Isolation and structural characterization of a novel sibutramine analogue, chlorosipentramine, in a slimming dietary supplement, by using HPLC-PDA, LC–Q-TOF/MS, FT-IR, and NMR

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Abstract

A novel sibutramine analogue was detected in a slimming formula by high performance liquid chromatography with a photo diode detector array (HPLC-PDA). The unknown compound exhibited an ultraviolet (UV) spectrum that was similar to that of chlorosibutramine, despite having a different HPLC retention time. Further analysis of the slimming formula by LC–quadrupole time-of-flight mass spectrometry (LC–Q-TOF/MS) showed that the unknown compound had the formula C_{18}H_{27}Cl_{2}N. To elucidate the structure of this new sibutramine analogue, the target compound in the slimming formula was isolated on a preparative-LC system equipped with a PDA. After analysis by fourier transform infrared (FT-IR) and nuclear magnetic resonance (NMR) spectroscopy, the unknown compound was identified as a sibutramine analogue in which the iso-butyl group on the side chain is replaced with an iso-pentyl group. This new sibutramine analogue was identified to be 1-(1-(3,4-dichlorophenyl)cyclobutyl)-N,N,N-trimethylpentan-1-amine and has been named chlorosipentramine.

1. Introduction

Obesity has become a serious chronic disease as well as a risk factor for several health problems such as type 2 diabetes, hypertension, metabolic syndrome, cardiovascular disease, stroke, and renal disease. Sibutramine (Meridia, Abbott Laboratories), which was originally developed as an antidepressant, was approved as an anti-obesity drug by the United States Food and Drug Administration (FDA) in 1997; sibutramine is an inhibitor of serotonin and noradrenaline re-uptake. In addition to reducing weight, sibutramine also improves cardiometabolic factors, such as plasma levels of glucose and lipids [1]. However, sibutramine is no longer approved for obesity treatment and has been withdrawn from the American, European, and Korean markets in 2010 owing to serious adverse cardiovascular events including tachyarrhythmia, hypertension, and death [2,3].

Recently, anti-obesity drugs, designer analogues, and natural weight-loss ingredients are being increasingly included in dietary supplements intended for rapid weight loss [4]. Moreover, some manufacturers have continued to synthesize structurally modified sibutramine analogues to circumvent government regulations. Sibutramine is rapidly metabolized in humans to pharmaceutically active N-desmethylsibutramine and N-didesmethysibutramine.

To date, the following sibutramine analogues have been identified: desmethylsibutramine, didesmethysibutramine, benzylsibutramine, chlorosibutramine, and homosibutramine [5–10]. Some studies have reported side-effects in men who use slimming foods adulterated with unsafe anti-obesity drugs, such as the sibutramine, rimonabant, and the phenethylamine class of drugs [11,12]. Most of these drugs have been withdrawn from the market owing to their adverse effects, including cardio-excitatory and psychostimulatory effects, and addiction. In addition, the safety and efficacy of their analogues have not been proven. Most of the slimming supplements sold on the internet have not been subjected to rigorous quality control. The number of dietary supplements entering markets in the Republic of Korea increases every year. For these reasons, the South Korean Ministry of Food and Drug Safety (MFDS) has been monitoring unapproved adulterants in a variety of foods since 2002.

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In this study, a new sibutramine analogue was isolated from a dietary slimming supplement. Its structural elucidation was performed by liquid chromatography–quadrupole-time-of-flight mass spectrometry (LC–Q–TOF/MS), nuclear magnetic resonance (NMR), and infrared (IR) spectroscopy. The analogue was named chlorosipentramine, and its proposed structure is shown in Fig. 1.

2. Materials and methods

2.1. Material and chemicals

The slimming supplement was submitted to the South Korean MFDS by the Seoul Institute of Health and Environment to analyze illegal compounds such as anti-obesity drugs and designer analogues. The sample was supplied as an unlabeled zip bag, containing an ivory powder intended as a slimming formula. For compound identification, sibutramine (MFDS S-3, purity 97.6%) was obtained from Hanni Pharmaceutical (Hwaseong, South Korea), while chlorosibutramine (MFDS C-22, purity 93.4%) and the proposed chlorosipentramine (MFDS C-22, purity 99.0%) were synthesized by the Korean MFDS. Stock solutions were dissolved in methanol at 1 mg/mL and stored at 4 °C prior to analysis. LC-grade methanol and acetonitrile were obtained from Merck (Darmstadt, Germany). Sodium 1-hexanesulfonate and formic acid were purchased from Sigma–Aldrich (St. Louis, MO, USA). Phosphoric acid was supplied by Wako Pure Chemical Industries Ltd. (Osaka, Japan). Ultrapure water at 18.2 mΩ was purified using a Thermo Scientific Barnstead Nanopure water purification system (Marietta, OH, USA). Before use, all solvents were filtered through a 0.45 μm pore polytetrafluoroethylene (PTFE) membrane filter purchased from Teknokroma (Barcelona, Spain).

2.2. Sample extraction

For screening, approximately 1.0 g of the powdered sample was dissolved in 50 mL of 70% methanol and extracted by sonication for 20 min. After centrifugation at 4000 rpm for 5 min at 5 °C, 1 mL of the supernatant was filtered through a 0.45 μm PTFE syringe filter (Teknokroma). The filtrate was finally injected into the HPLC–photodiode detector array (PDA) system.

2.3. HPLC–PDA analysis

The sample was analyzed using a Shiseido Nanospace SI–2 HPLC–PDA system (Tokyo, Japan). A Capcell Pak C18 MG II column (4.6 mm × 150 mm, 3 μm, Shiseido) was used, and the column temperature was set to 40 °C. Mobile phase A consisted of 0.5 mM sodium–1-hexanesulfonate and 0.1% phosphoric acid in distilled water, while mobile phase B contained acetonitrile/distilled water 95:5 (v/v). The following gradient elution program was applied: 0–6 min, 15% B; 6–15 min, 15–30% B; 15–30 min, 30–40% B; 30–32 min, 40% B; 32–40 min, 40–100% B; 40–50 min, 100% B; 50–52 min, 100–15% B; 52–60 min, 15% B. The injection volume was 10 μL. The flow rate of the mobile phase was 1.0 mL/min. Ultraviolet (UV) spectra were recorded by the PDA detector over the 190–400 nm range, and HPLC chromatograms were recorded at 220 nm.

2.4. Isolation of the unknown compound

To isolate the unknown compound, the fraction containing the target compound was collected using a Waters AutoPurification system with a 2767 sample manager, 2545 quaternary gradient module, and 2998 PDA (Milford, MA, USA), and a Waters Atlantis T3 Prep OBD C18 column (19 mm × 250 mm, 5 μm). Mobile phases A and B comprised distilled water and acetonitrile containing 0.1% formic acid, respectively. The column temperature was set to 30 °C, the injection volume was 3 mL, and the flow rate was 11 mL/min. The gradient conditions were as follows: 0–2 min, 15% B; 2–20 min, 15–100% B; 20–25 min, 100% B; 25–25.1 min, 100–15% B; 25.1–35 min, 15% B. Fractions containing the target compound were collected by an automatic collector based on a UV detection wavelength of 200–400 nm and HPLC chromatogram recorded at 220 nm. The collected fractions were combined and the solvent was removed by rotary evaporation, followed by freeze-drying at –80 °C.

2.5. LC–Q–TOF/MS analysis

LC quadrupole time-of-flight mass spectrometry (LC–Q–TOF/MS) analysis was carried out on a Waters SYNAP G2 system coupled with an ACQUITY UPLC. Separation was performed on a Waters ACQUITY BEH C18 column (2.1 mm × 50 mm, 1.7 μm) with 0.1% formic acid in water and 0.1% formic acid in acetonitrile as mobile phases A and B, respectively. The column temperature was set to 35 °C, the injection volume was 2 μL, and the flow rate was 0.4 mL/min. The gradient program was as follows: 0–1 min, 10% B; 1–7 min, 10–100% B; 7–8 min, 100% B; 8–8.1 min, 100–10% B; 8.1–10 min, 10% B. The conditions for MS measurement were as follows: ionization mode, positive; desolvation temperature, 350 °C; desolvation gas rate, 700 L/h; capillary voltage, 2.5 kV; cone voltage, 30 V; source temperature, 150 °C. Data acquisition was managed by the Masslynx software.

2.6. FT–IR analysis

The Fourier transform infrared (FT–IR) spectrum of the isolated compound was recorded on a Varian 640 FT–IR spectrometer (Palo Alto, CA, USA) in the 4000–650 cm⁻¹ range. Data acquisition and processing were using the Resolution Pro™ software.

2.7. NMR analysis

The isolated compound was dissolved in CDCl₃ for nuclear magnetic resonance (NMR) spectroscopic analysis. ³¹P-NMR
(500 MHz), $^1$H-NMR (125 MHz), distortionless enhancement by polarization transfer (DEPT) (125 MHz), correlation spectroscopy (COSY), heteronuclear multiple bond correlation (HMBC), and heteronuclear single quantum correlation (HSQC) spectra were recorded on a Bruker AVANCE III spectrometer (Rheinstetten, Germany) using Me$_4$Si as the internal standard in CDCl$_3$. Coupling constants ($J$ values) were measured in Hertz (Hz) and chemical shifts ($\delta$ values) are given in parts per million (ppm).

3. Results and discussion

A new sibutramine analogue was detected in a slimming formula. The chromatogram of the unknown compound exhibited an intense peak at 36.17 min. The UV spectrum of this unknown compound was similar to that of chlorosibutramine, with three UV absorption maxima at 203, 274 and 379 nm. As shown in Fig. 2, the retention times of chlorosibutramine and the unknown compound were 33.53 min.

![Fig. 2. HPLC chromatograms of chlorosibutramine (a) and the original methanol extract of the sample (b), and overlaid UV spectra (c).]
and 36.17 min, respectively. Although the two analytes showed different retention times in HPLC, the UV spectrum of the unknown compound was similar to that of chlorosibutramine. This suggests that the unknown compound is possibly a chlorosibutramine-related sibutramine analogue. In addition, the unknown compound was slightly more hydrophobic than chlorosibutramine.

An accurate mass determination of the unknown compound was performed by LC–Q-TOF/MS analysis. The protonated precursor [M+H]+ ion of the unknown compound was found at m/z 328.1603 (Fig. 3A) with a predicted molecular formula of C1bH2fCl2N (the calculated mass for [M+H]+ is 328.1599, and the mass error is 1.2 ppm). Compared with the molecular formula of chlorosibutramine (C17H25Cl2N), the unknown compound showed a mass increase of 14 Da, which likely represents the insertion of a methylene group. The MS2 spectra of [M+H]+ of unknown compound (m/z = 328) shows the prominent fragment ions at m/z 159 and 173 (Fig. 3B), and its dissociation pattern is similar to that of chlorosibutramine [8]. Once the unknown compound had been confirmed to be chlorosipentramine (vide infra), the fragmentations observed could be reconciled with its structure, as shown in Fig. 3C. Following LC–Q-TOF/MS analysis, the unknown compound was revealed to be a new sibutramine analogue related to chlorosibutramine. In order to completely characterize its structure, the isolated compound was analyzed further by NMR and IR spectroscopies.

To elucidate its accurate structure, approximate 1 mg of the unknown compound was isolated from 100 g of the powder sample using preparative LC-PDA, followed by IR and NMR spectroscopic analyses that included 1H-NMR, 13C-NMR, DEPT, COSY, HMBC, and HSQC techniques. As shown in Fig. 4 and Table 1, the IR spectrum of the unknown compound exhibits absorption bands characteristic of alkane (C–H) stretching at 2953 cm⁻¹, alkane C–H bending (CH₂) at 1471 cm⁻¹, alkane C–H bending (CH₃) at 1375 cm⁻¹, and aromatic in-plane bending at 1028 cm⁻¹.

The 1D and 2D NMR data of the isolated compound are listed in Table 2, while the spectra are displayed in Figs. 5–7. The 1H and 13C NMR data for sibutramine and chlorosibutramine are also provided in Table 2 for comparison with those of the unknown compound. A total of 18 peaks were observed in the 13C spectrum of the isolated compound, confirming the presence of 18 carbons (assuming no symmetry). The positions of the methine (CH), methylene (CH₂), and methyl (CH₃) carbons were determined by DEPT spectroscopy (Fig. 5). As shown in Table 2, four protons, namely H-2 [δH 7.45 (1H, d, J 8.5 Hz)], H-3 [δH 7.65 (1H, d, J 8.5 Hz)], H-5 [δH 7.65 (1H, d, J 8.5 Hz)], and H-6 [δH 7.45 (1H, d, J 8.5 Hz)] were observed for the para-substituted benzene ring of sibutramine, while chlorosibutramine and the unknown compound exhibited signals for three aromatic protons, namely H-2, H-5, and H-6. The 1H data for the unknown compound are consistent with the presence of an additional chlorine substituent, as well as the LC–Q-TOF/MS data (vide supra). The position of the additional chlorine on the benzene ring was determined by HMBC and HSQC spectroscopy. As shown in Fig. 6B, significant long-range correlations were observed

![Fig. 3. Mass pattern (a) and fragmentation pattern of the unknown compound in the ESI positive mode (b), and proposed fragmentation for chlorosipentramine (c).](image-url)
between the signal corresponding to C-7 and those corresponding to the aromatic protons H-2 and H-6, and between the proton signals of H5 and H-11, and C-1, indicating that a 3,4-dichlorophenyl group is bonded to C-7 of the cyclobutane ring. The significant interaction was observed between the proton signal of H-11 and the six carbon signals of C-13, C-17, C-18, C-1, C-8, and C-10. N-dimethyl peak was correlated with C-7. These correlations revealed that an N-dimethyl group and an iso-pentyl group are attached at C-11. The comparison of the molecular formula of the isolated compound with that of chlorosibutramine suggests that the iso-butyl group on the side-chain of chlorosibutramine has been replaced with the iso-pentyl group in the unknown structure.

Based on the analytical results, we synthesized chlorosipentramine standard. The retention time and UV spectrum of the unknown compound were identical to that of the synthesized chlorosipentramine standard, and its accurate mass data were in good agreement with the proposed molecular formula (data not shown). As shown in Fig. 7, the \(^1\)H- and \(^13\)C-NMR spectra of the compound isolated from the slimming formula coincided with those of the synthesized compound. In addition, the NMR spectra of the isolated compound spiked with synthesized products also showed no evidence of any difference between the isolated or synthesized compounds. Based on these analytical data, the International Union of Pure and Applied Chemistry (IUPAC) name for the unknown compound was identified as 1-{1-(3,4-dichlorophenyl)cyclobutyl}-N,N4-trimethylpentan-1-amine and its common name designated as chlorosipentramine, which is a new sibutramine analogue.

The skeletons of chlorosibutramine and chlorosipentramine are similar to that of sibutramine, and chlorosibutramine-related

### Table 1
IR absorption range and types of vibration.

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<th>Frequency (cm(^{-1}))</th>
<th>Types of vibration</th>
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<td>2953</td>
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<tr>
<td>1471</td>
<td>sp(^3) C-H bending (CH(_2))</td>
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<tr>
<td>1375</td>
<td>sp(^3) C-H bending (CH(_3))</td>
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<td>1028</td>
<td>Aromatic in-plane bending</td>
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### Table 2
NMR data for sibutramine, chlorosibutramine, and the unknown compound.

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<th>Compound</th>
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<th>(^13)C ((\delta_C))</th>
<th>(^1)H ((\delta_H))</th>
<th>(^13)C ((\delta_C))</th>
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<th>(^1)H COSY</th>
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<td>32.2</td>
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<td>H-2/H-6/H-8/H-10/H-12</td>
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<td>H-2/H-6/H-8/H-10/H-12</td>
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</table>

NMR data sibutramine and chlorosibutramine are shown for comparison with chlorosipentramine. Positions 1−18 indicate either a hydrogen or carbon signal.

\(^a\) NMR solvent: sibutramine (DMSO-d\(_6\)); chlorosibutramine (DMSO-d\(_6\)); unknown compound (CDCl\(_3\)).

\(^b\) Number of DEPT corresponded to the number of attached protons.
Fig. 5. $^1$H-NMR spectrum (a), $^{13}$C-NMR spectrum (b), and DEPT 135 spectrum (c) of the unknown compound.
Fig. 6. $^1$H-$^1$H-COSY spectrum (a), HMBC spectrum (b), and HSQC spectrum (c) spectra of the unknown compound.
sibutramine analogues exhibit common fragmentation patterns at m/z 159 and 173 in their mass spectra. Therefore, prominent fragment ions at m/z 159 and 173 are considered to be the main characteristics of chlorosibutramine-related sibutramine analogues. The results obtained from these spectroscopic analyses, using LC-PDA, MS, NMR, and IR techniques, serve as important references to identify and elucidate the structures of new sibutramine analogues.

4. Conclusions

In regular screening of illegally adulterated compounds, a novel sibutramine analogue was isolated from a slimming formula. In HPLC-PDA analysis, a large unknown peak had a similar UV spectrum to that of chlorosibutramine, although the unknown compound showed a different retention time to that of chlorosibutramine. LC–Q-TOF/MS analysis revealed that the unknown compound has the formula C_{18}H_{27}Cl_{2}N (M.W. 328.32). The new compound was identical to chlorosibutramine, except that the iso-butyl group on the side-chain of chlorosibutramine had been replaced by an iso-pentyl group in the unknown structure. The new sibutramine analogue was identified to be 1-(1-(3,4-dichlorophenyl)cyclobutyl)-N,N,4-trimethylpentan-1-amine and was named chlorosipentramine. Sibutramine analogues are not naturally present in food, and their safety and efficacy in humans have never been reported. We hope that this study will contribute to the management of food safety as it provides a useful method to identify the adulteration of a slimming formula using chlorosipentramine standard.

Conflict of interest

The authors declare that they have no conflict of interest.
Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jforsciint.2018.03.021.

References