Simultaneous Determination of Slimming Drugs in Dietary Supplements by Liquid Chromatography/Time-of-Flight Mass Spectrometry

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Abstract
A method for the simultaneous determination of 13 slimming drugs in dietary supplements by liquid chromatography/time-of-flight mass spectrometry was developed and applied to real-time surveys of imported health supplements. The target compounds were mutually well separated on a reversed-phase LC Shodex ODP2-HP 2D column using a combination of step-wise and linear gradient elution of a mixed mobile phase solution of ammonium formate buffer, methanol, and formic acid. The detection limits were in the range 1-50 ng/mL, and the lower limits of quantification were 2-100 ng/mL. The average recoveries of the 13 types of pharmaceutical ingredients in the diet supplements were 93-110%, and the RSD was within 7.1%. Applying the developed method to the survey of imported dietary supplements revealed that 32 out of the 39 Chinese products tested contained pharmaceutical ingredients such as sibutramine. On the other hand, out of 30 US products, synephrine was detected in 26 specimens, and yohimbine was detected in 8 specimens. These results proved that a number of products were contaminated by drugs, which was considered to be intentional. The developed method is applicable for the survey of imported health supplements suspected to be contaminated with pharmaceutical ingredients.

Keywords: Dietary supplements; LC/TOF-MS; Pharmaceutical ingredients; Slimming drugs

1. Introduction
In recent years, there has been an increasing demand for health supplements that provide benefits such as revitalizing, skin-beautifying, visual improvement, and slimming effects. Among them, so-called "slimming dietary supplements" often contain pharmaceutical ingredients belonging to the category of obesity drugs, and are mainly imported personally as health supplements via the Internet or other sources. The number of cases of adverse health effects as a result of inappropriately taking or abusing supplements containing these pharmaceutical ingredients is increasing, and the Ministry of Health, Labour and Welfare has published an alert regarding such substances [1]. Additionally, in the event of an adverse effect, it is necessary to promptly identify the causative substances using reliable analytical methods in order to treat patients and prevent further damage.

A relatively large number of analysis methods targeting PDE-5 inhibitors have been reported for supplements containing ergogenic ingredients [2-4]. However, although many analytical methods targeting individual slimming pharmaceutical ingredients have been reported, there are few methods to analyze many ingredients simultaneously. This is presumably because of the great number of slimming pharmaceutical ingredients added to health supplements.

Thin layer chromatography (TLC), which can provide an overview of all constituent components, has been reported to be useful for preliminary screening prior to confirmatory inspection by high-performance liquid chromatography (HPLC) [5-7]. However, in TLC, either chromogenic reactions or a TLC plate impregnated with a UV absorptive substance must be employed. The sensitivity of TLC is relatively low, and thus this technique carries a risk of false negatives. Additionally, dietary supplements are complex matrices with many contaminants, which makes it difficult
to separate and detect trace amounts of pharmaceutical ingredients by TLC unless sufficient cleanup is performed. Therefore, techniques such as two-dimensional TLC have also been tried [5]. In addition to screening by TLC and gas chromatography/mass spectrometry, pharmaceutical ingredients and analogues are often identified and quantified using HPLC and more sophisticated methods such as liquid chromatography/mass spectrometry (LC/MS), liquid chromatography/tandem mass spectrometry (LC/MS/MS), and direct analysis in real time/time-of-flight mass spectrometry (DART-TOF-MS) [7-12]. In order to accurately quantify and identify multiple medicinal ingredients and analogues, it is critically important to achieve adequate separation of their peaks. Although LC/MS/MS using MRM mode measurement offers high sensitivity and selectivity compared to other methods, it has the fatal disadvantage of being unable to detect ions other than those of a preset target compound. On the other hand, TOF-MS can accurately detect the masses of all ions with a high resolution (over 10000) for a certain range of mass-to-charge ratios. That is, accurate data for all masses are recorded in full scan mode at the time of measurement, making it possible to check for the presence or absence of new target compounds after the measurement of the sample.

Therefore, in this study, we attempted to construct an analytical method employing LC/TOF-MS, which can not only separate high polarity and low polarity components simultaneously, but can also capture their masses accurately for screening and confirmatory tests. Furthermore, the method was applied to the analysis of dietary supplements imported as health foods. A total of 13 frequently used pharmaceutical ingredients that have been detected in the past in health supplements, such as dietary supplements containing slimming drugs, were selected as the target compounds (Fig.1)[1].

2. Experimental

2.1. Materials and reagents

We purchased 39 dietary supplements made in China and 30 dietary supplements made in the USA, all of which claimed to have slimming effects.

Fenfluramine hydrochloride (biochemical grade), N-nitrosofenfluramine (racemic mixture; pharmacological agent), glibenclamide (biochemical grade), phenolphthalein (special grade), yohimbine (> 98%), and caffeine (> 99%) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Norephedrine (> 98%), phentermine (> 98%), and synephrine (> 98%) were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Ephedrine hydrochloride and methylephedrine hydrochloride (Japanese Pharmacopoeia grade) were purchased from Alps Pharmaceutical Industry Co., Ltd. (Gifu, Japan). Mazindol (> 99%) was purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA), and sibutramine hydrochloride–hydrate (> 99%) was purchased from Alexis Biochemicals (San Diego, CA, USA).

HPLC grade methanol and acetonitrile, special grade (> 97%) formic acid, ammonium formate, analytical grade ammonium hydroxide solution (25%), and biochemical grade leucine-enkephalin were purchased from Wako Pure Chemical Industries. Water was purified with a Milli-Q Gradient A10 system equipped with an EDS-PAK® polisher (Millipore Ltd., Bedford, MA). A cellulose-acetate membrane filter was used (pore diameter: 0.45 μm, filter housing diameter: 25 mm; Advantec Toyo Kaisha, Ltd. (Tokyo, Japan)).

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2.2. Preparation of the calibration solution and standard solutions

A 0.25 µg/mL leucine-enkephalin solution was prepared in 50% acetonitrile containing 0.1% formic acid, and stored in a refrigerator. Each standard was dissolved in methanol to make 100 µg/mL standard stock solutions. The working standard solutions were then prepared from the standard stock solutions by dilution with 50% methanol.

2.3. Preparation of sample solutions

Dietary supplements containing slimming pharmaceutical ingredients exist in various forms, such as tablets and capsules. One tablet or one capsule was used as a sample. In the case of a tablet, pulverization was carried out in a magnetic mortar. In the case of a soft capsule, a cut of approximately several mm was made with a knife, and as much as content as possible was taken out. After each sample was weighed accurately, methanol was added and the volume was adjusted to 10 mL. The sample was dissolved by applying mechanical vibration and ultrasonic irradiation for 20 min. Insoluble matter was removed by filtration through a membrane filter. The filtrate was diluted with 50% methanol/water, and this solution was used as the test solution for LC/TOF-MS analysis.

2.4. Apparatus and operating conditions

An Alliance HT 2795 HPLC system equipped with an LCT Premier XE TOF-MS (Waters Corporation, Milford, MA, USA) was used. LC separation was performed with a Shodex ODP2-HP 2D column (150 mm × 2.0 mm I.D., 5 µm; Showa denko, K.K.; Tokyo, Japan). The column temperature was maintained at 50°C. The mobile phase was a mixture of (A) 10 mmol/L ammonium formate buffer (pH 5.0), (B) methanol, and (C) 10 mmol/L formic acid aqueous solution, and was delivered by a combination of gradient elution and step-wise elution modes as shown below, at a flow rate of 0.2 mL/min. A 5 µL of the sample was injected into the system.

A/B/C = [60:40:0]/(0–4 min), [0:40:60]/(4.01–8 min), [0:50:60 to 0:55:50]/(8.01–12 min), [0:70:45 to 0:70:30]/(12.01–15 min)

The optimum working parameters for TOF-MS were as follows; ionization: electrospray ionization (ESI) positive mode, capillary voltage: 2500 V, aperture #1 voltage: 15 V, cone voltage: 50 V, desolvation temperature: 350°C, source temperature: 120°C, desolvation gas flow (N2): 750 L/h, cone gas flow (N2): 50 L/h.

Mass accuracy was maintained using Lock–Spray with the leucine-enkephalin [M+H]+ ion, m/z = 556.2771 as the lock mass. The resolution was at least 10000 as calculated by using the full width at half maximum method.

3. Results and discussion

3.1. Optimization of the LC conditions

Substances to be measured included highly polar caffeine and synephrine, which are difficult to retain on conventional silica gel based ODS columns because of their extremely low logPow values (logPow < 0). Therefore, a polymer based Shodex ODP2-HP 2D column, which is considered to have a relatively high retention even for highly polar compounds, was used for this study. To investigate the mobile phase conditions for LC, first, a gradient elution with a mixture of ammonium formate buffer and methanol was tried. However, when the pH of the mobile phase was neutral, some substances were strongly retained on the column, making simultaneous analysis within a short time difficult. However, when the pH of the mobile phase was lowered (pH 3.0), highly polar compounds such as synephrine and norephedrine were not sufficiently retained on the column. Therefore, a three-solvent ammonium formate/methanol/formic acid system was employed to achieve simultaneous analysis in a short time, allowing both organic solvent gradient and pH gradient elution.

The optimum conditions among those tested were found to be the following: A mixed solution of ammonium formate buffer (pH 5.0) and methanol was flowed from 0 to 4 minutes of the retention time to retain highly polar substances such as synephrine. After 4 minutes, the ammonium formate buffer was changed to an aqueous solution of formic acid, and compounds that were strongly retained on the column, such as phentermine, were eluted. After 8 minutes, the proportion of organic solvent was gradually increased to elute low polarity substances such as phenolphthalein. Thus, by combining stepwise and gradient elution, mutual separation of all 13 measured substances was achieved. In addition, the reproducibility of the retention times was good. Fig. 2 shows typical mass chromatograms of the 13 standards measured using the constructed LC conditions.

![Mass chromatograms](image)

**Fig. 2.** Mass chromatograms of slimming drugs obtained using LC/TOF-MS.
3.2. Optimization of the TOF-MS operating conditions

3.2.1. Monitoring ions

Ionization in the TOF-MS was performed via ESI using both positive and negative ion modes. In the negative ion mode, compounds other than phenolphthalein were not ionized and could not be detected, and thus, positive ion mode was employed for the measurements.

The mass spectra of the 13 compounds are shown in Fig. 3. For phentermine, norephedrine, and synephrine, proton adducts ([M+H-NH$_3$]+ or [M+H-H$_2$O]+) from which amino groups or hydroxyl groups are easily eliminated were used as monitoring ions. For the other 10 compounds, the proton adduct of each compound was used as the monitoring ion.

3.2.2. Capillary voltage and aperture #1 voltage

The optimum capillary voltage was examined in the range of 1500 to 3500 V. Although the peak intensity decreased slightly with increasing voltage from 1500 to 2000 V, it gave a plateau at over 2500 V, and the precise mass error was relatively small (< 5 ppm). Thus, a capillary voltage of 2500 V was employed. Aperture #1 voltages in the range of 5 to 25 V were tested. The optimum value was set to 15 V, where good peak intensity was obtained for all 13 target substances.

3.3. Method validation

The limit of detection (S/N = 3) and the calibration range of the 13 target compounds are shown in Table 1. Each calibration curve showed good linearity ($r > 0.998$) over the range. In addition, to examine the effect of sample concentration on the accuracy of the mass measurement, the precise mass error in the range of the calibration curve was measured and determined to be within 5 ppm.

Table 1. Method validation of slimming drugs by LC/TOF-MS

| Compounds          | LOD (ng/mL) | Range (ng/mL) | Coefficient of correlation ($r$) |
|--------------------|-------------|---------------|---------------------------------|
| Caffeine           | 2           | 4–200         | 0.999                           |
| Ephedrine          | 2           | 4–100         | 0.999                           |
| Fenfluramine       | 1           | 2–40          | 0.999                           |
| Glibenclamide      | 5           | 10–200        | 0.999                           |
| Mazindol           | 4           | 8–400         | 0.999                           |
| Methylephedrine    | 1           | 2–50          | 0.999                           |
| N-Nitrosofenfluramine | 5      | 10–200        | 0.999                           |
| Norephedrine       | 4           | 8–200         | 0.999                           |
| Phenolphthalein    | 50          | 100–200       | 0.999                           |
| Phentermine        | 20          | 40–400        | 0.999                           |
| Sibutramine        | 20          | 40–400        | 0.999                           |
| Synephrine         | 2           | 40–100        | 0.999                           |
| Yohimbine          | 1           | 2–200         | 0.999                           |

LOD: limit of detection (S/N = 3)

Fig. 3. Mass spectra of slimming drugs obtained using ESI positive ionization mode.
3.4. Recovery experiment

A commercially available tablet-type dietary supplement from the domestic market, which was previously confirmed to be free of the 13 target substances, was used as the sample. The 13 target substances were prepared in low and high concentration with the sample. The recovery tests were carried out with three replicates for each concentration. The recoveries were in the range of 93.0–110.6 %, and the relative standard deviations were within 7.1 % (Table 2).

Table 2. Recoveries of slimming drugs from dietary supplement

| Compounds        | Spiked amount (ng/mL) | Recovery (%) | RSD (%) |
|------------------|-----------------------|--------------|---------|
| Caffeine         | 20, 200               | 95.7, 93.0   | 1.9, 0.8|
| Ephedrine        | 10, 100               | 103.8, 99.3  | 2.6, 0.7|
| Fenfluramine     | 2, 20                 | 109.9, 101.4 | 4.3, 1.8|
| Glibenclamide    | 10, 100               | 110.6, 96.6  | 7.1, 4.4|
| Mazindol         | 20, 200               | 109.6, 103.2 | 2.5, 1.4|
| Methylephedrine  | 5, 50                 | 103.3, 99.5  | 2.3, 2.1|
| N-Nitrososulfuramin | 10, 100          | 100.8, 98.6  | 0.1, 4.8|
| Norephedrine     | 20, 200               | 103.0, 97.6  | 2.0, 1.5|
| Phenolphthalein  | 100, 1000             | 99.1, 100.9  | 4.6, 3.1|
| Phentermine      | 40, 400               | 100.3, 98.4  | 5.6, 0.3|
| Sibutramine      | 20, 200               | 105.9, 98.4  | 1.6, 6.2|
| Synephrine       | 10, 100               | 103.4, 95.1  | 4.0, 2.6|
| Yohimbine        | 20, 200               | 101.8, 97.7  | 0.6, 3.2|

LOD; limit of detection (S/N = 3)

3.5. Slimming drugs contained in Chinese dietary supplements

Among the 39 Chinese dietary supplements, five target pharmaceuticals were detected in 80% of products. Among them, sibutramine was detected in about 74% of products. Sibutramine is a serotonin and noradrenaline reuptake inhibitor (SNRI) that inhibits the uptake of serotonin and noradrenaline by neurons in the brain [13]. The sibutramine content of the supplements would result in a daily intake of 3.9 to 34.2 mg/day. Prescribed doses of sibutramine range from 5 to 15 mg/day [13], indicating that adverse effects such as hypertension and arrhythmia were a significant concern. While the detection rate of sibutramine in Chinese products was relatively remarkable, it was inferred that its slimming effect, low cost, and availability were the reasons for its wide use.

Additionally, about 36% products contained phenolphthalein. The content of phenolphthalein in the supplements would result in a daily intake in the range of 16.2 to 217.6 mg/day. Although phenolphthalein is not currently in circulation as a pharmaceutical product, considering the doses (30 to 200 mg/day) once prescribed as a laxative, this was an amount that could affect the living body.

In addition, caffeine was detected in about 20% of products, and fenfluramine and ephedrine were also confirmed in some products. Ephedrines (including norephedrine and methylephedrine) have been used as main ingredients in dietary supplements, since they have appetite suppression and hypermetabolism effects. However, due to occasional occurrence of adverse events such as myocardial infarction, FDA (US Food and Drug Administration) banned the use of ephedrines in dietary supplements [14].

3.6. Slimming drugs contained in dietary supplements from the USA

Synephrine was detected in 26 samples (about 87% frequency) from US products (30 samples), and caffeine was detected in nearly the same products. Synephrine is an alkaloid contained in Citrus aurantium, which is also called bitter orange, and is used as a substitute for ephedrine to health foods [15]. In addition, it has been suggested that the use of synephrine in combination with caffeine enhances such side effects due to the cardiotonic action of caffeine [16].

Yohimbine was also detected at a frequency of about 27%. Yohimbine is a component of Yohimbe, a plant of the Rubiaceae family, and belongs to the rauwolfia alkaloids. Since yohimbine is generally used as an energetic drug or an aphrodisiac, it is included in many health supplements. Although in recent years, yohimbine has also been included in dietary supplements for men [17,18], the evidence for its aphrodisiac action and effectiveness against erectile dysfunction is considered insufficient [19]. Yohimbine is classified a dietary supplement within the United States, but it is designated as an "Ingredient essence (raw material) used exclusively as medicine" in Japan. Therefore, its usage should be restricted due to safety considerations.

4. Conclusions

In this study, a method for the simultaneous determination of 13 pharmaceutical ingredients that are potential contaminants in dietary supplements using LC/TOF-MS was developed and applied to imported dietary supplement products. LC analysis conditions for the good mutual separation of the 13 target substances was achieved via simultaneous organic solvent and pH gradients using an ammonium formate buffer solution, methanol, and an aqueous formic acid solution.

In the analysis of real-world samples, pharmaceutical ingredients including sibutramine were detected from in 32 of the 39 Chinese products tested. Among the 30 US products, synephrine was detected in 26 specimens, and
yohimbine was detected in 8 specimens. The analytical method developed in this research is expected to be utilized in applications such as administrative inspection of imported health foods suspected of contamination with pharmaceutical ingredients.

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