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Determination of melatonin in sleep supplements by high-performance liquid chromatography

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The aim of this study was to quantify the actual melatonin content in commonly available over-the-counter melatonin sleep supplements marketed in Lithuania. For this purpose, a high-performance liquid chromatography (HPLC) method was optimised and validated. The calibration curve was linear ($R^2 = 0.9996$) over a concentration range of 0.5–50.0 mg/L, and the limit of detection was 0.17 mg/L. Recoveries of melatonin from spiked supplement samples ranged from 96.8 to 104.1%, with relative standard deviation values below 3.2%. Melatonin was quantified in 20 commercial sleep supplements representing 10 brands and three dosage forms (capsules, tablets and liquids), with two different lots analysed for each brand. Overall, the measured melatonin content ranged from –7 to +26% relative to the labelled amount, indicating a low variability among melatonin products sold in Lithuania. Only one product contained melatonin amount outside the ±20% margin of the labelled claim. The least variable products were liquid supplements, which generally contained the simplest ingredient compositions.

Keywords: high-performance liquid chromatography, melatonin, sleep supplements

INTRODUCTION

Melatonin (N-acetyl-5-methoxytryptamine) is an important hormone that regulates the body's sleep–wake cycle, known as the circadian rhythm [1]. In addition to regulating sleep, melatonin also plays an important role due to its antioxidant, anti-inflammatory and immune-modulating properties [2]. This hormone is naturally produced by the pineal gland, located in the middle of the brain. It is a chemical messenger that signals to your body that it is time to relax and prepare for rest. Melatonin levels in the body fluctuate throughout the day. During daylight hours, levels remain low, helping you stay awake and alert. As evening approaches and darkness sets in, melatonin production increases, gently signalling the body to transition into the sleep mode [3]. Unfortunately, natural melatonin production can be disrupted by various factors, including stress, poor sleep habits, jet lag, and exposure to artificial light during nighttime hours [4]. This is one reason why melatonin sleep supplements have grown in popularity over the past two decades. For example, in the United States, melatonin use increased from 0.4% of survey respondents in 1999–2000 to 2.1% in 2017–2018. That upward trend was similar among both men and women and across different age groups [5]. Rising melatonin consumption has driven an expansion in the variety of melatonin supplements offered by pharmaceutical companies. Today, melatonin is widely available over the counter in most pharmacies, with a diverse range of formulations, including flavoured liquids, rapid-dissolve tablets and strips, traditional liquid gels and solid tablets, as well as capsules containing complex combinations of vitamins, minerals, and whole plant extracts [6].

The increased popularity of melatonin-containing products has also brought into discussion the safety and quality of these products. Melatonin

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is generally considered safe for short-term use. However, long-term use or higher doses may cause side effects such as dizziness, nausea, headaches, muscle aches, mood changes, and daytime drowsiness, sometimes referred to as a 'melatonin hangover' [7]. Because melatonin has been suggested to be unstable under some storage conditions, and melatonin is known to occur naturally in medicinal plants, this raises potential pitfalls that may lead to a degradation or an increase in the melatonin content of these supplements, especially with such a wide diversity of matrices. In 2017, an analysis of 30 melatonin products sold in Canada reported melatonin content between –83 and 478% of that declared on the label [8]. A more recent investigation on melatonin gummy products sold in the United States reported melatonin content ranging from –4 to 347% of the labelled amount [9]. In another recent study, 110 supplement products labelled as containing melatonin and marketed towards children were purchased and analysed to quantify their melatonin content. Melatonin was identified in 108 of 110 products (98%), with measured contents ranging from 0 to 667% of the labelled amount [10]. Such high variability in melatonin content highlights the importance of accurate and rigorous quality control for these supplements.

The aim of this study was to quantify the actual melatonin content in commonly available over-the-counter melatonin sleep supplements marketed in Lithuania. For this purpose, a high-performance liquid chromatography (HPLC) method was optimised and validated. A total of 20 melatonin supplement products, representing different brands and dosage forms and labelled as containing melatonin, were purchased from local pharmacies and analysed.

EXPERIMENTAL

Reagents and solutions

Melatonin ($C_{13}H_{16}N_2O_2$, purity ≥99%), pyridoxine hydrochloride (vitamin B_6, $C_8H_{12}NO_3Cl$, purity ≥98%), serotonin (5-hydroxytryptamine, $C_{10}H_{12}N_2O$, purity ≥98%), tryptophan (2-amino-3-(1*H*-indol-3-yl)-propanoic acid, $C_{11}H_{12}N_2O_2$, purity ≥98%), nicotinamide (vitamin B_3, $C_6H_6N_2O$, purity ≥99.5%), o-phosphoric acid (H_3PO_4, 85% for HPLC) and methanol (CH_3OH, for HPLC, gradient grade, purity ≥99.9%)were purchased from Sigma-Aldrich (St. Lous, MO, USA). Ultrapure water was obtained from a Milli-Q water purification system from Millipore (Bedford, MA, USA).

A stock solution of melatonin (200 mg/L) was prepared in MeOH and stored at 4°C. Working solutions were freshly prepared by diluting the stock solution with MeOH/H_2O (1:1, v/v) containing 5 mmol/L H_3PO_4.

Sample preparation

All melatonin sleep supplements investigated in this study were purchased from local pharmacies in Vilnius. Supplements tested spanned 10 different brands and included a representative range of formulations (e.g. liquid, capsule and tablet). Two lots from each brand were analysed.

Five tablets were weighed, and the average weight per tablet was determined. The tablets were then ground into a fine powder. For capsules, the contents of five capsules were emptied, thoroughly mixed, and the average weight of the capsule contents was determined. For liquid supplements with a specified serving volume, the average serving weight was determined based on ten servings.

Samples were prepared according to a slightly modified procedure described in Ref. [10]. For solid samples, an amount equivalent to one dose was weighed and transferred to a 25 mL volumetric flask. For spiking experiments, an aliquot of melatonin standard solution was added to the flask. Subsequently, 15 mL of methanol was added, and the mixture was vortexed for at least 1 min. Additional methanol was added to bring the volume to 25 mL. The samples were kept at room temperature for 30 min, then mixed, and aliquots (approximately 2 mL) were filtered through a 0.22 μm PTFE membrane filter. Additional dilutions with MeOH/H_2O (1:1, v/v) containing 5 mmol/L H_3PO_4 were prepared to adjust the melatonin concentration to within the calibration range. Liquid samples were weighed, directly diluted with MeOH/H_2O (1:1, v/v) containing 5 mmol/L H_3PO_4, filtered and analysed. All samples were prepared in triplicate.

HPLC analysis

Chromatographic separations were performed on an Agilent 1290 Infinity II LC system (Agilent,

Waldbronn, Germany) equipped with a binary pump, a thermostatted column compartment, a photodiode array detector and an autosampler. Luna Phenyl-Hexyl column (3.0 × 150 mm, 3 μm, Phenomenex), maintained at 25°C, was used in the experiments. Separations were performed at a flow rate of 0.5 mL/min with a MeOH/water mobile phase containing 5 mmol/L H_3PO_4 under linear gradient elution conditions. The composition of the mobile phase was changed from 60% MeOH to 100% MeOH in 8 min. A 5 min post-run time was set to fully reequilibrate the systems. The injection volume was 10 μL and the detection wavelength was set at 220 nm. Data collection and management was performed by Agilent OpenLAB CDS software.

RESULTS AND DISCUSSION

HPLC separation

According to the literature, reversed-phase HPLC is the most commonly used technique for the determination of melatonin in a wide range of samples, including melatonin sleep supplements [8–13]. Many melatonin supplements also contain tryptophan and vitamins B_3 and B_6 to support the body's natural production of melatonin. Amino acid tryptophan is a precursor for melatonin, and certain vitamins are essential co-factors in its conversion [14]. Although serotonin, another precursor of melatonin, is strictly regulated and not permitted for sale as a supplement, it was detected in some melatonin supplements [8]. Because these structurally related compounds

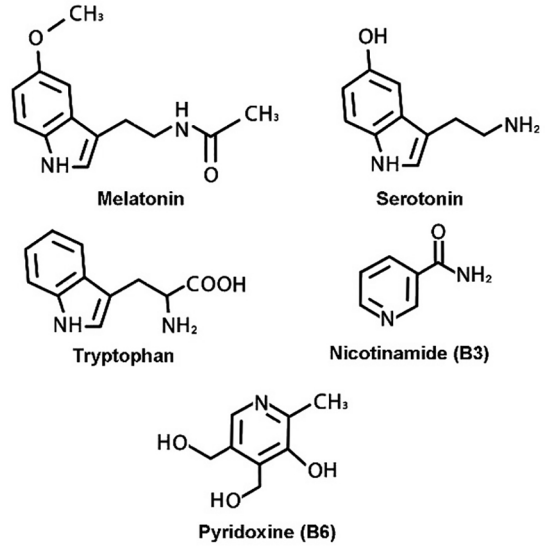

Fig. 1. Chemical structures of the studied compounds

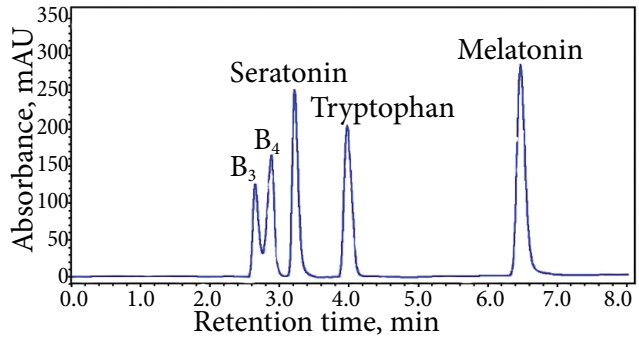

Fig. 2. Chromatogram of the standard mixture obtained under optimised conditions

(Fig. 1) may interfere with the quantification of melatonin, the HPLC conditions were initially selected to achieve their separation. As the present study focused exclusively on the determination of melatonin content, the complete separation of all compounds was not intended. The primary objective was to select conditions under which these compounds would not interfere with melatonin determination. Several experiments involving different mobile phase compositions and gradient programs were conducted to achieve a suitable separation. Because most of these compounds are pH-sensitive, the mobile phases were acidified with H_3PO_4. Figure 2 presents the chromatogram of a five-compound standard solution obtained under optimised conditions. The final conditions are presented in the HPLC analysis section.

Method validation

The HPLC method for the determination of melatonin was validated for some method performance parameters including linearity, limit of detection (LOD), limit of quantification (LOQ), precision and accuracy. Using seven melatonin standard concentrations (0.50–50.00 mg/L), each analysed in triplicate, the regression equation, correlation coefficient, and linear response range were determined. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated as signal-to-noise ratios of 3 and 10, respectively (Table 1).

Precision of the method was determined by repeatability (intra-day precision) and intermediate precision (inter-day precision). Repeatability was assessed analysing three levels of melatonin standard solutions: low (1.0 mg/L), medium (5.0 mg/L) and high (50.0 mg/L) on the same day. Intermediate precision was evaluated through the analysis of those standard solutions on three different days, in

Table 1. **Some performance characteristics of the HPLC method for the determination of melatonin ($n = 3$)**

Characteristic	Value
Linear regression equation	$y = 1.2409x - 0.3418$
R^2	0.9996
Linearity range, mg/L	0.50–50.00
LOD, mg/L	0.55
LOQ, mg/L	0.17

triplicate. The relative standard deviation (RSD) values for method repeatability at all three concentrations were below 0.85%. For the intermediate precision, the RSD values were below 1.42%. The results obtained demonstrate that the method exhibits the acceptable precision.

Method accuracy was evaluated using three types of supplement samples (liquid, capsule and tablet) fortified with melatonin standards at three concentration levels (50, 100 and 150% of the nominal sample concentration), with each sample analysed in triplicate. The obtained recoveries (Table 2) ranged from 96.8 to 104.1%, indicating that the method is sufficiently accurate and does not introduce significant errors that could interfere with the analytical results.

Table 2. **Accuracy results for the HPLC method ($n = 3$)**

Sample type	Spiked level, %	Mean recovery, %	RSD, %
Tablet	50	96.8	2.1
	100	101.5	1.9
	150	98.2	2.4
Capsule	50	96.9	2.5
	100	97.6	3.2
	150	104.1	1.8
Liquid	50	98.8	1.5
	100	97.3	1.7
	150	98.0	0.9

Sample analysis

The validated HPLC method was employed for the quantification of melatonin in commercial melatonin supplements. This study quantified melatonin in 20 commercial sleep supplements representing 10 brands and three dosage forms (capsules, tablets and liquids), with two lots analysed per brand. It should be noted that, unlike in

Table 3. **Results of melatonin content in sleep supplements tested ($n = 3$)**

Supplement code	Lot	Form	Labelled amount, mg	Determined amount, mg	RSD, %	Difference from labelled amount, %
C1	1	Capsule	1	1.26	2.5	26.0
	2		1	1.18	2.3	18.0
C2	1	Capsule	0.8	0.82	1.9	2.5
	2		0.8	0.81	2.6	1.3
C3	1	Capsule	0.4	0.45	2.8	12.5
	2		0.4	0.43	2.4	7.5
T1	1	Tablet	1	1.09	3.3	9.0
	2		1	1.13	2.8	13.0
T2	1	Tablet	1	1.14	2.9	14.0
	2		1	1.18	2.3	18.0
T3	1	Tablet	0.75	0.83	3.2	10.7
	2		0.75	0.81	3.0	6.7
T4	1	Tablet	0.5	0.53	4.4	6.0
	2		0.5	0.57	3.8	14.0
L1	1	Liquid	1	0.98	2.0	–2.0
	2		1	0.93	2.4	–7.0
L2	1	Liquid	1	1.03	1.5	3.0
	2		1	1.01	2.1	1.0
L3	1	Liquid	0.75	0.76	2.3	1.3
	2		0.75	0.73	1.8	–2.7

the USA and Canada, melatonin supplements in the European Union are typically manufactured to contain no more than 1 mg of melatonin per dose. The obtained results are presented in Table 3. Figure 3 shows the chromatograms of three different dosage form samples.

In general, the measured melatonin content ranged from –7 to 26% relative to the labelled amount. Compared with the data on melatonin supplements sold in the United States (from 0 to 667%) [10] and Canada (from –83 to 478%) [8], the variability in melatonin content among products sold in Lithuania is low. Most solid sleep supplements (capsules and tablets) were found to contain slightly more melatonin than declared on the label. The least variable products were liquid supplements, which generally contained the simplest ingredient compositions. Under general EU guidance for food supplements [15], a tolerance of approximately ±20% is commonly applied to vitamins and minerals. Accordingly, only one product had a melatonin content that fell outside a 20% margin of the labelled claim.

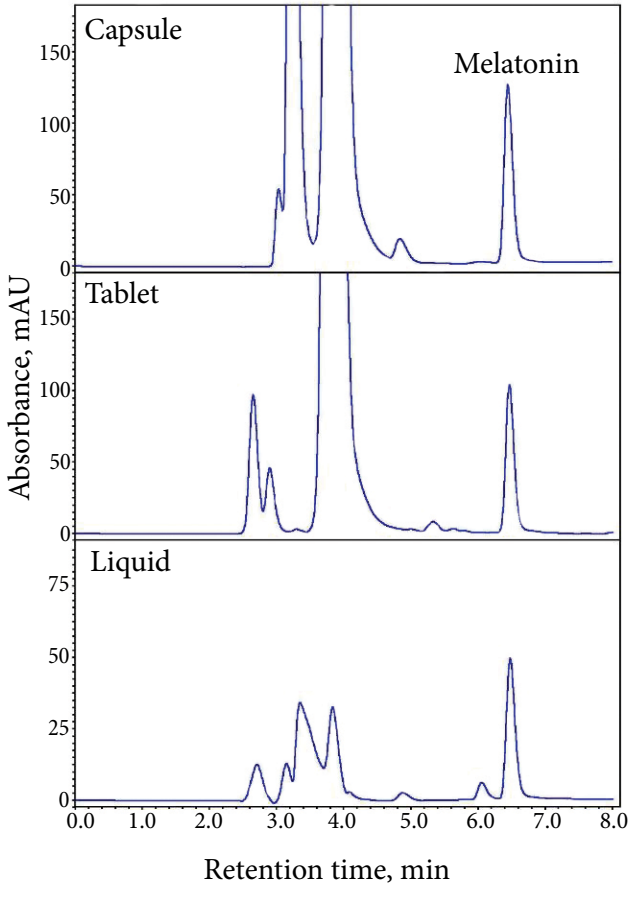

Fig. 3. Chromatograms of three different dosage form samples

CONCLUSIONS

This study reports, for the first time, the HPLC determination of melatonin content in sleep supplements marketed in Lithuania. The validated HPLC method demonstrated specificity and suitability for quantifying melatonin across different supplement formulations and complex matrices. The obtained results demonstrate a relatively low variability, ranging from –7 to +26% of the labelled melatonin content. The vast majority of products were accurately labelled, with only one product exceeding the declared amount beyond a ±20% margin of the labelled claim.

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MELATONINO KIEKIO NUSTATYMAS MIEGUI GERINTI SKIRTUOSE PAPILDUOSE EFEKTYVIOSIOS SKYSČIŲ CHROMATOGRAFIJOS METODU

S a n t r a u k a

Šio tyrimo tikslas buvo nustatyti faktinį melatonino kiekį Lietuvoje parduodamuose nereceptiniuose melatonino papilduose, skirtuose miegui gerinti. Tam buvo optimizuotas ir validuotas efektyviosios skysčių chromatografijos metodas. Kalibravimo kreivė buvo tiesinė (R^2 = 0,9996) melatonino koncentracijų intervale nuo 0,5 iki 50,0 mg/L, o melatonino aptikimo riba siekė 0,17 mg/L. Melatonino priedų išgavos iš papildų mėginių svyravo nuo 96,8 % iki 104,1 %, o santykinio standartinio nuokrypio vertės buvo mažesnės nei 3,2 %. Melatonino kiekis buvo nustatytas 20-yje komercinių miegui skirtų papildų, reprezentuojančių 10 skirtingų prekės ženklų ir tris dozavimo formas (kapsules, tabletes ir skysčius). Buvo analizuojamos dvi skirtingos kiekvieno prekės ženklo partijos. Nustatytas melatonino kiekis svyravo intervale nuo –7 % iki +26 % etiketėje nurodyto kiekio, rodančio nedidelį kiekybinį kintamumą Lietuvoje parduodamuose papilduose. Tik viename produkte nustatytas melatonino kiekis viršijo ±20 % ribą deklaruoto kiekio atžvilgiu. Mažiausi nukrypimai nustatyti paprasčiausios sudėties skystuose papilduose.