Printed in the Republic of Korea
ANALYTICAL SCIENCE & TECHNOLOGY
Vol. 32 No. 1, 24-34, 2019
https://doi.org/10.5806/AST.2019.32.1.24

# Detection of phenobarbital adulteration in dietary supplements: simultaneous analysis of 16 sedative-hypnotics and sleep-inducers by ultra-high-performance liquid chromatography with UV detection (UPLC-UV) and quadruple Orbitrap mass spectrometry (Q-Orbitrap-MS)

**Ji Hyun Lee†, Ji Yeon Choi†, Hanna Park, Ah Young Min, Nam Sook Kim, Seong Soo Park, Sung-Kwan Park, and Ho-il Kang★**

*Division of Advanced Analysis, National Institute of Food and Drug Safety Evaluation, Ministry of Food & Drug Safety, Osong Health Technology Administration Complex, 187 Osongsaengmyong2-ro, Osongeup, Heungdeok-gu, Cheongju-si, Cheongwoungcheongbuk-do 363-700, Korea*

(Received September 9, 2018; Revised November 18, 2018; Accepted January 15, 2019)

**Abstract:** The safety of food is occasionally questionable, as there have been some reports of products contaminated with illegal adulterants. In this study, the presence of 16 sedative-hypnotics and sleep inducers in dietary supplements was determined by ultra-high-performance liquid chromatography with UV detection (UPLC-UV) and quadruple Orbitrap mass spectrometry (Q-Orbitrap-MS). The UPLC method was validated, providing a linearity (R2) of more than 0.999, and LODs and LOQs that ranged from 0.2 to 0.5 and 0.6 to 1.5 μg $mL^{-1}$, respectively. The repeatabilities were 0.2-8.4 % (intra-day) and 0.3-4.5 % (inter-day), and the accuracies were 89.0-117.0 % (intra-day) and 87.8-111.9 % (inter-day). The mean recoveries of the spiked samples ranged from 98.7 to 107.3 %. The relative standard deviation (%RSD) of the stability was less than 2.4 %. Using the developed method, one sedative-hypnotic compound, phenobarbital, was detected in one of the nineteen samples tested. In addition, the major characteristic fragment ions of each target compound were confirmed using Q-Orbitrap-MS for higher accuracy. Monitoring the presence of these 16 sedative-hypnotics and sleep inducers in dietary supplements should be pursued in the interest of human health, and the results of this study confirmed that the developed method has value for this application.

**Key words:** Sedative-Hypnotics, Adulterants, UPLC-UV, Q-Orbitrap-MS, Method Validation

## 1. Introduction

Over the past few years, illegal adulteration with sedative-hypnotic and sleep-inducer compounds such as diazepam, chlordiazepoxide, nitrazepam, clonazepam and estazolam has been routinely detected in dietary supplements without labelling.[1-4] Sedative-hypnotic medications represent a variety of chemically distinct groups of compounds, including barbiturates, benzodiazepines, and selective non-benzodiazepine hypnotics.

★ Corresponding author
Phone : +82-(0)43-719-5301 Fax : +82-(0)43-719-5300
E-mail : kanghi79@gmail.com
†The first two authors contributed equally to this article.

This is an open access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons. org/licenses/by-nc/3.0) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

These medications shared a general sedative profile of clinical effects, but they differ in other pharmacological properties, including their therapeutic profile, safety, and potential to produce adverse behavioral effects, such as abuse or dependence. Benzodiazepines and selective hypnotics have a more favorable efficacy and safety profile, and their safety profile has led to a decrease in the prescription and clinical issues associated with the use of older agents, such as barbiturates.[5-6]

However, taking sedative-hypnotics and sleep inducers can induce drowsiness, dizziness, weakness, respiratory depression and other side-effects, while long-term use may lead to tolerance, dependence and addiction. These side effects have been used for the purposes of criminal activity, such as robberies and rape. But some manufacturers adulterate their merchandise with sedative-hypnotics and sleep inducers in order to achieve high profits and deceive consumers into thinking that they are experiencing the claimed health benefits by enhancing short-term effects.[7] Therefore, there is an urgent need to develop analytical methods with high sensitivity and high selectivity to screen for the presence of sedative-hypnotic and sleep inducer compounds in dietary supplements.

The analytical methods hitherto used for the detection of sedative-hypnotics and sleep inducers in dietary supplements include HPLC,[4,13] GC-MS[3,8,14] and LC-MS.[1,9,15] However, the simultaneous analysis of more than 16 compounds in dietary supplements using UPLC has not yet been reported.

Therefore, we conducted qualitative analyses with two 'state-of-the-art' instruments. First, qualitative analysis of 16 sedative-hypnotics and sleep inducers was conducted via UPLC. LC with UV detection has advantages including its low cost, and straight forward application and interpretation. The equipment is often standard in medicine control laboratories and readily available in most laboratories.[10] UPLC builds upon the well-established principles of LC, but uses sub-2-μm porous particles. These particles operate at elevated mobile phase linear velocities to produce rapid separation with increased resolution. These attractive features prompted us to develop a rapid, sensitive and specific UPLC assay method for the simultaneous determination of multiple sedative-hypnotics and sleep inducers as possible adulterants in dietary supplements. Second, we wanted to use quadruple Orbitrap mass spectrometry (Q-Orbitrap-MS) to identify the detected substances once again to perform an accurate and in-depth analysis. The major characteristic fragment ions were confirmed using Q-Orbitrap-MS for higher accuracy. Furthermore, after confirming the presence of the sedative-hypnotics and sleep inducer compounds, quantitative analysis was conducted using UPLC.

In this study, we developed and validated a UPLC-UV method for the identification and quantification of 16 sedative-hypnotics and sleep inducer compounds in illegally adulterated dietary supplements. In addition, most of the other studies have analysed single or a few compounds using UPLC. The advantages of our method is the first to carry out simultaneous analysis of more than 16 sedative-hypnotics and sleep inducer compounds using UPLC, which is the most commonly used for analysis. The results of this study are expected to be useful in confirming the safety and effectiveness of sedative-hypnotics and sleep inducer compounds.

## 2. Materials and Methods

### 2.1. Standards and reagents

Alprazolam, Clemastine, Clonazepam, Diphenhydramine, Estazolam, Flunitrazepam, Flurazepam, Hexobarbital, Lorazepam, Mequitazine, Midazolam, Pentobarbital, Phenobarbital, Temazepam, Triazolam and Zolpidem were obtained from the following companies: U.S. Pharmacopeia (Rockville, MD, USA), Lipomed AG (Arlesheim, Switzerland), Merck (Darmstadt, Germany), Cayman (Ann Arbor, MI, USA) and TRC (Toronto, Canada). Sodium phosphate and phosphoric acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). High-purity deionised water was prepared using a Milli-Q purification system (Millipore, Bedford, MA, USA). HPLC-grade methanol and acetonitrile (ACN) were obtained from Burdic and Jackson (Muskegon, MI, USA). Stock solutions (1000 μg $mL^{-1}$) were prepared by dissolving each

compound in methanol. Each stock solution was stored at 4 °C, and working mixtures were prepared daily by diluting the stock solutions in methanol to a concentration appropriate for the calibration curves and method validation.

### 2.2. UPLC conditions

A Waters Acquity UPLC system (Milford, MA, USA) equipped with binary pumps, a sample manager, a column oven and a photodiode array (PDA) detector was used for all analyses. Chromatographic separation was performed using an Acquity UPLC HSS $T_3$ column (2.1 × 150 mm, 1.8 μm; Waters, Milford, MA, USA), and the elution peaks were detected by UV absorption at 210 nm. The column temperature was maintained at 40°C, and the flow rate was 0.2 mL $min^{-1}$, with an injection volume of 1 μL. The mobile phase consisted of (A) 0.5 mM sodium phosphate in deionised water (DW) with 0.1 % phosphoric acid, and (B) 95 % ACN. The gradient program proceeded as follows: 0 min, 32 % B; 2 min, 32 % B; 15 min, 50 % B; 15.1 min, 100 % B; 17 min, 100 % B; 17.1 min, 32 % B; 20 min, 32 % B.

### 2.3. Sample preparation

Dietary supplements were purchased from various markets. Notably, these products were imported to South Korea or sold via online sites or offline stores. A total of 19 samples were obtained and analysed. The samples were homogenised with a blender. Each sample (1 g) was placed into a 50 mL volumetric flask and dissolved in 100 % methanol. The obtained mixture was extracted in an ultrasonic bath for 30 min and then further methanol was added to the 50 mL in volumetric flask after cooling. The extract was subsequently filtered through a 0.22 μm PTFE filter (Millipore) and injected into the UPLC and Q-Orbitrap-MS systems.

### 2.4. Method validation

A series of analyses to evaluate the specificity, linearity, stability, repeatability, limits of detection (LOD) and quantification (LOQ) were conducted to validate the performance of our method. Standard solutions containing the 16 sedative-hypnotic and sleep-inducer were prepared and diluted with methanol to appropriate concentrations for the construction of calibration curves. Calibration curves were developed by plotting the peak areas versus the corresponding concentrations of each analytes. The repeatability of the method was evaluated by analysing the 16 standard compounds. The %RSD of the peak areas was used to evaluate the repeatability of the method. The LOD and LOQ for each analyte were determined at a signal-to-noise ratio (S/N) of approximately 3 and 10, respectively. A recovery test was used to evaluate the accuracy of this method by adding the corresponding standard compounds at low (near the LOQ), medium (~10 fold above the LOQ) and high (~50 fold above the LOQ) concentrations to each dietary supplement, which had been analysed previously. The mixtures were extracted and analysed using the aforementioned method in triplicate.

### 2.5. Mass spectrometry conditions

The experiment was performed with a Q-Exactive Orbitrap mass spectrometer (Thermo Scientific, San Jose, CA) coupled to a Thermo Dionex UltiMate 3000 LC. Target compounds were separated with a BEH-C18 (100 × 2.1 mm i.d., 1.7 μm) column maintained at 40°C. The mobile phases consisted of 0.1% formic acid in both DW (A) and acetonitrile (B). The injection volume was 1 μL and the flow rate was 0.25 $mLmin^{-1}$. Elution was conducted with the following gradient profile: 0.0-2.0 min (A: 80 %, B: 20 %), 2.0-7.0 min (A: 80-0 %, B: 20-100 %), 7.0-11.0 min (A: 0 %, B: 100 %), 11.0-11.1 min (A: 0-80 %, B: 100-20 %), and 11.1-13 min (A: 80 %, B: 20 %).

Full MS/dd$MS^2$ (data-dependent $MS^2$) was implemented as the mass analysis mode. The mass calibration was performed according to the manufacturer's specifications. Data were obtained using Xcalibur 3.0 software. The mass conditions were as follows: HESI ion source; positive ion mode, except for phenobarbital and pentobarbital; spray voltages of 3.5 kV(+) and 3.0 kV(−); capillary temperature of 320°C; sheath gas flow at 42 arbitrary units; auxiliary

*Table* 1. Recovery of each compound under different extraction conditions

| Compound | Extraction solvent | | Methanol concentration (%) | | | Sonication time (min) | | | Sample amount (g) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Methanol | Ethanol | 100 | 70 | 50 | 10 | 30 | 60 | 1 | 2 | 3 |
| | Mean ± RSD[a](%) | Mean ± RSD (%) | Mean ± RSD(%) | Mean ± RSD(%) | Mean ± RSD(%) | Mean ± RSD(%) | Mean ± RSD(%) | Mean ± RSD(%) | Mean ± RSD(%) | Mean ± RSD(%) | Mean ± RSD(%) |
| Zolpidem | 108.9±1.9 | 113.6±1.7 | 114.0±0.7 | 109.2±6.5 | 102.7±1.2 | 108.3±1.0 | 114.0±0.7 | 110.6±1.9 | 114.0±0.7 | 107.6±0.6 | 108.5±1.5 |
| Phenobarbital | 117.6±1.7 | 117.5±0.9 | 117.6±1.7 | 116.4±1.5 | 115.6±0.9 | 98.3±1.8 | 117.6±1.7 | 100.0±2.3 | 117.6±1.7 | 125.4±1.3 | 129.7±0.5 |
| Midazolam | 116.4±2.2 | 95.0±3.0 | 116.4±2.2 | 117.2±1.7 | 92.1±4.0 | 85.8±3.2 | 116.4±2.2 | 91.1±2.6 | 116.4±2.2 | 117.2±2.9 | 123.0±3.2 |
| Flurazepam | 111.1±1.1 | 110.3±1.3 | 111.1±1.1 | 102.5±0.4 | 102.4±0.9 | 109.6±1.1 | 111.1±1.1 | 107.1±1.0 | 111.1±1.1 | 106.3±1.3 | 105.6±0.8 |
| Diphenhydramine | 102.4±2.5 | 125.8±3.0 | 102.4±2.5 | 93.5±1.3 | 90.9±1.4 | 97.0±2.8 | 102.4±2.5 | 97.6±2.4 | 102.4±2.5 | 120.3±0.5 | 127.9±7.4 |
| Pentobarbital | 116.3±3.6 | 110.1±1.0 | 116.3±3.6 | 108.8±0.4 | 105.8±0.7 | 106.9±1.2 | 116.3±3.6 | 107.4±0.7 | 116.3±3.6 | 118.0±1.0 | 126.1±1.4 |
| Hexobarbital | 113.2±0.9 | 112.5±0.8 | 113.2±0.9 | 104.7±0.9 | 105.0±0.2 | 98.7±0.8 | 113.2±0.9 | 104.5±0.8 | 113.2±0.9 | 111.3±1.0 | 111.4±0.9 |
| Estazolam | 114.2±2.9 | 105.6±1.6 | 114.2±2.9 | 96.2±1.2 | 102.3±2.3 | 101.7±2.4 | 114.2±2.9 | 93.4±1.2 | 114.2±2.9 | 97.7±0.6 | 96.2±0.8 |
| Alprazolam | 114.4±2.2 | 112.0±0.8 | 114.4±2.2 | 108.3±1.1 | 104.1±0.9 | 104.9±0.7 | 114.4±2.2 | 106.2±1.2 | 114.4±2.2 | 115.1±1.8 | 118.5±0.9 |
| Lorazepam | 105.7±0.3 | 111.9±1.1 | 105.7±0.3 | 97.0±0.4 | 96.4±0.7 | 100.3±1.4 | 105.7±0.3 | 104.0±1.0 | 105.7±0.3 | 101.6±2.5 | 103.7±0.7 |
| Clonazepam | 109.0±1.0 | 108.7±1.3 | 116.1±0.2 | 113.0±0.8 | 112.5±0.4 | 112.9±1.2 | 116.1±0.2 | 113.7±0.4 | 116.1±0.2 | 118.7±0.3 | 122.9±0.7 |
| Triazolam | 116.1±0.2 | 118.8±1.0 | 114.4±0.4 | 117.1±0.3 | 114.5±0.2 | 98.4±0.7 | 114.4±0.4 | 98.7±0.5 | 114.4±0.4 | 126.2±0.4 | 129.7±0.4 |
| Mequitazine | 114.4±0.4 | 115.9±0.4 | 114.8±0.4 | 112.5±0.9 | 110.6±0.7 | 79.7±1.0 | 114.8±0.4 | 81.2±1.3 | 114.8±0.4 | 118.9±0.4 | 123.4±0.6 |
| Flunitrazepam | 114.8±0.4 | 115.4±1.0 | 115.2±0.5 | 103.9±0.4 | 101.8±0.9 | 110.7±0.7 | 115.2±0.5 | 110.2±1.1 | 115.2±0.5 | 108.7±1.3 | 109.7±0.5 |
| Temazepam | 115.2±0.5 | 114.2±1.0 | 107.8±1.8 | 89.6±4.7 | 83.1±1.3 | 83.8±4.1 | 107.8±1.8 | 84.2±2.9 | 107.8±1.8 | 89.0±2.7 | 84.7±0.4 |
| Clemastine | 107.8±1.8 | 87.7±1.5 | 113.1±0.2 | 115.6±0.6 | 113.6±0.6 | 101.7±0.7 | 113.1±0.2 | 102.3±1.0 | 113.1±0.2 | 124.6±0.3 | 128.4±0.3 |

gas flow at 10 arbitrary units; probe heater temperature set to 350 °C(+) and 300 °C(−); S-lens RF level of 50; resolution of 70,000 (full scan) and 17,500 (MS/MS); automatic gain control (AGC) target of $3 \times 10^6$ (full scan) and $1 \times 10^5$ (MS/MS); scan range from m/z 50 to 1000; maximum infusion time (IT) of 100 ms (full scan) and 50 ms (MS/MS); single microscan count; loop count of 5; MSX count of 1; Top N of 5; isolation window of 4 m/z; underfill ratio of 1.0 %; intensity threshold of $2.0 \times 10^4$; isotope exclusion; dynamic exclusion of 10.0 s.

## 3. Results

### 3.1. Optimization of sample preparation

In order to develop the optimal sample preparation conditions, the extraction parameters, solvent proportion, sample weight and sonication time were considered. The recovery under each set of conditions was determined as the fortified standard area, where the 16 sedative-hypnotic and sleep-inducers were fortified with a precisely known amount prior to extraction.

The recovery efficiency was greater with methanol extraction than with ethanol. The use of 100 % methanol led to a better recovery than either 70 % or 50 % methanol (v/v). The sonication time was varied from 10 to 60 min; sonication of most compounds for 30 min led to superior recovery. The sample preparation optimization results are shown in *Table* 1.

### 3.2. Optimization of UPLC conditions

To achieve a successful separation of the 16 sedative-hypnotics and sleep-inducers, we evaluated several mobile phases and buffers, including sodium phosphate, phosphoric acid and potassium phosphate. In phosphoric acid and potassium phosphate buffers, we observed that an excellent resolution was achievable in 0.5 mM sodium phosphate in deionized water (DW) with 0.1 % phosphoric acid.

We also evaluated several stationary phase columns, and we found that the Acuity UPLC HSS T3 column gave better separation, peak shapes, and resolution compared with either a BEH C8 (1.7 μm, 2.1 × 150 mm; Waters, Milford, MA, USA) or an HSS C18 column (1.8 μm, 2.1 × 100 mm; Waters, Milford, MA, USA); the latter two both showed broad peaks with poor analyte resolution. Therefore, we chose the HSS T3 column for further analysis. The UPLC

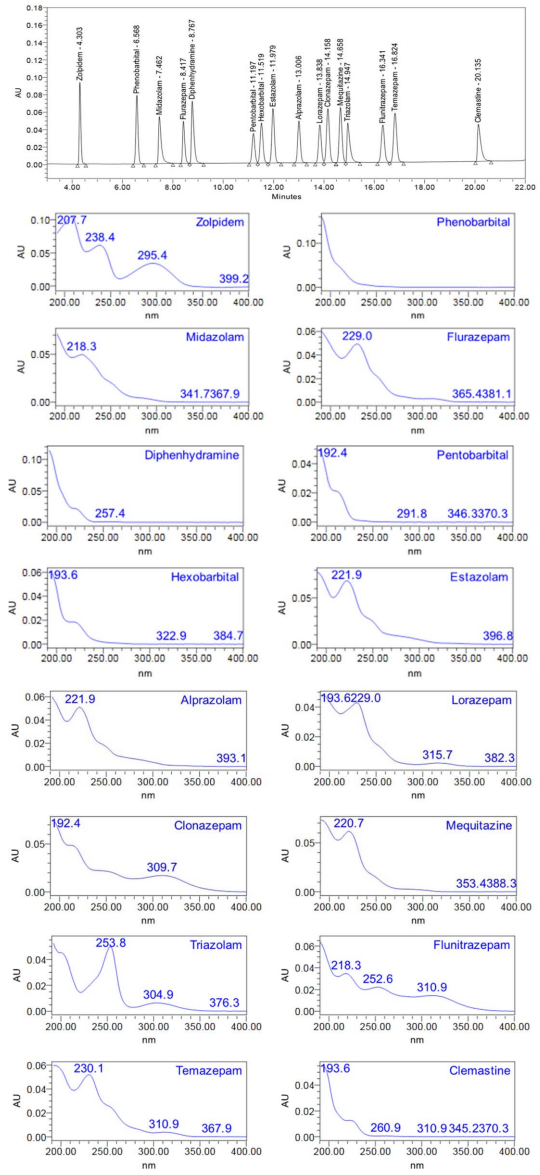


*Fig.* 1. LC chromatogram of the 16 sleep inducers analysed in this study and the individual PDA spectra of each compound.

chromatogram and UV spectra of a standard mixture of the 16 sedative-hypnotics and sleep-inducers are shown in *Fig.* 1.

### 3.3. Method validation

The developed method was validated in accordance with the guidelines established by the AOAC, CODEX, FDA and at the ICH. The performance of the method was evaluated by estimation of the specificity, LOD, LOQ, linearity, recovery, and repeatability.

#### 3.3.1. Specificity

The specificity of the method was guaranteed by comparing the retention times (RTs) of the samples with those of reference materials in blank samples. *Fig.* 1 shows the individual chromatograms of the 16 sedative-hypnotics and sleep-inducers, which do not exhibit significant matrix interferences at their respective RTs. The developed UPLC method is capable of separating all analytes under the given gradient conditions within 20 min.

#### 3.3.2. Linearity, LOD, and LOQ

The calibration plots based on linear regression analysis revealed good linear relationships between the response and six different concentrations between 0.6-30 μg $mL^{-1}$, based on the LOQ of the 16 sedative-hypnotics and sleep-inducers. Acceptable linearity with $R^2$ values between 0.9990 and 1.0000 (*Table* 2) is obtained.

The LODs and LOQs for all analytes were defined at a S/N of 3:1 and 10:1, respectively. The LOD and LOQ values are presented in *Table* 2. The LODs and LOQs of 16 sleep-inducing compounds range from 0.20 to 0.50 and 0.60 to 1.50 μg $mL^{-1}$, respectively.

#### 3.3.3. Repeatability and accuracy

The intra-day and inter-day repeatabilities were assessed using the relative standard deviation (RSD, %) at low, medium and high concentrations. The accuracy of the method was determined as the recovery (%) of the theoretical concentration of the target compounds. Intra-day assays were carried out in triplicate using samples of low, medium and high concentrations on the same day, and inter-day assays were carried out in triplicate using samples of low, medium and high concentrations on three separate days. The intra- and inter-day repeatability values are 0.2-8.4 % and 0.3-4.5 %, respectively, and the intra- and inter-day accuracy values are 89.0-117.0 % and 87.8-111.9 %, respectively (*Table* 3).

*Table* 2. Summary of the calibration curves, limits of detection (LOD), and limits of quantification (LOQ) for 16 sleep inducers by UPLC

| Compound | Calibration curve | $R^2$ | Range (μg/mL) | LOD (μg/mL) | LOQ (μg/mL) |
|---|---|---|---|---|---|
| Zolpidem | y = 33,530.89x + 3640.99 | 0.9998 | 0.6-12.0 | 0.20 | 0.60 |
| Phenobarbital | y = 37,344.12x + 5,189.10 | 0.9999 | 0.6-12.0 | 0.20 | 0.60 |
| Midazolam | y = 37,213.08x − 3,917.44 | 0.9990 | 1.5-30.0 | 0.50 | 1.50 |
| Flurazepam | y = 25,640.19x + 2,098.93 | 0.9992 | 1.5-30.0 | 0.50 | 1.50 |
| Diphenhydramine | y = 39,457.66 x + 1,443.55 | 0.9999 | 0.9-18.0 | 0.30 | 0.90 |
| Pentobarbital | y = 20,642.18 x + 3,737.40 | 0.9990 | 0.9-18.0 | 0.30 | 0.90 |
| Hexobarbital | y = 27,170.63 x + 803.31 | 0.9996 | 1.2-24.0 | 0.40 | 1.20 |
| Estazolam | y = 37,347.73 x + 4,455.82 | 1.0000 | 0.9-18.0 | 0.30 | 0.90 |
| Alprazolam | y = 30,943.42 x + 653.32 | 0.9996 | 1.2-24.0 | 0.40 | 1.20 |
| Lorazepam | y = 27,873.12 x + 3,557.51 | 0.9990 | 1.5-30.0 | 0.50 | 1.50 |
| Clonazepam | y = 41,880.01 x + 5,515.29 | 0.9999 | 0.9-18.0 | 0.30 | 0.90 |
| Triazolam | y = 40,497.13 x + 6,029.67 | 1.0000 | 0.9-18.0 | 0.30 | 0.90 |
| Mequitazine | y = 30,257.52 x − 4,396.89 | 0.9991 | 1.5-30.0 | 0.50 | 1.50 |
| Flunitrazepam | y = 32,502.18 x + 250.54 | 0.9996 | 1.2-24.0 | 0.40 | 1.20 |
| Temazepam | y = 41,251.99 x + 5,137.69 | 1.0000 | 0.9-18.0 | 0.30 | 0.90 |
| Clemastine | y = 33,430.43 x + 1,383.65 | 0.9994 | 1.5-30.0 | 0.50 | 1.50 |

*Table* 3. Intra-and inter-day variation in the three concentrations of the 16 sleep inducers using UPLC

| Analyte | Conc. (μg $mL^{-1}$) | UPLC | | | |
|---|---|---|---|---|---|
| | | Intra-day | | Inter-day | |
| | | Recovery (%) | RSD (%) | Recovery %) | RSD (%) |
| Zolpidem | 0.6 | 106.0 | 6.8 | 104.7 | 3.1 |
| | 3.0 | 110.7 | 2.0 | 111.9 | 2.3 |
| | 12.1 | 109.3 | 0.6 | 109.6 | 1.3 |
| Phenobarbital | 0.6 | 90.8 | 8.4 | 95.8 | 0.7 |
| | 3.0 | 106.7 | 2.0 | 107.9 | 1.8 |
| | 12.1 | 106.0 | 0.3 | 106.3 | 1.2 |
| Midazolam | 1.5 | 107.7 | 1.6 | 98.5 | 2.1 |
| | 7.4 | 102.0 | 3.2 | 95.8 | 1.2 |
| | 29.7 | 101.5 | 1.3 | 97.7 | 0.9 |
| Flurazepam | 1.5 | 93.3 | 2.2 | 91.5 | 0.6 |
| | 7.6 | 100.3 | 2.3 | 95.8 | 1.4 |
| | 30.3 | 100.3 | 0.8 | 97.6 | 1.0 |
| Diphenhydramine | 1.0 | 102.4 | 2.4 | 101.1 | 2.9 |
| | 5.0 | 98.9 | 1.2 | 100.5 | 1.5 |
| | 19.0 | 99.8 | 0.3 | 101.0 | 1.5 |
| Pentobarbital | 1.5 | 89.0 | 3.8 | 87.8 | 1.3 |
| | 7.6 | 98.2 | 0.3 | 95.8 | 2.8 |
| | 30.5 | 98.3 | 0.8 | 96.6 | 1.5 |
| Hexobarbital | 1.4 | 90.11 | 0.8 | 89.7 | 0.5 |
| | 7.1 | 93.3 | 0.2 | 92.1 | 1.5 |
| | 28.3 | 91.5 | 0.8 | 90.4 | 1.4 |
| Estazolam | 0.9 | 97.8 | 3.6 | 94.2 | 3.3 |
| | 4.6 | 102.4 | 1.1 | 100.2 | 1.9 |
| | 18.5 | 101.0 | 0.8 | 99.3 | 1.5 |

*Table* 3. Continued

| Analyte | Conc. (μg $mL^{-1}$) | UPLC | | | |
|---|---|---|---|---|---|
| | | Intra-day | | Inter-day | |
| | | Recovery (%) | RSD (%) | Recovery %) | RSD (%) |
| Alprazolam | 1.2 | 100.1 | 2.2 | 99.0 | 1.6 |
| | 6.0 | 102.3 | 1.5 | 100.3 | 1.8 |
| | 24.0 | 101.3 | 0.4 | 99.8 | 1.4 |
| Lorazepam | 1.6 | 92.3 | 0.8 | 91.8 | 2.9 |
| | 7.8 | 93.4 | 1.9 | 98.6 | 3.5 |
| | 31.1 | 90.6 | 0.5 | 98.8 | 1.3 |
| Clonazepam | 0.9 | 96.0 | 1.5 | 96.0 | 3.0 |
| | 4.5 | 100.5 | 0.7 | 99.8 | 2.5 |
| | 18.0 | 99.8 | 0.8 | 99.9 | 3.1 |
| Triazolam | 0.9 | 97.7 | 1.5 | 93.2 | 4.5 |
| | 4.6 | 102.4 | 1.3 | 100.4 | 1.8 |
| | 18.4 | 101.7 | 0.8 | 99.9 | 1.5 |
| Mequitazine | 1.6 | 95.8 | 0.5 | 96.3 | 2.4 |
| | 7.8 | 99.0 | 0.6 | 98.5 | 0.9 |
| | 31.1 | 101.3 | 0.4 | 101.1 | 0.3 |
| Flunitrazepam | 1.2 | 99.7 | 3.6 | 99.7 | 0.3 |
| | 6.1 | 101.5 | 1.3 | 99.9 | 1.3 |
| | 24.6 | 99.9 | 0.3 | 98.9 | 0.9 |
| Temazepam | 0.9 | 93.1 | 4.1 | 92.0 | 2.8 |
| | 4.6 | 105.3 | 1.3 | 98.0 | 2.6 |
| | 18.2 | 105.6 | 0.3 | 98.0 | 3.1 |
| Clemastine | 1.5 | 93.5 | 2.0 | 92.9 | 3.7 |
| | 7.5 | 98.7 | 1.1 | 99.0 | 2.6 |
| | 30.0 | 99.9 | 0.7 | 100.7 | 1.6 |

*Table* 4. The recovery efficiency of the 16 sleep inducers from dietary supplement samples using UPLC

| Compound | Recovery (Mean±RSD), % | |
|---|---|---|
| | Solid | Liquid |
| Zolpidem | 103.6±0.3 | 103.9±0.4 |
| Phenobarbital | 100.8±0.3 | 100.2±0.9 |
| Midazolam | 99.3±1.0 | 98.7±0.8 |
| Flurazepam | 100.4±1.1 | 101.7±2.4 |
| Diphenhydramine | 105.0±1.6 | 104.9±0.7 |
| Pentobarbital | 101.4±1.7 | 100.3±1.4 |
| Hexobarbital | 101.4±1.0 | 100.8±0.6 |
| Estazolam | 102.1±1.8 | 101.0±1.2 |
| Alprazolam | 99.8±1.6 | 100.5±1.2 |
| Lorazepam | 100.9±2.4 | 100.4±1.3 |
| Clonazepam | 100.5±0.2 | 100.1±0.3 |
| Triazolam | 100.7±1.2 | 100.6±0.7 |
| Mequitazine | 107.3±1.9 | 107.0±1.8 |
| Flunitrazepam | 100.8±0.8 | 101.1±0.5 |
| Temazepam | 100.3±0.9 | 100.8±1.0 |
| Clemastine | 104.2±2.3 | 104.1±1.4 |

*Table* 5. Stability of the 16 sleep inducer compounds over 48 h analysed with UPLC.

| Compounds | | 0[a] h | 24 h | 48 h |
|---|---|---|---|---|
| | | RSD[b] (%) | | |
| Zolpidem | Low | 2.90 | 0.62 | 5.69 |
| | Medium | 0.31 | 2.51 | 3.70 |
| | High | 0.55 | 1.16 | 1.83 |
| Phenobarbital | Low | 2.45 | 6.51 | 8.00 |
| | Medium | 0.09 | 2.10 | 4.02 |
| | High | 0.47 | 1.14 | 1.81 |
| Midazolam | Low | 0.27 | 2.58 | 1.58 |
| | Medium | 0.20 | 1.58 | 3.20 |
| | High | 0.89 | 1.16 | 2.73 |
| Flurazepam | Low | 0.76 | 0.21 | 1.02 |
| | Medium | 0.70 | 1.23 | 3.36 |
| | High | 1.03 | 1.39 | 2.71 |
| Diphenhydramine | Low | 3.20 | 3.61 | 7.37 |
| | Medium | 1.40 | 0.68 | 1.39 |
| | High | 0.40 | 1.59 | 3.56 |
| Pentobarbital | Low | 0.01 | 0.52 | 1.58 |
| | Medium | 0.54 | 2.85 | 4.33 |
| | High | 1.07 | 1.07 | 2.89 |
| Hexobarbital | Low | 0.80 | 1.32 | 1.48 |
| | Medium | 0.26 | 1.64 | 2.34 |
| | High | 0.05 | 1.14 | 1.86 |
| Estazolam | Low | 0.76 | 3.11 | 5.70 |
| | Medium | 0.09 | 2.24 | 2.42 |
| | High | 0.44 | 1.61 | 3.62 |
| Alprazolam | Low | 0.31 | 1.71 | 2.08 |
| | Medium | 0.42 | 1.89 | 3.11 |
| | High | 0.13 | 1.75 | 2.52 |
| Lorazepam | Low | 0.19 | 1.68 | 3.62 |
| | Medium | 0.24 | 1.63 | 4.55 |
| | High | 0.99 | 0.84 | 2.47 |
| Clonazepam | Low | 3.37 | 5.28 | 7.05 |
| | Medium | 0.63 | 1.73 | 2.74 |
| | High | 0.56 | 1.55 | 3.80 |
| Triazolam | Low | 2.11 | 5.40 | 8.40 |
| | Medium | 0.68 | 1.73 | 2.34 |
| | High | 0.23 | 1.68 | 3.93 |
| Mequitazine | Low | 3.59 | 2.48 | 1.76 |
| | Medium | 1.22 | 0.15 | 3.06 |
| | High | 0.40 | 0.40 | 1.83 |
| Flunitrazepam | Low | 0.45 | 0.26 | 1.99 |
| | Medium | 0.24 | 1.49 | 2.71 |
| | High | 0.19 | 0.95 | 1.73 |
| Temazepam | Low | 2.29 | 4.61 | 5.65 |
| | Medium | 0.74 | 1.81 | 2.68 |
| | High | 0.29 | 1.79 | 3.99 |
| Clemastine | Low | 1.45 | 1.69 | 3.52 |
| | Medium | 0.54 | 0.92 | 3.08 |
| | High | 0.62 | 0.77 | 2.67 |

[a]Stored for 6 h at room temperature after making the solution.
[b]%RSD (relative standard deviation) is defined as the standard deviation of a group of values divided by their mean.

### 3.3.4. Recovery

The recovery (%) of each compound was calculated by comparing it to the response for the true concentration of the liquid and solid reference standards. Each sample was analysed at the same concentration three times. As shown in *Table* 4, the mean recoveries of the solid and liquid samples are 99.3-105.0 % and 98.7-107.0 %, respectively. The %RSD values are less than 2.4 %, which is within the acceptable limit (15 %; UNODC 2009). In solid and liquid samples, the recoveries tend not to be affected by matrix effects.

### 3.3.5. Stability

The stability of each compound was measured several times over 48 h, after incubation in the sample solution. The stability was assessed using three different concentrations of samples stored at room temperature for 6 h, and samples in autosampler vials stored at 4 °C for 48 h. All stability samples were analysed in triplicate.

The %RSD values were taken as indicators of the stability of the compounds analysed by the analytical method. The %RSD values are < 8.4 % (*Table* 5). The results indicate that the prepared sample solutions are sufficiently stable at room temperature for 6 h. Also, the sample solutions could be stored at 4 °C for up to 48 h, and no stability-related problems would be expected during routine analysis of the 16 sedative-hypnotics and sleep-inducers.

### 3.3.6. Confirmation by Q-Orbitrap

For the 16 sedative-hypnotics and sleep-inducers, protonated $[M+H]^+$ and deprotonated $[M-H]^-$ molecules were observed in Q-Orbitrap in positive and negative ion mode. The exact masses and RTs of the 16 sedative-hypnotics and sleep-inducers were obtained from a database. It is necessary to identify the major characteristic fragment ions present in the mass

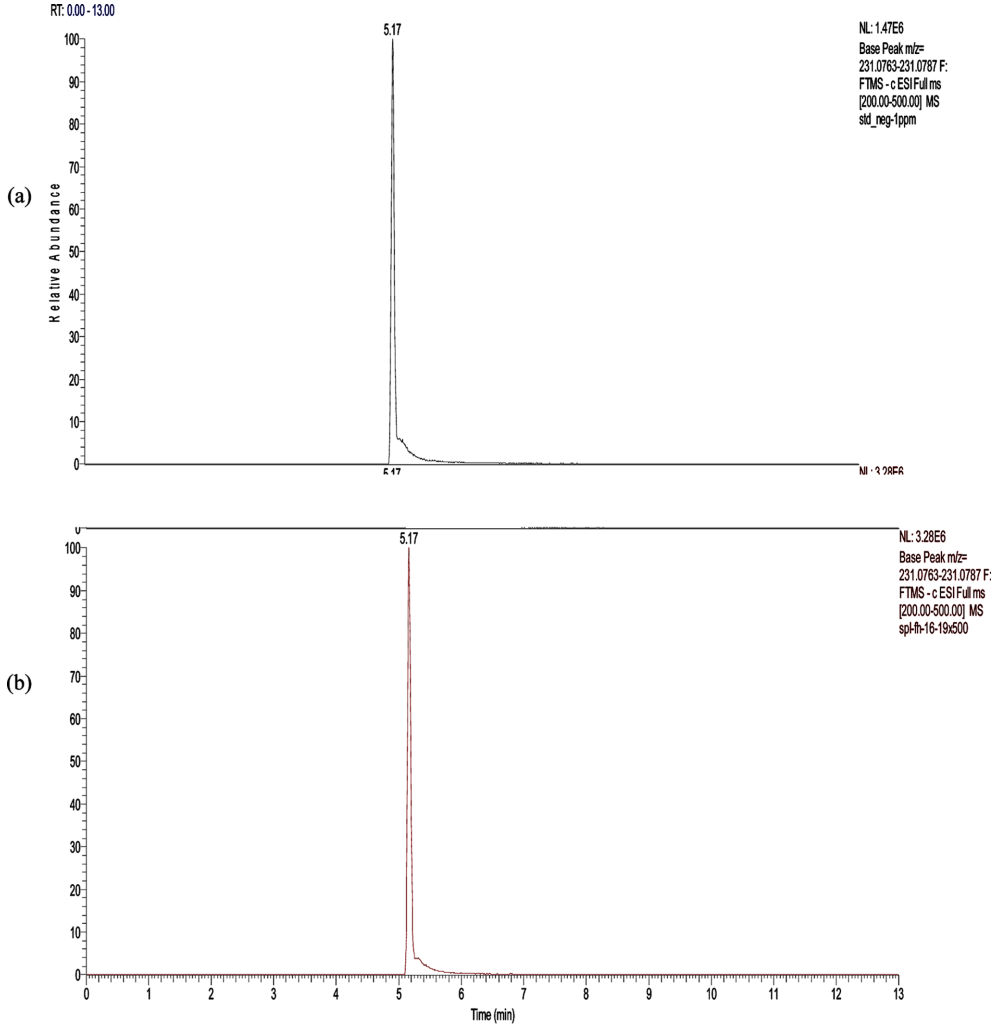


*Fig.* 2. XIC of a phenobarbital standard (a) and 500-fold diluted positive sample (b).

spectra, if these spectra are to be used for qualitative analysis. Towards this end, reference MS spectra of the target compounds were studied and the fragmentation pathways leading to the formation of the major characteristic fragment ions were investigated. The Q-Orbitrap method was demonstrated as suitable for detecting the target compounds, evaluated using a combination of RT, mass accuracy, and fragmentation.

### 3.3.7. Application in real samples

19 samples were collected from online and offline markets; their adulteration with 16 sedative-hypnotics and sleep-inducers were evaluated using the UPLC-UV and Q-Orbitrap methods. First, qualitative analysis of the sleep-inducing compounds in the food samples was conducted via UPLC-UV. After qualitative analysis for screening of the presence of the sleep inducers in food samples by UPLC-UV, confirming the result of qualitative analysis by Q-Orbitrap. The risk of a false-positive was minimised by applying several criteria, such as RT, mass accuracy and $MS^2$ product ions employed to identify the sedative-hypnotics and sleep inducer compounds present in dietary supplements. *Fig.* 2 show the representative XICs of a phenobarbital standard and phenobarbital in a positive sample. The MS spectra of the samples that

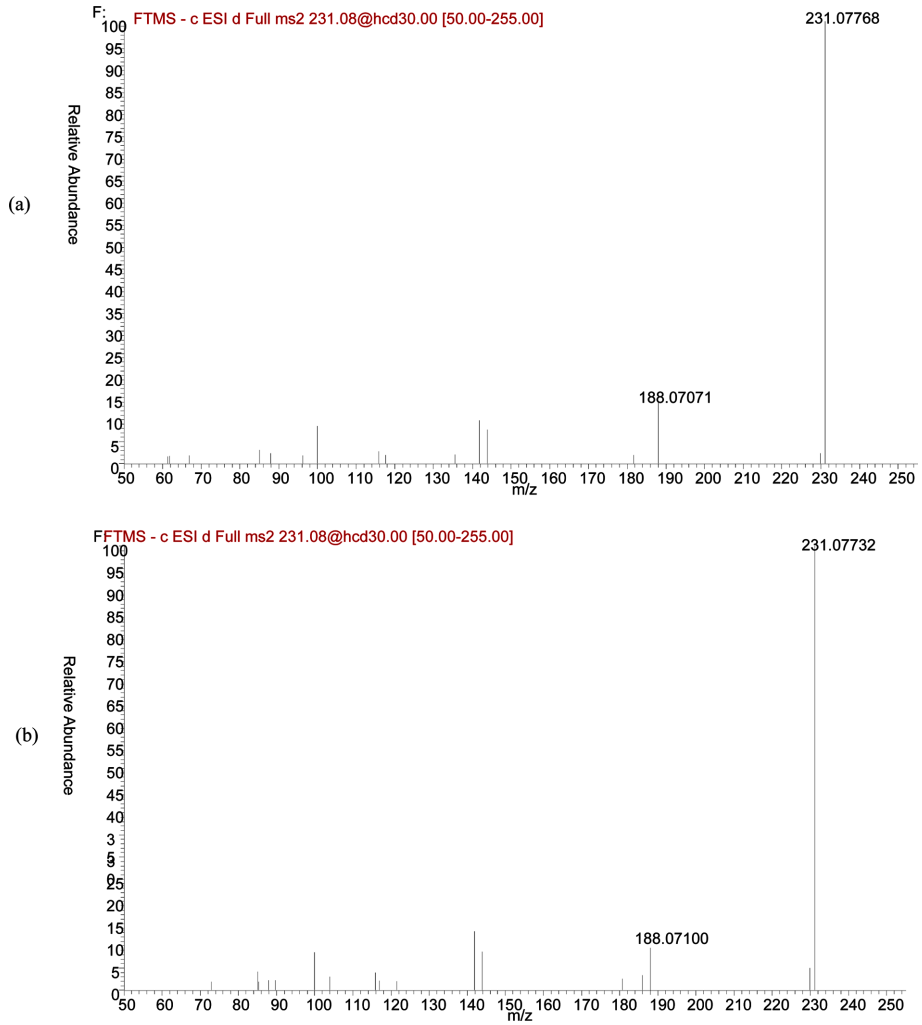


*Fig.* 3. Mass spectra of a phenobarbital standard (a) and a sample that contains phenobarbital (b).

contained one sleep-inducing compound, phenobarbital, and phenobarbital standards are shown in *Fig.* 3.

Phenobarbital was detected as an illegal adulterant. The RT, accurate mass and $MS^2$ product ions confirmed the presence of phenobarbital in the positive samples. After confirming the presence of the sleep inducing compounds in food samples, quantitative analysis was conducted using UPLC-UV. It is important to note that the positive samples were illegally adulterated with phenobarbital at high levels (24.45 mg $g^{-1}$) in this study. Therefore, for safety, these screening results should be publicised. Furthermore, these results suggest that this sort of health and food product monitoring should be continued in order to safeguard human health.

## 4. Discussion

Previously, most of the other studies have analysed single or a few sedative-hypnotics and sleep-inducing compounds using UPLC; these methods can simultaneously determine of sedative-hypnotics and sleep-inducers in illegally adulterated dietary supplements. In this method, the successful separation produced by UPLC-UV, in addition, the major characteristic fragment ions were confirmed using Q-Orbitrap-MS for higher accuracy. The UPLC-UV separation was achieved on a HSS T3 column by using mobile phase of 0.5 mM sodium phosphate in deionized water (DW) with 0.1 % phosphoric acid and acetonitrile of a gradient elution mode. The optimized method was validated for specificity, linearity, LOD, LOQ, repeatability, accuracy, recovery and stability according to ICH guideline. The developed method was successfully applied to determine sedative-hypnotics and sleep-inducing compounds in dietary supplements without any interference. The results demonstrated that the values were within the acceptable range.

## 5. Conclusions

We attempted to detect the presence of 16 sedative-hypnotics and sleep inducers in dietary supplements advertised to improve sleep functions, by developing and fully validating a sensitive, accurate, and selective UPLC-UV method. Nineteen representative samples were screened by UPLC-UV and Q-Orbitrap. Only one sample was tested positive for illegal adulteration with a sleep-inducing compound, phenobarbital, at high levels. The novel UPLC-UV method has been proven to be a very promising and powerful method for routine screening of illegal adulterated sedative-hypnotics and sleep inducers in dietary supplements, ensuring food safety and public human health.

### Funding

This work was supported by the Ministry of Food and Drug Safety [grant number 15181MFDS521].

## References

1. Q. Gao, B. Che, Z. Zhang, H. Dai, and H. Guo, *J. Chin. Pharm. Univ.*, **39**, 142-146 (2008).
2. B. Zhu, Z. Zhao, Z. Wang, Q. Xue, and L. Zhao, *Chin. J. Pharm. Anal.,* **30**, 745-751 (2010).
3. J. Liu, L. Xiao, X. Guan, Y. Xiong, T. Wang, and J. Li, *Chin. J. Pharm. Anal.*, **31**, 376-379 (2011).
4. P. Jin, Z. Wang, W. Wang, D. Zou, J. Ma, and X. Hu, *Chin Pharm. J.,* **47**, 462-466 (2012).
5. L. J. Greenfield, S. H. Zaman, M. L. Sutherland, S. C. Lummis, M. I. Niemeyer, E. A. Barnard, and R. L. Macdonald, *Neuropharmacology*, **42**, 502-521 (2002).
6. C. Gottesmann, *Neuroscience*, **111**, 231-239 (2002).
7. J. Wang, B. Chen, and S. Yao, *Food addit. Contam*., **25**, 822-830 (2008).
8. Z. Wang, J. Zhang, and Y. Zhang, *J. Chin. Mass Spec. Soc.*, **30**, 282-286 (2009).
9. A. Yan, X. Li, C. Xi, L. Zhang, S. Xia, G. Wang, B. Tang, and Z. Mu, *Chinese J. Anal. Chem.*, **41**, 509-516 (2013).
10. E. Deconinck, P. Y. Sacré, P. Courselle, and J. O. De Beer, *J. Chromatogr. Sci*., **51**, 791-806 (2013).
11. AOAC Official Methods of Analysis. Internationalconference on harmonization of harmonized tripartiteguideline, validation of analytical procedures: text andmethodology.
12. AOAC International, Rockville, MD (2005).
13. ICH International conference on Harmonization. Harmo-

nized tripartite guideline, validation of analyticalprocedures: text and methodology Q2
14. (R1). European Medicines Agency (EUMA), London, UK .
15. A, El-Gindy, *Il Farmaco*, **60**, 745 (2005).
16. R. A. Menck, D. S. De Lima, S. C. Seulin, V. Leyton, C. A. Pasqualucci, D. R. Munoz, M. D. Osseltore, M. Yonamine, *J. Sep. Sci.*, **35**, 3361-3368 (2012).
17. X. Zhang, Z. Lin, J. Li, Z. Huang, Y. Rao, H. Liang, J. Yan, and F. Zheng. *Drug Testing and Analysis*, **9**, 588-595 (2017).

## Authors' Positions

Ji Hyun Lee: Research Scientist
Ji Yeon Choi: Research Scientist
Hanna Park: Research Scientist
Ah Young Min: Research Scientist
Nam Sook Kim: Research Scientist
Seong Soo Park: Research Scientist
Sung-Kwan Park: Research Scientist
Ho-il Kang: Research Scientist