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Development and Validation of a High-Performance Thin-Layer Chromatography Method for Detection of Sibutramine in Dietary Supplements

Vanya R. Kozhuharov[1], Dzhevdet Chakarov[2], Stanislava Ivanova[1,3], Kalin Ivanov[1]

[1] Department of Pharmacognosy and Pharmaceutical Chemistry, Faculty of Pharmacy, Medical University of Plovdiv, Plovdiv, Bulgaria

[2] Department of Propedeutics of Surgical Diseases, Section of General Surgery, Faculty of Medicine, Medical University of Plovdiv, Plovdiv, Bulgaria

[3] Research Institute at Medical University of Plovdiv, Plovdiv, Bulgaria

Corresponding author: Vanya R. Kozhuharov, Department of Pharmacognosy and Pharmaceutical Chemistry, Faculty of Pharmacy, Medical University of Plovdiv, 15A Vassil Aprilov Blvd., 4002 Plovdiv, Bulgaria; Email: vanya.kozhuharov@mu-plovdiv.bg

Abstract

Introduction: In the period between 1997 and 2010, sibutramine-containing drugs were widely prescribed for obesity and over-weight management. Due to safety concerns, in 2010 all medicines containing sibutramine were urgently withdrawn from the USA and European pharmaceutical market. Although sibutramine is no longer available in pharmaceutical products, there have been numerous reports of mislabeled weight-loss dietary supplements containing sibutramine.

Aim: This work aimed to develop and validate an accurate and sensitive high-performance thin-layer chromatographic method for the detection of sibutramine.

Materials and methods: The method was developed using a CAMAG HPTLC system. Silica gel 60 F254 aluminum HPTLC plates were used as stationary phase and toluene:ethyl acetate:methanol (7:2:1 v/v/v) as mobile phase.

Results: The calibration curve was built in the range of 0.250–1.250 µg/band. The method provided satisfactory linearity, specificity, precision, and accuracy. The LOD and the LOQ were 0.0765 µg/band and 0.2318 µg/band, respectively.

Conclusions: The method allows for the simultaneous analysis of multiple samples as well as the rapid and sensitive monitoring of sibutramine levels in dietary supplements.

Keywords

INTRODUCTION

Obesity and overweight are today regarded as some of the most pressing global health issues. Furthermore, over the last 30 years, the prevalence of obese and overweight people has reached pandemic proportions. Pharmaceutical interventions have played a significant role in the pursuit of effective treatments.[1,2] One such medication that gained considerable attention was sibutramine. Between 1997 and 2010, sibutramine was widely prescribed to treat obesity. However, due to safety concerns, it was withdrawn in 2010.[3,4]

PENSOFT.

Sibutramine is a centrally-acting serotonin-norepinephrine reuptake inhibitor (SNRI). Its primary mode of action involves altering neurotransmitter levels in the brain, resulting in appetite suppression and increased thermogenesis. By targeting the central nervous system, sibutramine aims to aid individuals in achieving weight loss and maintaining long-term weight management.[5]

Clinical studies demonstrated that sibutramine, combined with a low-calorie diet and exercise, can lead to significant weight loss in obese individuals. Its efficacy was particularly notable in patients with a body mass index (BMI) exceeding 30 kg/m² or a BMI above 27 kg/m² with comorbidities such as dyslipidemia, hypertension, or type 2 diabetes. The medication exhibited promising results by promoting sustained weight loss and improving metabolic parameters.[6]

However, despite its initial success, concerns regarding the safety profile of sibutramine emerged over time. Later, it was announced that the intake of sibutramine increases the risk of cardiovascular events such as heart attacks, strokes, and arrhythmias.[5,7-9]

In 2010, all medicines containing sibutramine were urgently withdrawn from the USA and European pharmaceutical market. Although sibutramine is no longer available in pharmaceutical products, there have been numerous reports of adulterated weight-loss dietary supplements (DSs) containing this compound without proper labeling.[10-12]

One of the main reasons for sibutramine's presence in DSs is the lack of appropriate and obligatory quality control for DSs.[13] As a result, consumers may unknowingly consume high doses of sibutramine, which can increase the risk of adverse effects, especially when combined with other medications.[14] Something more, cases of sibutramine overdose from adulterated DS were reported as well.[15]

Dietary supplements aimed at weight loss, according to Wrobel et al., are among the most commonly purchased supplements on the Internet.[16] However, the missing quality control and the high demand for these products expose consumers at high risk. The quality control of the active compounds in DSs and the screening for adulterants are critical for ensuring the safety of consumers. There are only a few published analytical methods for detecting sibutramine[10-12,17], the majority of which rely on high-performance liquid chromatography and gas chromatography.

AIM

The aim of the current study was to develop a simple and rapid high-performance thin-layer chromatography (HPTLC) method for the identification and quantification of sibutramine.

HPTLC is an advanced and more sophisticated version of the classical thin-layer chromatography (TLC), designed to provide higher separation efficiency, faster and more precise analysis, improved resolution, and enhanced sensitivity.[18,19]

HPTLC offers several advantages over other chromatographic techniques, such as HPLC and GC: it is more cost-effective and time-saving. HPTLC allows multiple samples to be analyzed simultaneously on a single plate, reducing the overall cost per analysis (up to 75 samples simultaneously).[20] It is a versatile technique that can be used to analyze a variety of compounds, including organic and inorganic substances, natural products, pharmaceuticals, DS, and others. It can be adapted to various sample matrices and compound classes, making it suitable for diverse applications. HPTLC techniques seem to be much more appropriate for the detection of sibutramine in DSs than other techniques because of the possibility for simultaneous screening of multiple samples.

MATERIALS AND METHODS

Standards, reagents, and samples

Standard of sibutramine hydrochloride monohydrate was purchased from Sigma-Aldrich, Steinheim, Germany. The toluene, ethyl acetate, and methanol were of analytical grade purchased from Sigma-Aldrich, Steinheim, Germany. Bismuth subnitrate, potassium iodide, glacial acetic acid were purchased from Sigma-Aldrich, Steinheim, Germany. Forty DSs were purchased in pharmacies, herbal shops, and via the Internet.

Sample preparation

The stock solution of sibutramine was prepared by diluting with methanol in concentration of 1 mg/mL. Better dilution was accomplished using ultrasonic bath (BANDELIN, Berlin, Germany).

Dragendorff's reagent was prepared by dissolving 0.85 g of bismuth subnitrate in a mixture of 40 mL of water and 10 mL of glacial acetic acid (Solution A) and dissolving 8 mg of potassium iodide in 20 mL of water (Solution B). Five milliliters of both solutions were diluted with 20 mL of glacial acetic acid and 100 mL of water.[21]

The samples of DSs were prepared by diluting 250 mg of each sample with 10 mL methanol. After that they were homogenized by vortex. The next step involved the use of an ultrasonic bath for 30 minutes and then the samples were filtered through 0.45 µl PTFE Syringe filters (GVS North America Sanford, USA).

Apparatuses

The method was developed using a CAMAG HPTLC system (CAMAG, Muttenz, Switzerland). The configuration of the HPTLC system consisted of Limomat 5; Automatic Developing Chamber 2, and TLC Visualizer 2. The system was controlled by VisionCATS version 3. An ultrasonic bath (BANDELIN, Berlin, Germany) and a dipping chamber (Biostep-Desaga, Burkhardtsdorf Germany) were also used.

Chromatographic conditions

The analyses were carried out using silica gel 60 F254 aluminum HPTLC plates, 10×20 cm, 200 µm layer thickness (E. Merck KGaA, Darmstadt, Germany). The mobile phase comprised toluene, ethyl acetate, and methanol at a ratio of 7:2:1 v/v/v. The volume of the mobile phase was 10 mL. Application type: band. Front: 70 mm. Time for development: 25 minutes. Drying: 5 minutes. After development, derivatization was performed with Dragendorff's reagent in a dipping chamber for 20 seconds. The plate was observed with the CAMAG TLC visualizer at 254 nm.

RESULTS AND DISCUSSION

Method development

To achieve good linearity, accuracy, precision, robustness, limit of detection, and limit of quantification different chromatographic conditions were tested, including a reversed-type chromatography, using RP-18 modified silica gel-coated aluminum plates, with F254 fluorescent indicator.

However, the best results were achieved using silica gel 60 F254 aluminum HPTLC plates, 10×20 cm, 200 µm layer, and mobile phase – toluene : ethyl acetate : methanol at a ratio of 7:2:1 v/v/v.

Compared to the classical TLC, this technique requires a smaller volume of the mobile phase (10 mL). The standard solutions and the samples were applied as bands. All steps of the progress development were carefully monitored using specialized software. The time for the development was 25 minutes. After reaching a front of 70 mm, the plates were dried for 5 minutes. In the HPTLC techniques, the drying process is independent of human interaction, it is much safer for the researcher who performs the analysis, and it is performed automatically in a specific place of the development chamber. The next step involved derivatiza-

tion with Dragendorff's reagent in a dipping chamber for 20 seconds. The plate was observed with the CAMAG TLC visualizer after 5 minutes at 254 nm.

Method validation

The optimized HPTLC method was validated for linearity, accuracy, precision, limit of detection and quantification, and robustness according to the guidelines of the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH).[22]

Linearity

According to the ICH guidelines, the linearity must be evaluated using a minimum of five concentrations. The calibration curve was built using concentrations of sibutramine standard solutions in range from 0.250 to 1.250 µg/band.

A stock solution of sibutramine 100 µg/ml was prepared in methanol. Different volumes of the stock solution were applied by automatic sampler on HPTLC plate to obtain concentrations of 0.250 µg/band, 0.500 µg/band, 0.750 µg/band, 1.000 µg/band, and 1.250 µg/band.

After the development and derivatization, the plate was observed with the CAMAG TLC visualizer at 254 nm (**Fig. 1**).

The peak areas of the standard solutions were measured with CAMAG HPTLC system. Isometric profile of sibutramine in different concentrations is represented in **Fig. 2**. The linear regression line was y=0.0055933+0.000995. R²=0.9977. The concentration range 0.250–1.250 µg/band showed a good linearity.

Accuracy

Accuracy was assessed as a percentage of recovery. Recovery was evaluated using 3 known concentrations and 6 replicates for each concentration level. For accuracy tests from each examined substance were used 3 different quality con-

Figure 1. HPTLC chromatogram of sibutramine after derivatization with Dragendorff's reagent under 254 nm. Concentrations 250 ng/band, 500 ng/band, 750 ng/band, 1000 ng/band, and 1250 ng/band.
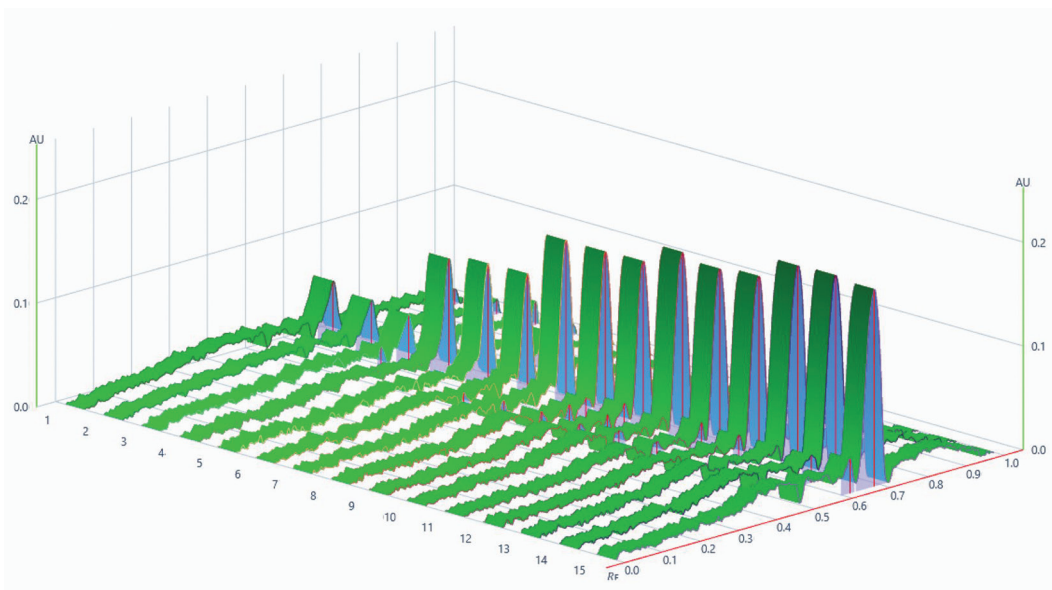
Figure 2. Isometric profile of sibutramine in different concentration levels.

trol (QC) levels: lower QC (LQC; 0.500 μg/band), middle QC (MQC; 0.750 μg/band), and high QC (HQC; 1.000 μg/band) with six replicates. The results from the accuracy evaluation are presented in **Table 1**.

Precision

The interday and intraday precision of the method were determined by evaluating the coefficient of variation for the 3 known level concentrations, each concentration with 6 replicates **(Table 2)**.

Limit of detection and limit of quantification

The detection limit and quantification limit were expressed by the standard deviation of the slope (s) and the slope of the calibration curve (S). We used the following formulas: LOD=3.3 s/S and LO-Q=10 s/S. The limit of detection was 0.0765 μg/band, and the limit of quantification was 0.2318 μg/band.

Comparison of the proposed method with other HPTLC/TLC methods

The study by Phattanawasin et al. was the first validated TLC method for quantification of sibutramine in DSs. The analysis was carried out using silica gel 60 F254 TLC plates, where the mobile phase comprised toluene-n-hexane-diethylamine, and for visualizing the spots Dragendorff's reagent was used. The calculated LOD and LOQ were 190 ng/spot and 634 ng/spot respectively.[10]

Later on, Hayun et al. developed a TLC-densitometry method using TLC silica gel 60 F254 aluminum plate, tol-

Table 1. Results on the accuracy of the developed HPTLC method for detection of sibutramine in dietary supplements

Concentration (μg/band)	Mean (μg/band)±SD	Recovery, %	CV%
0.500	0.5164±0.0135	103.278	2.606
0.750	0.7697±0.01599	102.622	2.078
1.000	0.9991±0.0209	99.911	2.092

Table 2. Results on the precision of the developed HPTLC method for detection of sibutramine in dietary supplements

Concentration (μg/band)	Intraday precision			Interday precision		
	Mean (μg/band)±SD	Standard error	CV%	Mean (μg/band)±SD	Standard error	CV%
0.500	0.5076±0.0105	0.0043	2.073	0.5241±0.0152	0.0062	2.892
0.750	0.7619±0.0151	0.0062	1.984	0.7589±0.0220	0.0090	2.900
1.000	1.0140±0.0192	0.0078	1.891	1.0015±0.0315	0.0128	3.140

uene-diethylamine (10:0.3) as mobile phase, and densitometric scanning at 227 nm. Lower amounts of LOD/LOQ were reported, 217.5 ng and 724.9 ng/spot, respectively.[23]

With the advent of new improved techniques based on the principles of thin-layer chromatography, HPTLC instruments were developed. HPTLC represents a significant advancement in the TLC methodology, owing to the incorporation of several enhancements: automatic and improved sample application, automatic development and drying, automatic monitoring of the analytical procedure, increased resolution, etc. All these essential improvements lead to more precise results and better safety for the researchers.[18,19]

Ariburnu et al. developed and reported much sensitivity HPTLC technique compared to previous TLC methods, where the LOD and the LOQ were 77.34 and 257.79 ng respectively. HPTLC was performed with glass plates silica gel 60 F254, n-hexane-acetone-ammonia as a mobile phase, where the densitometric observation was set at 225 nm.[24]

The discussed studies reported not only novel methods for sibutramine detection but also, positive samples adulterated with sibutramine in various concentrations, reaching up to 35 mg per capsule.[10,23,25-27] A comparison between previous HPTLC/TLC techniques for the detection of sibutramine in DSs is presented in **Table 3**.

The method established and validated in this study demonstrated excellent linearity, accuracy, and precision. The accuracy of sibutramine was between 99.911% and 103.278%. The limit of detection was 0.0765 µg/band, and the limit of quantification was 0.2318 µg/band which shows good sensitivity of the proposed method. The HPTLC assay for the determination and quantification of sibutramine could be used for the analysis of DSs.

In contrast to prior approaches used for analysis, derivatization was performed in a dipping chamber. Dipping allows for a more controlled and even application of the sample onto the HPTLC plate. Dipping provides better resolution of the separated spots on the HPTLC plate compared to spraying. The controlled application of the sample by dipping ensures that the spots remain well-defined and do not merge, leading to more accurate identification and quantification of the analytes.

Analysis of dietary supplements and confirmation of the method

The research carried out by Hachem et al. investigated 164 dietary supplements, of which 43 samples had sibutramine adulteration, with concentrations varying from 0.1 mg to 22 mg.[28] In 2016, a study conducted by Adela Krivohlavek and her team revealed that sibutramine was present in 20% of the 123 analyzed supplements, with the highest recorded level reaching 26.41 mg/g.[29] A comparable investigation was conducted by Zeng et al., who analyzed 447 weight loss dietary supplements, among which 55 out of 119 contaminated samples were found to contain sibutramine.[30] In 2014, Kim et al. discovered that 29 out of 188 DSs contained sibutramine, with quantities ranging from 0.03 mg/g to 132.40 mg/g.[11]

The results indicated that a large number of DSs contain undeclared sibutramine.

For determining sibutramine in DSs, the validated HPTLC method was used. The samples and standard solutions were spotted on the same plate and analyzed. Bands of dietary supplements were identified by comparing the Rf value, after dipping in Dragendorff's reagent, with those from standard of sibutramine. Forty DSs were tested and the presence of sibutramine in 5 dietary supplements was confirmed. The chromatogram and isometric profile of sibutramine standards and 5 dietary supplements are presented in **Figs 3, 4**, respectively.

Table 3. Comparison between previous HPTLC/TLC techniques for analyzing sibutramine in dietary supplements

Technique	Mobile phase	Wavelength	Reagent	Sibutramine concentration detected	LOD/LOQ	Ref.
HPTLC	N-hexane-acetone-ammonia (10:1:0.1)	225 nm	-	-	77.34/257.79 ng/spot	(24)
HPTLC	Toluene-methanol (9:1)	225 nm	-	Amounts reach 35 mg per capsule	-	(25)
TLC	Toluene-diethylamine (10:0.3)	227 nm	-	Range from 2.45 to 26.24 mg in a single dosage	217.5/724.9 ng/spot	(23)
HPTLC	MTBE-toluene-methanol (9:1:1)	225 nm	-	-	-	(17)
HPTLC	Toluene-ethyl format-formic acid (5:4:1)	254 nm 366 nm	Dragendorff's reagent	-	-	(27)
TLC	Methanol: ammonia (100:1.5)	245 nm	Dragendorff's reagent	4.38 mg/capsule and 26.37 mg/capsule		(26)
TLC	Toluene-n-hexane-diethylamine (9:1:0.3)		Dragendorff's reagent	Range from 6 mg to 24 mg per single dosage	190/634 ng/spot	(10)

Figure 3. Chromatogram of sibutramine standard in concentrations from 0.250 µg/band to 1.250 µg/band and 5 dietary supplements.
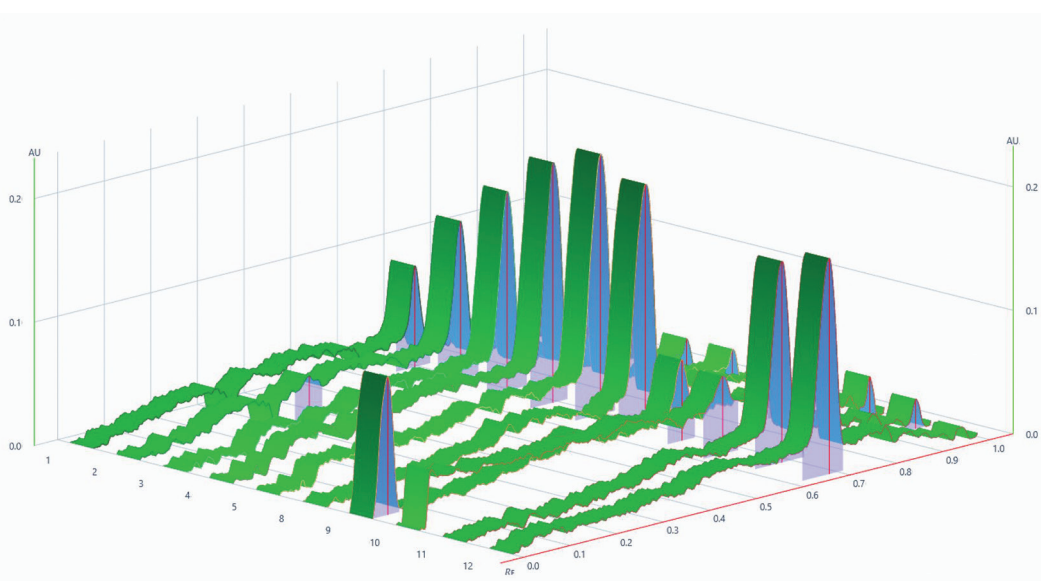
Figure 4. Isometric profile of sibutramine standards and 5 dietary supplements. 1. Sibutramine 0.250 µg/band. 2. Sibutramine 0.500 µg/band. 3. Sibutramine 0.750 µg/band. 4. Sibutramine 1.000 µg/band. 5. Sibutramine 1250 µg/band. 8–12 DSs.

Three DSs showed a higher amount of sibutramine. The results from HPTLC were confirmed through GC-MS analysis by a previously validated method.[12]

Despite the high level of consumption of DSs, the lack of regulations is currently a critical issue.[31] The majority of DS users believe these products are safe and represent a healthy lifestyle. In general, consumers are unaware of the risks associated with the intake of DSs.[32] Unintentional intake of sibutramine from DSs can affect levels of norepinephrine and serotonin in the body, resulting in an increased heart rate and tachycardia. These effects raise the risk of cardiovascular events such as heart attacks, strokes, and arrhythmias.[3,5,7,9] Moreover, sibutramine intake can cause sleep disorders, anxiety, dizziness, and restlessness.[33] People with cardiovascular disease, including those with a history of heart attack, stroke, arrhythmias, congestive heart failure, or uncontrolled hypertension, are at a higher risk of adverse cardiovascular events when using sibutramine. Sibutramine intake can potentially increase the likelihood of cardiovascular events, as mentioned above.[3]

Furthermore, DSs are essential components of professional athletes' diets and may offer advantages like quick recovery from strenuous exercise regimens, improved exercise performance, and dietary enrichment.[34] However, unintentional doping can occur after intake of DSs, if an athlete unknowingly consumes substances that are prohibited by the World Anti-Doping Agency (WADA). Sibutramine falls into this category because it is classified as a banned substance by WADA.[35] The reason for its prohibition is primarily due to appetite suppression and weight loss.

The five contaminated DSs, from our study, could expose consumers not only to serious health side effects but also expose many professional athletes to a significant risk of unintentional doping.

CONCLUSIONS

The presence of sibutramine in weight-loss DSs is a serious concern. DSs, unlike prescription medications, are not

subject to the same testing procedures and regulations, putting consumers' safety at risk. The presence of sibutramine in DSs without labeling could result in the consumption of high doses of this compound, increasing the risk of adverse effects, especially when combined with other medications. The current study reported on the development of a simple and rapid HPTLC method for the identification and quantification of sibutramine. The method was subsequently validated and showed good linearity, accuracy, and precision over a concentration range of 0.250–1.250 μg/band. The LOD and LOQ were determined as 0.0765 μg/band and 0.2318 μg/band, respectively. Furthermore, the developed method is sensitive, rapid, dependable, and precise, making it useful for detecting sibutramine in dietary supplements. The method was applied for the analysis of 40 DSs. Five of the samples were contaminated with sibutramine in different concentrations. The method contributes to the ongoing efforts in monitoring and controlling the undeclared presence of sibutramine in DSs.

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Author contributions

Conceptualization: V.K., S.I., and K.I.; methodology: V.K., K.I., and S.I.; validation: V.K. and S.I.; investigation: V.K., K.I., D.C., and S.I.; resources: V.K.; data curation: V.K. and S.I.; writing original draft: V.K., K.I., and S.I.; review and editing: K.I., D.C., and S.I.; supervision: K.I. and S.I. All authors have read and agreed to the published version of the manuscript.

Competing Interests

The authors have declared that no competing interests exist.

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Folia Medica

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Разработка и валидация метода высокоэффективной тонкослойной хроматографии для обнаружения сибутрамина в пищевых добавках

Ваня Кожухаров[1], Джевдет Чакаров[2], Станислава Иванова[1,3], Калин Иванов[1]

[1] *Кафедра фармакогнозии и фармацевтической химии, Факультет фармации, Медицинский университет – Пловдив, Пловдив, Болгария*

[2] *Кафедра пропедевтики хирургических болезней, Секция общей хирургии, Факультет медицины, Медицинский университет – Пловдив, Пловдив, Болгария*

[3] *Научно-исследовательский институт при Медицинском университете – Пловдив, Пловдив, Болгария*

Адрес для корреспонденции: Ваня Кожухаров, Кафедра фармакогнозии и фармацевтической химии, Факультет фармации, Медицинский университет – Пловдив, бул. „Васил Априлов" № 15А, 4002 Пловдив, Болгария; Email: vanya.kozhuharov@mu-plovdiv.bg

Резюме

Введение: В период 1997-2010 гг. сибутраминсодержащие препараты широко назначались для лечения ожирения и избыточной массы тела. Из соображений безопасности в 2010 году все препараты, содержащие сибутрамин, были срочно изъяты с фармацевтического рынка США и Европы. Хотя сибутрамин больше не доступен в фармацевтических продуктах, были многочисленные сообщения о фальсифицированных пищевых добавках для снижения веса, содержащих сибутрамин, которые не были маркированы.

Цель: Целью данной работы было разработать и апробировать точный и чувствительный высокоэффективный тонкослойный хроматографический метод обнаружения сибутрамина.

Материалы и методы: Метод разработан с использованием системы CAMAG HPTLC. Алюминиевые пластины для HPTLC с силикагелем 60 F254 использовали в качестве неподвижной фазы, а толуол:этилацетат:метанол (7:2:1 объём/ объём/ объём) в качестве подвижной фазы.

Результаты: Построена калибровочная кривая в диапазоне 0.250–1.250 µg/лента. Метод обеспечил удовлетворительную линейность, специфичность, скрупулёзность и достоверность. LOD и LOQ составляли 0.0765 µg/лента и 0.2318 µg/лента соответственно.

Заключение: Метод позволяет проводить одновременный анализ нескольких образцов, а также быстрый и чувствительный мониторинг уровня сибутрамина в пищевых добавках.

Ключевые слова

пищевые добавки, высокоэффективная тонкослойная хроматография, HPTLC, сибутрамин

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