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Method Validation for the Analysis of Multiple Weight Loss Drugs in Dietary Supplement Materials by LC-MS/MS

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Abstract

A method validation is reported for the detection and quantification of multiple weight loss drugs in dietary supplement materials. The sample preparation was followed as described in LIB 4549 and the mass spectral measurements were accomplished using liquid chromatography tandem mass spectrometry with electrospray ionization. A method validation was performed at various fortification levels (250 and 1000 μg/g) for all 11 drug compounds. The overall recoveries with standard deviation averaged 93.8 ± 6.65% with the majority of individual recoveries in the range of 76% - 110%. The overall limits of quantification (LOQ) for analytes averaged at 10 ng/g. An overall indicator of linearity (R2) averaged at 0.9993 (0.0 250.0 ng/mL). The precision of five replicates of the 50 ng/mL level averaged at 98.4± 0.41% with < 1%.

Keywords:
weight loss drugs, sibutramine, LC-MS/MS

1. Introduction

Under the Dietary Supplement Health and Education Act (DSHEA) of 1994, a dietary supplement in the U.S. is defined as a product that is intended to supplement the diet which may include vitamins, minerals, herbs or other botanical, amino acids, or concentrates, and extracts of these. However, prescription drugs such as weight loss and other drugs are being detected in dietary supplement products at the FDA and other labs. These illegal products can cause serious problems when consumers take them without knowing the presence of drugs [1, 2, 3, 4]. Furthermore, according to the National Institute of Technology (NIST) and National Institute of Health (NIH), approximately 75% of the U.S population takes dietary supplements either by pills, juices or both representing an annual expenditure of more than $20 billion [5]. There are many dietary supplement products coming in US markets every year, promoted and available on the internet. To prevent adulterated products and to meet with the challenge of testing too many products, our laboratory has developed a method to analyze dietary supplements for multiple weight loss drugs including sibutramine, which is one of the most common adulterants in dietary supplements sold for weight loss purposes [4]. Here we report our results of this method validation on dietary supplements provided by CFSAN. The results indicate our developed method is suitable for confirmation and quantification of selected adulterated drugs including sibutramine and ten others in dietary supplements.

2. Experimental

Method validation was accomplished by evaluation of selectivity, precision, standard calibration linearity, standard calibration accuracy, method detection limit and method limits of quantification. The performance of the method validation was evaluated using the method validation criteria outlined by FDA, Guidelines for the Validation of Chemical Methods for the FDA Foods Program. These criteria are listed in Table 1.

All mass spectral measurements were performed on an Agilent 6490 LC-MS/MS system with an electrospray source equipped with an Agilent 1260 LC system. The LC-MS/MS data were processed using Agilents Mass Hunter software.

2.1. Materials and Apparatus

The methanol and acetonitrile were HPLC high purity grade (Burdick & Jackson, Morristown, NJ, USA). Ammonium formate was purchased from Fisher Scientific (Waltham, MA, USA). Milli-Q water was used throughout the validation (Millipore, 18.2 Ω). Drug standards were obtained with high purity grade (98% pure) from Sigma Aldrich (St. Louis, MO, USA), Toronto Research Chemicals (Toronto, Ontario, Canada) and from U.S.

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Table 1. Method validation criteria

Criteria		Range
Selectivity	LC Retention time	within 5% of RT
	MS/MS Transition	Three transitions per analyte
	MS/MS Ion Response Ratio	within 20 %
Linearity	Calibration STD Linearity (7 levels)	$R^2 > 0.995$
Accuracy	Calibration STD Accuracy (IC, ICV, CCV)	80 – 120 %
	Fortification Recovery	75 – 120 %
Precision	Five Replicates at Certain Concentration	RSD < 20 %
Specificity	Solvent Blank	No residue should be detected
Detection Limits	Limits of Quantification	10 ng/mL

Table 2: LC-MS/MS conditions and parameters used.

LC Conditions	LC Column	Zorbax C18, 100 x 2.1 mm, 1.8 µm (Agilent)
	Column Temp.	40 °C
	Mobile Phases	Pump A: Water with 2 mM ammonium formate and 0.05 % formic acid
		Pump B: Methanol with 2 mM ammonium formate and 0.05 % formic acid
	Injection Volume	1.0 µL
	Injection Mode	Injection with needle flush (30s)
	LC Flow Rate	0.3 mL / min
	LC Gradient	Time (min) B (%)
		0.0 20
		8.5 95
		13.0 95
		13.5 20
		15.0 20
MS/MS Conditions	Scan Type	Dynamic MRM
	Polarity	Positive
	Ion Source	ESI + Agilent Jet Stream
	Detection Window	RT ± 0.1 min
	Dry Gas Temp.	200 °C
	Dry Gas Flow	11 L/min
	Nebulizer Pressure	45 psi
	Sheath Gas Temp.	350 °C
	Sheath Gas Flow	11 L/min
	Capillary Potential	3500 V
	NozzleVoltage	500 V

Pharmacopeia (Rockville, MD, USA). Individual stock solutions for the 11 drug compounds were prepared in acetonitrile at 1000μg/mL. The standard solutions were stored in a refrigerator.

2.2. Calibration Standards

The injection of various calibration standards in acetonitrile/ H_2O (1:1) was used to construct the calibration curves (concentration versus peak areas). A series of dilutions were made in methanol to prepare analytical standards containing 0, 10, 20, 40, 80, 100 and 250 ng/mL concentrations.

2.3. Sample Preparation and Extraction

Five capsules or tablets were typically used to make sample composites. The tablet samples were ground into a fine powder to prepare a composite; capsule samples were emptied and mixed together. About 100 mg of composite was weighed for each product and then extracted using 20 mL of acetonitrile in 50 mL centrifuge tubes (30 x 115 mm polypropylene conical Falcon tubes, Becton-Dickinson Labware). To determine the spike recoveries of the analytes, spiking standards were added to portions of the sample composites before addition of the extraction solvent. The samples were shaken on a Burrell wrist-action shaker (Burrell Scientific, Pittsburgh, PA) for 30 mins then centrifuged at 4000 rpm for 5 mins (Thermo Scientific Multifuge X3R). The supernatant was diluted for injection with acetonitrile/H_2O (1:1). The samples and high-level-spike samples were diluted 133 fold. On the other hand, low-level-spike samples were diluted to 33 fold. All samples were then filtered using 0.2 μm PTFE membranes in vials (GE Mini-Uni Prep PTFE Filter Media/Vials, 0.2 μm pore size). A 1 μL sample volume was injected onto the HPLC-MS. For each sample

Table 3: Acquisition parameters

Compound Name	Precursor Ion	Product Ion	Collision	Dwell	RT (min)
Desmethylsibutramine	266.17	153.0	9	35.10	7.33
Desmethylsibutramine	266.17	139.0	5		
Desmethylsibutramine	266.17	125.1	33		
Didesmethylsibutramine	255.15	179.1	9	73.31	7.49
Didesmethylsibutramine	255.15	153.0	5		
Didesmethylsibutramine	255.15	139.0	5		
Fenfluramine	232.01	159.0	17	124.10	4.97
Fenfluramine	232.01	119.1	50		
Fenfluramine	232.01	109.1	50		
Fluoxetine	310.01	279.1	8	39.95	7.38
Fluoxetine	310.01	163.0	20		
Fluoxetine	310.01	129.0	32		
Lorcaserin	196.01	129.1	33	124.14	4.56
Lorcaserin	196.01	128.0	50		
Lorcaserin	196.01	115.1	49		
Orlistat	496.01	337.1	20	165.78	11.71
Orlistat	496.01	319.2	9		
Orlistat	496.01	301.0	20		
Phenolphthalein	319.31	225.0	21	93.43	6.31
Phenolphthalein	319.31	141.0	50		
Phenolphthalein	319.31	115.0	49		
Phentermine	150.01	133.1	5	165.71	3.21
Phentermine	150.01	91.0	24		
Phentermine	150.01	65.0	48		
Rimonabant	463.01	363.0	33	165.40	10.07
Rimonabant	463.01	300.1	50		
Rimonabant	463.01	164.0	50		
Sertraline	306.01	275.0	9	39.95	7.39
Sertraline	306.01	158.9	37		
Sertraline	306.01	123.0	49		
Sibutramine	280.18	153.0	13	49.01	7.13
Sibutramine	280.18	138.9	13		
Sibutramine	280.18	125.0	29		

Table 4: The instrument calibration curves of 0, 10, 20, 40, 80, 100, and 250 ng/mL.

Compound Name	R^2 Value		Average	Overall
Desmethylsibutramine	0.9998	0.9996	0.9997	0.9993
Didsemethylsibutramine	0.9990	0.9992	0.9991	
Fenfluramine	0.9999	0.9999	0.9999	
Fluoxetine	0.9997	0.9999	0.9998	
Lorcaserin	0.9999	0.9999	0.9999	
Orlistat	0.9948	0.9966	0.9957	
Phenolphthalein	0.9997	0.9999	0.9998	
Phentermine	0.9998	0.9999	0.9999	
Rimonabant	0.9999	0.9974	0.9987	
Sertraline	0.9997	0.9997	0.9997	
Sibutramine	0.9998	0.9992	0.9995	

matrix studied, a matrix blank, matrix spike and matrix spike duplicate were prepared by weighing the same amount of sample and performing the extraction procedures as described above. Each sample was fortified at two different concentrations (250 and 1000 μg/g) prior to extraction.

2.4. Calculation

Sample results:

$amount found/capsule = \frac{(mg.found)}{mg.composite} x \frac{total.wt.of.composite}{number.of.capsule}$

where: mg found is the amount obtained from LCMS; mg composite is the weight-out amount for extraction; total wt. of composite is the total weight of all capsules used

Spike results:

$\%spike.recovery = \frac{mg.found - mg.from.capsule}{mg.std.added} x100\%$

where: mg from capsule is defined as (mg composite x(amount found/capsule)

Calculation for RPD:

$RPD = \frac{spk1-spk2}{(spk1+spk2)/2} x100\%$

All statistical calculations were determined using Microsoft Office Excel 2010. These include calculations of average, standard deviation (STDEV), relative standard deviation (RSD = STDEV÷ Average x 100)

2.5. LC-MS/MS Determiantion

The LC-MS/MS system used for this validation consisted of Agilent 1260 LC interfaced to an Agilent 6490 LC-MS/MS with an electrospray ionization source in the positive ionization mode. The LC column was an Agilent 100 x 2.1 mm Zorbax C18, 1.8 μm particle size. Gradient elution was performed using 2 mM of ammonium formate in water with 0.05% formic acid as mobile A and 2 mM of ammonium formate in methanol with 0.05% formic acid as mobile B. Gradient elution started with 80% B, increased to 95% B at 8.5 min and held for 4.5 min, the changed to 20% B at 13.5 min. The flow rate was 0.3 mL per minute and the runtime of the method was 15 min. The injection volume was 1 μL. Detailed LC-MS/MS conditions and parameters are listed in Table 2. The acquisition parameters for each drug compound are listed in Table 3. The collision energies were optimized with standard references.

3. Validation Results

3.1. Selectivity

Two official samples of dietary supplements received from CFSAN were subjected for this validation. They represented samples as real as consumers may purchase and intake. They are sold in the stores and all over internet as promising dietary supplements for weight loss. Analysis selectivity using LC-MS/MS is obtained by monitoring two or more multiple reaction monitoring (MRM) transitions, comparing and evaluating selected MRM response ratios, and by measurement of the chromatographic retention time (RT) of each analyte. Under conditions set for this validation, all peaks appeared well within 5% of RT according to CVM Guidance for Industry [6]. The ratio of three MS/MS transitions for sample and average of standards was also within 20% [6].

3.2. Linearity

Calibration linearity was determined by evaluation of the correlation coefficients, R^2. Working standards in methanol were prepared to construct the instrument calibration curves of 0, 10, 20, 40, 80, 100, and 250 ng/mL. As shown in Table 4, the overall R2 for all compounds was 0.9993 with the average of individual R^2 of each compound ranging from 0.9957 to 0.9999.

3.3. Accuracy

Initial calibration (IC), continuing calibration verification (CCV) and initial calibration verification (ICV) were prepared at 50 ng/mL. For spike recovery study, each sample matrix was fortified at two different concentrations. Calculated amounts of spike solutions were added prior to the extraction. Results obtained for all elements in this validation for analysis accuracy were within the successful ranges of criteria. The statistical data for the overall recoveries are summarized in Table 5. The average recoveries with standard deviation (STDEV) were from 76.0 ± 7.40% to 110.6 ± 2.44%, respectively. The overall average recovery and overall STDEV were 93.8 ± 6.65 % for all analytes in these sample matrices. Desmethylsibutramine was detected in both matrix blanks. Thus, amounts of desmethylsibutramine recoveries reported in Table 5 are results after the deduction of found amounts. Finding of desmethylsibutramine was reported in a package of sample analyses. Desmethylsibutramine is not a common adulterant but lately found in many dietary supplements [7]. On the other hand, sibutramine is the common adulterant in dietary supplements sold for weight loss [4]. It is an FDA-approved drug in 1997 used as an appetite suppressant for weight loss and was withdrawn in 2010 due to associated cardiovascular risks [1].

3.4. Precision

The within-batch precision was determined by analyzing five replicate analyses of a solvent standard solution (50 ng/mL). The recoveries of five analyses were from 97.66% to 98.90%. The average recovery with STDEV was 98.4± 0.41% (RSD<1%).

3.5. Detection Limits

The method detection limit (MDL or LOD) and limits of quantification (LOQ) should be demonstrated in practice by acquiring signal/noise ratios>3 and acceptable recovery data at the claimed LOQ. The LOQ was estimated at 10 ng/mL in this validation. The estimated LOQ level in this study was in fact 100+ times lower than the typical levels of concern for these types of products. When added to dietary supplements, these types of weight loss drugs are usually present at therapeutic dosages which are typically in milligram/g.

4. Conclusion

In summary, the proposed method is fast and suitable for the analysis of 11 weight loss drugs in dietary supplements. This method is validated for LC-MS/MS amendable drug substances in dietary supplements. This method extends the capability of the FDAs dietary supplement program to identify and quantify of adulterated weight loss drugs in dietary supplements using LC-MS/MS.

Table 5: The statistical data for the overall spike recoveries

Compound Name	Spike Recoveries (%)				Average (%)	STDEV	RSD
	Sample 1		Sample 2				
	S1	S2	S1	S2			
Desmethylsibutramine*	97	102	99	108	101.5	4.79	4.72
Didesmethylsibutramine	102.3	104.7	93.8	95.3	99.0	5.30	5.35
Fenfluramine	93.8	98.9	93.3	89.6	93.9	3.82	4.07
Fluoxetine	79.1	92.4	71.2	72.8	78.9	9.65	12.23
Lorcaserin	92.4	92.4	75.0	73.8	83.4	10.40	12.47
Orlistat	107.5	113.3	110.1	111.4	110.6	2.44	2.21
Phenolphthalein	111.2	114.3	99.0	98.3	105.7	8.24	7.80
Phentermine	91.5	92.1	73.6	73.9	82.8	10.43	12.60
Rimonabant	107.7	110.0	101.5	104.1	105.8	3.77	3.56
Sertraline	78.2	85.5	71.2	69.2	76.0	7.40	9.74
Sibutramine	84.9	99.3	94.3	100.0	94.6	6.96	7.36
				Overall	93.84	6.65	7.46

** Amounts shown after subtracting the amount found in each samples*

5. Declaration of Conflicting Interest

The authors declare that there is no conflict of interest. Research was funded by U.S. Food and Drug Administration.

6. Disclaimer

The views expressed are those of the authors and should not be construed to represent the views or policies of the U.S. Food and Drug Administration. Any reference to a specific commercial product, manufacturer, or otherwise, is for the information and convenience of the public and does not constitute an endorsement, recommendation or favoring by the U.S. Food and Drug Administration.

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8. Article information

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