Simultaneous Determination of Sibutramine and *N*-Di-desmethylsibutramine in Dietary Supplements for Weight Control by HPLC–ESI-MS

Ziqiang Huang[1,2,*], Song Xiao[2], Dan Luo[2], Bo Chen[2,†], and **Shouzhuo Yao[1,2,†]**

[1]State Key Laboratory of Chemo/Biological Sensing & Chemometrics, Hunan University, Changsha 410082, PR China; [2]Key Laboratory of Chemical Biology & Traditional Chinese Medicine Research, Ministry of Education, Hunan Normal University, Changsha 410081, PR China

Abstract

A high-performance liquid chromatographic method, coupled with UV detection and electrospray ionization mass spectrometry (HPLC–UV-ESI-MS), is developed for the simultaneous determination of the illegal additives sibutramine and its metabolite *N*-di-desmethylsibutramine in dietary supplements for weight control. The separation is achieved on a Spherisorb C8 reversed-phase column, employing acetonitrile and an aqueous 0.2% formic acid solution containing 20mM ammonium acetate as mobile phases in a gradient mode. UV detection is used for quantitation at a wavelength of 223 nm. Identification of target compounds is completed by ESI-MS using selected ion recording at *m/z* 280 for sibutramine and *m/z* 252 for *N*-di-desmethylsibutramine. Calibration curves are linear over the range of 0.025–1.0 mg/mL for sibutramine and *N*-di-desmethylsibutramine. Correlation coefficients are better than 0.9990. The intra- and inter-day precision and accuracy for sibutramine and *N*-di-desmethylsibutramine are acceptable. The method is successfully applied to the analysis of natural dietary supplement samples.

Introduction

Sibutramine, *N*-[1-(4-chloro phenyl cyclobutyl)-3-methyl-butyl]-*N*-*N*-dimethyl amine, is a tertiary amine (1). The U.S. Food and Drug Administration (FDA) approved sibutramine for the treatment of obesity (2). Now, it is a recommended drug to help those who are obese to lose weight. After being administered to animals and humans, sibutramine is rapidly metabolized to *N*-mono-desmethylsibutramine and *N*-di-desmethylsibutramine. The in vivo effects of sibutramine are mainly due to the actions of these two metabolites (2,3). But in excessive amounts, sibutramine might cause a series of side effects such as palpitation, chest pain, insomnia, diabetes, anorexia, and abnormal liver (4–6).

A dietary supplement (or functional food) is a product taken by mouth that contains a "dietary ingredient" intended to supplement the diet. The "dietary ingredients" in these products may include: vitamins, minerals, herbs or other botanicals, amino acids, and substances such as enzymes, organ tissues, glandulars, and metabolites. The dietary supplement manufacturer is responsible for ensuring that a dietary supplement is safe before it is marketed. The FDA is responsible for taking action against any unsafe dietary supplement product after it reaches the market. Dietary supplements for weight control should be safe without causing any danger to health. However, illegal dealers add some drugs such as sibutramine or its analogs such as *N*-di-desmethylsibutramine into the dietary supplements to enhance the weight-reducing effect of the products. These illegal products may endanger people's health. Among illegal additives, the drug analogs such as *N*-di-desmethylsibutramine are more covert because these types of components are always out of the monitoring range. Therefore, the drug analogs are more frequently added to the products by illegal dealers. To ensure the quality of dietary supplements for weight control and to protect people's health, it is important to develop a method to determine these illegal additives.

For the analysis of sibutramine and *N*-di-desmethylsibutramine, a number of methods such as high-performance liquid chromatography (HPLC) and liquid chromatography electrospray ionization mass spectrometry (LC–ESI-MS) (2,7–9) have been developed. Among these methods, most of them have been used to determine sibutramine and its metabolites in plasma samples. Recently, some analytical methods for the determination of sibutramine and its metabolites in herbal products have been reported (10,11). For the identification of sibutramine, a urine sample and a Chinese herbal capsule sample were analyzed by gas chromatography–MS and HPLC–diode array detection.

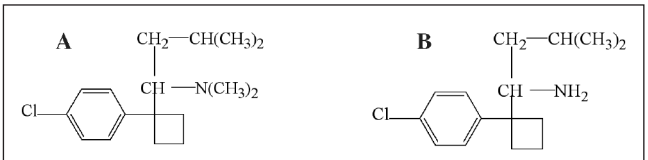

Figure 1. Chemical structures of sibutramine (A) and *N*-di-desmethylsibutramine (B).

* Current location: Hunan Entry-Exit Inspection and Quarantine Bureau.
† Authors to whom correspondence should be addressed: Bo Chen, email dr-chenpo@vip.sina.com; Shouzhou Yao, email szyao001@sina.com.

However, the authors only analyzed sibutramine (11). In 2007, Zou et al. presented an analytical method of LC coupled with triple quadrupole MS and time-of-flight MS. This method successfully identified sibutramine, its two metabolites, and one analogue in an herbal product for weight loss. However, there was no quantitative analytical result (10).

In the present work, an accurate and fast method for the simultaneous determination of sibutramine and *N*-di-desmethylsibutramine in dietary supplement products is proposed. The method showed some merits such as specificity, sensitivity, and simplicity in sample preparation.

Experimental

Materials and chemicals

The HPLC system used was Waters (Milford, MA) alliance 2695 module, which was interfaced to a Waters 2487 dual absorbance detector. The detection wavelength for the quantitation of sibutramine and *N*-di-desmethylsibutramine was set at 223 nm. The MS used was a Micromass ZQ 2000 (Manchester, UK) equipped with an ESI probe and a quadrupole analyzer. The control of system and data acquisition was performed with a Masslynx3.5 workstation (Waters).

Sibutramine hydrochloride and *N*-di-desmethylsibutramine were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The purities for the standards were higher than 99% (HPLC). The chemical structures of these compounds are shown in Figure 1.

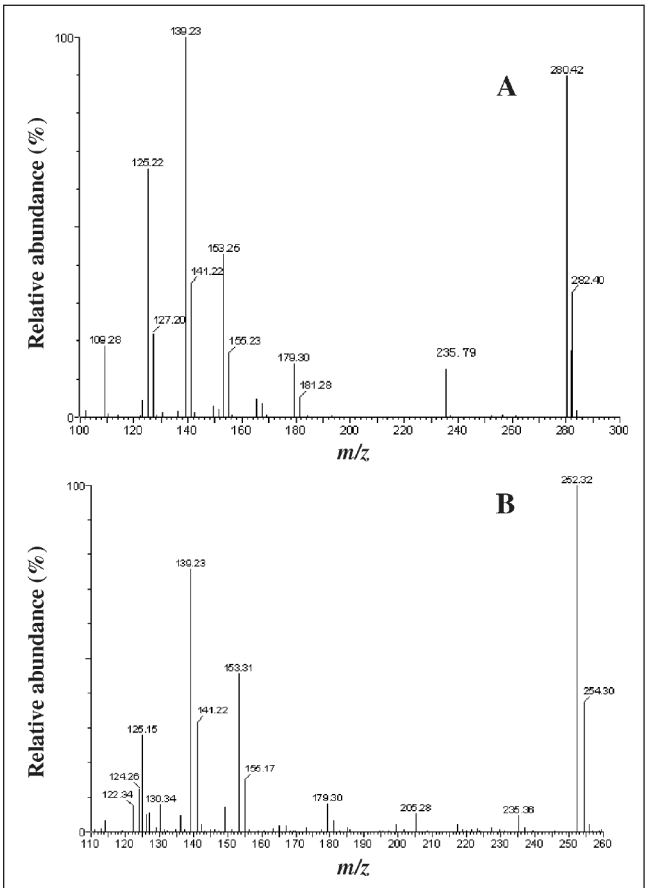

Figure 2. ESI mass spectrum of sibutramine (A); ESI mass spectrum of *N*-di-desmethylsibutamine (B).

Acetonitrile and methanol were of HPLC-grade from Tedia Company (Fairfield, OH). Formic acid (analytical-grade reagent) was purchased from Shanpu Chemical Corporation (Shanghai, China). Ammonium acetate (analytical-grade) was obtained from Shanghai Chemical Company (Shanghai, China). Ultrapure water was prepared by a Millipore Milli-Q purification system (Millipore Corp., Bedford, MA). The samples were purchased from supermarkets (Changsha, China). All of these products are natural dietary supplements for weight control. Drugs are forbidden to be added in these products according to Chinese law. The samples included two oral liquid formulations, eight capsules, and five tealeaf preparations (their herbal constituents are listed in Table I).

Preparation of standard solutions

Stock solutions of sibutramine and *N*-di-desmethylsibutramine were prepared in methanol, respectively. The concentration of each compound was 10.0 mg/mL.

Ten milliliters of these stock solutions were transferred to a volumetric flask to produce the mixed standard solution. This solution was then diluted using methanol to yield a series of working standard solutions for validation and calibration. The final concentrations of the compounds were: 0.025, 0.05, 0.10, 0.25, 0.50, and 1.0 mg/mL, respectively.

Preparation of sample solutions

The solid sample (0.5 g) was crushed and extracted with methanol in an ultrasonic bath for 10 min. After filtering, the sample solution was diluted to 10 mL with methanol. Five microliters of the sample solution were injected into the HPLC system.

Table I. Main Herbal Constituents Contained in Samples

Sample	Plant sources
Oral liquid preparation 1	*Nelumbo nucifera* leaves, Whitethorn, Ginkgo leaves
Oral liquid preparation 2	Whitethorn, Cassia Seeds, Chrysanthemum
Capsule preparation 1	Green tea, Seaweed, Cellulose
Capsule preparation 2	*Nelumbo nucifera* leaves, Tea polyphenols
Capsule preparation 3	Whitethorn, *Nelumbo nucifera*, Cellulose
Capsule preparation 4	Ginkgo leaves, Cellulose
Capsule preparation 5	*Cannabis sativa* L. seeds, Chrysanthemum
Capsule preparation 6	Whitethorn, *Nelumbo nucifera* leaves, Cellulose
Capsule preparation 7	Alisma orientalis (Sam.) Juzep., Whitethorn
Capsule preparation 8	Tea polyphenols, Seaweed
Tealeaf preparation 1	Green tea, *Nelumbo nucifera* leaves, Ginseng
Tealeaf preparation 2	Green tea, *Nelumbo nucifera* leaves, Cassia Seed
Tealeaf preparation 3	Green tea, *Nelumbo nucifera* leaves,
Tealeaf preparation 4	Green tea, Whitethorn
Tealeaf preparation 5	Green tea, Whitethorn, *Nelumbo nucifera* leaves

The liquid sample was filtered through a 0.45-µm nylon membrane, and 5 µL of the filtrate were injected into the HPLC system.

HPLC–MS analysis

A column packed with 5 µm C8 bonded to silica (4.6 mm × 250 mm) (Johnson, Dalian, China) was used for the separation. The mobile phase consisted of acetonitrile (A) and an aqueous solution with 0.2% formic acid and 20mM ammonium acetate (B). The gradient elution was programmed as follows: A was maintained at 4% within the first 3 min, linearly increased to 40% during the following 7 min, then maintained at 40% for another 10 min. The flow rate was set at 1 mL/min and the column temperature was maintained at 30ºC.

ESI was operated in the positive mode to generate $[M+H]^+$ ions at *m/z* 280 for sibutramine and *m/z* 252 for *N*-di-desmethylsibutramine. Nitrogen was used as the desolvation gas at a flow rate of 250 L/h and cone gas, the flow rate was set to 50 L/h. The desolvation temperature was maintained at 250ºC. The source temperature was 110ºC. Capillary and cone voltages were 3.5 kV and 30 V, respectively. The ionization source was set to 105ºC. The extractor voltage was set at 4 V, and 0.5 V for RF lens. The outflow of the UV detector was split with only 0.2 mL/min portion of the column eluent delivered to the MS.

Linearity, limit of detection, limit of quantitation

The mixed standard solutions (the working solutions) at each concentration level were injected in triplicate. Calibration curves were constructed by plotting the average peak areas of the standard compounds against the corresponding concentrations. The limit of detection (LOD) for UV detection was evaluated as the concentration giving a signal equal to three times of noise (S/N = 3); the limit of quantitation (LOQ) was determined as the mass giving a signal equal to ten times of noise (S/N = 10).

Results and Discussion

HPLC–ESI-MS

To improve the peak shape and MS detection sensitivity for target compounds, different mobile phase modifiers such as acetic acid, formic acid, triethylamine, and ammonium acetate were investigated. The results show that triethylamine dramatically impairs the MS sensitivities of sibutramine and *N*-di-desmethylsibutramine. After optimizing the HPLC conditions, ammonium acetate and formic acid were adopted as the mobile phase modifiers. In order to improve the separation efficiency, a gradient mode was applied, and good separation of the analytes was obtained in 9 min.

The mass spectra of sibutramine and *N*-di-desmethylsibutramine are shown in Figure 2. Different cone voltages including 20, 30, 40, 50, 60, 70, 80, and 90 V were investigated, respectively. The results showed that the most intense protonated molecular could be obtained by using a cone voltage of 30 V. The protonated molecules of sibutramine and *N*-di-desmethylsibutramine ($[M+H]^+$) were at *m/z* 280 and 252, respectively. After increasing the cone voltage, collision-induced dissociation (CID) was generated. The fragment ions of the protonated molecules were observed. Protonated molecules of sibutramine and *N*-di-desmethylsibutramine lose $HN(CH_3)_2$ and NH_3, respectively, to give a common fragment ion at *m/z* 235 which has a side chain of $–CH=CH-CH-(CH_3)_2$. This fragment ion can be further fragmented to product-ions at *m/z* 179 and 153. By monitoring the protonated molecules and CID fragments, a highly sensitive and specific identification of the drugs can be accomplished. The typical in-source CID tracks of the protonated molecules are shown in Figure 3. It indicates that sibutramine and *N*-di-desmethylsibutramine have same mother structure.

Table II. Linearity, LOD, and LOQ (*n* = 3)

Component	Regression equation*,‡	r^2*	Linear range (mg/mL)*	LOD (ng)	LOQ (ng)
Sibutramine	$y = 19939x + 10$	0.9991	0.025–1.0	15*, 0.010†	50*
N-Di-desmethylsibutramine	$y = 82383x + 133$	0.9995	0.025–1.0	6.25*, 0.005†	20*

* Shows the result with detection at 223 nm.
† Shows the result with the selected ion recording mode.
‡ *Y* means peak area (µV/s), *X* means concentration (mg/mL).

Table III. Precision and Recoveries (*n* = 3)*

	Intra-assay			Inter-assay		
Actual conc. (mg/mL)	Measured conc. (mg/mL)	Recovery (%)	RSD (%)	Measured conc. (mg/mL)	Recovery (%)	RSD (%)
Sibutramine						
0.050	0.049 + 0.006	97.4	11.3	0.048 + 0.007	95.0	15.2
0.100	0.104 + 0.009	103.5	8.31	0.106 + 0.008	105.7	9.93
0.200	0.209 + 0.015	104.3	7.33	0.214 + 0.018	106.7	8.38
0.500	0.486 + 0.037	97.1	7.58	0.479 + 0.040	95.8	8.29
0.750	0.724 + 0.050	96.5	6.86	0.789 + 0.066	105.2	8.31
***N*-Di-desmethylsibutramine**						
0.050	0.047 + 0.006	94.6	13.1	0.047 + 0.008	93.6	16.7
0.100	0.105 + 0.010	104.9	9.37	0.093 + 0.010	93.3	10.4
0.200	0.189 + 0.017	94.3	9.17	0.207 + 0.021	103.7	9.37
0.500	0.478 + 0.041	95.7	8.47	0.484 + 0.041	96.7	8.37
0.750	0.735 + 0.055	97.9	7.47	0.722 + 0.057	97.2	7.87

* The results were obtained from UV detection at 223 nm.

Method validation

Linearity of the two analytes was obtained over a concentration range from 0.025–1.0 mg/mL for sibutramine and *N*-di-desmethyl-

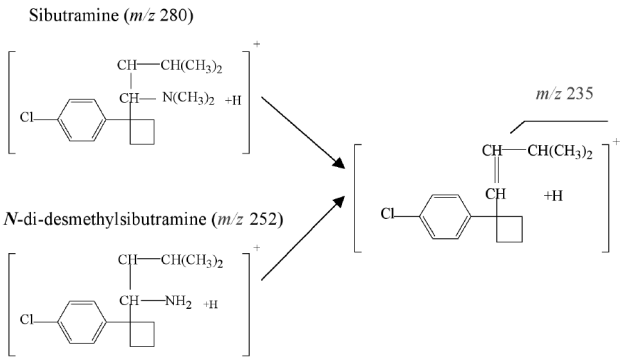

Figure 3. In-source CID tracks of the protonated molecules.

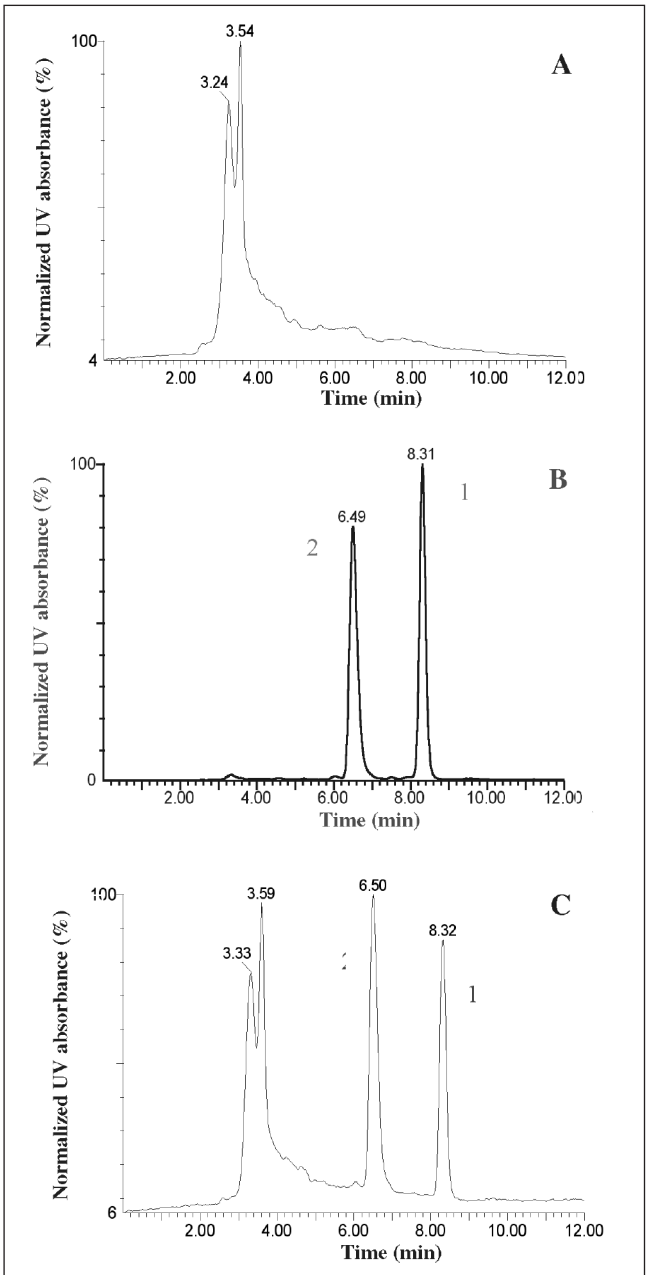

Figure 4. Chromatogram of a blank sample (A); chromatogram of sibutramine (peak 1) and *N*-di-desmethylsibutramine (peak 2) standards (B); chromatogram of the sample spiked with sibutramine (peak 1) and *N*-di-desmethylsibutramine (peak 2) standards (C).

sibutramine, respectively. Results are shown in Table II. Because there is phenyl moiety in the target compounds, the higher molar absorptivity of the phenyl structure results in a lower limit of UV detection. Hence the UV detection method has sufficient sensitivity and can be used for conventional analysis of these compounds even without mass spectrometric detection. However, the LOD for MS of these compounds was found to be lower. The proposed HPLC–ESI-MS method is advantageous in trace analysis of these compounds and can partly provide structural information for the fast screening for the illegal additives in the products even if no standards are available.

The accuracy of the method was evaluated by calculating the mean recovery of the target compounds after adding standards to a blank sample (a capsule preparation) at low, medium, and high levels. Each sample with the same concentration was analyzed 3 times. The results are summarized in Table III. From this table, it can be seen that the mean recovery for two compounds was 93.3%–106.7%. These results for precision and accuracy met the acceptable criteria (12).

Application

Most of the samples examined in the present work were complicated preparations, including nelumbo nucifera leaves, whitethorn, ginkgo leaves, cassia seeds, chrysanthemum, green tea, seaweed, etc. The compositions of all these herbs are rather complicated. However, under the previously given conditions, no interference from these herbs was observed. Figure 4 shows the chromatograms of a capsule preparation before (Figure 4A) and after (Figure 4C) adding sibutramine and *N*-di-desmethylsibutramine standards. It can be seen that no interfering components co-eluted with the two drugs. Among the 15 examined samples, two samples (oral liquid preparation 1 and tealeaf preparation 1) were found to contain sibutramine; the concentrations were 0.51 mg/mL and 2.17 mg/g, respectively. Two samples (capsule preparation 5 and capsule preparation 8) were found to contain *N*-di-desmethylsibutramine; the concentrations were 1.73 mg/g and 0.23 mg/g, respectively. A typical chromatogram of a sample (tealeaf preparation 1) is shown in Figure 5.

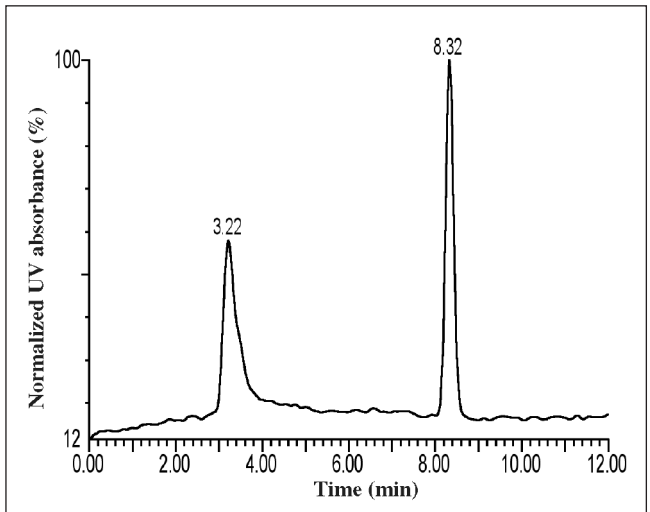

Figure 5. Chromatogram of tealeaf preparation 1.

Conclusion

With the improvement in production technology of sibutramine and its analogous compounds, these compounds are becoming more available and their prices are on the decline, hence even more of these compounds are being illegally added to dietary supplements. The method presented in this paper is useful for the simultaneous monitoring of sibutramine and *N*-didesmethylsibutramine. It can be employed to ensure people's safety by inspecting dietary supplements which may contain these illegal substances. The developed method has the advantage of simplicity, rapidity, and accuracy.

Acknowledgment

This work was financially supported by the National Foundation of Key Technologies for Food Safety, China (2003AA2Z3515, 2001BA804A21, 03JJY1002, 2001BA804A39).

References

1. M. LeBlanc and L. Thibault. Effect of sibutramine on macronutrient selection in male and female rats. *Physiol. Behav.* **80:** 243–252 (2003).
2. S.D. Glick, R.E. Haskew, I.M. Maisonneuve, J.N. Carlson, and T.P. Jerussi. Enantioselective behavioral effects of sibutramine metabolites. *Eur. J. Pharmacol.* **397:** 93–102 (2000).
3. I.P. Connoley, Y.L. Liu, I. Frost, I.P. Reckless, D.J. Heal, and M.J. Stock. Thermogenic effects of sibutramine and its metabolites. *Brit. J. Pharmacol.* **126:** 1487 (1999).
4. C.A. Luque and J.A. Rey. The discovery and status of sibutramine as an anti-obesity drug. *Eur. J. Pharmacol.* **440:** 119–128 (2002).
5. I.D. Hind, J.E. Mangham, S.P. Ghani, R.E. Haddock, C.J. Garratt, and R.W. Jones. Sibutramine pharmacokinetics in young and elderly healthy subjects. *Eur. J. Clin. Pharmacol.* **54:** 847–9 (1999).
6. K.M. Walsh, E. Leen, and M.E.J. Lean. The effect of sibutramine on resting energy expenditure and adrenaline-induced thermogenesis in obese females. *Int. J. Obes.* **23:** 1009–1015 (1999).
7. T. Radhakrishna, C.L. Narayana, D.S. Rao, K. Vyas, and G.O. Reddy. LC method for the determination of assay and purity of sibutramine hydrochloride and its enantiomers by chiral chromatography. *J. Pharm. Biomed. Anal.* **22:** 627–639 (2000).
8. J. Chen, W. Lu, Q. Zhang, and X. Jiang. Determination of the active metabolite of sibutramine by liquid chromatography–electrospray ionization tandem mass spectrometry. *J. Chromatogr. B* **785:** 197–203 (2003).
9. L. Ding, X. Hao, X. Huang, and S. Zhang. Simultaneous determination of sibutramine and its *N*-desmethyl metabolites in human plasma by liquid chromatography–electrospray ionization-mass spectrometry method and clinical applications. *Anal. Chim. Acta* **492:** 241–248 (2003).
10. P. Zou, S.S. Oh, K.H. Kiang, M.Y. Low, and B.C. Bloodworth. Detection of sibutramine, its two metabolites and one analogue in an herbal product for weight loss by liquid chromatography triple quadrupole mass spectrometry and time-of-flight mass spectrometry. *Rapid Commun. Mass Spectrom.* **21:** 614–618 (2007).
11. J. Jung, M. Hermanns-Clausen, and W. Weinmann. Anorectic sibutramine detected in a Chinese herbal drug for weight loss. *Forensic Sci. Int.* **161:** 221–222 (2006).
12. US. Department of Health and Human Services, Food and Drug Administration. *Guidance for Industry: Bioanalytical Method Validation, 1st ed.* Food and Drug Administration, 2001, pp. 4–6.

Manuscript received March 4, 2007;
revision received November 18, 2007.