Simultaneous Identification of Hydroxythiohomosildenafil, Aminotadalafil, Thiosildenafil, Dimethylsildenafil, and Thiodimethylsildenafil in Dietary Supplements Using High-Performance Liquid Chromatography-Mass Spectrometry

(Received September 6, 2012)

Takaomi Tagami*, Akihiro Takeda, Akiko Asada, Airin Aoyama, Takahiro Doi, Keiji Kajimura and Yoshiyuki Sawabe

Osaka Prefectural Institute of Public Health:
1–3–69 Nakamichi, Higashinari-ku, Osaka 537–0025, Japan
* Corresponding author

We developed a method for the separation and identification of illegal adulterants (hydroxythiohomosildenafil, aminotadalafil, thiosildenafil, dimethylsildenafil, and thiodimethylsildenafil) from dietary supplements using high-performance liquid chromatography-mass spectrometry. The separation was achieved on a C18 column: the mobile phase consisted of 5 mM ammonium formate (pH 6.3)–acetonitrile (75 : 25, v/v) and acetonitrile, with gradient elution at a flow rate of 0.2 mL/min. The proposed method could also be used to separate vardenafil, homosildenafil, and dimethylsildenafil, all of which have the same molecular weight. Furthermore, the proposed method could simultaneously separate hydroxythiohomosildenafil, aminotadalafil, thiosildenafil, dimethylsildenafil, thiodimethylsildenafil, vardenafil, and homosildenafil. Thus, this method may be useful to identify medicinal ingredients for erectile dysfunction and their analogs and to control the quality of dietary supplements.

Key words: LC-MS; dietary supplement; erectile dysfunction; medicinal ingredients

Introduction

In recent years, adverse effects of dietary supplements that contain medicinal ingredients has become a social problem1. To prevent impairment of health by medicinal ingredients present in dietary supplements, it is necessary to analyze the components commercial of dietary supplements. Inhibitors of phosphodiesterase type 5 (PDE-5) such as sildenafil, tadalafil, and vardenafil are administered to treat erectile dysfunction and have recently been detected in dietary supplements2,3. The known side effects of PDE-5 inhibitors include headaches and visual abnormality. Mortality could also result from the combined use of PDE-5 inhibitors and nitric monoxide donors4. 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 supplements4–7. Sometimes, more than one kind of medicinal ingredient or analog is detected in a single dietary supplement8. For example, in 2011, we detected 5 analogs (hydroxythiohomosildenafil (HT; 13 mg/capsule), aminotadalafil (AT; 10 mg/capsule), thiosildenafil (TS; 5 mg/capsule), dimethylsildenafil (DS; 53 mg/capsule), and thiodimethylsildenafil (TDS; 10 mg/capsule)) in a single dietary supplement8.

Generally, medicinal ingredients and analogs are quantified by HPLC-UV and identified by LC-MS. It is critically important to separate the peaks of multiple medicinal ingredients and analogs in order to accurately quantify (by HPLC-UV) and identify (by LC-MS) them. It is also undesirable to use counter ions for the LC-MS mobile phase. In this study, we developed a method to separate 5 analogs using LC-MS.

Materials and Methods

Chemicals and reagents

LC-MS grade acetonitrile, methanol, and formic acid were purchased from Wako Pure Chemical Industries (Osaka, Japan). LC-MS grade ammonium formate, aqueous ammonia solution, and ammonium bicarbonate were purchased from Sigma-Aldrich (St. Louis, MO, USA). The HT, DS, and TDS standards were purchased from TLC Pharma Chem (Ontario, Canada). The TS standard was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).
from Toronto Research Chemicals (Ontario, Canada). The vardenafil standard was provided by the National Institute of Health Sciences in Japan. Standard stock solution of homosildenafil (75.5 ppm) was provided by the Tokyo Metropolitan Institute of Public Health, Department of Pharmaceutical Sciences.

Instrumentation and chromatographic conditions

The LC-electrospray ionization-MS experiments were performed using a Prominence UFLC liquid chromatography system and an LCMS-2020 quadrupole mass spectrometer (Shimadzu, Kyoto, Japan). An Inertsil ODS-3 column (5 mm particle size, 2.1 mm i.d. × 150 mm) (GL Science, Tokyo, Japan) was used. The following gradient system was used with mobile phase A (5 mM ammonium formate (pH 6.3)–acetonitrile (75 : 25, v/v)) and mobile phase B (acetonitrile) delivered at 0.2 mL/min; A : B, 100 : 0 (0–3 min); 70 : 30 (13–20 min); and 50 : 50 (30–50 min). The injection volume was 1 mL. The column temperature was 40°C. The instrument parameters were as follows: source temperature, 350°C; desolvation temperature, 250°C; and desolvation gas flow, 600 L/hr. The mass range of the spectra was m/z 100–800 in the scan mode analysis. All LC/MS was performed in both positive and negative modes.

Preparation of standard solutions

Each standard was dissolved in methanol to make 100–500 µg/mL standard stock solutions. The working standard solutions (1 µg/mL) were then prepared from the standard stock solutions.

Sample preparation

The dietary supplement (10 mg) investigated was either a powdered tablet or the contents of a capsule. Spiking solution (each medicinal ingredient: 10 µg) was added. After 30 min, the sample was extracted with 10 mL of methanol by sonicking for 10 min. The solution was then filtered through a 0.45-µm pore size membrane prior to LC-MS analysis.

Results and Discussion

Optimization of chromatographic separation conditions

Except for the mobile phase A and the flow rate, chromatographic conditions were based on the methods of the National Institute of Health Sciences in Japan. The method has previously been used to analyze 20 different medicinal ingredients and analogs using 5 mM ammonium formate (pH 3.5)–acetonitrile (75 : 25, v/v) as mobile phase A. Though this method is very useful, it does not measure HT, DS and TDS. Initially, 5 mM ammonium formate (pH 3.5)–acetonitrile (75 : 25, v/v) was used as mobile phase A to analyze the standard solutions of all 5 analogs (HT, AT, TS, DS, and TDS, each at a concentration of 1 µg/mL). To plot the mass chromatogram, m/z 521 (HT), 491 (TS), 489 (DS), and 505 (TDS) in the positive mode and 389 (AT) in the negative mode were extracted as major peaks of the 5 analogs. Our results showed that HT and TDS were not separated. This was unexpected, since previous reports have shown that the retention time of TDS is very close to those of TS and HT in HPLC analysis. Takahashi et al. achieved separation of TDS and DS using HPLC/UV and 10 mM ammonium bicarbonate (pH 10.0)–acetonitrile (70 : 30, v/v) as a mobile phase under isocratic conditions. We therefore attempted to use alkaline conditions to separate the 5 analogs. When 10 mM ammonium bicarbonate (pH 10.0)–acetonitrile (75 : 25, v/v) was used as the mobile phase A, the chromatogram pattern changed markedly. But, although HT and TDS were separated, HT and DS were not.

To improve the separation of the 5 analogs, we investigated the retention time of each analog when 5 mM ammonium formate (pH 3.5)–acetonitrile (75 : 25, v/v), 5 mM ammonium formate (pH 6.3)–acetonitrile (75 : 25, v/v), 10 mM ammonium bicarbonate (pH 7.9)–acetonitrile (75 : 25, v/v), 10 mM ammonium bicarbonate (pH 9.0)–acetonitrile (75 : 25, v/v), and 10 mM ammonium bicarbonate (pH 10.0)–acetonitrile (75 : 25, v/v) were used as mobile phase A (Fig. 1). We found that the 5 analogs were separated when 5 mM ammonium formate (pH 6.3)–acetonitrile (75 : 25, v/v) was used as mobile phase A.

Detection limit

The molecular ions ([M+H]+, [M−H]−) were the major peaks for each compound in both the positive and negative modes. The detection limit of each compound was estimated based on a signal-to-noise ratio of 3. The detection limits were 50 ppb (TS), 250 ppb (AT), 10 ppb (TDS), 25 ppb (DS), and 75 ppb (HT). To plot the mass chromatogram, m/z 521 (HT), 491 (TS), 489 (DS), and 505 (TDS) in the positive mode and 389 (AT) in the negative mode were extracted as major peaks of the 5 analogs. Our results showed that HT and TDS were not separated. This was expected, since previous reports have shown that the retention time of TDS is very close to those of TS and HT in HPLC analysis. Takahashi et al. achieved separation of TDS and DS using HPLC/UV and 10 mM ammonium bicarbonate (pH 10.0)–acetonitrile (70 : 30, v/v) as a mobile phase under isocratic conditions. We therefore attempted to use alkaline conditions to separate the 5 analogs. When 10 mM ammonium bicarbonate (pH 10.0)–acetonitrile (75 : 25, v/v) was used as the mobile phase A, the chromatogram pattern changed markedly. But, although HT and TDS were separated, HT and DS were not.

To improve the separation of the 5 analogs, we investigated the retention time of each analog when 5 mM ammonium formate (pH 3.5)–acetonitrile (75 : 25, v/v), 5 mM ammonium formate (pH 6.3)–acetonitrile (75 : 25, v/v), 10 mM ammonium bicarbonate (pH 7.9)–acetonitrile (75 : 25, v/v), 10 mM ammonium bicarbonate (pH 9.0)–acetonitrile (75 : 25, v/v), and 10 mM ammonium bicarbonate (pH 10.0)–acetonitrile (75 : 25, v/v) were used as mobile phase A (Fig. 1). We found that the 5 analogs were separated when 5 mM ammonium formate (pH 6.3)–acetonitrile (75 : 25, v/v) was used as mobile phase A.

Detection limit

The molecular ions ([M+H]+, [M−H]−) were the major peaks for each compound in both the positive and negative modes. The detection limit of each compound was estimated based on a signal-to-noise ratio of 3. The detection limits were 50 ppb (TS), 250 ppb (AT), 10 ppb (TDS), 25 ppb (DS), and 75 ppb (HT). To plot the mass chromatogram, m/z 521 (HT), 491 (TS), 489 (DS), and
505 (TDS) in the positive mode and \( m/z \) 389 (AT) in the negative mode were selected. In terms of sample preparation, the detection limits of the 5 analogs were below 250 \( \mu \text{g/g} \). Thus, the proposed method has sufficient sensitivity to detect medicinal ingredients, since the prescribed dosage of sildenafil, tadalafil, and vardenafil is over 5 mg.

**Recovery and precision**

The proposed method was evaluated using the standard addition method to 2 capsules and 1 tablet, which did not contain the 5 analogs. Recovery rates of the 5 analogs when standards were spiked at 10 \( \mu \text{g/10 mg} \) were between 66.7% and 146.3%, and the relative standard deviations (RSD) were between 1.7% and 29.7% (Table 1).

The recovery rates and RSD were not satisfactory to quantify the amounts of the 5 analogs. Generally, medicinal ingredients and analogs are quantified by HPLC-UV. LC-MS is usually used to identify compounds, and not to quantify them. Therefore, the recovery rates and RSD were satisfactory for the identification of the 5 analogs.

**Table 1. Recovery of 5 analogs from 3 dietary supplements**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Capsule 1</th>
<th></th>
<th>Capsule 2</th>
<th></th>
<th>Tablet 1</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean recovery (%)</td>
<td>RSD (%)</td>
<td>Mean recovery (%)</td>
<td>RSD (%)</td>
<td>Mean recovery (%)</td>
<td>RSD (%)</td>
</tr>
<tr>
<td>TS</td>
<td>84.8</td>
<td>11.2</td>
<td>83.7</td>
<td>12.9</td>
<td>72.4</td>
<td>17.2</td>
</tr>
<tr>
<td>AT</td>
<td>66.7</td>
<td>17.6</td>
<td>146.3</td>
<td>29.7</td>
<td>109.5</td>
<td>21.3</td>
</tr>
<tr>
<td>TDS</td>
<td>92.8</td>
<td>1.7</td>
<td>93.6</td>
<td>6.0</td>
<td>79.2</td>
<td>3.4</td>
</tr>
<tr>
<td>DS</td>
<td>100.9</td>
<td>3.0</td>
<td>98.6</td>
<td>3.2</td>
<td>100.4</td>
<td>3.9</td>
</tr>
<tr>
<td>HT</td>
<td>101.8</td>
<td>2.5</td>
<td>91.9</td>
<td>3.4</td>
<td>76.8</td>
<td>1.9</td>
</tr>
</tbody>
</table>

\((n = 3)\)

![Extract ion chromatograms of a dietary supplement containing all 5 analogs](image)

**Fig. 2.** Extract ion chromatograms of a dietary supplement containing all 5 analogs

\(a\) \( m/z \): 389, 489, 491, 505, 521; \(b\) \( m/z \): 389
Simultaneous Identification of 5 Adulterants in Dietary Supplements

Application of the proposed method

We then attempted to apply the proposed method to a dietary supplement containing all 5 analogs. The sample solution was diluted 10-fold with methanol prior to LC-MS. The peaks of all 5 analogs were separated and clearly observed (Fig. 2).

It is very important to separate the peaks of medicinal ingredients and analogs, especially when a dietary supplement contains compounds possessing the same molecular weight. Vardenafil, homosildenafil, and DS have the same molecular weight, and the molecular ion (m/z 489) was the major peak for each compound in the positive mode. Standard solutions (each compound: 1 µg/mL) of the 3 compounds were therefore analyzed using the proposed method. The compounds were clearly separated (data not shown). Two peaks of homosildenafil and DS were not separated when 5 mM ammonium formate (pH 3.5)–acetonitrile (75 : 25, v/v) was used as mobile phase A (data not shown). Therefore, the proposed method is also useful for distinguishing vardenafil, homosildenafil, and DS. A standard solution of 7 compounds (HT, AT, TS, DS, TDS, vardenafil, and homosildenafil; concentration of each compound, 1 µg/mL) was separated into 7 peaks using our proposed method (Fig. 3). Thus, the proposed method could identify at least 7 compounds simultaneously suggesting that it may be applicable in quality control procedures of dietary supplements.

Conclusion

In this study, different LC-MS separation conditions were tested to develop a method for the separation of multiple medicinal ingredients and/or their analogs. The method developed could simultaneously identify HT, TS, DS, TDS, and AT in dietary supplements. The proposed method could also distinguish vardenafil, homosildenafil, and DS, all of which have the same molecular weight. Furthermore, HT, AT, TS, DS, TDS, vardenafil, and homosildenafil could be analyzed simultaneously. The proposed method may be useful for the separation and identification of medicinal ingredients and analogs and for quality control of dietary supplements.

Acknowledgements

The authors are grateful to Dr. Goda (National Institute of Health Sciences in Japan) for helpful suggestions and for providing reference materials. The authors also thank the Tokyo Metropolitan Institute of Public Health, Department of Pharmaceutical Sciences for providing standards.

References


