P-ISSN 2586-9000
E-ISSN 2586-9027
Homepage : https://tci-thaijo.org/index.php/SciTechAsia
Science & Technology Asia
Vol.28 No.1 January - March 2023
Page: [206-216]
*Original research article*

# Development of Dried Urine Samples for Simultaneous Quantitative Detection of Sibutramine and Its Active Metabolites by Liquid Chromatography/Mass Spectrometry

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Received 14 November 2021; Received in revised form 28 September 2022
Accepted 3 October 2022; Available online 20 March 2023

**ABSTRACT**

Sibutramine is an unsafe compound adulterated in weight-loss supplements. Quantitative detection of sibutramine and its active metabolites in urine can signify its ingestion and predict sudden unexpected death due to toxicity following overdose. However, the requirement for cold storage and restrictions on the amount of urine specimens limit downstream procedures. In this study, dried urine spot (DUS) coupled with liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used for the detection of sibutramine hydrochloride and its metabolites. Spiked drug-free urine was used to prepare DUS. Analytes were accordingly extracted and analyzed by LC-MS/MS with optimum instrumental conditions. The results exhibited that the $R^2$ of sibutramine, desmethyl sibutramine and didesmethyl sibutramine were 0.9993, 0.9980 and 0.9993, respectively, and that the limits of detection (LOD) were 0.02, 0.02 and 0.03 ng/mL, respectively. Analytical characteristics and stability affirmed the usability of this newly developed method. These findings favor the application of this analytical protocol toward quantitative detection of sibutramine and its active metabolites using DUS microsampling and LC-MS/MS.

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**Keywords:** Desmethyl sibutramine; Didesmethyl sibutramine; Dried urine spot; Liquid chromatography/mass spectrometry; Sibutramine

## 1. Introduction

Sibutramine is a selective serotonin-norepinephrine reuptake inhibitor (SSNRI) as well as a dopamine reuptake inhibitor [1-8]. It is considered as a potential antidepressant drug according to its mechanisms of action. However, the effect of weight-loss seen in early trials in obese depressed individuals favored an alternate research scheme for obesity management [9-10]. Sibutramine was approved by the U.S. Food and Drug Administration (FDA) as an appetite-suppressant drug in 1997 and was prescribed to nearly 20 million people with a dose of 10-15 mg daily in over 80 countries [11-14]. The molecular mechanisms underlying the weight-loss include satiety enhancement via increased serotonergic and noradrenergic activity and thermogenesis through increased energy expenditure [15-17]. Sibutramine Cardiovascular Outcomes (SCOUT) trials aimed to investigate the long-term effects of sibutramine in overweight and obese individuals [12, 13]. Despite the absence of lethal side effects, elevation of blood pressure and heart rate during the use of sibutramine was found to pose a high risk of cardiovascular disease [18, 19]. These findings prompted the European Medicines Agency (EMA), the U.S. FDA, and parts of Asia to reassess sibutramine. In Thailand, sibutramine had been prescribed as a controlled substance to treat obesity for a short period of time before it was withdrawn due to safety concerns in 2010. To date, sibutramine is categorized as a psychotropic type I substance, for which production, import, export, possession, and consumption are forbidden. Nevertheless, sibutramine has been illegally used and intentionally adulterated in several weight-loss dietary supplements. Sudden cardiac death has been reported as a serious event of sibutramine intake and many new cases continuously occur [20, 21]. The average half-life of sibutramine and its metabolites is 1.1 hrs in the body (plasma). However, 68-95% of sibutramine and its metabolites can still be detected by specific methods in urine for up to 15 days after ingestion [22]. Therefore, blood (plasma) and urine are two most common samples used for testing of sibutramine and its metabolites in people. Sibutramine is absorbed rapidly from the gastrointestinal tract to plasma and advances to hepatic metabolism to form two active metabolites, desmethyl sibutramine (M1) and didesmethyl sibutramine (M2), which are further hydroxylated and conjugated to form inactive metabolites, M5 and M6, before renal excretion. With this, only its metabolites are detectable in urine. Although M5 and M6 are mainly identified in urine over a couple of weeks, M1 and M2 can be measured via highly sensitive liquid chromatography-mass spectrometry (LC/MS) and gas chromatography-mass spectrometry (GC/MS) approaches [23, 24]. Generally, the methods for determination of sibutramine and its derivatives requires one to two milliliters of urine sample for pretreatment with a solid phase extraction.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been widely used to determine illegal compounds and adulterants in dietary supplements and biological samples with high sensitivity and specificity. In some cases; however, the specimen is inadequate for LC-MS/MS analysis and required a cold storage unit for urine transportation. Dried urine spot (DUS) is one of microsampling precedures that allows the collection of a fixed urine sample and can be applied to many analytical methods especially for chemical compounds detection in urine [25-29]. Moreover, urine collection is a non-invasive sampling procedure and there have been no reports of misidentification of sibutramine and its metabolites due to microbial or fungal

contamination. Considering all of this, urine is one of the best biological samples for testing of sibutramine and its metabolites.

To investigate the potential of urine microsampling DUS for the detection of sibutramine and its active metabolites using LC-MS/MS, the optimal parameters for DUS preparation, DUS extraction, and LC-MS/MS were studied.

## 2. Materials and Methods

### 2.1 Chemicals

Sibutramine hydrochloride was kindly provided by Associate Professor Dr. Prapin Wilairat from the National Doping Control Centre, Mahidol University, Bangkok, Thailand. Desmethyl sibutramine (D292171) and didesmethyl sibutramine (D441165) were purchased from Toronto Research Chemicals (Toronto, ON, Canada). Methanol hypergrade for LC-MS (106035), acetonitrile hypergrade for LC-MS (100029), LC-MS grade water (115333), and formic acid (111670) were purchased from Merck (Darmstadt, Germany). Ammonium formate (01209) was purchased from Loba Chemie (Mumbai, India). Certified drug-free urine was purchased from UTAK Laboratories (Valencia, CA, USA).

### 2.2 Dried urine spot

Standard sibutramine and its derivatives (sibutramine, desmethyl sibutramine and didesmethyl sibutramine) were prepared in methanol. Then, the standard mixture was spiked into certified drug-free urine to prepare working solutions at the final concentrations of 0.5, 1, 5, 10, and 20 ng/mL. To prepare a dried urine spot (DUS), a total of 30 µL of spiked urine was spotted within the circle dashed area of Whatman 903 Protein Saver Card (GE Healthcare, Cardiff, UK). The urine spots were allowed to dry at different conditions including air-drying at 25°C for 3 hours, drying by desiccator at 25°C for 3 hours under 50% relative humidity, and drying by hot-air oven (Universal oven UF110; Memmert, Schwabach, Germany) at 30°C, 40°C, and 50°C for 3 hours. Each condition was tested in triplicate. The condition that presented the highest percentage of recovery was chosen for further experiments.

### 2.3 Analyte extraction

Each DUS was cut along the dashed line with clean scissors and placed into a sterile 1.5 mL microcentrifuge tube. To extract sibutramine, desme-thyl sibutramine, and dides-methyl sibutramine, the cut DUS was soaked in a total of 300 µL methanol and sonicated under 35 kHz at 30°C for 5 minutes using an ultrasonic bath (Sonorex Digitec DT512H; Bandelin, Berlin, Germany). Finally, the supernatant was transferred into an HPLC vial for analysis using LC-MS/MS.

### 2.4 The instrumental condition of LC-MS/MS

LC-MS/MS analysis was performed using ultra-high-performance liquid chromate-graphy (Nexera X2, Shimadzu, Kyoto, Japan) coupled to tandem mass spectrometer. The liquid chromatography was equipped with LC-30AD binary pump, DGU-20A5R degasser, CTO-20AC column oven, SIL-30AC autosampler, and an FCV-20AH2 valve unit. The separation was performed on a C-18 reverse-phase Shim-pack XR-ODS II 150mm x 2.0 mm, 2.2 µm (Shimadzu, Kyoto, Japan) analytical column. The column temperature was fixed at 40°C. The mobile phase consisted of mobile phase A (10 mM ammonium formate with 0.1% v/v formic acid in water) and mobile phase B (10 mM ammonium formate with 0.1% v/v formic acid in methanol). The gradient program with proportion of mobile phase B was applied at specific time intervals. The mobile phase was maintained at 0.3 mL/min and injection volume was set to 10 µL. LCMS-8060 model triple quadrupole mass spectrometer (LCMS-8060, Shimadzu, Kyoto, Japan) equipped with electrospray ionization source (ESI) operating in positive ion mode was used as a detector. The optimum condition of

ESI source was determined under interface voltage -4,000 V, interface temperature 300°C, DL temperature 250°C, nebulizing gas flow 3 L/min; heating gas flow 10 L/min, and drying gas flow 10 L/min. The LC-MS/MS data were collected and analyzed with LabSolution software version 5.93 (Shimadzu, Kyoto, Japan). Multi-reaction monitoring (MRM) was used to quantify sibutramine and its derivatives.

### 2.5 Stability

To evaluate stability, DUSs with the indicated concentrations of sibutramine mixture were prepared in triplicate and kept in the dark at 25°C for 4 consecutive weeks. Sibutramine derivatives were extracted from the DUSs each week and injected into the LC-MS/MS for analysis. The percentage of recovery was compared to the freshly prepared DUS.

### 2.6 Statistical analysis

Statistical analysis was performed using unpaired Student's t-test by GraphPad Prism version 9.1.0 (GraphPad Software, San Diego, CA, USA). Data are presented as mean ± standard deviation (SD).

## 3. Results and Discussion

### 3.1 Optimal conditions for DUS preparation and extraction

The maximum volume of the spiked urine that could be spotted onto the Whatman 903 Protein Saver Card was 30 µL. Five different conditions for drying the spotted papers were studied, in triplicate. The supernatants of standard analytes extracted from the prepared DUSs using absolute methanol were then injected into the LC-MS/MS. The percentage of recovery was calculated with the acceptable range being between 80-120%. Of these, the drying methods that provided the acceptable percentage of recovery were by a desiccator under 50% relative humidity at 25°C, and by a hot-air oven at 30°C for 3 hours (Fig. 1a-c). Therefore, the desiccator protocol was chosen for further experiments involving DUS preparation in which DUS was prepared using 30 µL urine and allowed to be dried by a desiccator under 50% relative humidity at 25°C for 3 hours. This drying method was ease of use and portable when compared to the hot-air oven method. Moreover, using a desiccator is more compatible with use in rural areas. Additionally, to investigate the applicability of other cellulose paper grades, Whatman qualitative filter paper, Grade 1 (GE Healthcare, Buckinghamshire, UK) was substituted for the Whatman 903 Protein Saver Card. However, this test was terminated due to the robust increase in HPLC column pressure. Furthermore, acetonitrile, a non-protic solvent, was considered as an alternative substance for improving extraction capacity. The results revealed that acetonitrile contributed a hump peak on the chromatogram when compared with methanol, a protic solvent (Fig. 2a, b). Finally, Whatman 903 Protein Saver Card and methanol were used for DUS preparation and extraction, respectively, in this study.

### 3.2 LC-MS/MS analytical procedure

The mass to charge (m/z) ratio of the precursor ions of sibutramine, desmethyl sibutramine, and didesmethyl sibutramine were evaluated using LC-MS/MS. Each standard analyte was prepared in absolute methanol at a final concentration of 1,000 ng/mL and then injected into the LC-MS/MS without column. The results revealed that the m/z ratio of sibutramine, desmethyl sibutramine, and didesmethyl sibutramine were 280.10, 266.05, and 252.00, respectively (Table 1). In addition, the m/z ratio of fragment ions was explored using different collision energies, pre-quadrupole 1 (pre-Q1) and 3 (pre-Q3) voltage, and different dwell times. Variables that provided robust signals were chosen as the optimal multi-reaction monitoring (MRM) mass spectrometer parameters for further experiments. All of the most favorable

parameters used in this study are shown in Table 1. Furthermore, variables in the liquid chromatography testing, such as retention time and mobile phase ratio, were optimized to obtain individual peaks for sibutramine, desmethyl sibutramine, and didesmethyl sibutramine (Fig. 3). The optimal variables for the LC-MS/MS used in this study are shown in Table 2. Lastly, analytical characteristics including linearity ($R^2$) and limit of detection (LOD) were examined. The optimum conditions of the LC-MS/MS were applied to analyze the mixed standard solution of sibutramine, desmethyl sibutramine, and didesmethyl sibutramine dissolved in methanol and then subsequently prepared as DUS protocol at the final concentration of 0.5, 1, 5, 10, and 20 ng/mL. The results showed that the $R^2$ of sibutramine, desmethyl sibutramine, and didesmethyl sibutramine were 0.9993, 0.9980, and 0.9993 and the LODs were 0.02, 0.02, and 0.03 ng/mL, respectively. These analytical procedures were applicable for simultaneous quantitative detection of sibutramine, desmethyl sibutramine, and didesmethyl sibutramine in spiked urine.

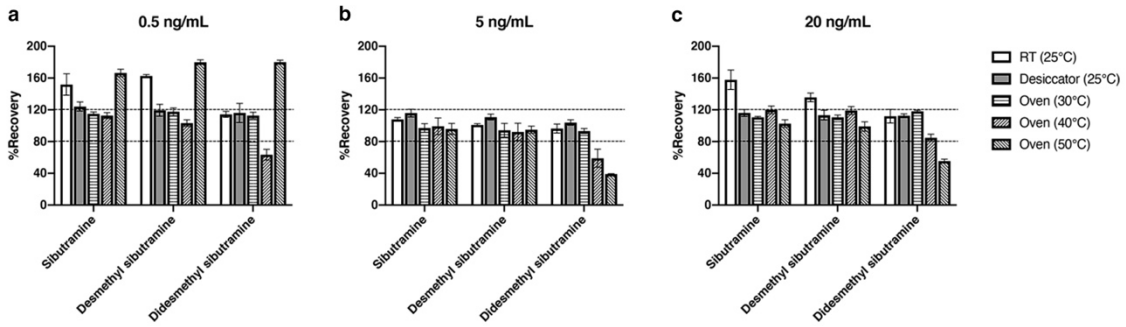


**Fig. 1.** Percentage of recovery (%recovery) after DUS preparation by different methods. DUS was prepared in triplicate with fabricated urine mixture including 0.5 ng/mL (a), 5 ng/mL (b) and 20 ng/mL (c) of sibutramine, desmethyl sibutramine and didesmethyl sibutramine. Horizontal dash lines indicate acceptable window of %recovery ranging from 80 to 120. Data are presented as mean ± SD. RT, room temperature.

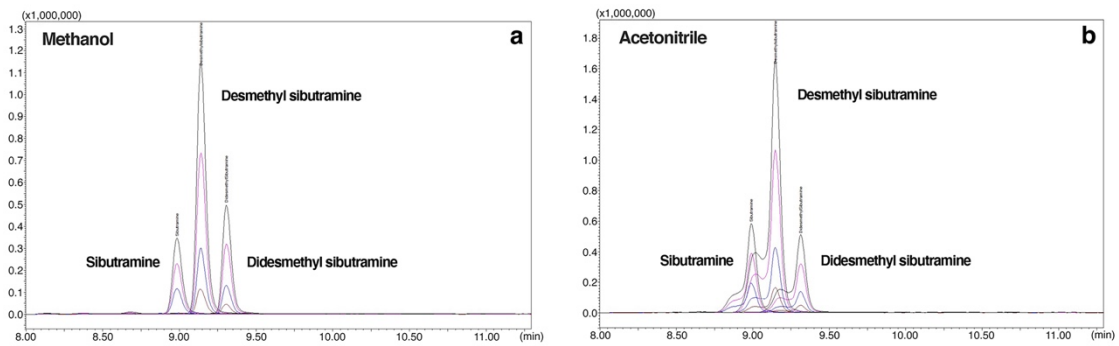


**Fig. 2.** LC-MS/MS chromatogram of sibutramine, desmethyl sibutramine, and didesmethyl sibutramine after absolute methanol (a) and acetonitrile (b) extraction.

### 3.3 Stability

Stability was evaluated with the analytes kept in the dark at room temperature for four consecutive weeks. Analytes were extracted from the prepared DUSs and quantitatively measured by LC-MS/MS each week. All analytes were qualitatively measured throughout the four consecutive weeks as well. The percentage of recovery was computed with the acceptable range being 80-120%. Shortly after (week 0) and one week after DUS preparation, the percentage recovery of all substances fell within the acceptable window (Figs. 4a-c). This suggested that all analytes were stable in DUS form and capable of being quantitatively measured by LC-MS/MS upon one week after the upkeep of the DUSs.

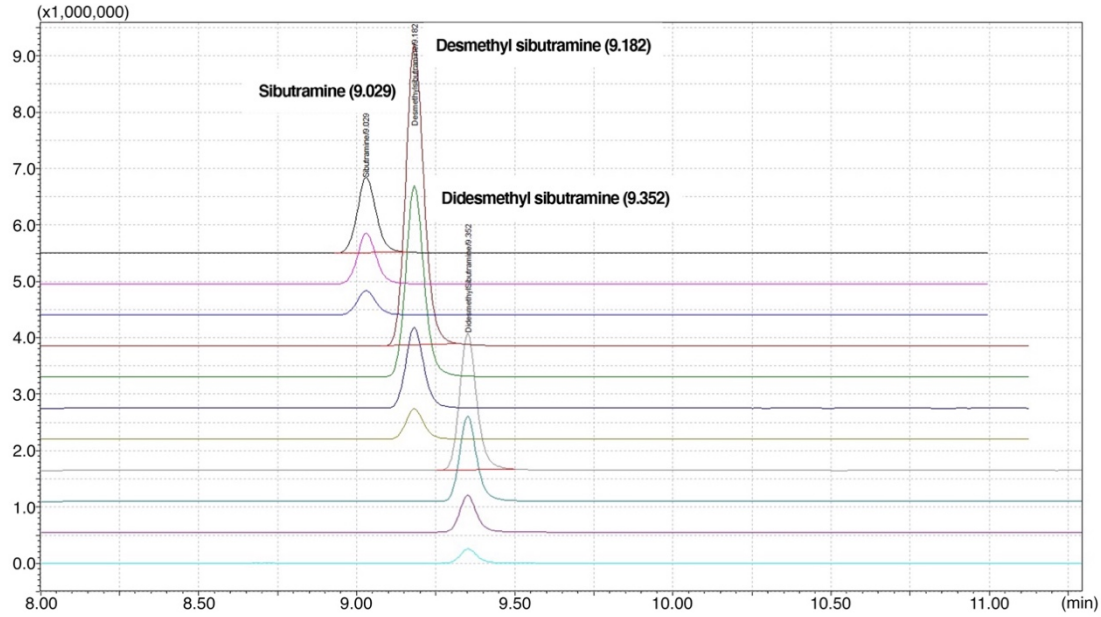


**Fig. 3.** LC-MS/MS chromatogram of sibutramine, desmethyl sibutramine, and didesmethyl sibutramine generated in this study.

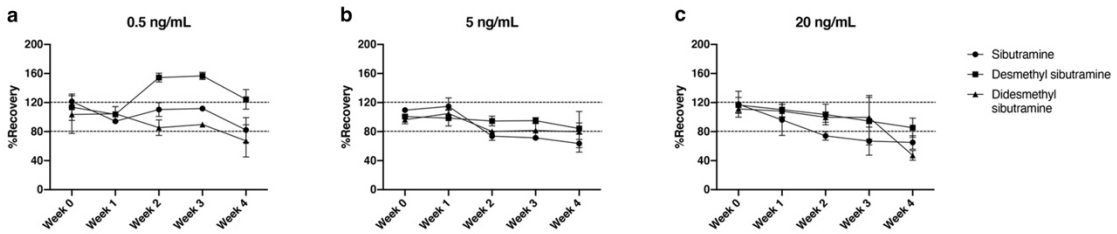


**Fig. 4.** Percentage of recovery (%recovery) during stability test. DUS was prepared in triplicate with fabricated urine mixture including 0.5 ng/mL (a), 5 ng/mL (b) and 20 ng/mL (c) of sibutramine, desmethyl sibutramine, and didesmethyl sibutramine and kept in the dark at room temperature for four consecutive weeks. Horizontal dash lines indicate acceptable window of %recovery ranging from 80 to 120. Data are presented as mean ± SD.

**Table 1.** Multiple reaction monitoring mass spectrometer (MRM-MS) parameters used in this study.

| Analyte | Precursor ion (m/z) | Pre-Q1 bias (V) | Collision energy (eV) | Pre-Q3 bias (V) | Fragment ion (m/z) | Dwell time (msec) |
|---|---|---|---|---|---|---|
| Sibutramine | 280.05 | -21.0 | -25 | -24.0 | 124.75 | 50 |
| | 280.05 | -21.0 | -15 | -15.0 | 138.85 | 50 |
| Desmethyl sibutramine | 266.05 | -29.0 | -23 | -23.0 | 138.90 | 25 |
| | 266.05 | -19.0 | -14 | -29.0 | 124.90 | 25 |
| | 266.05 | -20.0 | -13 | -30.0 | 153.00 | 25 |
| Didesmethyl sibutramine | 252.00 | -18.0 | -21 | -24.0 | 124.95 | 50 |
| | 252.00 | -17.0 | -11 | -29.0 | 139.25 | 50 |
| | 252.00 | -13.0 | -9 | -16.0 | 152.75 | 50 |

Interface was electrospray ionization (ESI). Interface temperature was 300°C. Desolvation line temperature was 250°C. Nebulizing gas flow was 3 L/min. Heating gas flow was 10 L/min. Drying gas flow was 10 L/min. m/z, mass to charge; Pre-Q1, pre-quadrupole 1; Pre-Q3, pre-quadrupole 3; V, volt; eV, electronvolt; msec, millisecond.

**Table 2.** Liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) parameters used in this study.

| | | | |
|---|---|---|---|
| Sample temperature | 5°C | | |
| Injection amount | 10 µL | | |
| Column | Shim-Pack XR-ODS II 2.0x150 mm, Particle 2.2 µm | | |
| Column temperature | 40°C | | |
| Mobile phase A | 10 mM ammonium formate and 0.1%v/v formic acid in water | | |
| Mobile phase B | 10 mM ammonium formate and 0.1%v/v formic acid in methanol | | |
| Flow rate | 0.3 mL/min | | |
| Gradient elution system | Time (min) | Mobile phase A (%) | Mobile phase B (%) |
| | 0.01 | 95 | 5 |
| | 1 | 95 | 5 |
| | 10 | 5 | 95 |
| | 13 | 5 | 95 |
| | 15 | 95 | 5 |
| | 20 | 95 | 5 |

### 3.4 Discussion

Sibutramine used to be approved for obesity treatment. Currently though, it is prohibited in many countries around the globe due to resulting severe cardiovascular adverse events. However, sibutramine has been illegally used as an adulterant in weight-loss products and many sudden unexpected deaths caused by sibutramine overdose toxicity continue to be documented globally [20, 21]. Detection of sibutramine in the bodies of those deceased is perhaps insufficient to estimate the cause of death. Instead, sibutramine metabolites are considered to be better indicators of sibutramine ingestion as they are only produced and excreted from the body after oral administration of sibutramine. To date, detection of sibutramine by various methods can signify contamination in suspected products [30-32]. Unfortunately, sibutramine levels are undetectable in biological samples of the users; instead, only inactive metabolites (M5 and M6) are predominantly detectable. To confirm and estimate the toxicity of sibutramine, its active metabolites including desmethyl sibutramine (M1) and didesmethyl sibutramine (M2) should be quantified. Many highly sensitive approaches such as liquid chromatography mass spectrometry (LC/MS) and gas chromatography mass spectrometry (GC/MS) have been developed to quantitatively identify small amounts of M1 and M2 in urine sample [23, 24]. Here, this study demonstrated the possibility of using dried urine spot (DUS) for quantifying sibutramine and its active metabolites by LC-MS/MS. The DUS was prepared by blotting a total volume of the 30 μL urine on Whatman 903 Protein Saver Card and then were allowed to completely dry by desiccator under 50% relative humidity at 25°C for 3 hours. Analytes were then extracted from the DUSs using 300 μL of absolute methanol under sonication. The aqueous part was subjected to LC-MS/MS analysis using well-developed conditions. Sibutramine, desmethyl sibutramine, and didesmethyl sibutramine were able to be quantified within acceptable percentage of recovery by this optimal protocol and were stable for up to one week when kept in the dark at room temperature. Beyond one week; however, the inaccuracy of percentage of recovery was observed throughout the rest of the three consecutive weeks. This may be due to chemical or physical alterations of the analytes, such as degradation or loss of the analytes during the process. To illustrate the possible causes, internal standards should be applied along with the experiments. Although the quantitative detection of sibutramine and its active metabolites was limited by one week, the analytes were able to be detected for up to four consecutive weeks. This suggests a desirable stability and qualitative detection capacity of the analytes in DUS. However, to extend the detection capacity of sibutramine and its active metabolites in DUS, putative reagents such as preservatives or stabilizers may need to be supplemented.

## 4. Conclusion

This study successfully produced protocols for the detection of sibutramine and its active metabolites in fabricated urine microsampling by LC-MS/MS and provided scientific proof that DUSs with various concentrations of sibutramine and its active metabolites are applicable for quantitative and qualitative investigation. Furthermore, application of this approach to some cases of unexplained death can also benefit the inquiry process of forensic autopsy and toxicology.

## Acknowledgements

The authors would like to thank Associate Professor Dr. Prapin Wilairat from National Doping Control Centre, Mahidol University, Bangkok, Thailand for providing sibutramine hydrochloride.

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