Simultaneous determination of sibutramine and its derivatives in weight loss dietary supplements by LC-MS/MS

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(Received: 27/3/2020; Accepted: 19/6/2020)

Abstract

The liquid chromatography tandem mass spectrometry method (LC-MS/MS) was used to determine the content of sibutramine (SB), N-desmethyl sibutramine (DSB) and N-didesmethyl sibutramine (DDSB) illegally mixed in weight loss dietary supplements. Sibutramine and its derivatives were extracted by methanol; impurities in the extract were removed by graphitized carbon black (GCB) adsorbent. The chromatographic separation of analytes took place on C18 column (100 mm x 2.1 mm, 3.5 µm) with a gradient mobile phase of acetonitrile and 2 mM ammonium acetate in 0.1% formic acid solution. Multiple reaction monitoring (MRM) in the positive mode was used to detect and quantify SB, DSB and DDSB at m/z 279.9/124.8; 266.0/124.8 and 252.1/125.0, respectively. The method was validated following the AOAC requirements for specificity, repeatability and recovery. Calibration curves lineared from 0.002 to 0.1 µg/mL for SB, DSB and DDSB. The method was successfully applied to determine the content of SB, DSB and DDSB in weight loss dietary supplements that were randomly collected from phamacies in Hanoi of three formulations of hard capsule, soft capsule and teabag. The results shown that six samples had SB and DSB with the content in the range of 0.817 - 31.7 mg/g.

Keywords*: sibutramine, N-desmethyl sibutramine, N-didesmethyl sibutramine, weight lossdietary supplement, LC-MS/MS.*

1. INTRODUCTION

Sibutramine (SB) acts by inhibiting the re-uptake of neurotransmitter serotonin, norepinephrine and dopamine and enhancing satiety. Consequently, it prevents compulsive eating and inhibits the sensation of hunger in obese patients [1-4]. After being administered to animals and humans, sibutramine is rapidly metabolized to N-mono-desmethyl sibutramine (DSB) and N-didesmethyl sibutramine (DDSB) [5-6]. The in vivo effects of sibutramine are mainly due to the actions of these two metabolites [4, 7, 8]. Undersirable effects of SB are excessive stimulation of the central nervous system, some side effects such as restlessness, dry mouth, headache, paralysis, paresthesias [9]. Furthermore, it is linked to cardiovascular risk such as increased blood pressure, heart rate and stroke risk [10].

Sibutramine is one of the most popular anesthetic drugs found in herbal based weight loss foods. However, SB has been banned from circulating on the European market since January 21, 2010 due to side effects [11]. In Viet Nam, SB containing products have been withdrawn

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from circulation registration numbers since April 2011. Dietary supplements for weight control should be safe without causing any danger to health. However, illegal dealers add some drugs such as sibutramine's analogs including N-desmethyl sibutramine and N-didesmethyl sibutramine into the dietary supplements to enhance the weight-reducing effect of the products. The presence of SB and its derivatives in dietary supplements for weight control caused serious health problems for consumers [5].

Recently, there have been a number of analytical methods to determine the illegally mixed drugs in herbal supplements such as infrared spectrometry (IR), raman spectroscopy, high performance liquid chromatography (HPLC) - diode array detection (DAD) [12], etc. Sabina Strano and his colleagues developed the method to detect the metabolites of sibutramine in urine by GC-MS [13], all SB' metabolites were clearly detectable in a range of concentrations between 10 and 50 ng/mL, satisfying the minimum required performance limits of the world Anti - Doping Agency [14]. Sibutramine and its two metabolites and one analogue in an herbal product for weight loss were detected by liquid chromatography triple quadrupole mass spectrometry and time of flight mass spectrometry by Zou P and his colleagues [15]. It is important for health authorities and health professionals to detect and identify synthetic drugs and their analogues in slimming products [15]. Venkata developed a method to quantify sibutramine and its two metabolites in human plasma by LC-ESI-MS/MS, the method was applied in a bioequivalence and pharmacokinetic study [4].

In Vietnam, the HPLC method to determine the content of sibutramine in dietary supplement for weight control was validated [16]. However, there has not been any studies on the simultaneous determination of the content of SB and its derivatives yet. Therefore, the goal of this research was to develop a method to simultaneously determine the content of SB and its derivatives. LC-MS/MS was chosen as an analytical method, the method after being validated will be applied to control the SB, DSB and DDSB illegally mixed in weight loss dietary supplement.

2. MATERIALS AND METHODS

2.1. Materials

The common formulation of weight loss dietary supplements are hard capsules, soft capsules and teabags, which were randomly collected from pharmacies in Hanoi.

2.2. Standards and Reagents

Sibutramine hydrochloride, N-desmethyl sibutramine and N-didesmethyl sibutramine were purchased from Sigma Aldrich (purities ≥ 99%). HPLC grade methanol (MeOH), acetonitrile (ACN), ethanol (EtOH), acetone, formic acid, hydroxide ammonium (NH_4OH) were obtained from Merck (Germany). Ammonium formate, Ammonium acetate of analytical reagent grade were obtained from Merck (Germany). Solid-phase extraction (SPE) strong cation exchange (SCX) cartridge was purchased from Waters. Graphitized Carbon Black (GCB) adsorbent from Agilent, US was used for cleaning impurities in this study.

2.3. Instrumentation

Shimadzu's LC20AD high performance liquid chromatography system coupled with the 5500 QQQ (triplequad mass spectrometer of AB-SCIEX, USA) was used in this method. SB, DSB and DDSB were separated on the C18 Xbridge column (100 mm x 2.1 mm, 3.5µm, Waters). The gradient elution contains Acetonitril (A) and 0.1% formic acid in 2 mM ammonium acetate

(B). The injection volume was 10 µL. The sample was introduced into ESI source at a flow rate of 0.4 mL/min to enable the targeted qualitative determination of SB, DSB and DDSB.

2.4. Methods

2.4.1. Mass spectrometry optimization

Using flow injection analysis (FIA) to directly inject the 10 ng/mL mixed standard solution of sibutramine, N-desmethyl sibutramine and N-didesmethyl sibutramine into mass spectrometry without liquid chromatographic separation. Electrospray ionization (ESI) technology in positive mode was selected to investigate the precursor and product ion. Based on the signal of analyte on the chromatogram to select the optimal parameters for MS and MS/MS analysis.

2.4.2. Sample prepration

2.4.2.1. Investigation of extraction solvent

Four extraction solvents including (1) MeOH, (2) ACN, (3) EtOH and (4) acetone were investigated for the extraction of SB, DSB and DDSB from spiked samples. After adding the extraction solvent, the sample was sonicated for 20 minutes, followed by centrifugation at 4.000 rpm for 10 minutes. Sample was then diluted with appropriate ratio before being injected into LC-MS/MS.

2.4.2.2. Investigation of cleanup step

It is always necessary to remove impurities in the matrix of the samples to reduce interferents and protect the LC-MS/MS system. The samples were cleaned by 3 procedures as in Table 1. The most effective cleaning technique was chosen based on the recovery of spiked samples.

***Table 1**. Procedures of sample cleaning*

Procedure 1	***Procedure 2***	***Procedure 3***
Filter immediately through 0.25 µm filter membrane	- Filter extraction solution. - Activate the column: 6 mL MeOH, 6 mL H_2O. - Transfer samples on column: 4 mL extraction. - Clean the impurities: 3 mL H_2O, 3 mL MeOH. - Elution: 4 mL MeOH: NH_4OH (95/5, v/v).	- Filter extraction solution. - Pipette 15 mL of extraction into centrifuge tube containing 25mg GCB. - Shake about 30 - 60 second - Centrifuge the tubes at 2000 rpm, 5 minutes. Transfer 1 mL of the supernatant into vial. - Inject sample into LC-MS/MS

2.4.2.3. Mobile phase composition preparation

Based on a number of references [5, 10, 17] and the actual conditions of the laboratory, 05 mobile phase systems were selected of gradient separation, including:

- System 1: channel A: ACN, channel B: 2 mM ammonium acetate;
- System 2: channel A: MeOH, channel B: 2 mM ammonium acetate;
- System 3: channel A: ACN, channel B: 2 mM ammonium formate;
- System 4: channel A: ACN, channel B: 2 mM ammonium formate + 0.1% formic acid;
- System 5: channel A: ACN, channel B: 2 mM ammonium acetate + 0.1% formic acid/H_2O.

2.4.3. Method validation

2.4.3.1. Specificity

The specificity of the method was evaluated by comparing the chromatograms of the analytes in the blank with standard and spiked samples. The specificity of the method was further guaranted by a confirmation method based on the IP (identification point) and ion ratio according to the regulations of the European Council. For LC-MS/MS, the method is specific when the IP score was 4.

2.4.3.2. Linearity and calibration curves

Prepared a mixed standard solution of sibutramine, N-desmethyl sibutramine and N-didesmethyl sibutramine, diluted with appropriate ratio to obtain 06 concentrations: 2.0 ng/mL, 5.0 ng/mL, 10 ng/mL, 20 ng/mL, 50 ng/mL and 100 ng/mL, respectively. The calibration curve was constructed based on the relation between the concentration and the peak area of the corresponding standards. The calibration curve is linear when the correlation coefficient R^2 was higher than 0.99.

2.4.3.3. Limit of detection (LOD) and limit of quantification (LOQ)

LODs are the lowest SB, DSB, DDSB concentration in a sample that can be detected from the background noise but cannot be quantitated. LOD was determined when the signal - to - noise ratio (S/N) of 3/1 was reached. LOQ is defined as the lowest concentration of an analyte that can be determined with acceptable precision and accuracy. LOQ was determined as the concentration that provides the signal - to - noise ratio (S/N) of 10/1 [4].

2.4.3.4. Recovery and repeatability

The method repeatability and recovery were evaluated by measuring the spiked samples of hard capsule, soft capsule and teabag formulations at three concentration of 10 µg/kg, 20 µg/kg and 50 µg/kg with six replicates per concentration.

2.4.4. Application to analysis of dietary supplement for weight loss

The validated method was applied for analysis of real samples that were taken from Hanoi markets. Accurately weigh approx 1.0 gram of the sample into a falcon tube (50 mL capacity) and added 20 mL of suiltabe solvent, mix the contents thoroughly, ultrasound about 20 minutes, transfered the supernatant into a volumetric flask, 2 times repeatly. Adjusted the volume to 50 mL, removed the impurities, and injected the sample on LC-MS/MS equipment.

3. RESULTS AND DISCUSSION

3.1. Selection of mass spectrometry conditions

Mass spectrometry analysis was performed in positive mode (ESI). The optimization in source parameters were as follows: curtain gas (CUR), 25 psi; ion spray voltage (IS), 5.500 V; ion source gas 1 (GS 1), 20 psi; ion source gas 2 (GS 2), 20 psi; and temperature (TEM), 400°C. The CAD (Collision-induced dissociation) gas was fixed at 9 psi. For optimal MS parameters, the highest intensity ion was used as quantitative ion and lower intensity ion were used as confirmation ion. The MS/MS conditional results for SB, DSB and DDSB analysis were presented in Table 2.

***Table 2**. MS/MS conditions for qualitative and quantitative analysis of SB, DSB, DDSB*

Analyte	*Molecular weight*	*Retention time (min)*	*Precursor ion (m/z)*	*Product ion (m/z)*	*Collision energy (V)*	*Collision Cell Exit Potential (V)*
SB	278.9	6.37	279.9	124.8	22	26
				138.4	16	26
DSB	265.0	6.33	266.0	124.8	24	28
				138.8	12	28
DDSB	251.1	6.27	252.1	208.1	47	11
				268.2	47	11

3.2. Investigation of extraction solvent

Sibutramine, N-desmethyl sibutramine and N-didesmethyl sibutramine were spiked to 03 matrices including hard capsules, soft capsules and teabags. After being extracted with different solvents, the recoveries of SB, DSB and DDSB were shown in Table 3.

***Table 3**. Recoveries of SB, DSB and DDSB using different extraction solvents*

Solvents	*Hard capsule*			*Soft capsule*			*Teabag*		
	SB	*DSB*	*DDSB*	*SB*	*DSB*	*DDSB*	*SB*	*DSB*	*DDSB*
MeOH	110.6	102.1	105.2	99.3	98.7	109.1	98.8	104.1	97.2
ACN	97.6	97.8	89.2	93.4	92.1	85.3	95.1	94.4	83.5
EtOH	84.2	87.8	85.1	95.2	85.3	83.2	82.1	84.5	79.2
Acetone	78.3	81.9	75.4	81.3	71.6	75.4	81.5	78.7	89.8

Comparing the content of analytes obtained from the use of different extraction solvents in different matrices (Table 3), methanol (MeOH) is the extraction solvent that provides the highest extraction efficiency. Therefore, MeOH was the solvent of choice in the study.

3.3. Selection of cleanup steps

The recovery efficiencies of blank samples spiked with standards: sibutramine, N-desmethyl sibutramine and N-didesmethyl sibutramine after being cleaned by different cleaning techniques are presented in Table 4.

***Table 4.** Recovery efficiencies of SB, DSB, DDSB using different procedures*

Procedure	*Recovery efficiency (%)*		
	Sibutramine	*N-desmethyl sibutramine*	*N-didesmethyl sibutramine*
Procedure 1	90.7	93.7	85.0
Procedure 2	93.9	98.7	91.5
Procedure 3	94.8	107.0	100.0

Food supplements are usually prepared from herbs, so the post-extracted solution is often chromatic. The extract was passed through filter paper and a solid-phase extraction column, but the color of the solution still remains Using GCB decreased the color of extract solution which

is the reason for the higher recovery efficiency compared with using solid phase extraction column and the membrane filtration method. This is explained that activated carbon is capable of adsorbing pigments and commonly used on the laboratory scale to purify solutions containing unwanted colored organic impurities.

3.4. Optimization of the amount of graphitized carbon black (GCB)

Graphitized carbon black has ability to adsorb color of solution, but using too much or too little of activated carbon also leads to a change in the recovery efficiency of the analyte. The results on the influence of activated carbon amount on the recovery efficiency were shown in Figure 1.

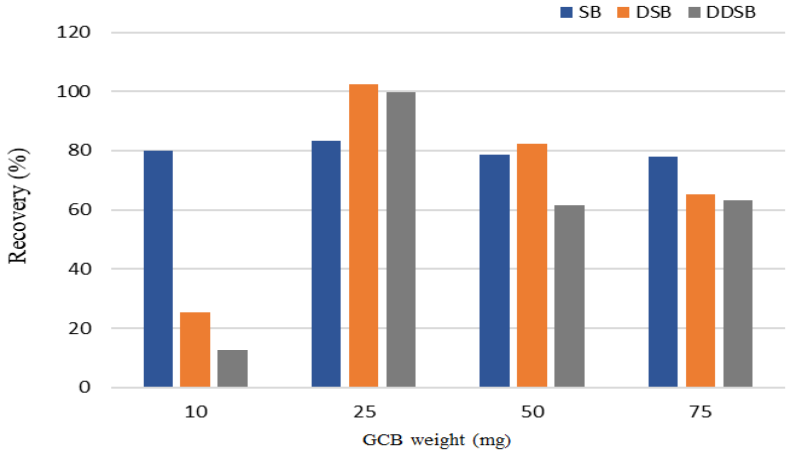

Figure 1. *Effects of the amount of GCB on recovery of SB, DSB, DDSB (teabag matrix)*

As can be seen in figure 1, using 25 mg of GCB for 10 mL of sample solution gave the highest recovery efficiency of the analytes in teabag sample. 10 mg of GCB was not enough to adsorb all impurities, so the recovery efficiency was lowest, when the amount of GCB increased, the recovery efficiency decreased. GCB was coal with an extremely large surface area per unit volume and it is a network of pores under the membrane where absorption took place. With 50 and 75 mg of GCB, the recoveries of SB, DSB and DDSB were lower, these are explained possible because GCB may adsorb both impurities and analytes.

3.5. Mobile phase composition

Mobile phase affected the ability to separate analytes in the mixture, different mobile phases give differen signals for analytes, mobile phase programs of 5 systems were similar. The dependence of peak area of sibutramine, N-desmethyl sibutramine and N-didesmethyl sibutramine on different mobile phases is presented in Table 5. System 1 to system 5 was detailed in 2.4.1.3.

Table 5. *Influence of mobile phase components*

Mobile phase system	***Sibutramine***		***N-desmethyl sibutramine***		***N-didesmethyl sibutramine***	
	Peak area	***RT (*)***	***Peak area***	***RT (*)***	***Peak area***	***RT (*)***
System 1	5.17×10^5	6.50	6.94×10^5	6.39	4.21×10^5	6.35
System 2	5.01×10^5	8.55	6.54×10^5	6.65	3.89×10^5	6.67
System 3	8.25×10^5	6.45	9.01×10^5	6.40	4.72×10^5	6.34
System 4	6.29×10^5	7.11	6.78×10^5	7.16	3.11×10^5	7.18
System 5	9.09×10^5	6.29	1.25×10^5	6.27	9.61×10^5	6.23

RT (*): Retention time

Mobile phase system 5 is the gradient of channel A: ACN, channel B: 2 mM ammonium acetate + 0.1% formic acid/H_2O provided the highest peak area of sibutramine, N-desmethyl sibutramine and N-didesmethyl sibutramine. Using this mobile phase with gradient program in Table 6, we obtained the chromatogram of SB, DSB and DDSB as presented in Fig. 2.

***Table 6**. Gradient program*

Time (min)	%A	%B
0.01	100	0
3	90	10
5	5	95
7	5	95
8	90	10
10	90	10

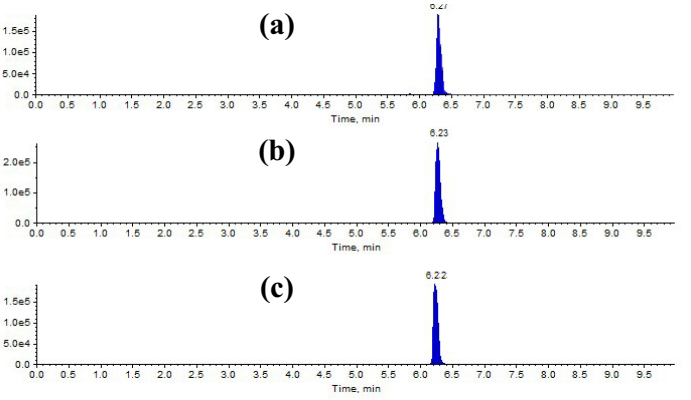

***Figure 2**. Chromatogram of SB (a), DSB (b), DDSB (c) in optimal mobile phase and gradient condition*

3.6. Method validation

3.6.1. Specificity

Analysis of blank samples, standard samples and blank samples were spiked with standards at 5 ng/mL to all 3 matrices including hard capsules, teabags and soft capsules. An example of chromatograms of SB are shown in Figure 3.

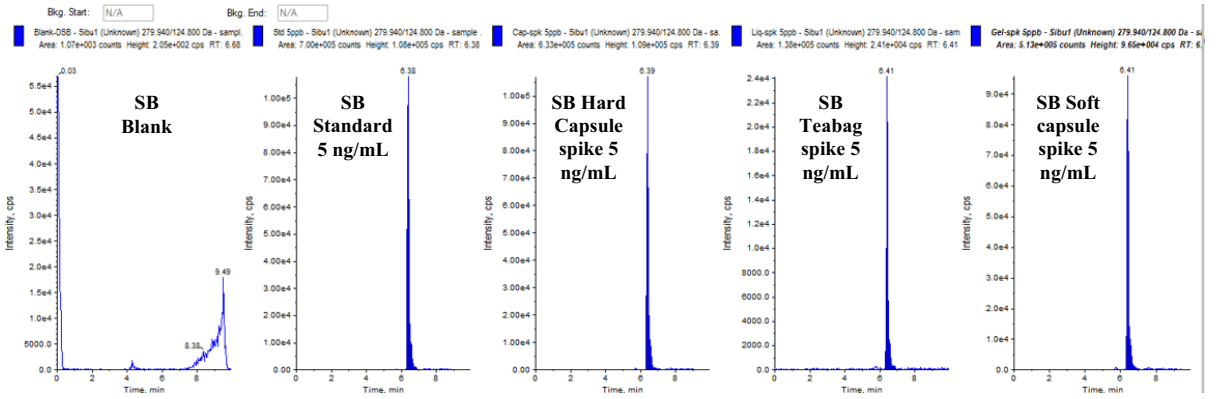

***Figure 3**. Chromatograms of blank sample, standard solution and spiked blank samples for SB*

On the chromatograms, the blank samples did not show the signal of the SB, while the blank spiked with standards have peaks of sibutramine with retention time similar to that of corresponding standard. This confirmed that the method was specific.

Table 2 showed that each SB, DSB and DDSB precusor ions were bombarded into 2 daughter ions, so the total number of identification points of the method corresponding to each substance was 4 conformable with the regulation 2002/657/EC of the European Council, which confirmed the LC-MS/MS method in this study was specific.

3.6.2. Linearity and calibration curves

The standard solution of SB, DSB and DDSB from 2 ng/mL to 100 ng/mL was analyzed to determine the linearity. Calibration equations, correlation coefficients and bias of SB, DSB

and DDSB in this range were shown in Table *7*.

***Table** 7. Calibration equations and correlation coefficients of SB, DSB and DDSB*

Analytes	*Calibration equations*	*Correlation coefficients (R^2)*	*Bias (%)*
Sibutramine	$y = 5.08 \times 10^4x - 5.35 \times 10^3$	0.9982	4.52 - 12.1
N-desmethyl sibutramine	$y = 6.41 \times 10^4x - 7.18 \times 10^4$	0.9996	3.81 - 10.7
N-didesmethyl sibutramine	$y = 3.69 \times 10^4x - 6.12 \times 10^4$	0.9984	2.52 - 9.83

As can be seen from Table 7, correlation coefficients of three calibrations were higher than 0.990, bias was smaller than 15%, which met the criteria of AOAC requirements and proved the high linearity between the peak area and concentration of analyte.

Figure 4 is an example of SB calibration curve.

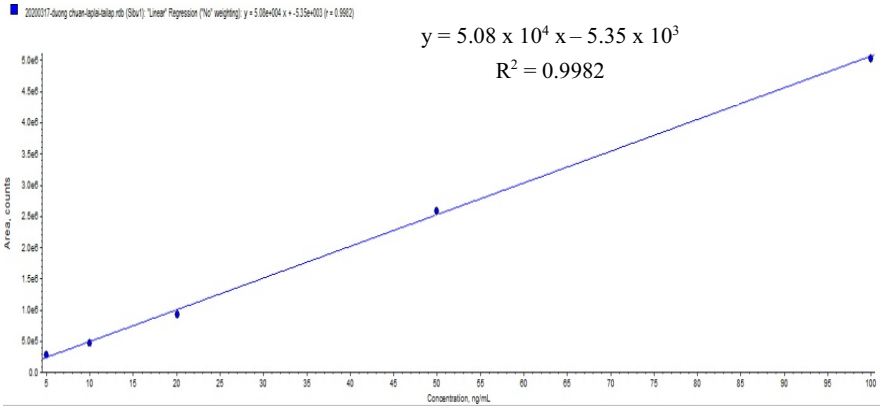

***Figure 4**. Calibration curve of SB*

3.6.3. Limit of detection (LOD) and limit of quatification (LOQ)

After optimizing analytical conditions for determination of SB and its derivative on LC-MS/MS equipment, the limit of detection (LOD) and limit of quantification (LOQ) of SB, DSB and DDSB were determined. Results were presented in Table 8.

***Table 8**. Limit of detection (LOD) and limit of quantification (LOQ) of the analytes*

Analytes	*LOD (µg/kg)*	*LOQ (µg/kg)*
Sibutramine	0.05	0.15
N-desmethyl sibutramine	0.10	0.30
N-didesmethyl sibutramine	0.10	0.30

The method's LOQs of SB, DSB and DDSB at 0.15, 0.3, 0.3 µg/kg, respectively confirmed that it has sensitivity enough to analyze the dietary supplement for food control samples on the market.

3.6.4. Recovery and repeatability

The recovery and repeatability of the method were assessed by analyzing spiked samples at three different concentration levels of 10, 20, 50 µg/kg with six replicates at each concentration. The results were shown in Table 9.

Table 9. *Recovery (R %) and repeatability (RSD %) of SB, DSB and DDSB at three concentration in different matrices*

Dosage form	*Analytes*	*10 µg/kg, n = 6*		*20 µg/kg, n = 6*		*50 µg/kg, n = 6*	
		R (%)	*RSD (%)*	*R (%)*	*RSD (%)*	*R (%)*	*RSD (%)*
Hard capsule	SB	90.6	8.90	99.0	6.42	100.1	6.32
	DSB	92.7	7.30	107.3	5.25	101.6	4.98
	DDSB	100.1	4.52	84.9	5.01	94.7	3.75
Soft capsule	SB	100.4	6.32	100.5	5.85	105.7	5.01
	DSB	100.5	10.5	100.5	7.85	107.0	7.01
	DDSB	102.6	8.34	104.1	7.56	105.0	4.85
Teabag	SB	100.5	5.34	109.5	8.72	100.5	6.09
	DSB	109.0	4.78	87.7	7.34	93.6	4.01
	DDSB	101.8	6.03	108.3	5.85	102.3	4.79

The recoveries of SB, DSB and DDSB of three dosage forms at three different concentrations were found in the range of 90.6 - 109.5%, 92.7 - 109.0% and 84.9 - 108.3%, respectively. Standard deviation (RSD %) were found in the range of 5.01 - 8.90%, 4.01 - 10.5 %, 3.75 - 8.34%, respectively. The recoveries and RSD meet the requirements of AOAC (recovery of 80 - 100%, relative standard deviation ≤ 15% at 10 µg/kg concentration).

3.7. Application for analysis of real samples

The validated method was used successfully to quantify SB, DSB and DDSB in 30 weight loss dietary supplements samples taken from some pharmacies in Hanoi. 04 samples of hard capsule and 02 samples of soft capsule were detected to contain sibutramine with contents between 0.817 - 31.4 mg/g, 02 samples of hard capsule, 02 samples of soft capsules contain N-desmethyl sibutramine with contents in the range of 0.27 - 3.10 mg/g. There was no sample detected with N-didesmethyl sibutramine.

4. CONCLUSIONS

In this study, an analytical method using LC-MS/MS for simultaneous determination of SB, DSB and DDSB in weight loss dietary supplement was developed and fully validated. All the parameters meet the acceptance criteria for method validation according to the AOAC. The method showed good specificity and linearity. The developed method is rapid, sensitive and can be used to quantify SB and its derivative as well as can be transfered to laboratories equipped with mass spectrometry liquid chromatography equipment. The method helps to alert the authorities of the quality of weight loss dietary supplements.

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Xác định đồng thời hàm lượng sibutramine và một số dẫn xuất trong thực phẩm bổ sung hỗ trợ giảm cân bằng sắc ký lỏng khối phổ hai lần (LC-MS/MS)

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Tóm tắt

Phương pháp sắc ký lỏng khối phổ hai lần được sử dụng để xác định hàm lượng của sibutramine (SB), N-desmethyl sibutramine (DSB) và N-didesmethyl sibutramine (DDSB), là những chất trộn trái phép trong thực phẩm bổ sung hỗ trợ giảm cân. Sibutramine và dẫn xuất của nó được chiết bởi dung môi methanol, sau đó tạp chất được loại bằng carbon hoạt tính (GCB). Quá trình tách sắc ký được thực hiện trên cột sắc ký C18 (100 mm x 2,1 mm, 3,5 µm). Pha động là gradient của 2 kênh: Kênh A là Acetonitril, kênh B là hỗn hợp ammoni acetate 2 mM + acid formic 0,1%. Kỹ thuật ghi phổ MRM ở chế ion dương được sử dụng để phát hiện SB, DSB và DDSB với số khối tương ứng m/z là 279,9/124,8; 266,0/124,8 và 252,1/125,0. Phương pháp đã được thẩm định và đáp ứng yêu cầu của AOAC về độ đặc hiệu, độ tái lặp và độ thu hồi. Đường chuẩn được xây dựng tuyến tính trong khoảng 0,002 - 0,100 µg/mL đối với SB, DSB và DDSB, hệ số tương quan lớn hơn 0,990. Phương pháp đã được ứng dụng để xác định hàm lượng SB, DSB và DDSB trong thực phẩm bổ sung hỗ trợ giảm cân, mẫu được lấy ngẫu nhiên tại các cửa hàng thuốc trên địa bàn Hà Nội ở cả 3 dạng viên nang cứng, viên nang mềm và trà túi lọc. Kết quả phát hiện sáu mẫu chứa SB, và DSB có hàm lượng nằm trong khoảng 0,817 - 31,7 mg/g.

***Từ khóa**: sibutramine, N-desmethyl sibutramine, N-didesmethyl sibutramine, thực phẩm bổ sung, giảm cân, LC-MS/MS.*