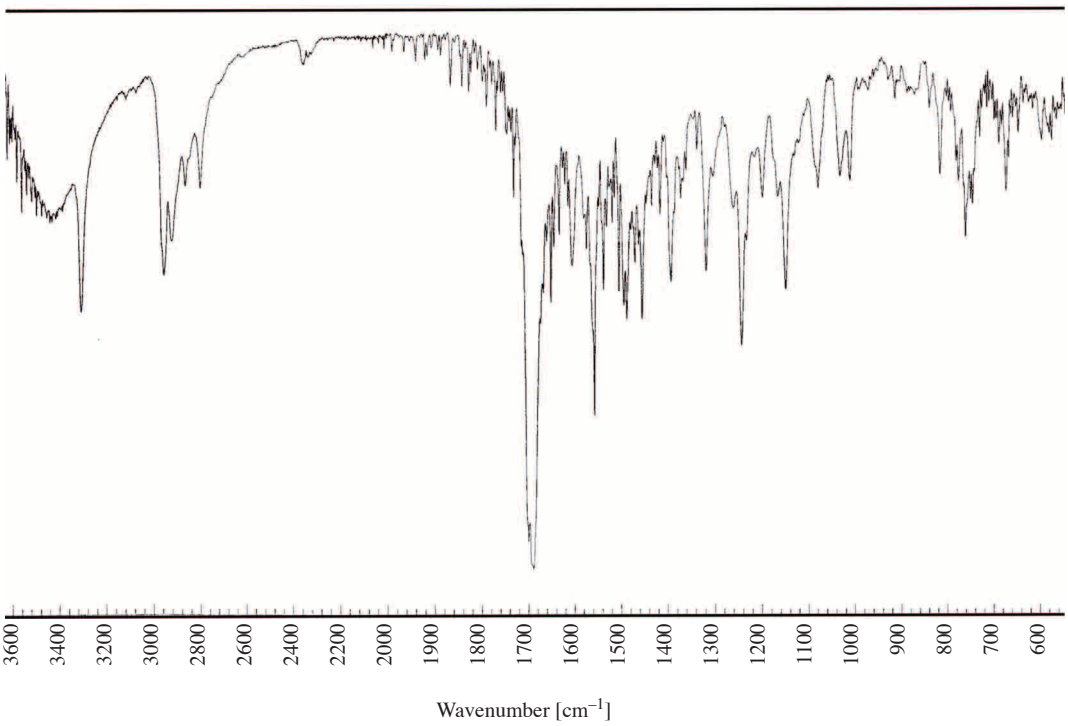


Figure 3. IR spectrum of compound **1**.

The Table shows the ^{1}H-NMR, ^{13}C-NMR, DEPT, COSY, HSQC, and HMBC spectral data of isolated compound **1**, which were similar to those of acetildenafil. The spectroscopic numbering used is given in Figure l.

The major difference between compound **1** and acetildenafil is related to piperazine protons. Homo-COSY and HSQC experiments show that the signals at δ 1.83 and δ 2.9 are the 2 geminal protons, while the signal at 3.02 δ is the methine proton. C-26,28 at δ 50.7 correlates with the proton at δ 3.02, which shows that this proton is the ring methine proton, and C-25,29 at δ 60.9 correlates with the protons at δ 2.9 and δ 1.83, which shows that these are methylene protons. The signal at δ 2.9 is regarded as the equatorial because of the deshielding effect of the C=0 group. A similar situation was established by Reepmeyer and Avignon[5] for methisosildenafil, which also has a 3,5-dimethyl piperazine ring and its 2D-NOESY experiments demonstrate the proximity of H-15 and H-17 to geminal piperazine protons. However, in our described compound there is a methylene group (C-23) between the nitrogen atom of piperazine and the C=0 group. In order to find a similar correlation in our compound **1**, we also carried out a NOESY experiment. A very strong correlation was observed between the

Table. NMR data of dimethylacetildenafil.

No.	^{13}C	^{1}H	DEPT[a]	COSY	HMBC
3	146.9	—			C-3/H-12 C-3/H-11
5	147.6	—			C-5/H-15
6	–	10.85 (br.s, 1H)			—
7	153.9	—			—
8	124.7	—			C-8/H-10
9	138.7	—			C-9/H-11
10	38.4	4.28 (s, 3H)	3		—
11	27.9	2.93 (t, 2H, J=7.2 Hz)	2	H-11/H-12	C-11/H-13, C-11/H-12
12	22.5	1.89 (m, 2H, J=7.6 Hz)	2	H-12/H-11, H-12/H-13	C-12/H-13, C-12/H-11
13	14.2	1.04 (t, 3H, J=7.6 Hz)	3	H-13/H-12	C-13/H-12, C-13/H-11
14	130.1	—		–	C-14/H-18
15	132.6	9.15 (d, 1H, J_m=2 Hz)	1	H-15/H-17	C-15/H-17
16	120.3	—			C-16/H-18
17	132.8	8.16 (dd, 1H, J_o=8.8 Hz, J_m=2 Hz)	1	H-17/H-18, H-17/H-15	C-17/H-15
18	112.8	7.09 (d, 1H, J_o=8.8 Hz)	1	H-18/H-17	—-
19	159.9	—			C-19/H-15, C-19/H-17
20	65.9	4.37 (q, 2H, J=7.2 Hz)	2	H-20/H-21	C-20/H-21
21	14.8	1.63 (t, 3H, J=6.8 Hz)	3	H-21/H-20	C-21/H-20
22	195.4	—			C-22/H-23, C-22/H-15, C-22/H-17
23	65.0	3.79 (s, 2H)	2		—
25,29	60.96	1.83 (t, 2H, J=10.4 Hz, axial) & 2.9 (dd, 2H,J=10.4 Hz, J=1.6 Hz equatorial)	2	H-25, H-29 axial/H-26, H-28	C-25,29/H-30,31 C-25,29/H-23
26,28	50.7	3.02 (m, 2H, J=3.6 Hz, axial)	1	H-26, H-28/H-25, H-29 axial H-26, H-28/H-30, H-31	C-26,28/H-30,31
30,31	19.9	1.05 (d, 6H, J=6.8 Hz)	3	H-30, H-31/H-26, H-28	—

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

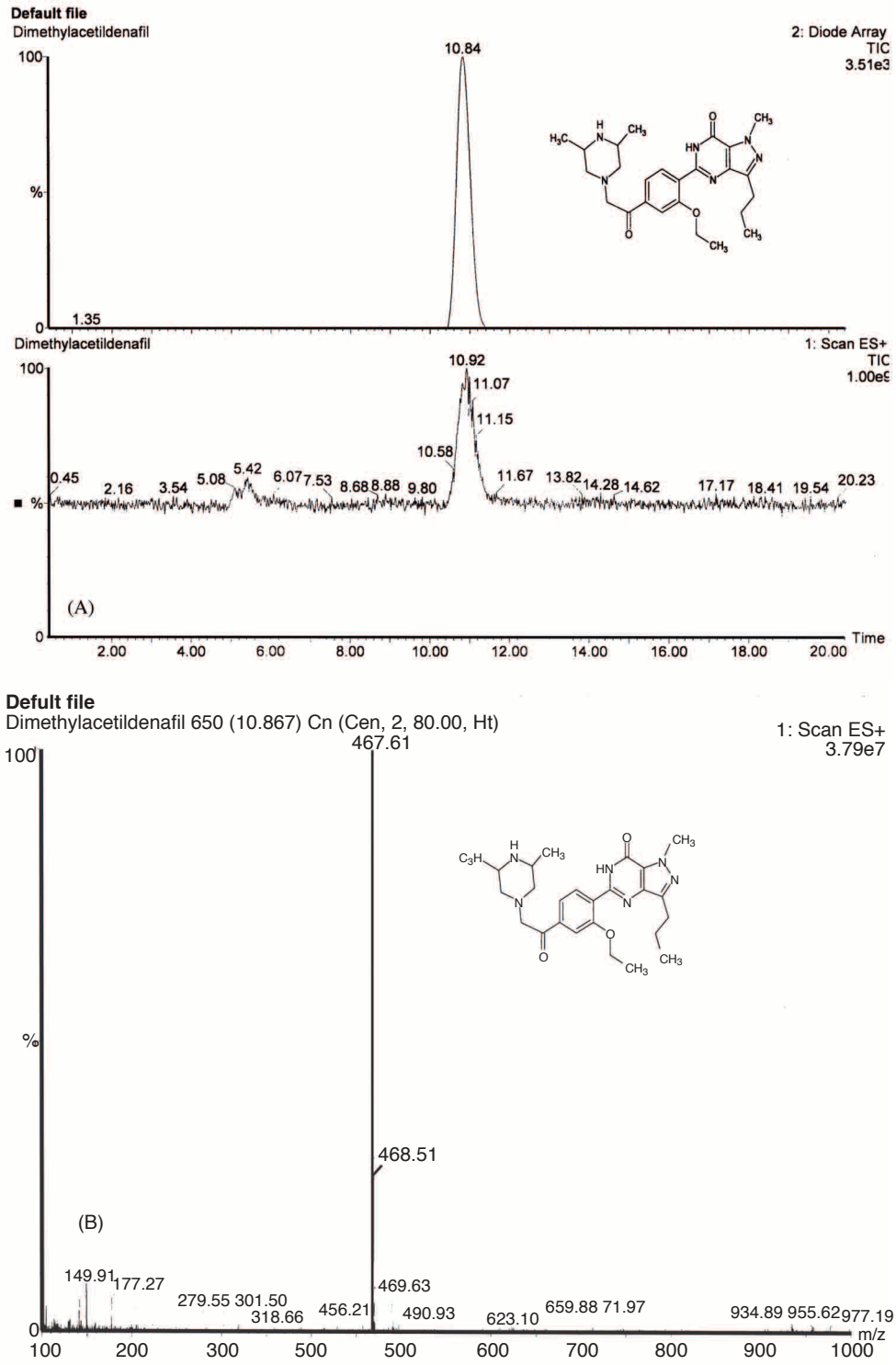


Figure 4. Mass spectrometric characterization of compound **1**. (A) HPLC and total ion chromatogram, (B) Full scan ESI+ mass spectrum for compound **1**.

H-23 and aromatic protons H15-17. Weaker NOE occurs between the geminal piperazine (equatorial stronger than axial) protons and aromatic H-15. Both of these correlations indicate that the piperazine ring tends to be oriented toward the other heterocyclic ring in the molecule as it has been suggested by Reepmeyer and Avignon.[5] Otherwise it was not possible to see the same pharmacological effects with methisosildenafil and acetildenafil analogues for binding to the same active site of phosphodiesterase type 5 enzyme (PDE-5). The 3,5-dimethyl protons of piperazine were observed at δH 1.05 (d, 6H) as expected; in the HMBC spectrum the correlation of H-30,31/C-25,29 and C-26,28, DEPT, and HSQC results indicated that the dimethyl group was attached to C26-28. Since the 2,6-diequatorial methyl groups would be in lower energetic form, the configuration is established as a cis diequatorial methyl configuration as shown in Figure 1 as it is in methisosildenafil.[5]

Conclusion

Based on the mass, UV, IR spectrum, and NMR spectroscopic data, the structure of compound **1** was determined as 5-(2-ethoxy-5-{[4-(3,5-dimethyl)piperazine-1-yl]-acetyl}phenyl)-1,6-dihydro-1-methyl-3-propyl-7$H-$pyrazolo[4,3-d]pyrimidin-7-on, a new acetildenafil analogue (Figure 1), which, when compared with acetildenafil, showed that the N-ethylpiperazine was switched with an 3,5-dimethylpiperazine. This compound must be included in the inspection list for illegal health-related substances because of the unknown safety and toxicity profile.

Acknowledgement

The Central Laboratory of the Pharmacy Faculty of Ankara University provided support for the acquisition of the NMR, mass spectrometer, and elemental analyzer used in this work.

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