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To cite this article: Yen-Chun Huang, Hui-Chun Lee, Yun-Lian Lin, Ya-Tze Lin, Chia-Fen Tsai & Hwei-Fang Cheng (2016) Identification of a new sildenafil analogue adulterant, desethylcarbodenafil, in a herbal supplement, Food Additives & Contaminants: Part A, 33:11, 1637-1642, DOI: 10.1080/19440049.2016.1236402

To link to this article: http://dx.doi.org/10.1080/19440049.2016.1236402
Identification of a new sildenafil analogue adulterant, desethylcarbodenafil, in a herbal supplement

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ABSTRACT
In a maca-containing herbal supplement claimed to remedy erectile dysfunction, a new sildenafil analogue was found using adulterant screening with TLC, GC-MS and LC-MS/MS. This compound was isolated by column chromatography and HPLC, and identified by extensive 1D- and 2D-NMR and mass spectral analyses. The structure of this new compound was established as 5-[(2-ethoxy-5-(piperazine-1-carbonyl)phenyl]-1-methyl-3-propyl-1,6-dihydropyrazolo[4,3-d]pyrimidin-7-one, and was named desethylcarbodenafil.

ARTICLE HISTORY
Received 23 June 2016
Accepted 11 September 2016

KEYWORDS
Sildenafil analogue; desethylcarbodenafil; adulterated food supplements

Introduction
The rate of detection of adulteration of supplement products for sexual enhancement has been the highest in adulterant screening, and the adulterants detected are often PDE-5 inhibitor drugs and their analogues. Analogues of the first-approved PDE-5 inhibitor sildenafil have been reported more frequently than other approved ones. In 2014, more than 40 analogues of sildenafil were identified, and some had two or more common names (Patel et al. 2014). Based on the modification of pyrazolo pyrimidine-7-one and sulfonamide moieties, those analogues could be classified into four groups: (1) analogues with both moieties (as sildenafil) like the first reported analogue homosildenafil (Shin et al. 2003; Lai et al. 2006); (2) analogues with just pyrazolo pyrimidine-7-one, but without the sulfonamide moieties (mostly with carbonyl group) like acetildenafil and carbodenafil; (3) analogues with pyrazolo pyrimidine-7-thione like propoxyphenyl hydroxyethylsildenafil (Liao et al. 2013) and dithiodimethylcarbodenafil (Ge et al. 2011); and (4) others with miscellaneous moieties.

In this study, a new sildenafil analogue (compound YJ-09; Figure 1) was detected in a herbal product from the market which was claimed to contain maca root extract, cordyceps extract, rhodiola rosea, zinc gluconate and black pepper. The structure of the sildenafil analogue was elucidated by spectroscopic analysis including UV, NMR and accurate MS.

Materials and methods

Materials
A carbodenafil standard was purchased from TLC Pharmachem, Inc. (Vaughan, ON, Canada). Acetonitrile (ACN), n-butanol, methanol, chloroform and ethyl acetate (HPLC grade) were purchased from J.T. Baker Avantor (Center Valley, PA, USA). Ethanol (95%) was produced by Taiwan Tobacco and Liquor Corporation (Taipei, Taiwan). Analytical reagent-grade formic acid was obtained from Sigma Aldrich (St. Louis, MO, USA). Dimethylsulfoxide-d<sub>6</sub> (DMSO-d<sub>6</sub>) used in NMR experiments and silica gel (Kieselgel 60, 70–230 mesh) were supplied by Merck (Darmstadt, Germany).

Detection of the new compound
Products from the market were screened for adulterants by TLC, GC-MS and LC-MS/MS following the ‘method of test for adulterants in Chinese medicine and foods’ (TFDA 2012). When the chromatogram showed obvious spots on TLC plates or unusually high peaks in GC-MS or LC-MS/MS, but the detected compounds could not...
be identified with our drug and analogue library of MS fragments, they would be further studied.

**Isolation of compound YJ-09**

The contents of 20 red capsules (13.6 g) were extracted three times with 50 ml methanol, and the combined crude extract was evaporated (657 mg) and partitioned with chloroform and water. The dried organic layer (429 mg) was fractionated by column chromatography on silica gel eluted by chloroform–methanol (19:1, v/v) to yield five fractions. All fractions were tested by TLC analysis, and fraction 2 containing YJ-09 was selected for further purification by HPLC (Hitachi Elite LaChrom HPLC System with Hitachi Diode Array systems) on a Purospher®STAR RP-18 end-capped column (10 × 250 mm, 5 μm; Merck) with a mobile phase of ACN–water (54:46, v/v) to give a purified compound YJ-09 (10.5 mg).

**TLC and UV analysis**

TLC was performed on a Kieselgel 60 F254 TLC plates (0.20 mm; Merck). After development with chloroform–methanol (9:1, v/v), TLC plates were observed under UV light at 254 and 366 nm, and then sprayed with Dragendorff’s spray. Dragendorff’s spray reagent was prepared as follows: solution A (2 g bismuth subnitrate and 25 ml in 100 ml water) and solution B (40 g potassium iodide in 100 ml water) were mixed in equal volumes. The UV spectra were measured in 95% ethanol on a Varian Cary 300 Bio UV/Vis spectrophotometer.

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**Figure 1.** Structures of carbodenafil and YJ-09.
**LC-MS/MS analysis**

An Agilent 6530 Accurate-Mass Q-TOF/MS with 1290 infinity LC was used for high-resolution MS analysis. Carbodenafil standard and purified YJ-09 were dissolved in methanol, and separated by an Agilent Poroshell 120 SB-C18 column (4.6 × 150 mm, 2.7 μm) with a linear gradient of solvent A (0.1% formic acid solution) and solvent B (ACN containing 0.1% formic acid) from 90% to 0% solvent A in 20 min and maintained for 5 min, and reversed to the original condition at 30 min at the flow rate of 0.5 ml min⁻¹. The MS experiments were performed in positive-ion mode with fragmentor energy at 150 V and gas temperature at 280°C. The mass scan range (m/z) was from 100 to 1700 Da, and the MS scan rate was 2.00 spectra s⁻¹ for MS data. The mass scan range (m/z) was from 100 to 800 Da with collision energy at 20 eV for MS/MS data. Data acquisition and qualitative analysis were performed using an Agilent LC-Q-TOF/MS MassHunter Workstation Software (Ver. B.05.00, Agilent Technologies, Palo Alto, CA, USA).

**NMR spectroscopy**

1D- and 2D-NMR spectra (¹H, ¹³C, DEPT, homocOSY, HMQC, HMBC and TOCSY) were obtained using Bruker AVIII 500 MHz FT-NMR spectrometer in DMSO-d₆. Chemical shifts are reported in δ (ppm) values with the solvent peak used as a reference, and coupling constants (J) were reported in Hertz (Hz).

**Results and discussion**

Compound YJ-09 was detected in a sex-enhancing product by adulterant screening because of the unusually high amount present, and showed the same colour as sildenafil on TLC plates with Dragendorff’s spray. After purification, the UV spectrum of YJ-09 (Figure 2), especially the shoulder at about 230 nm, showed a strong similarity to carbodenafil but not sildenafil.

Accurate MS data of purified YJ-09 were compared with those of carbodenafil standard in LC-Q-TOF/MS (Figure 3(a)). The peak of YJ-09 at 8.30 min was separated from that of carbodenafil at 8.67 min. The mass of YJ-09 was m/z 425.2311 for [M + H]⁺, corresponding to a molecular formula of C₂₂H₂₈N₆O₃. The mass of YJ-09 is less than that of carbodenafil at about 27. The MS/MS spectrums of the parent ion m/z 425 (YJ-09) and m/z 453 (carbodenafil) are shown in Figure 3(b); both had the major fragments ion at m/z 339.1 and 311.0. The fragment ion at m/z 339.1 could be formed by the cleavage of C-16 and N-17, and that at m/z 311.1 could be formed by further loss of the O-linked ethyl group, which indicated that the structural difference between YJ-09 and carbodenafil might be the modification of the piperazine ring.

The NMR data of YJ-09 are listed in Table 1. With the ¹H-NMR spectrum, three aromatic proton signals at δ7.63 (1 H, d, J = 2.3 Hz), δ7.50 (1 H, dd, J = 8.7, 2.3 Hz) and δ7.17 (1 H, d, J = 8.7 Hz) indicated a 1,3,4-trisubstituted benzene. Another methyl proton signal at δ4.14 (3 H, s) with a high chemical shift implied a methyl group conjugated with an adjacent nitrogen atom. Based on the COSY spectrum (Figure 4(a)), the H-H correlation of the signals at δ4.16 (2 H, q, J = 7.0 Hz) and δ1.32 (3 H, t, J = 7.0 Hz) indicated an ethyl group connected to an oxygen atom. The H-H correlation of the signals at δ2.76 (2 H, t, J = 7.4 Hz), δ1.72 (2 H, q, J = 7.4 Hz) and δ0.92 (3 H, t, J = 7.4 Hz) indicated a propyl group. All the above NMR data showed that YJ-09 had consistent pyrazolo pyrimidine-7-one and ethoxyphenyl moieties. Furthermore, TOCSY correlation of H-18/22 and H-19/21 in Figure 4(b) and their high chemical shift suggested a piperazine moiety, and HMBC correlations of H-13 and H-15 with C-16 in Figure 4(c) and Table 1 showed the linkage of a carbonyl group. However, there were no signals for the ethyl group that connected to the nitrogen atom of the piperazine ring as carbodenafil.
Therefore, YJ-09 was identified as 5-[2-ethoxy-5-(piperazine-1-carbonyl) phenyl]-1-methyl-3-propyl-1,6-dihydro-pyrazolo[4,3-d]pyrimidin-7-one, and since YJ-09 had a similar skeleton to carbodenafil but without the ethyl group in the piperazine ring, its trivial name is desethylcarbodenafil.

**Conclusions**

A new sildenafil analogue isolated from a herbal product was characterised by UV, LC-Q-TOF/MS and NMR. These data showed that the new analogue had a structural similarity with carbodenafil, but no ethyl...
Table 1. NMR data of compound YJ-09 (in DMSO-$d_6$ at 500 MHz for $^1$H, and 125 MHz for $^{13}$C).

<table>
<thead>
<tr>
<th>Number $^a$</th>
<th>$\delta^b_\text{H}$</th>
<th>$\delta^b_\text{C}$</th>
<th>COSY</th>
<th>HMBC</th>
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<tr>
<td>1</td>
<td>–</td>
<td>153.6</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>–</td>
<td>124.2</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>–</td>
<td>137.9</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>5</td>
<td>–</td>
<td>148.9</td>
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<td>–</td>
</tr>
<tr>
<td>9</td>
<td>–</td>
<td>144.9</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>10</td>
<td>–</td>
<td>122.5</td>
<td>–</td>
<td>–</td>
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<tr>
<td>11</td>
<td>–</td>
<td>157.2</td>
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<td>–</td>
</tr>
<tr>
<td>12</td>
<td>7.17 (1H, d, $J = 8.7$ Hz)</td>
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<td>H-13</td>
<td>C-10, C-11, C-14</td>
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<tr>
<td>13</td>
<td>7.30 (1H, dd, $J = 8.7$, 2.3 Hz)</td>
<td>130.8</td>
<td>H-12, H-15</td>
<td>C-11, C-15, C-16</td>
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<tr>
<td>14</td>
<td>–</td>
<td>127.8</td>
<td>–</td>
<td>–</td>
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<tr>
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<td>16</td>
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<td>18/22</td>
<td>3.42 (4H, br s)</td>
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<tr>
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<tr>
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<td>C-11, C-25</td>
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<tr>
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<td>28</td>
<td>0.92 (3H, t, $J = 7.4$ Hz)</td>
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<td>C-26, C-27</td>
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<td>6-NH</td>
<td>11.90 (1H, br s)</td>
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<td>20-NH</td>
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<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Notes: $\delta = \text{ppm}$; $J = \text{Hz}$.

$^a$See Figure 1 for atom numbering.

$^b$br, Broad signal; d, doublet; dd, doublet of doublet; q, quartet; s, singlet; t, triplet.

$^c$Signals are missing.

Figure 4. (a) Correlations of H-24 and H-25 (dotted lines), and correlations of H-26, H-27 and H-28 (dot–dash lines) (COSY-NMR spectrum); (b) correlation of H-18/22 and H-19/21 (TOCSY-NMR spectrum); and (c) correlations of H-13 and H-15 with C-16 (HMBC-NMR spectrum).
group in the piperazine ring. This newly identified compound was named as desethylcarbodenafil.

**Acknowledgements**

The authors gratefully thank the financial support from Taiwan Food and Drug Administration and the technical assistance of NMR spectra acquirement by the instrumentation Center of National Taiwan University.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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