Simultaneous Identification of 18 Illegal Adulterants in Dietary Supplements by Using High-Performance Liquid Chromatography-Mass Spectrometry

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We developed a method for the identification of 18 illegal adulterants in dietary supplements for erectile dysfunction by using high-performance liquid chromatography-mass spectrometry. The separation was achieved on a Cosmosil 3C18-EB column. The mobile phase consisted of 0.1% formic acid solution and 0.1% formic acid in acetonitrile, with gradient elution at a flow rate of 0.15 mL/min. The proposed method may be useful for the identification of illegal adulterants and for quality control of dietary supplements.

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Key words: dietary supplement; erectile dysfunction; medicinal ingredient; simultaneous identification

Introduction

In recent years, impairment of health owning to ingestion of dietary supplements that contain medicinal ingredients has become a social problem. Since this may be due to the presence of illegal adulterants, it is necessary to analyze commercial dietary supplements to confirm their purity. Inhibitors of phosphodiesterase type 5 (PDE-5), such as sildenafil, tadalafil, and vardenafil are administered to treat erectile dysfunction and pulmonary arterial hypertension, and they have recently been detected in dietary supplements for erectile dysfunction. The known side effects of PDE-5 inhibitors include headaches and visual abnormalities. The combined use of PDE-5 inhibitors and nitric monoxide donors could be fatal. Various ingredients whose structures have been modified from those of medicinal ingredients, such as sildenafil, in order to avoid identification have also been detected in dietary supplements. To efficiently analyze the many adulterants that may be present in commercial dietary supplements, a simultaneous identification method is needed. In this study, we developed a method to identify simultaneously 18 adulterants in dietary supplements by using LC/MS.

Materials and Methods

Chemicals and reagents

LC/MS grade acetonitrile, methanol, acetic acid, and formic acid were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). LC/MS grade ammonium formate and ammonium acetate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hydroxythiohomosildenafil, dimethylsildenafil, acetalacid, thiohomosildenafil, thiodimethylsildenafil, and chloropretadalafil were purchased from TLC Pharma Chem (Ontario, Canada). Hydroxyhomosildenafil, thiosildenafil, pseudovardenafil, gendein, and neoneosildenafil were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Amidotadalafil was purchased from Toronto Research Chemicals (Ontario, Canada). Sildenafil, tadalafil, vardenafil, xanthoanthrafil, and a positive control containing approximately 18% acetildenafil were kindly provided by the National Institute of Health Sciences in Japan. The positive control of acetildenafil was used as a standard. Standard stock solution of homosildenafil (approximately 75.5 ppm) was provided by the Department of Pharmaceutical Sciences, Tokyo Metropolitan Institute of Public Health. The chemical structures of compounds used in this study are shown in Fig. 1.

Instrumentation and chromatographic conditions

The LC-electrospray ionization-MS experiments were performed using a Prominence Ultra Fast Liquid Chromatograph (UFLC) system and an LCMS-2020 quadrupole mass spectrometer (Shimadzu, Kyoto, Japan). A Cosmosil 3C18-EB column (particle size, 3 μm; i.d., 2.0 mm; length, 250 mm, Nacalai Tesque, Kyoto, Japan) was used. The following gradient system was used. Mobile phase A consisted of 0.1% formic acid solution and mobile phase B 0.1% formic acid in acetonitrile. The gradient elution program, with a constant flow rate of 0.15 mL/min, was started with solution A at 70%, followed by...
Fig. 1. Chemical structures of the 18 adulterants examined
a linear decrease to 30% in 50 min. The injection volume was 1 μL. The column temperature was maintained at 40°C. The values of the instrument parameters were as follows: source temperature, 350°C; desolvation temperature, 250°C; and desolvation gas flow, 600 L/h. In the scan mode analysis, the mass range of the spectra was m/z 100–800. All LC-MS runs were performed in the positive mode.

**Preparation of standard solutions**

The standards were each dissolved in methanol to make standard stock solutions. The working standard solution was then prepared from the standard stock solutions.

**Sample preparation**

The dietary supplement (10 mg) to be investigated was either a powdered tablet or the contents of a capsule. The sample was extracted with 10 mL of methanol

<table>
<thead>
<tr>
<th>Compound</th>
<th>m/z</th>
</tr>
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<tbody>
<tr>
<td>Gendenafil</td>
<td>355</td>
</tr>
<tr>
<td>Acetil-acid</td>
<td>357</td>
</tr>
<tr>
<td>Tadalafil</td>
<td>390</td>
</tr>
<tr>
<td>Xanthoanthrafil</td>
<td>390</td>
</tr>
<tr>
<td>Aminotadalafil</td>
<td>391</td>
</tr>
<tr>
<td>Chloropretadalafil</td>
<td>427</td>
</tr>
<tr>
<td>Nornosildenafil</td>
<td>460</td>
</tr>
<tr>
<td>Pseudovardenafil</td>
<td>460</td>
</tr>
<tr>
<td>Acetildenafil</td>
<td>467</td>
</tr>
<tr>
<td>Sildenafil</td>
<td>475</td>
</tr>
<tr>
<td>Dimethylsildenafil</td>
<td>489</td>
</tr>
<tr>
<td>Homosildenafil</td>
<td>489</td>
</tr>
<tr>
<td>Vardenafil</td>
<td>489</td>
</tr>
<tr>
<td>Thiosildenafil</td>
<td>491</td>
</tr>
<tr>
<td>Hydroxyhomosildenafil</td>
<td>505</td>
</tr>
<tr>
<td>Thiodimethylsildenafil</td>
<td>505</td>
</tr>
<tr>
<td>Thiohomosildenafil</td>
<td>505</td>
</tr>
<tr>
<td>Hydroxythiohomosildenafil</td>
<td>521</td>
</tr>
</tbody>
</table>

**Fig. 2**. Extracted ion chromatograms of standard solution of aminotadalafil (concentration: 1 μg/mL)

A: mobile phase 1, B: mobile phase 2, C: mobile phase 3
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by sonication for 10 min using an 8510J-MT ultrasonicator (BRANSON, Danbury, CT, USA) according to the previous reported method. The solution was then filtered using a 0.45-μm pore size membrane. The eluate was diluted 10-fold with methanol to form the sample solution.

Results and Discussion

Selection of chromatographic separation conditions

Initially, the composition of the mobile phase was investigated to enable separation of all 18 compounds. Three kinds of mobile phases (mobile phase 1: mobile phase A, 0.1% formic acid solution and mobile phase B, 0.1% formic acid in acetonitrile; mobile phase 2: mobile phase A, 0.1% formic acid in 5 mM ammonium formate and mobile phase B, 0.1% formic acid in acetonitrile; and mobile phase 3: mobile phase A, 0.1% acetic acid in 5 mM ammonium acetate and mobile phase B, 0.1% acetic acid in acetonitrile) were used to analyze the standard solutions of all 18 compounds (each at a concentration of 1 μg/mL). The concentration of each compound was 1 μg/mL.

The m/z values shown in Table 1 were selected to plot the extracted ion chromatograms. Our results showed that the sensitivity of aminotadalafil was low and the peak of aminotadalafil (1 μg/mL) was not detected in mobile phases 2 and 3 (Fig. 2). Thus, mobile phase 1 was selected as the mobile phase.

Secondly, the gradient condition was investigated. The gradient elution program, with a constant flow rate of 0.15 mL/min, was started with solution A at 80% (gradient program 1), 70% (gradient program 2), or 60% (gradient program 3), followed by a linear decrease to 20% (gradient program 1), 30% (gradient program 2), or 40% (gradient program 3) in 50 min. The gradient program 2 was selected because the retention times of the compounds were widely distributed (Fig. 3).

Extracted ion chromatograms of standard solution including all 18 compounds (each at a concentration of 0.5 μg/mL) are shown in Fig. 4.

Comparison of columns

To choose the best column, the standard solution including all 18 compounds (each at a concentration of 0.5 μg/mL) was analyzed by the proposed method using a Cosmosil 3C18-EB column and a conventional octadecylsilica gel column (Cosmosil 3C18-MS-II column; particle size, 3 μm; i.d., 2.0 mm; length, 250 mm; Nacalai Tesque). It is very important to separate the peaks of different illegal adulterants, especially when a dietary supplement contains multiple compounds possessing the same molecular weight. Hydroxyhomosildenafil, thiodimethylsildenafil, and thiohomosildenafil have the same molecular weight, and the molecular ion (m/z 505) was the major peak for each compound in the positive mode. Hydroxyhomosildenafil, thiohomosildenafil, and thiodimethylsildenafil were clearly separated (the resolutions were 49.0 and 3.3), and sharp peaks were obtained on the 3C18-EB column (Fig. 5A). On the other hand, thiohomosildenafil and thiodimethylsildenafil were not clearly separated on the conventional ODS column (Fig. 5B). The proposed method could also separate vardenafil, homosildenafil, and dimethylsildenafil (the resolutions were 17.3 and 3.2 at m/z: 489, tadalafil, and xanthoanthrafil (the resolution was 4.2 at m/z: 390), and pseudovardenafil and norneosildenafil (the resolution was 40.4 at m/z: 460). Thus, it was consid-
Considered that the proposed method is suitable for distinguishing the peaks of compounds possessing the same molecular weight.

**Detection limit**

The detection limit of each compound was estimated based on a signal-to-noise ratio of 3. The detection limit of each compound was lower than 0.1 μg/mL. Depending on the sample preparation, the detection limits of the 18 compounds were below 1 mg/g. Thus, the proposed method has sufficient sensitivity for detecting illegal adulterants, since the prescribed dosage of sildenafil, tadalafil, and vardenafil is over 5 mg.

**Application of the proposed method**

We applied the proposed method to analyze 6 dietary supplements (A to F), which were examined in 2011 and were found to contain one or more illegal adulterants. We bought the dietary supplements via the Internet in 2011. Dietary supplements A and B contained sildenafil. Dietary supplement C contained pseudovardenafil. Dietary supplement D contained hydroxyhomosildenafil and aminotadalafil. Dietary supplement E contained thiohomsildenafil, thiosildenafil, and homosildenafil. Dietary supplement F contained hydroxythiohomsildenafil, aminotadalafil, thiosildenafil, dimethyl sildenafil, and thiodimethylsildenafil. The peak derived from each compound was clearly detected in all cases. Extracted ion chromatograms of dietary supplement F are shown in Fig. 6. Thus, the proposed method is adequate for the detection of illegal adulterants in commercial dietary supplements.

**Conclusion**

In this study, we developed a method to identify 18 illegal adulterants in dietary supplements. The proposed method can distinguish the peaks of multiple compounds possessing the same molecular weight, and is suitable to identify harmful illegal adulterants in dietary supplements.
Acknowledgements

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References

3) Zhang, Y., Huang, Z., Ding, L., Yan, H., Wang, M., Zhu, S. Simultaneous determination of yohimbine, sildenafil, vardenafil and tadalafl in dietary supplements using high-performance liquid chromatography-tandem mass...


