

Determination of the Sibutramine Content of Dietary Supplements Using LC-ESI-MS/MS

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SUMMARY. Obesity is recognized as a public health problem throughout the world. Dietary supplements have been used as alternative treatments for obesity, and many of these supplements allegedly contain “natural products”. This paper describes the development of an LC-MS/MS method for the analysis of sibutramine, which is an adulterant found in dietary supplements. The samples were prepared using a simple extraction process, and the analytical run time was approximately 10 minutes. A reverse-phase column was used to resolve the contents of the supplements, and gradient elution using acetonitrile and water (1:1, V/V) acidified with 0.1% formic acid was performed at a flow rate of 0.4 mL/min. The identification of the relevant compounds was performed using multiple reaction monitoring (MRM) ratios in positive ionization mode. The method described in this work was validated, and our analysis indicates that this method is accurate, precise, easily performed and sensitive. The limit of quantification was 4.0 ng/mL, and the limit of detection was 1.3 ng/mL. The linear range for the data was 5.0-30.0 ng/mL, and in this range, the correlation coefficient (r) calculated by the least squares method was 0.99848. The proposed method can be used for routine analysis.

INTRODUCTION

Obesity is recognized as a public health problem that affects populations worldwide ¹. It is a chronic disease associated with many comorbidities, and its major consequences include conditions such as diabetes mellitus, systemic hypertension, dyslipidemia, cardiovascular disease, some types of cancer, sleep apnea and osteoarthritis ². Therefore, many options have been proposed for the treatment of obesity, including dietary modifications (a balanced healthy diet), increased physical activity and pharmacological or surgical treatment ³.

There has been a continuous search for alternative treatments for obesity based on phytotherapeutic formulations and food supplements (which may be formulated for athletes or may be simple dietary supplements) ^{4,5}. There are many safety and legal issues surrounding the use of such supplements. For example, false or misleading claims, the inclusion of unspeci-

fied synthetic drugs ⁶⁻⁹ and toxicity have been associated with food supplements (vitamins and minerals) and herbal formulations. These issues have become increasingly common and may result in serious adverse effects ¹⁰. Carvalho *et al.* ⁴ reviewed several important cases and described the adulteration of phytotherapeutic formulations for weight loss by synthetic pharmaceuticals. These cases occurred in several European countries, as well as in Asia, Brazil and the USA. Sibutramine (Sb) is an anorectic that has been prescribed frequently by physicians to facilitate weight loss. However, the use of this compound has become controversial due to the risk of exacerbating cardiovascular disease in obese patients already diagnosed with cardiovascular disease or type 2 diabetes ¹¹. In the literature, there are many reports concerning the detection of Sb, as well as its metabolites and analogs, in herbal supplements marketed for weight loss ¹²⁻¹⁷.

KEY WORDS: Adulterant, Dietary supplement, LC-MS/MS, Sibutramine.

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In Brazil, legislation controlling the use of therapeutic drugs changed in 2010¹⁸, resulting in stricter regulation of the prescription of Sb. Sb is a racemic mixture of a tertiary amine, specifically a cyclic alkylamine¹⁹. Although this compound was originally developed as an antidepressant¹⁹, it has been used worldwide as an anti-obesity agent for nearly 15 years²⁰. Sb is a combined serotonin-norepinephrine reuptake inhibitor that acts on the central nervous system. The main effects of Sb include the intensification of satiety and a subsequent decrease in food intake^{21,22}. The long-term use of Sb has been associated with cardiovascular risks because Sb also has peripheral sympathomimetic effects¹⁹.

In Brazil, there are several laws that address the protection of health as a basic human right. The Federal Constitution says that health is a right of all, and therefore, the State is responsible for protecting public health through disease prevention, surveillance and consumer protection. Each manufacturer is primarily responsible for the quality of its products, but the State is not exempt from the responsibility of evaluating the quality of products subject to health surveillance, ensuring that drugs and foods comply with the quality and safety requirements and preventing the distribution and sale of counterfeit or adulterated products²³.

The Adolfo Lutz Institute is an Official Laboratory for Public Health of the Department of Health of the State of São Paulo-Brazil, which participates in the Programs of Monitoring the Quality of Drugs/Foods to promote the improvement of the market. As part of this participation, the Institute receives samples from the Health Surveillance Agency for analysis. Recently, the Institute received samples of dietary supplements in capsule form, including two black soy supplements and five orange peel supplements, all of which were from the same manufacturer. These samples were suspected to contain unauthorized Sb and were obtained from food markets and drugstores located in São Paulo. The present study describes the development of an LC-MS/MS method for the detection of Sb as an adulterant in dietary supplements.

MATERIAL AND METHODS

Sibutramine hydrochloride (100.9% purity) was supplied by Abbot Laboratory S.A. (Brazil). LC-grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). Analytical reagent-grade formic acid was obtained

from Fluka Analytical (Steinheim, Germany). High purity water was prepared using a Millipore Milli-Q water purification system.

Instrumentation

A mass spectrometer (MS) was used for the identification of Sb in samples. The MS analysis was performed on a model 3200 MS/MS (Applied Biosystems, Concord, Ontario, Canada). An electrospray ionization (ESI) source was used in positive mode for this study. An Agilent 1200 series LC system, which consisted of a G4208A system controller, a 1311A quaternary pump, a G1322A degasser and an HIP-ALSG 1367B auto sampler, was used. Separation was performed on an Agilent Eclipse XDB-C18 column (150 x 4.6 mm, 5 µm) at room temperature using a mobile phase composed of acetonitrile and water (1:1, V/V) acidified with 0.1% formic acid. The flow rate was 0.4 mL/min. The gradient used normal phase conditions. An injection volume of 5 µL was used. All prepared solutions were filtered through a 0.22 µm Millipore® (Millex HV) hydrophilic membrane before injection into the chromatographic system. Multiple reaction monitoring (MRM) spectra obtained in positive ion mode were used to detect the specified analyte, and three diagnostic transitions were monitored simultaneously. The optimized ion source parameters were as follows: curtain gas (CUR), 18 psi; ion spray voltage (IS), 4500 V; ion source gas 1 (GS 1), 50 psi; ion source gas 2 (GS 2), 45 psi; and temperature (TEM), 750 °C. The CAD (collision-induced dissociation) gas was fixed at 8 psi. Calculations of the peak areas and peak area ratios were performed using Analyst® 1.5.1 (AB Sciex®) software.

Preparation of the standard solutions

A stock solution of Sb (0.16 mg/mL) was prepared in methanol. A calibration curve was constructed using data obtained for six different concentrations (5-30 ng/mL) of Sb stock solution. The linearity was evaluated by the least squares method in triplicate for each concentration level. Working solutions were prepared from the stock solutions.

Preparation of the sample solutions

Samples A, B, C, D and E were prepared from orange fiber capsules, and samples F and G were prepared from ground black soybean capsules. According to the literature²⁴, no analytical characterization of unknown or unlabeled constituents of these supplements has been per-

formed by LC-MS/MS. A review of studies on vegetable-based supplements indicated that there are no naturally occurring compounds with chemical structures similar to that of Sb in these samples. In addition, certified mixtures of herbal materials and synthetic adulterants are not available commercially ¹⁰.

Initially, a 100 mg sample of the capsule contents, which was prepared from twenty capsules, was transferred to a 50 mL conical flask containing 25 mL of methanol. Next, the flask was vortexed vigorously for 10 min and then sonicated for 10 min. The flask was left standing for 30 min, and then, solution was decanted and subsequently diluted in methanol to reach a final concentration of 800 ppb.

Precision

The intra- and interday precisions were determined by analyzing solutions of A, B and C and the standard. For this evaluation, five samples of the standard solution and ten samples of A, B and C were prepared on a single day and analyzed over the next 3 days ²⁵.

Recovery

The percent recovery was evaluated by adding a known amount of Sb standard to a sample solution. This parameter was evaluated at three concentration levels, 2.5 ng/mL, 5.0 ng/mL and 10.0 ng/mL. Analyses were performed in triplicate at each concentration level ²⁶.

Detection limit (DL) and quantitation limit (QL)

The DL and QL were determined based on the standard deviation of the response and the slope of the regression equation for the calibration curve ²⁷.

RESULTS AND DISCUSSION

Sb (Fig. 1) was initially injected at known concentrations for MRM optimization, and the corresponding collision energies were stored in the method file. The retention time, the precursor and product ions and the corresponding collision energies for the detection of the three transitions for Sb were monitored using this LC-MS/MS method and are shown in Table 1.

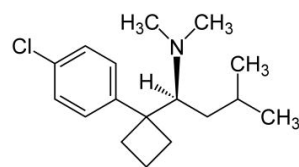


Figure 1. Structure of sibutramine hydrochloride.

A reverse-phase LC-ESI-MS/MS method was developed to assess the presence and quantity of Sb using MRM ratios. MRM was employed to monitor the LC effluent using three known transitions for Sb, thus increasing the reliability of this method. The chromatographic conditions were adjusted to allow efficient analysis. The selection of the mobile phase was based on the time needed for resolution and the ease of preparation. An Eclipse XDB column was used at a low pH and provided excellent resolution of the peaks during LC-MS/MS analysis. Fig. 2 shows the chromatograms obtained for a methanol blank, a standard solution of Sb and a sample solution. Each injection of a standard or sample solution was followed by the injection of a blank (methanol) to determine whether carryover had occurred. The observed retention time (4.2 min) allowed the rapid determination of the Sb concentration.

The calibration curve for Sb was constructed by plotting the sample concentration versus the mean peak area. Good linearity was observed in the concentration range of 5-30 ng/mL. The least squares regression yielded a correlation coefficient of $r = 0.99848$, which indicated a good fit. The limit of detection studies demonstrated that concentrations of Sb in the range of nanograms per milliliter could be detected, indicating that this method has good sensitivity for the detection of Sb in herbal supplements (Table 2).

The intra- and interday precisions were determined and are reported as the relative standard deviation (RSD) for a series of measurements, as shown in Table 3.

The results shown in Table 3 indicate that this method is sufficiently precise for routine analysis. According to Jenke ²⁸, precision reflects the reproducibility of a procedure rather than the procedure's ability to obtain the correct val-

Compound	Rt*	MS/MS transition (m/z)	CE (V)
Sb	4.6 min	208→125, 139, 153	33, 23, 21

Table 1. MS parameters for the detection of Sb using LC-ESI-MS/MS. *Rt = retention time.

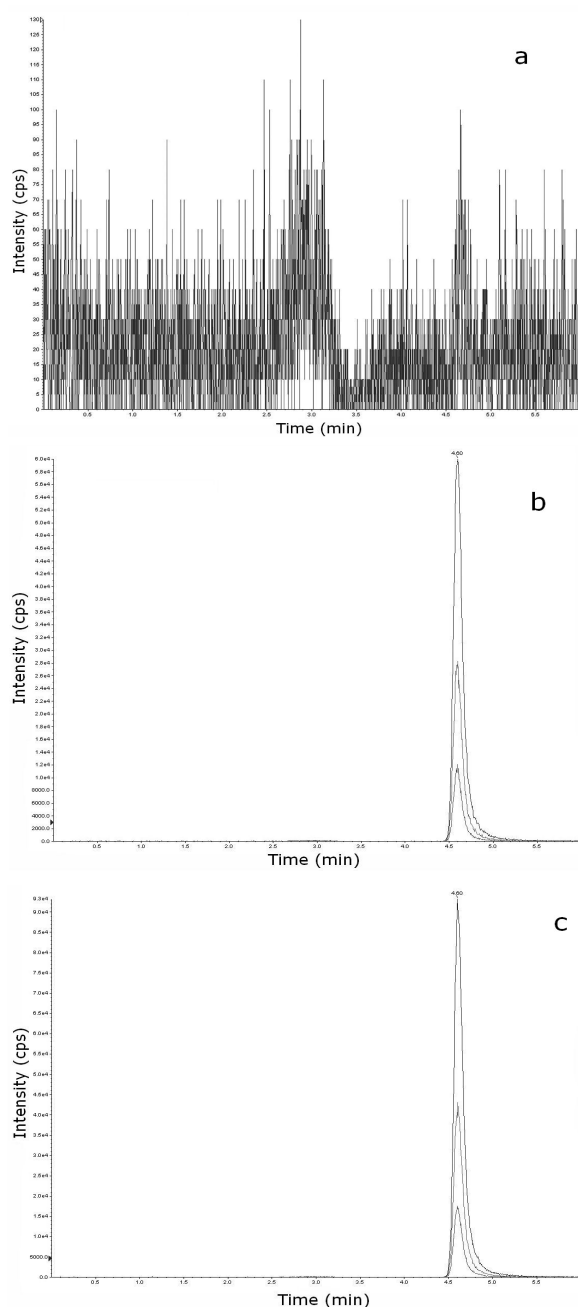


Figure 2. LC-ESI-MS/MS chromatograms of a methanol blank (a), a standard solution of Sb (b) and a sample solution (c).

Statistical parameters	Sb
Concentration range (ng/mL)	5-30
Number of points	6
Regression equation	$Y = 21631x + 73181$
Correlation coefficient (r)	0.99848
*DL (ng/mL)	1.3
**QL (ng/mL)	4.0

Table 2. Overview of the linearity of the Sb data. *DL = Detection limit; **QL = Quantitation limit.

ues. On the first day of our experiment, 3.75 mg/caps of Sb was detected for sample A, but on the other two days, the amount was 4.23 mg/caps. This small difference in the results for the three days was acceptable because the same conditions were used for analysis. The literature-accepted minimum for the RSD of biological or discovery samples is 10%²⁸. The percent recovery (%R) was expressed as the percentage of the standard recovered from the sample matrix. All concentrations used in the analysis were within the limits of the analytical curve because samples A, B and C all contained Sb. The percent recovery in the recovery studies was always higher than 97%, which indicated that this method is accurate. The results are shown in Table 4.

In six out of seven samples of the analyzed supplements, Sb was successfully identified using the proposed method. The results of the quantification of Sb (mg/capsule) in the samples were as follows: sample A = 4.04 mg/capsule, B = 4.17 mg/capsule, C = 3.81 mg/capsule, D = 4.81 mg/capsule, E = 0.000184 mg/capsule, F = not detected and G = 0.0165 mg/capsule. When analyzed in positive ionization mode, samples A-D showed identical MRM transitions to those in the Sb standard solution. The amounts of Sb were low in samples D and G and were thus likely the result of contamination during the manufacturing process. Sb was not detected in sample F. The observed limits of detection (Table 2) showed that the level of sensitivity was more than sufficient for the detection of adulterants¹⁰.

The adulterated products claimed to be an "Oriental Natural Blend- Balance and healthy for the body" and allegedly only contained natural ingredients, such as black soy and orange fibers; Sb was not mentioned on the labels. The adulteration and contamination of supplements with Sb is a critical issue in the worldwide market of herbal medicine and dietary supplements^{4,7,10,29-31}. Because there is no age restriction for the use of dietary supplements, the potentially harmful unspecified compound(s) present in these products could be administered to children. Anti-obesity pharmacological agents are not recommended for children because of a dearth of evidence regarding their effects on this age group². These findings indicate that the composition of vegetal supplements must be monitored by regulatory authorities, especially when products claim to be natural and therefore safe. The pur-

Parameter	LC-ESI-MS/MS		
	Sample A	Sample B	Sample C
*Intraday reproducibility			
Day 1, mg/caps	3.75 ± 0.02	4.10 ± 0.13	3.69 ± 0.08
RSD, %	0.67	4.41	3.01
Day 2, mg/caps	4.25 ± 0.05	4.20 ± 0.03	3.87 ± 0.03
RSD, %	1.73	1.06	0.94
Day 3, mg/caps	4.22 ± 0.07	4.20 ± 0.03	3.98 ± 0.02
RSD, %	2.44	1.07	0.71
**Interday reproducibility			
Days 1-3, mg/caps	4.07 ± 0.09	4.17 ± 0.04	3.81 ± 0.04
RSD, %	6.04	2.83	2.99

Table 3. Precision of the results and statistical data obtained for the analysis of Sb in vegetal supplement samples. *n = 10; calculated after statistical treatment by analysis of variance. ** n = 30; the calculation was based on the arithmetic mean. Caps = capsule.

Sample	LC-ESI-MS/MS		
	Sb, ng/mL		
	Added	Found	Recovery, %
A	2.50	2.64	105.7%
	5.00	5.10	102.7%
	10.00	10.46	104.6%
B	2.50	2.52	100.8%
	5.00	5.21	104.1%
	10.00	10.58	105.8%
C	2.50	2.61	104.5%
	5.00	4.90	98.0%
	10.00	9.72	97.2%

Table 4. Recovery of Sb added to sample solutions.

pose of the Health Surveillance Agency is to prevent the sale or supply of products that may pose risks to the consumer. Good manufacturing practices (GMPs) are intended to standardize processes and procedures to ensure the quality of the final product. Despite laws and regulation, some suppliers could adulterate products, resulting in poisonings and health risks. If suppliers are found to be adulterating their products, they must pay a fine, and they may also have their operating license suspended or even revoked.

The method described herein for the detection and quantitation of Sb as an adulterant in dietary supplements is simple, fast and sensitive.

CONCLUSIONS

This LC-MS/MS method can be used to screen for and confirm the presence of contaminants and adulterants. An LC-MS/MS system with an ESI ion source and a triple quadrupole mass analyzer was used to identify and quantify Sb in dietary supplements. This method features a simple sample preparation process, a short run time (6 min) and confirmation using MRM ratios. The validation of this method suggests that it could be used to detect Sb contamination and adulteration during quality control surveys of dietary supplements.

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