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Short communication

Comparative determination of sibutramine as an adulterant in natural slimming products by HPLC and HPTLC densitometry

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ABSTRACT

A new validated method for the identification and quantification of the sibutramine was developed by HPTLC-densitometry at 225 nm and advantages and disadvantages compared with HPLC-FLD at 225 nm emission and 316 nm excitation. Both methods were applied to the analysis of three natural slimming products in the market for the quantitative analysis of illegally added sibutramine. HPTLC separations were performed on (20 cm × 10 cm) glass HPTLC plates coated with silica gel 60 F_{254} using a mobile phase, *n*-hexane–acetone–ammonia (10:1:0.1, v/v/v). For HPLC analysis, a phenyl column (5.0 μm, 150 mm × 4.6 mm, i.d.) and an isocratic mobile phase of acetonitrile–water–formic acid (pH 3.0; 0.19 M) (45:55:0.78, v/v/v) was used. The calibration curve area versus concentration was found to be linear in the range of 250–2000 ng/spot^{-1} and 5–200 μg/ml for HPTLC and HPLC, respectively. Both methods were validated for accuracy, precision, linearity, selectivity, recovery and short term stability. As a conclusion, these methods were found to be useful for the routine analysis of illegally added sibutramine in the marketed products.

1. Introduction

Obesity, a worldwide health problem, may have negative impacts on life quality and in fact on life span. Greater energy intake than energy expenditure, decrease in physical activity, abnormal feeding regulations, neurogenic abnormalities and genetic factors are the known reasons for obesity [1]. WHO's latest report indicates that approximately 1.5 billion adults over the age of 20 were classified as overweight; of these, in fact, more than 200 million male and nearly 300 million female were obese. They further estimate that by 2015, these figures will increase roughly 2.3 billion for overweight and over 700 million for obese. On the other hand, nearly 43 million children under the age of 5 years were overweight globally in 2010 [2].

In the treatment of obesity main approaches are dieting and physical activity, while anti-obesity drugs are also used to support applications. Natural slimming products as an alternative for synthetic drugs are preferred because of the assumption that they are safe [3]. On the other hand, it is a fact that, the potency of these products is lower than synthetic slimming agents. Therefore, to enhance their efficiency they are frequently adulterated with potent chemical slimming agents such as orlistat, cetilistat, sibutramine and rimonabant, which are the most common additives in health food [4].

Sibutramine (Fig. 1) is a serotonin–noradrenalin reuptake inhibitor, which promotes and maintains weight loss in obese people. The pharmacological effects of sibutramine are reducing appetite, feeling of satiety and inducing thermogenesis [5]. However, the danger in using sibutramine is that it simultaneously overexcites the central nervous system and several side effects like nervousness, xerostomia, headache, numbness and paraesthesia have been reported. Also, it has been associated with increased cardiovascular events, *i.e.*, increase blood pressure and pulse rate and eventually enhance the risk of heart attacks and strokes [6]. Due to these risks, recently, it has been withdrawn from the market by European Medicines Agency (EMA) since 21st January 2010 [7].

Sibutramine and its metabolites are often added illegally into natural slimming products. Hence, detection and quantification of sibutramine plays crucial role for public health. For this purpose, several chromatographic methods such as GC–MS [8] and mostly liquid chromatography *i.e.*, HPLC-DAD [4,6], HPLC-CEAD [9], HPLC–MS/MS [10], HPLC-ESI–MS [11], HPLC-ESI–MS/MS [12], UPLC/Q-TOF MS [13] have been previously reported. In addition to chromatographic methods, DOSY ^{1}H NMR, a spectroscopic method [14], and X-ray powder diffractometry [9] have been developed to provide a wealth of information on the molecular structure of complex mixture ingredients. Also, recently, several papers have published simple and convenient analytical methods such as TLC

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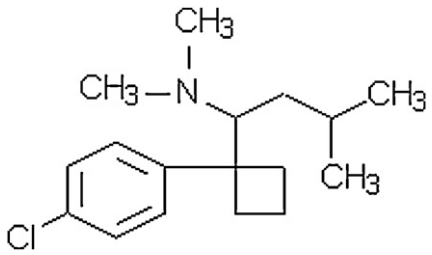

Fig. 1. Structure of the sibutramine.

and HPTLC for rapid detection of sibutramine in formulations [15,16].

The purpose of this research reported here was to develop a new, alternative, rapid, validated, sensitive and selective HPTLC method and compare of the results and also draw conclusions on the advantages and disadvantages between HPTLC and HPLC methods for the qualitative and quantitative analysis of sibutramine in natural slimming products.

2. Experimental

2.1. Materials

Solvents used for preparation of mobile phase for HPLC analysis were ultrapure water which is obtained from Sartorius Arium 611/61315 (Göttingen, Germany), acetonitrile (HPLC grade) and formic acid (analytical grade), purchased from Sigma Aldrich and Merck, Turkey, respectively. HPLC grade of acetone, *n*-hexane and analytical grade of ammonia from Sigma Aldrich and Merck, Turkey, respectively used in HPTLC analysis while preparing of mobile phase. Chemicals, basic bismuth nitrate, glacial acetic acid and potassium iodide, used to prepare Dragendorff reagent were purchased from Sigma and Riedel-De Hoen, respectively.

Sibutramine hydrochloride monohydrate, supplied from Matrix Laboratories Ltd., India, was used without further purification and certified to contain 99.84% (w/w) on anhydrous basis. Membrane filters (Sartorius model 0.45 μm PTFE filter) were used for the filtration of the mobile phase in HPLC analysis and the samples.

2.2. Instrumentation and analytical conditions

2.2.1. For HPTLC method

HPTLC analysis was performed on (20 cm × 10 cm) glass HPTLC plates coated with silica gel 60 F_{254} (E. Merck, Darmstandt, Germany). Samples were applied to the plates as 8-mm bands, 20 mm apart, by means of a Camag (Switzerland) Linomat V sample applicator fitted with a 100 μL syringe (Hamilton, Bonaduz, Switzerland) and used constant application rate of 0.2 μL s^{-1}. The plates were developed to a distance of 9 cm in a (20 cm × 10 cm) twin-through glass chamber (Camag, Muttenz, Switzerland) which had been pre-saturated with mobile phase vapors (*n*-hexane–acetone–ammonia 10:1:0.1, v/v/v). The optimized chamber-saturation time was 1 h and 15 min at room temperature.

Densitometric scanning was performed at 225 nm with Camag TLC scanner III in reflectance–absorbance mode and operated by CATS software (1.4.4 6337, Camag). Source of radiation was deuterium lamp emitting a continuous UV spectrum between 200 nm and 400 nm. The slit dimensions were 8 mm × 0.4 mm and the scanning speed was 10 mm s^{-1}. The intensity of the diffused light was used for the determination of concentrations of the substances chromatographed. Peak areas were determined with linear regression [17].

HPTLC plates were sprayed with Dragendorff reagent and the images of spots were captured with the CAMAG TLC visualizer by using white light.

2.2.2. For HPLC method

HPLC system (Agilents Technologies, USA), equipped with Chemstations 10.02 software (Agilents Technologies, USA) consist of a quaternary solvent delivery pump, an online vacuum degasser, an auto sampler, a thermostated compartment, and a fluorescence (FLD) detector, were used for the chromatographic analysis. All separations were carried out on ACE Phenyl column (5.0 μm, 150 mm × 4.6 mm, i.d.), Sem Laboratories Ltd, Turkey.

Mobile phase was acetonitrile–water–formic acid (pH 3.0; 0.19 M) (45:55:0.78, v/v/v). It was filtered and ultrasonically degassed before use. The flow rate was 1.0 ml/min, column temperature was maintained at 25 °C, effluent was monitored at 225 nm emission, 316 nm excitation, and injection volume was 5 μL. Identification of the peaks were based on the retention time and the FLD spectrum presented in the chromatogram.

2.3. Preparation of standards

40 mg sibutramine was weighed as an analytical standard by using an Ohaus Explorer 214C model analytical balance, which was calibrated to ensure accuracy. Then, it was dissolved in 50 ml in acetonitrile–water (1:1, v/v). The standard was mixed by using a vortex (Heidolph Reax Top 2010 model) and the final volume was completed to 200 ml with acetonitrile–water (1:1, v/v). Stock standards were prepared by serial dilution of 0.2 mg/ml standard in acetonitrile–water (1:1, v/v) using volumetric pipettes and flasks. All standards were stored protected from light at 5 °C for the duration of the study. Quality control samples were prepared from independent stock solutions at 5, 10, 20, 50, 100 and 200 μg/ml in acetonitrile–water (1:1, v/v). Each stock solution was filtered by using Sartorius model 0.45 μm PTFE filter before application to HPLC-FLD detection system and HPTLC system.

2.4. Preparation of samples

Samples used in these experiments were sold as natural slimming products in the market, belong to three different companies which were encoded as PT, PTCN and MAU.

Prior to weighing the capsules, balance was calibrated to ensure accuracy. 6 different capsules of first slimming product were weighted and mean value was found as 242.78 mg. After mixing sample powder homogenously 242.8 mg was weighted twice. Both of were extracted with 50 ml acetonitrile–water (1:1, v/v). After mixing the sample solutions 1 min by using vortex, they were filtered through a 0.45 μm PTFE filter. Finally they were diluted to 1/25 proportion with acetonitrile–water (1:1, v/v).

For the second and third slimming product, the same procedure was applied. 6 different capsules were weighted and mean value found 218.983 mg and 373.9 mg, respectively. For preparation of samples 219 mg and 374 mg was weighed twice. 50 ml acetonitrile–water (1:1, v/v) was used for the extraction. After mixing the sample solutions 1 min by using vortex, they were filtered through a 0.45 μm PTFE filter. Second slimming product was diluted to 1/100 and the third one was diluted to 1/2 proportion with acetonitrile–water (1:1, v/v). While, these three diluted samples were used in the HPLC analysis, stock samples (without dilution) were applied in the HPTLC analysis.

2.5. Preparation of 80%, 100%, 120% spiked samples for the recovery

Each capsule of different company includes different amount of sibutramine. After determination of the sibutramine for each

Table 1
Intra/inter-day linear regression data of investigated compounds by HPTLC and HPLC.

HPTLC	Linearity range (μg/ml)	Regression equation	Correlation coefficient (r^2)
Intra-day			
PT	0.25–2	$y = 163.5 + 1.314x$	0.996
PTCN	0.25–2	$y = 161.5 + 1.344x$	0.995
MAU	0.25–2	$y = 183.8 + 1.414x$	0.996
Inter-day			
PT	0.25–2	$y = 154.5 + 1.638x$	0.995
PTCN	0.25–2	$y = 183.8 + 1.545x$	0.995
MAU	0.25–2	$y = 177.5 + 1.954x$	0.995
HPLC			
1. Batch	5–200	$y = 7.2454e^{-1}x + 2.7071e^{-1}$	0.997
2. Batch	5–200	$y = 7.2588e^{-1}x + 2.0043e^{-1}$	0.997
3. Batch	5–200	$y = 7.2037e^{-1}x + 2.4691e^{-1}$	0.997

different company, spiked samples were prepared according to peak areas of the samples of different company by using 0.2 mg/ml sibutramine standard. 3, 5 and 8 ml of 0.2 mg/ml sibutramine were added into 2 ml PT samples and diluted to 10 ml to prepare 80%, 100% and 120 spiked samples, respectively. 2.5, 3.1 and 3.75 ml of 0.2 mg/ml sibutramine were added into 1 ml PTCN and 5 ml MAU and diluted to 10 ml, to prepare 80%, 100% and 120 spiked samples, respectively. These spiked samples were used in both HPLC and HPTLC method.

3. Results

3.1. HPTLC analysis and validation

The optimized HPTLC method was validated for linearity, precision, limits of detection and quantification, robustness, specificity, and accuracy.

3.1.1. Linearity and range

The calibration curve area versus concentration (ng/spot^{-1}) was found to be linear in the range of 250–2000 ng/spot^{-1}. 100 μg/ml of sibutramine was used as a stock solution and 2.5, 3.5, 5, 10 and 20 μl were spotted on HPTLC plates to obtain 250, 350, 500, 1000 and 2000 ng per spot of sibutramine, respectively. The linear regression data for the calibration curve for the three samples, PT, PTCN, MAU showed a good linear relationship over the concentration with respect to peak area, $r^2 = (0.996 \pm 0.001)$, as shown in Table 1.

3.1.2. Precision

The intra-day precision and inter-day precision of the method was evaluated by using linear regression data for the calibration curve analyzing sibutramine repeatedly at a concentration range 250–2000 ng per spot. The intra- and inter-day ($n = 4$) precisions are summarized in Table 2.

3.1.3. Limits of detection and quantification

The limits of detection (LOD) and quantification (LOQ) were determined by calculation of the signal to noise ratios (S/N) were 3 and 10, respectively. LOD and LOQ were determined by measuring the magnitude of analytical background (N), by spotting a blank, then calculating S/N for different amounts of sibutramine after application a series of solution (100 ng, 200 ng, 250 ng, 300 ng). As a result, according to S/N ratio (9.698) the minimum concentration (250 ng) which the analyte can be reliably detected was calculated as 77.34 ng (LOD) and 257.79 ng (LOQ).

3.1.4. Robustness

HPTLC method' robustness was determined during the method development. The HPTLC method was robust for (20 cm × 10 cm) glass HPTLC plates coated with silica gel 60 F_{254}, constant application rate of 0.2 μL s^{-1}, mobile phase *n*-hexane–acetone–ammonia (10:1:0.1, v/v/v) and the optimized saturation time was 1 h and 15 min.

Table 2
Intra- and inter-day precision of sibutramine (mg) in 1 capsule.

HPTLC $n = 4$	PT $n = 4$	MAU $n = 4$	PTCN
Intra-day	17.30	7.91	30.83
Intra-day	18.27	6.83	28.19
Intra-day	18.61	6.63	28.67
Inter-day	17.34	6.96	30.75
Average	17.88	7.08	29.61
SD	0.66	0.57	1.38
% RSD	3.70	8.02	4.65
HPLC			
Intra-day	19.35	7.35	29.43
Intra-day	19.47	7.33	28.88
Intra-day	19.58	7.36	29.57
Inter-day	19.43	7.24	29.90
Average	19.46	7.32	29.45
SD	0.01	0.05	0.43
% RSD	0.49	0.75	1.44

3.1.5. Specificity

The specificity of the method was determined by analysis of drug standard and test samples. The identity of the sibutramine spot from the samples was confirmed by comparison of its R_F (0.56 ± 0.01) and spectrum with those from a standard (Fig. 2).

3.1.6. Accuracy

The accuracy of the method was assessed by spiking of sibutramine standard in the diluted solutions of PT, PTCN and MAU. Amount of application of 80%, 100% and 120% recovery solutions on HPTLC (previously prepared for HPLC) was adjusted to approximately 600–750 ng due to the unknown matrix effect of capsules and migration problem of the high amount of sibutramine (Table 3A).

Recovery was estimated according to following formula [18]:

$$\text{Recovery}\,(\%) = \frac{\text{amount found} - \text{original amount}}{\text{amount spike}} \times 100\%$$

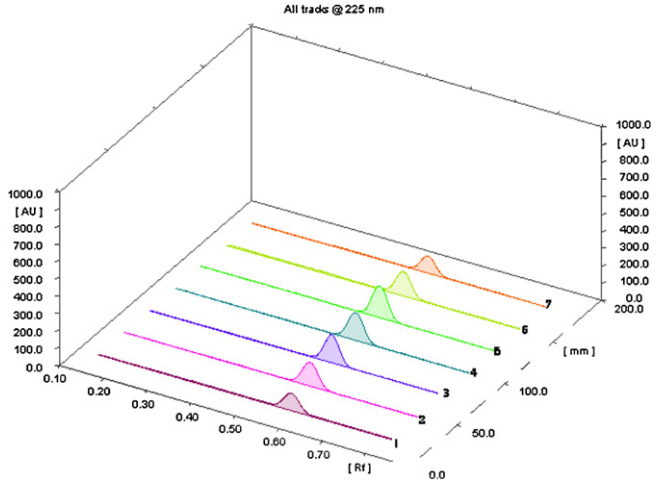

Fig. 2. HPTLC-densitogram at 225 nm: (1), (2) and (7) sibutramine, (3) PT, (4) PTCN, and (5) MAU.

Table 3A
Recovery of sibutramine by HPTLC.

Average	Original amount (ng)	Added sibutramine (ng)	Recovery (%)
PT			
653.29	143.072	600	85.03
713.16	100.15	700	88
682.67	57.23	640	97.73
PTCN			
601.4	71.06	600	88.39
696.13	65.14	682	92.57
682.18	53.3	675	101.06
MAU			
688.67	425.124	300	87.85
795.91	425.124	372	99.67
721.23	354.27	375	97.86

3.2. HPLC analysis and validation

3.2.1. Linearity and range

The calibration curve area versus concentration (μg/ml) was found to be linear in the range of 5–200 μg/ml ($n = 6$, average 98–114%, SD 0.1–0.9 and %RSD 0.1–2). The linear regression data for the calibration curve showed a good linear relationship over the concentration with respect to peak area, $r^2 = (0.997)$, shown in Table 1.

3.2.2. Precision

The intra-day precision and inter-day precision of the method was evaluated by using linear regression data for the calibration curve analyzing sibutramine repeatedly at a concentration range 5–200 μg/ml. The intra- and inter-day ($n = 4$) precisions are summarized in Table 2.

3.2.3. Limit of quantification

Limit of quantification (LOQ) was determined by series dilution of the least concentration of the standard solutions and was calculated as 1.71 ± 0.14 μg/ml.

3.2.4. Robustness

HPLC method' robustness was determined during the method development. The HPLC method was robust for column ACE Phenyl column (5.0 μm, 150 mm × 4.6 mm, i.d.), column temperature 25 °C, flow rate 1.0 ml/min and mobile phase acetonitrile–water–formic acid (pH 3.0; 0.19 M) (45:55:0.78, v/v/v).

3.2.5. Specificity

The specificity of the assay was checked by analyzing blank and the least concentration of the standard solutions. Sibutramine retention time (4.7 ± 0.1) was not detected on the blank chromatography (Fig. 3).

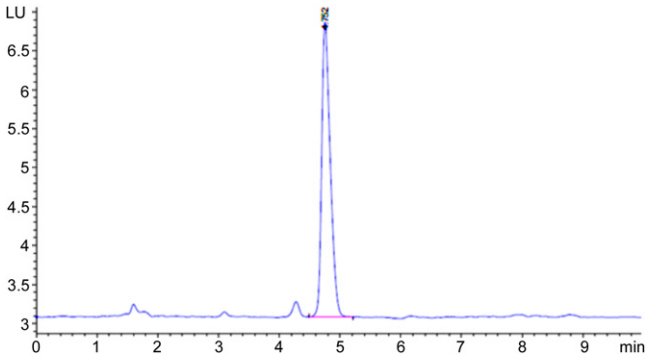

Fig. 3. HPLC chromatogram of sibutramine (50 μg/ml).

Table 3B
Recovery of sibutramine by HPLC.

Sample	Added sibutramine (μg)	Average $n = 4$	Recovery (%)	RSD (%)
PT	60	55.68 ± 0.31	93.43 ± 0.51	0.55
	100	96.10 ± 0.58	96.10 ± 0.58	0.60
	160	162.20 ± 3.3	101.37 ± 2.09	2.06
PTCN	50	49.30 ± 0.23	98.59 ± 0.47	0.47
	62	62.15 ± 0.46	100.24 ± 0.74	0.74
	75	75.04 ± 0.55	100.05 ± 0.73	0.73
MAU	50	45.96 ± 0.76	91.91 ± 1.53	1.66
	62	58.50 ± 2.17	94.35 ± 3.51	3.72
	75	70.79 ± 1.52	94.38 ± 1.45	1.53

Table 4
Short term stability of sibutramine by HPLC.

Concentration (μg/ml)	Stability	SD	RSD (%)
5	100.69	2.60	2.58
10	100.54	0.24	0.23
20	99.66	2.63	2.64
50	100.03	0.22	0.22
100	98.03	0.41	0.41
200	100.94	0.18	0.18

3.2.6. Recovery

Recovery was defined as the ratio of the experimentally determined result to the known added amount of the compound. The sibutramine stock solution (200 μg/ml) was spiked into the previously analyzed solutions with three concentrations of the drug, corresponding to 80, 100 and 120%. The results of recovery were illustrated in Table 3B.

3.2.7. Stability

3.2.7.1. Short term stability. Evaluation of the short-term stability of the sibutramine was performed between first day and the third day of the HPLC analysis (Table 4).

4. Discussion and conclusion

The worldwide consumption of natural slimming products for the weight loss has increased markedly due to misbelieve that they are safe. While the efficiency is rather weak comparing to synthetic drugs, they are frequently adulterated with chemical slimming agents such as sibutramine to increase their potency [3].

Due to permission of these products for marketing only based on the self-declaration of the content by the company of these products, they are not analyzed before or during marketing by the official authorities in Turkey. Recently, life threatening effects of sibutramine have been announced and unfortunately, this adulteration drives the public health into danger. Eventually, several deaths have been reported in Turkey by using these products particularly those imported from China.

In our study, sibutramine was detected in the three of these marketed products which were classified as natural slimming products and were not labeled to contain any synthetic adulterants. However on the labels of the products analyzed following contents were given; for PT: red pepper, *Glycyrhiza glabra*, green tea, ginger, wheat grass, vitamin C; for PTCN: red pepper; and for MAU: a thermogenic mixture, *Garcinia* extract, L-Carnitine, *Glycyrhiza glabra* and a proprietary blend composed of lycopene, green tea, ginger, wheat grass, silica, magnesium stearate.

Development of chromatographic methods for identification and quantification of such undeclared ingredients in these borderline products plays important role for public health. TLC is an alternative, easy handling, rapid, specific and low cost method for detection of such ingredients in these products. Eventually after the preliminary identification of sibutramine on TLC plates in the three herbal slimming products, a HPLC method was developed and validated by using fluorescence detector. In order to perform a precise comparison of the sibutramine content in these products, same samples were used in HPLC and HPTLC analysis.

As a conclusion, for the quantitative analysis of sibutramine both HPLC and HPTLC methods were found to be efficacious. Validation studies have revealed that both were specific, sensitive, fast, reliable and useful for routine analysis of illegally added sibutramine in herbal slimming products. However, there are some advantages and disadvantages between the sibutramine analysis methods by HPTLC and HPLC. Prominent advantages of HPTLC method over HPLC may be summarized as follows; shorter analysis time may be warranted due to rapid densitometric scanning of sibutramine. Moreover, higher number of samples can simultaneously be analyzed by HPTLC. Also sensitivity of HPTLC was much higher than HPLC (0.25 versus 5 μg/ml). In addition, since HPTLC plates are disposed after each analysis no risk for artificial peaks has occurred due to retained components inside the column from previous applications. However, there are several disadvantages of HPTLC methods comparing to HPLC. One of which was recovery; unknown matrix effect of capsule content caused difficulty in migration of higher amounts of sibutramine on plates and thus induced tailing problem in HPTLC analysis, while no such tailing problem was occurred and better recovery rate was secured by HPLC.

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