Structural Elucidation of a Tadalafil Analogue Found in a Dietary Supplement

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A tadalafil analogue was detected in a dietary supplement marketed for tonic effect, along with hydroxyhomosildenafil and aminotadalafil. The tadalafil analogue was isolated by preparative thin layer chromatography (TLC) and its structure was elucidated using high-performance liquid chromatography (HPLC), liquid chromatography electrospray ionization-mass spectrometry (LC-ESI-MS), Fourier transform-ion cyclotron resonance-mass spectrometry (FT-ICR-MS) and nuclear magnetic resonance (NMR) spectroscopy. The compound was determined to be methyl-1-(1,3-benzodioxol-5-yl)-2-(chloroacetyl)-2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indole-3-carboxylate. This is the first report of detection of this compound in a dietary supplement.

Key words: tadalafil analogue; tonic effect; dietary supplement; HPLC; NMR

Introduction

Dietary supplements are widely used in Japan. There are many dietary supplements advertised for weight reduction, hyperglycemia and tonic effect, but some are adulterated with synthetic drugs1,4, and associated health hazards have been reported in the past 5 years1,4. It is potentially dangerous for consumers to take adulterated dietary supplements. Moreover, not only prescription drugs but also their analogues such as N-nitroso-fenfluramine5, homosildenafil6, acetildenafil7, hydroxyhomosildenafil7 and aminotadalafil8 have been detected. We have already established a HPLC screening method for synthetic drugs in dietary supplements, such as tonic medicines, anorexic agents, hypoglycemic agents, and so on2. Inspections of 41 dietary supplements marketed for tonic effect were carried out in 2007. During the inspection, a tadalafil analogue was detected in a dietary supplement, along with hydroxyhomosildenafil and aminotadalafil. Here we report a newly found tadalafil analogue, which was isolated by preparative TLC and identified by means of HPLC, LC-ESI-MS, FT-ICR-MS and NMR.

Materials and Methods

Sample

Capsules containing “khaki powder”, marketed for tonic effect, were purchased through the internet.

Standard and reagents

Tadalafil was purchased from Toronto Research Chemicals, Inc. (North York, ON, Canada). Aminotadalafil and hydroxyhomosildenafil were obtained from the National Institute of Health Sciences (Tokyo, Japan). HPLC grade acetonitrile, methanol and all other reagents (analytical grade) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Dimethylsulfoxide (DMSO-d6) used for NMR analysis was purchased from Isotec, Inc. (Miamisburg, OH, U.S.A.).

Mixed standard solutions

A mixed standard solution of aminotadalafil, hydroxyhomosildenafil and tadalafil (30 μg/mL each) was prepared in methanol and used for HPLC and LC-ESI-MS.

Sample preparation for HPLC and LC-ESI-MS analysis

The sample powder (10 mg) was ultrasonically extracted in 10 mL methanol for 15 min. The extract was filtered through a 0.45 μm PTFE filter (Toyo Roshi Kaisha, Ltd., Tokyo, Japan) and the filtrate was applied to HPLC and LC-ESI-MS.

HPLC analysis

HPLC analysis was performed using a Waters LC system equipped with a model 616 pump, a model CHM
column oven, and a model 996 PDA detector (Waters Co., Milford, MA, U.S.A.). A TSKgel ODS-80Ts column (150×4.6 mm i.d., 5 µm, Tosoh Co., Tokyo, Japan) was used. The mobile phase was 0.1% (v/v) phosphoric acid aqueous solution (eluent A) and acetonitrile containing 0.1% (v/v) phosphoric acid (eluent B). The gradient elution began at 80% A and linearly decreased to 35% A in 35 min. The flow rate of the mobile phase was 1.0 mL/min and the injection volume was 20 µL. The column temperature was 40°C. The wavelength of PDA was 210 to 400 nm; and monitoring of chromatograms was performed at 280 nm.

**LC-ESI-MS analysis**

LC-ESI-MS analysis was performed in positive ionization mode using a Waters Alliance 2695 separation module and ZQ mass spectrometer (Waters Co.). An Atlantis dC18 column (150×2.1 mm i.d., 3 µm, Waters Co.) was used. The mobile phase was 0.1% formic acid aqueous solution (eluent A) and acetonitrile containing 0.1% formic acid (v/v) (eluent B). The gradient elution began at 80% A and linearly decreased to 20% A in 50 min. The flow rate of the mobile phase was 0.2 mL/min and the injection volume was 10 µL. The column temperature was 40°C. The instrument parameters were: source temperature, 120°C; desolvation temperature, 350°C; capillary voltage, 3 kV; cone voltage, 30 V; and desolvation gas flow, 500 L/hr. The mass range of the spectra was from m/z 100 to m/z 800.

**Purification**

The sample powder (240 mg) was ultrasonically extracted twice in 25 mL methanol for 15 min. The extract was filtered and the filtrate was concentrated to 2 mL under vacuum. Then, the residual solution was applied to three silica gel 60 F254 TLC plates (20×10 cm with 2.0 mm thickness, Merck, Darmstadt, Germany) in bands. The plates were developed with a chloroform/ethyl acetate mixture (2 : 1) to a distance of about 7 cm. After air-drying, the plates were examined under ultraviolet (UV) light (wavelength: 254 nm). The band of the unknown compound, Rf value 0.92, was taken and dissolved in 50 mL methanol. The methanol solution was filtered, the filtrate was evaporated to dryness, and the residue was reconstituted in 3 mL methanol. This solution was filtered and the filtrate was evaporated to dryness. The residue was dissolved in diethyl ether, the solution was filtered, and the filtrate was evaporated to dryness. The purity of the compound was determined by normalization of the peak areas detected by HPLC with UV detection at 210 nm and 280 nm.

**NMR analysis**

1H and 13C NMR data were acquired on a JNM ECA-800 (JEOL Ltd., Tokyo, Japan) operating at 800 MHz and 200 MHz, respectively. DMSO-d6 was used as a solvent. Chemical shifts are expressed in ppm values relative to tetramethylsilane as an internal reference; coupling constants (J) are given in Hz. Conventional pulse sequences were used for correlated spectroscopy (COSY), heteronuclear multiple quantum coherence (HMOC), heteronuclear multiple bond correlation (HMBC) and rotating frame nuclear Overhauser effect spectroscopy (ROESY).

**FT-ICR-MS analysis**

Accurate mass of the unknown compound was measured with an LTQ Orbitrap XL instrument (Thermo Fisher Scientific K.K., San Jose, CA, U.S.A.) with direct infusion in ESI positive ion mode, under conditions of solvent flow rate 5 mL/min, sheath gas flow rate 20 arb, auxiliary gas flow rate 10 arb, spray voltage 5 kV, capillary temperature 275°C, capillary voltage 4 V, and tube lens voltage 60 V. Tyrosin 1,3,6 standard was used as a mass calibrant of the FT mass analyzer (resolution =100,000), and tyrosin 3 standard was used as a lock mass ion (m/z 508.20783) during the measurement. Theoretical mass and delta values (mmu) were calculated by using the elemental composition tool of Xcalibur/Quail Browser software.

**Results and Discussion**

HPLC chromatograms of a mixed standard solution and the sample extract are shown in Fig. 1 and the corresponding UV spectra are shown in Fig. 2. Three major peaks were detected in the sample extract at 14.47 min (peak 1), 19.38 min (peak 2) and 33.22 min (peak 3). The compounds eluted at peak 1 and peak 2 were identified as hydroxyhomosildenafil and aminotadalafil, respectively, by comparing retention time, UV spectra (Fig. 2) and mass spectra (data not shown) with those of reference standards. Although the UV spectrum of peak 3 exhibited a similar spectrum to those of tadalafil and aminotadalafil, the retention time of peak 3 (unknown compound) was later than those of tadalafl and aminotadalafil. As shown in Fig. 3, in LC-ESI-MS analysis, the retention time of peak 3 was 25.4 min. The accurate mass of the unknown compound was measured as m/z 508.20783 during the measurement. Theoretical mass and delta values (mmu) were calculated by using the elemental composition tool of Xcalibur/Quail Browser software.
analysis, the base peak of the unknown compound was observed at m/z 427 and this ion was inferred to be the [M + H]$^+$ ion.

In the purification using preparative TLC, three well-separated bands, the $R_f$ values of which were 0.92 (unknown compound), 0.75 (aminotadalafil) and 0.32 (tadalafil), were observed. The $R_f$ values of the unknown compound, aminotadalafil and tadalafil were 0.92, 0.75 and 0.32, respectively.

**Figure 2.** UV Spectra of the peaks in the HPLC chromatograms of mixed standard solution and sample extract

**Figure 3.** LC-ESI-MS spectra of tadalafil and the unknown compound

**Table 1.** NMR data of tadalafil and the unknown compound

<table>
<thead>
<tr>
<th>Position no.</th>
<th>$^1$H (δH)</th>
<th>$^{13}$C (δC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.75 (1H, s)</td>
<td>51.3</td>
</tr>
<tr>
<td>2</td>
<td>5.19 (1H, d, $J=6.9$)</td>
<td>52.3</td>
</tr>
<tr>
<td>3</td>
<td>3.07 (1H, d, $J=6.9$, 14.7)</td>
<td>21.0</td>
</tr>
<tr>
<td>4</td>
<td>3.45 (1H, d, $J=14.7$)</td>
<td>21.0</td>
</tr>
</tbody>
</table>

$a)$ All measurements were made in DMSO-$d_6$.
(hydroxyhomosildenafil), were obtained from the sample solution. The band of the unknown compound afforded approximately 1.7 mg yellow powder, the purity of which was determined to be more than 96%, based on HPLC with detection at both 210 and 280 nm.

The structure of the unknown compound was further elucidated using NMR analysis. NMR spectral data of tadalafil and the unknown compound are shown in Table 1. The signals of tadalafil were in agreement with published data.

The 1H NMR and 13C NMR spectroscopic data of the unknown compound were similar to those of tadalafil except for the lack of a N-methyl group and the a methylene protons of the glycine moiety in the dioxopiperazine ring. The 1H NMR signals at δ 4.44 (1H, d, J = 13.8 Hz), 4.83 (1H, d, J = 13.8 Hz) and 3.02 (3H, s) indicated the presence of a chloromethylene group and a carbomethoxy group. This suggests that the dioxopiperazine ring was cleaved into a carboxylic methylester group and chloromethylene group. The connectivity of these groups was deduced from the HMBC correlations (Fig. 5). The methylene protons (H2-4), methine proton (H-3), and methyl proton (H3-CH3) correlated to an ester carbon (COOCH3). In addition, the methine proton (H-1), methine proton (H-3), and chloromethylene protons (CH2Cl) showed correlations to a carbonyl carbon (COOCH3Cl). The relative stereochemistry between H-1 and H-3 was determined by its ROESY correlation (Fig. 6). A ROESY correlation between H-1 and H-3 indicated that the two protons, H-1 and H-3, were oriented cis to each other. This is the same as the stereochemistry of tadalafil. The absolute configuration of the unknown compound remains to be determined.

The FT-ICR-MS measurement was carried out in order to confirm the molecular formula. Accurate FT-ICR-MS measurement on the unknown compound revealed the [M+Na]+ ion at m/z 449.0874 (calc. 449.0880) (base peak) and [M+H]+ ion at m/z 427.1055 (calc. 427.1061) (relative intensity: 30%), corresponding to the molecular formula of C22H19ClN2O5.

From these results, the unknown compound was determined to be methyl-1-(1,3-benzodioxol-5-yl)-2-(chloroacetyl)-2,3,4,9-tetrahydro-1H-pyrindole-3-carboxylate. Literature search indicated that the planar structure of this compound corresponds to a tadalafil synthetic precursor. This compound was detected for the first time in a dietary supplement.

Tadalafil is a PDE-5 inhibitor and is used to treat penile erectile dysfunction. Pharmacological studies indicate that interaction between PED-5 inhibitors and nitrates may cause severe blood pressure reduction. Although there have been no systematic studies on the efficacy and toxicology of PDE-5 inhibitor analogues, one case of liver function impairment in Japan may have been due to the use of products containing hydroxyhomosildenafil. It is potentially dangerous for consumers to take dietary supplements adulterated with PED-5 inhibitors analogues. Therefore, inspection of synthetic drugs in dietary supplements is important and should be carried out in the future.

Acknowledgments

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References

1) Kojima, T., Kishi, M., Sekita, S., Satake, M. Origin of sennosides in dietary supplements containing senna

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**Fig. 4.** Chemical structure of tadalafil

**Fig. 5.** 1H-1H COSY and selected HMBC correlations of the unknown compound

**Fig. 6.** Selected ROESY correlation of the unknown compound


