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Development and Validation of an HPLC-FLD Method for the Determination of Pyridoxine and Melatonin in Chocolate Formulations—Digestion Simulation Study

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Abstract: The undeniable value of sleep is commonly accepted and essential for all aspects of human existence. The aim of the present study was to develop and validate an applicable and sensitive HPLC-FLD method for the quantitative determination of pyridoxine (PYR) and melatonin (MEL) in a chocolate formulation that is suitable for the treatment of insomnia. The proposed chromatographic process was performed on an Aqua column (250 mm × 4.6 mm, 5 mm) at 45 °C, with a mobile phase consisting of two solvents (A, water with 0.2% formic acid; and B, acetonitrile with 0.2% formic acid) in a gradient elution and with a flow rate of 1.2 mL/min. Method validation was carried out according to ICH specifications in terms of linearity (R^2 > 0.999), precision, and repeatability (%RSD < 2). The limits of detection (LODs: PYR = 0.09 ng/mL; MEL = 0.92 ng/mL) and quantification (LOQs: PYR = 0.29 ng/mL; LOQ_{MEL} = 3.04 ng/mL) for both analytes were also determined. A mixture D-Optimal methodology of experimental design technique was applied for the quantitative recovery of the two APIs from chocolate (% recovery > 97.4%). Finally, to simulate the digestion process of the new preparation, three phases in vitro, a digestion method was applied. According to the study, 73.8% of pyridoxine and 55.9% of melatonin (on the indicated dose) were released from the formulation and ended up in the intestine.

Keywords: HPLC-FLD; chocolate pharmaceutical formulation; melatonin; pyridoxine; in vitro digestion; mixture D-Optimal

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1. Introduction

The problems of insomnia are mentioned in numerous scientific publications and are indisputable, since it causes related disorders that harm both physical and mental health. Indirectly, lack of sleep can lead to cardiovascular diseases [1], diabetes [2], hypertension [3], chronic pain [4], and higher body mass index [5]. Regarding mental health, good sleep has been shown to have a positive effect, as impaired sleep quality and quantity [6] is almost always present in all mental disorders [7–10].

Pharmaceutical science can play a key role in ameliorating the problem for many people by developing new drugs and formulations and contributing to the improvement of public health. More specifically, pyridoxine and melatonin are two Active Pharmaceutical Ingredients (APIs) with beneficial abilities and a synergistic effect in combating insomnia.

Vitamin B6 (pyridoxine) appears to enhance dream quantity, as well as the individual's ability to recall dreams [11]. In a related study, Huang and coworkers report that good sleepers tend to consume a higher intake of B6, compared to bad sleepers [12]. However, the most important contribution of B6 to sleep is that it acts as a coenzyme in the biosynthesis of melatonin [13]. Melatonin regulates circadian rhythms, such as wakefulness, as well as neuroendocrine rhythms [14]. Its exogenous administration is a good alternative for the treatment of sleep disorders, as taking melatonin supplements is well tolerated and does not present obvious side-effects [15]. For this reason, the presence of melatonin alone or with pyridoxine is reported in a multitude of formulations, some of which do not have the classic form and belong to the category of edible materials (e.g., gummies) [16–18].

In order for a new pharmaceutical preparation to be approved for industrial-scale production, pharmacokinetic and bioequivalence studies must be conducted. Regarding pharmacokinetics, the initial stage of the study is considered to be the release of its active ingredients in the gastrointestinal tract [19–21]. In vitro digestion is a three-phase technique (oral, gastric, and intestinal phase) that aims to simulate the process of digestion as best as possible, under simulated gastrointestinal conditions [22,23]. The process of consuming such formulations begins in the oral cavity, where they are chewed and mixed with saliva to proceed towards the stomach. In the stomach, gastric fluids cause chemical and mechanical decomposition. The next stage of digestion takes place in the small intestine, where most nutrients are absorbed. From there and before their final elimination, they pass to the large intestine [24].

In the present study, the two APIs (melatonin and pyridoxine) were incorporated into an edible substrate, *stevia*-sweetened couverture chocolate. The choice of such a substrate as a carrier was based on the fact that it is particularly loved by people of all ages, and therefore the patient easily complies with its administration [25]. *Stevia* is a non-calorie sweetener that is 100–300 times sweeter than sucrose, and it belongs to the genus *Stevia* of the *Asteraceae* family [26]. It is a natural sweetener and is used because of its benefits for diabetics and obese people [27]. Accordingly, couverture chocolate was chosen instead of milk chocolate or the particularly popular gummies that are widely used commercially as insomnia medications, because it is considered a vegan-friendly edible product [28,29].

From an analytical point of view, there are several reports on the determination of melatonin or pyridoxine by HPLC-FLD, either individually or in combination with other substances [30–33]. Most of them concern their quantitative determination with UV spectrophotometric [34] or LC-MS [35] techniques.

For the simultaneous determination of the two compounds, most studies are carried out with by gas chromatography–mass spectrometry [36], by multivariate calibration techniques (Partial Least Squares, Principal Component, and Artificial Neural Network) [37,38], or Voltametric methods [39,40]. A brief report of the techniques that have been used as well as their comparison with the method proposed in this work is given in Table 1.

However, according to literature data and to the best of our knowledge, their analysis with HPLC and fluorescence detector is not reported anywhere. Therefore, the development of such a method, which has a low cost and presents a high sensitivity, would be a good alternative proposal.

The aim of this work was to develop a reliable, sensitive, and accurate HPLC-FLD method for the quantitative determination of melatonin and pyridoxine in chocolate dosage form and digestion fluids. The Liquid Extraction method of the two APIs from the chocolate substrate was designed using experimental design methodology and the mixture D-Optimal technique. Subsequently, the release of both APIs in the gastrointestinal tract was studied, with an in vitro three-phase digestion protocol (oral, gastric, and intestinal phase). The overall effort is considered new, since both the composition of melatonin and

pyridoxine in chocolate, as well as the methods of its purification and analysis, are original. In this way, the science of medicine can open new bridges of communication with patients who are not compliant with their medication.

Table 1. Reference to published analytical techniques for the simultaneous determination of PYR and MEL.

Reference Title	Comments
"Simultaneous determination of melatonin and pyridoxine in tablets by gas chromatography–mass spectrometry" [36]	LODs: 5 ng/mL MEL; 2 ng/mL PYR LOQs: 10 ng/mL MEL; 5 ng/mL PYR Specialized software knowledge is required, time-consuming method, and complex and expensive instrument
"Simultaneous spectrofluorimetric and spectrophotometric determination of melatonin and pyridoxine in pharmaceutical preparations by multivariate calibration methods" [37]	LODs in FLD: 0.035 µg/mL MEL; 0.075 µg/mL PYR LOQs in FLD: 0.08 µg/mL MEL; 0.12 µg/mL PYR LODs in UV: 0.5 µg/mL MEL; 0.4 µg/mL PYR LOQs in UV: 1.3 µg/mL MEL; 1.2 µg/mL PYR Specialized software knowledge is required. Can only be used if no interfering substances that absorb or fluoresce coexist
"Simultaneous determination of melatonin–pyridoxine combination in tablets by zero-crossing derivative spectrophotometry and spectrofluorimetry" [41]	LODs in FLD: 287 ng/mL MEL; 42 ng/mL PYR LOQs in FLD: 957 ng/nL MEL; 141 ng/mL PYR LODs in UV derivative: 5.3 ng/mL MEL; 66 ng/mL PYR LOQs in UV derivative: 18 ng/mL MEL; 220 ng/mL PYR Can only be used if no interfering substances that absorb or fluoresce coexist
"Comparative ANNs with Different Input Layers and GA-PLS Study for Simultaneous Spectrofluorimetric Determination of Melatonin and Pyridoxine HCl in the Presence of Melatonin's Main Impurity" [42]	Low sensitivity Specialized software knowledge is required Can only be used if no interfering substances that fluoresce coexist
"Spectrophotometric Estimation of Melatonin and Pyridoxine Hydrochloride in Combined Dosage Forms" [43]	Dynamic range: 1.2–15.6 µg/mL MEL; 12–32 µg/mL PYR
"Spectrophotometric determination of melatonin and pyridoxine HCl in binary mixture using first derivative of the ratio spectra method" [38]	Dynamic range: 1–30 µg/mL MEL; 1–22 µg/mL PYR LODs: 0.17 µg/mL MEL; 0.12 µg/mL PYR LOQs: 0.55 µg/mL MEL; 0.41 µg/mL PYR
"Simultaneous Analysis of Pyridoxine and Melatonin in Tablet Formulation by Derivative Ultraviolet Spectroscopy" [44]	Dynamic range: 0.5–3.5 µg/mL MEL; 2–10 µg/mL PYR LODs: 0.05 µg/mL MEL; 0.26 µg/mL PYR
"Determination of melatonin and pyridoxine in pharmaceutical preparations for health-caring purposes by capillary electrophoresis with electrochemical detection" [45]	Dynamic range: 2.5–1000 µmol/L MEL; 5–1000 µmol/L PYR LODs: 1.3 µmol/L MEL; 2.7 µmol/L PYR Treatment of the electrode
"Simultaneous determination of melatonin and pyridoxine in tablet formulations by differential pulse voltammetry" [46]	Dynamic range: 20–80 µM MEL; 20–400 µM PYR LODs: 5.86 µM MEL; 2.45 µM PYR LOQs: 19.5 µM MEL; 8.15 µM PYR
"Voltammetric determination of melatonin and pyridoxine (vitamin B6) in tablets" [40]	Dynamic range: 5–50 µM MEL; 5–50 µM PYR LODs: 490 nM MEL; 185 nM PYR LOQs: 860 nM MEL; 300 nM PYR Chemically modified electrode that its preparation is time-consuming
"Voltammetric Method for the Simultaneous Determination of Melatonin and Pyridoxine in Dietary Supplements Using a Cathodically Pretreated Boron-doped Diamond Electrode" [39]	Dynamic range: 1–100 µg/mL MEL; 10–175 µg/mL PYR LODs: 0.14 µg/mL MEL; 1.35 µg/mL PYR LOQs: 0.47 µg/mL MEL; 4.49 µg/mL PYR RSDs > 2% Pretreatment of the electrode is required

2. Materials and Methods

2.1. Material and Solutions

Acetonitrile (ACN) and methanol, both of HPLC-gradient grade, were obtained from VWR Chemicals (Radnor, PA, USA). Water of high purity (18.2 MΩ cm resistivity) was

produced by a B30 water purification system (Adrona SIA, Riga, Latvia). Formic acid, FA (Sigma Aldrich, St. Louis, MI, USA), was in the mobile phase, in a concentration of 0.2%.

Melatonin (MEL; purity > 98.0%) and pyridoxine hydrochloride (PYR; purity > 98.0%) (Figure 1), from TCI (Zwijndrecht, Belgium), were used as standard APIs. For the chocolate formulation, a classic chocolate couverture with sweeteners from *Stevia* plant, purchased from a local shop in Thessaloniki, was used.

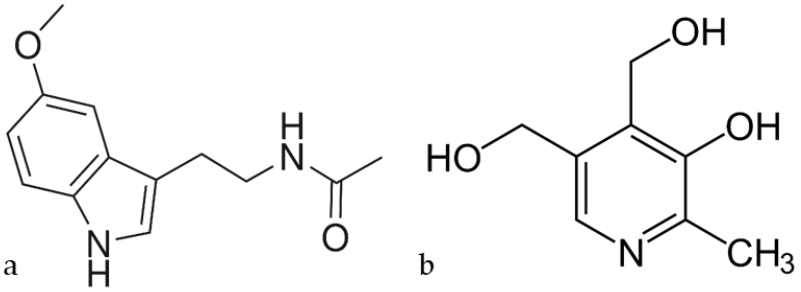

Figure 1. Chemical structure of melatonin (**a**) and pyridoxine (**b**).

2.1.1. Standard Solutions

Standard solutions were prepared according to the following procedure: 40.0 mg of melatonin and 4.0 mg of pyridoxine hydrochloride were accurately weighed and dissolved in 50 mL of methanol. Three successive dilutions (1 mL to 25 mL, 1 mL to 10 mL, and 0.3 mL to 10 mL) were made using ACN with 0.2% formic acid as diluent for the first two dilutions and water for the last one. The final dilution was the stock solution from which 6 standard solutions were obtained, with concentrations ranging from 0.3 to 9.6 ng/mL for PYR and from 3.2 to 96 ng/mL for MEL.

2.1.2. Stimulated Digestion Fluids

In order to study the release of the two APIs, PYR and MEL, from the chocolate formulation, three types of digestion fluids (simulating the GI system) were used: Simulated Salivary (SSF), Simulated Gastric (SGF), and Simulated Intestinal Fluid (SIF) [22]. For their preparation, all salts were purchased from Honeywell (Charlotte, NC, USA) and Chemlab (Zedelgem, Belgium). Enzymes were bought from Sigma (St. Luis, WI, USA). The 1M hydrochloride solution was prepared by diluting 8.33 mL of 37% HCl solution (Merck, Darmstadt, Germany) in 100 mL of water.

Briefly, for the preparation of SSF, a mixture of the appropriate salts at a specific final concentration were used. The salts and their concentrations were as follows: KCl, 15.1 mmol/L; KH_2PO_4, 3.7 mmol/L; $NaHCO_3$, 13.6 mmol/L; $MgCl_2 \times 6\ H_2O$, 0.15 mmol/L; $(NH_4)_2CO_3$, 0.06 mmol/L; and $CaCl_2 \times 2\ H_2O$, 1.5 mmol/L. pH was adjusted to the value of 7 using HCl 1M solution.

Similarly, for SGF, the final concentrations of the salts were as follows: KCl, 6.9 mmol/L; KH_2PO_4, 0.9 mmol/L; $NaHCO_3$, 25 mmol/L; NaCl, 47.2 mmol/L; $MgCl_2 \times 6\ H_2O$, 0.1 mmol/L; $(NH_4)_2CO_3$, 0.5 mmol/L; and $CaCl_2 \times 2\ H_2O$, 0.15 mmol/L. pH was adjusted to the value of 3 using the HCl solution.

Finally, for SGF, the final concentrations of the salts were as follows: KCl, 6.8 mmol/L; KH_2PO_4, 0.8 mmol/L; $NaHCO_3$, 85 mmol/L; NaCl, 38.4 mmol/L; $MgCl_2 \times 6\ H_2O$, 0.33 mmol/L; and $CaCl_2 \times 2\ H_2O$, 8.4 mmol/L. pH was adjusted to 7 using the HCl 1M.

For the oral-phase solution, 3.525 mL of SSF was added, along with 0.5 mL salivary α-amylase solution of 1500 U/mL (diluent SSF) and 975 μL of water.

For the gastric phase solution, the oral bolus was mixed with 7.505 mL of SGF, 1.6 mL porcine pepsin stock solution of 25,000 U/mL (diluent SGF), 0.2 mL of 1 M HCl, and 695 μL of water.

For the intestinal-phase solution, the gastric chyme was mixed with 11.04 mL of SIF, 5.0 mL of a pancreatin solution 800 U/mL (diluent SIF), 2.5 mL fresh bile (160 mM in fresh bile), and 1.31 mL of water. The final pH was adjusted at 7.

It is also worth mentioning that $CaCl_2 \times 2\ H_2O$ was added at the end, in the oral/gastric/intestinal-phase solution, and before the addition of water.

2.2. Instrumentation and Chromatographic Conditions

Chromatographic separations were performed using a Shimadzu HPLC system, consisting of two LC-20AD pumps, a DGU 14A degasser, a SIL-10AD autosampler (injection volume 30 μL), and a fluorescence detector, RF20-A (Shimadzu, Tokyo, Japan). Detector was set at Gain × 16 and a high sensitivity. The two analytes were detected with two pairs of excitation and emission values ($\lambda_{exc}/\lambda_{em}$): 290/390 nm for PYR and 602/674 nm for MEL. The analytical column was a reversed-phase Aqua Evosphere Fortis® (250 cm × 4.6 mm, 5 μm), thermostatically controlled with a CTO-10AS VP heated chamber (Shimadzu, Tokyo, Japan) at 45 °C.

The gradient elusion was performed with two mobile phases: A, water with formic acid 0.2%; and B, acetonitrile with formic acid 0.2%, and a flow rate of 1.2 mL/min. Data processing was carried out using the LC solution software version 1.25 SP4.

For the in vitro digestion study and the APIs release rate (from the formulation), a Thermostatic Shaking Water Bath set at 37 °C from Witeg (Wertheim, Germany) was used. In addition, during the process, the samples were centrifuged in a Labofuge® 400 R centrifuge from Thermo (Waltham, MA, USA) and lyophilized with a Virtis Advantage Plus device from SP Scientific (Warminster, PA, USA).

2.3. Formulation

For practical reasons, it was considered appropriate that the new chocolate formulation prepared in the laboratory should contain quantities of the two active ingredients equal to those existed in a corresponding commercial formulation, Zquil®, which is marketed in gum form (1 mg MEL and 1.4 mg PYR).

Therefore, to cover the total requirements of the planned experiments, 25 dosage units were prepared according to the following procedure: 25.0 mg of accurately weighed MEL was quantitatively mixed with 10 mL of 20% *w*/*w* propylene glycol [39]; meanwhile, in a separate container, 35.0 mg of PYR was dissolved in 3 mL of water. These were then mixed, and after homogenization, 50 g of melted couverture was gradually added (while mixing). Throughout the process, both mixtures were maintained in a water bath at 45 °C. The final chocolate composition, before solidification, was divided into 25 pre-weighed silicone molds (2 g/dose unit), which were placed at 2 °C.

2.4. Sample Pretreatment

The sample processing for the quantitative recovery of both MEL and PYR, from the chocolate composition (2.0 g), included the following steps: Initially, a pre-weighed chocolate bar (equivalent to one dosage form) was placed in a water bath at 45 °C, until melted. Then, 25 mL of extract diluent (ACN FA 0.2% -MeOH FA 0.2% 49.8: 0.2 *v*/*v*) was added, and the sample was subjected to 2 consecutive cycles of sonication and agitation for a total duration of 38 min. Subsequently, the sample was frozen (15 min), centrifuged (3 min at 6000 rpm), and placed in the freezer for 8 h. Then, 200 μL of the supernatant was diluted with water to a final volume of 10 mL, filtered (0.45 μm PTFE filter), and analyzed with the proposed HPLC-FLD analytical method.

2.5. In Vitro Digestion Method

The protocol for the in vitro digestion method, proposed by M. Minekus and coworkers, involves 3 sub-phases: oral, gastric, and intestinal phase [23].

In order to simulate the chewing process, the preparation was cut into small chocolate flakes. Then, 5 mL of the oral solution was added to each dosage form (chocolate flakes), and the mixture was placed in the shaking water bath, at 37 °C, for 2 min. After the first stage was complete, 10 mL of gastric solution was added to the oral bolus. The mixture was left for 2 h in the shaking water bath at 37 °C (gastric phase) and then used for the intestinal phase. In the final digestion phase, gastric chyme was mixed with 19.85 mL intestinal solution (pH 7) and left for 2 h in the shaking water bath (37 °C).

During the study, samples (200 μL) were taken at regular intervals of 2 min (oral phase); 1st and 2nd hour (gastric phase), at 2.5 h; and 3rd and 4th hour (intestinal phase). When the experiment was completed, the total final mixture (solution and residue) was frozen (10 min) and centrifuged (10 min, 45,000 rpm), alternately, in two cycles of repetitions of a total time of 40 min. The supernatant was removed by pouring, and the precipitate was collected in order to determine the two APIs incorporated into the chocolate. Next, 25 mL of extract diluent (ACN FA 0.2%-MeOH FA 0.2% 49.8: 0.2 v/v) was added, and the sample was subjected to 2 consecutive cycles of sonication and agitation for a total duration of 38 min. Subsequently, the sample was frozen (15 min), centrifuged (3 min at 6000 rpm), and placed in the freezer for 8 h. Then, 200 μL of the supernatant was diluted with water to a final volume of 10 mL, filtered (0.45 μm PTFE filter), and analyzed with the proposed HPLC-FLD analytical method.

The pretreatment of the samples (collected from the samplings) was necessary to remove the digestion salts. Thus, initially they were lyophilized and then reconstituted by adding 500 μL of extraction diluent. The samples were placed in the ultrasonic bath (38 min), then for 15 min in the freezer, 3 min of centrifugation (6000 rpm), and another 8 h of freezing. Then, 300 μL of the supernatant, after being collected from each sample, was diluted with water to a final volume of 3 mL and filtered. After that, 100 μL of the filtrate was diluted to a final volume of 1 mL and analyzed with the proposed HPLC method.

The cumulative release rate of pyridoxine and melatonin was calculated based on the following equation:

$$Q\ (\%) = 100\ \times\ \frac{(C_t \times V_0) + (\Sigma C_i \times V)}{M_{total}}$$

where Q (%) is the cumulative release rate (%) at time t, C_t is the concentration of the drug in the release medium at the current time point t (mg/mL), V_0 is the total volume of the release medium (mL), C_i is the concentration of the drug in the release medium at each previous sampling time point (mg/mL), V is the volume of the aliquot (mL), and M_{total} is the total amount of the pyridoxine and melatonin initially loaded in the system.

3. Results and Discussion

3.1. HPLC Method Optimization

In HPLC analysis methods, the degree of difficulty for the simultaneous determination of two or more analytes depends mainly on their different lipophilicity (LogP) and molecular weight. The task becomes even more complicated when one of them is very polar, so it does not interact with the stationary phase and elutes with the solvent front. In the present study, this behavior was exhibited by PYR, which is a highly polar compound (logP = −0.57) with a small size (Supplementary Table S1) that is poorly retained on reversed-phase columns [40].

Regarding the ionization, based on the pka values of the two analytes, acidic pH does not affect them, and they remain ionized in terms both of their basic and acidic group. The

only case in which it would be possible to have a differentiation in their chromatographic behavior is to use a mobile phase at pH > 6, where the basic group of PYR does not ionize, while the acidic one remains ionized. However, in a preliminary study of the fluorometric behavior of the substance, it was found that at pH = 6.17, the intensity of its emission signal decreased (approximately 40%), which was not desirable. A corresponding study of melatonin shows approximately the same behavior at both alkaline and acidic pH levels. From a chromatographic point of view at pH = 6.17 (adjusted with phosphate buffers), the retention time of PYR was delayed by 4 min, while that of MEL remained the same. Taking into account all of these data and especially the fact that PYR gives a significant emission signal only under specific conditions, acidic pH was chosen as the optimal pH value of the mobile phase.

Therefore, it was necessary to find a mobile phase with a low elution power at the beginning to delay PYR and then find another one strong enough to elute MEL (relatively lipophilic). Thus, the use of a gradient elution mobile phase was inevitable. In the proposed analytical method, factors that could improve the shape of the chromatographic peaks, the resolution of the system, and the elution times of the analytes were investigated. A combination of acetonitrile with water and 0.2% formic acid (in gradient elution) was initially used as a binary mobile phase system. The addition of formic acid was considered necessary because it improved the shape of the chromatographic peaks and kept melatonin stable [47]. Formic acid was chosen over other buffers because it gave less noise in the baseline.

Regarding the stationary phase, six columns were tested in order to find the one that could separate the polar pyridoxine from the solvent front but would also elute melatonin quickly. Specifically, a C18 Supelco Discovery HS column (250 mm × 4.6 mm, 5 μm), a C8 Waters Spherisorb® (125 mm × 4.6 mm, 5 μm), a CN Waters Spherisorb® (250 cm × 4.6 mm, 5 μm), a Phenyl-ACE® (150 mm × 4.6 mm, 5 μm), an Aqua Evosphere Fortis® (250 cm × 4.6 mm, 5 μm), and a SeQuant® ZIC®-HILIC (150 mm × 4.6 mm, 5 μm) were used. Of these, the phenyl, CN, and C8 columns were not effective and were rejected from the beginning, since in addition to a low separation efficiency (Rs < 1), they eluted PYR with the solvent front. Hilic, being a column that interacts with hydrophilic substances, works in reverse and retains the polar pyridoxine more strongly, compared to melatonin. But in this column, the problem was reversed: melatonin eluted with the solvent front. The separation problem was improved by applying a C18 column with larger dimensions (250 mm × 4.6 mm, 5 μm), but PYR continued to elute with the solvent front (90% aqueous mobile phase). This observation led to the use of an Aqua column that interacts more significantly with polar molecules and can be used even with 100% aqueous mobile phase [42]. Thus, by first applying a 100% aqueous mobile phase, pyridoxine was sufficiently separated from the solvent front. Then, it was necessary to increase the concentration of the organic solvent, linearly applying a gradient elution in order to quickly elute melatonin as well. The rate of increase was investigated in a concentration range of 20–70%. Of these values, 30% was chosen as the optimal value, with a low background signal (Supplementary Table S2).

The mobile-phase flow rate was set at 1.2 mL/min, and the oven at 45 °C, to reduce the total analysis run time and keep the system pressure low (<1800 psi). A factor that also had to be taken seriously into consideration, because it determined the presence or absence of additional peaks when applying gradient elution, was the diluent of the analytes. Many diluents were studied and in various combinations and ratios, such as water; water with 0.2% formic acid; acetonitrile; acetonitrile with 0.2% formic acid; and water with 0.2% formic acid and acetonitrile with 0.2% formic acid in a ratio of 1: 1 v/v, in a ratio of 0.3:0.7 v/v, and in a ratio of 0.8:0.2 v/v without formic acid. The worst result was given

with methanol and 0.2% formic acid, while the best was given with water or water and acetonitrile (0.2:0.8 *v*/*v*). Of the two diluents, the first was chosen to be used as the simpler.

Finally, it was important to choose the appropriate wavelength pairs ($\lambda_{exc}/\lambda_{em}$) for the detection and emission of the analytes. In the case of pyridoxine, the 290/390 nm pair was chosen as optimal, as the compound gave a strong fluorescence signal. Similarly, for melatonin, various $\lambda_{exc}/\lambda_{em}$ wavelengths were investigated: 290/390, 300/380, 300/390, 300/410, and 674 nm. The problem was that when the $\lambda_{exc}/\lambda_{em}$ was not so selective, melatonin gave a very high signal, but at the same time, there was instability in the baseline. In contrast, in the excitation pair at 602 nm and emission at 674 nm, the melatonin peak was clean, without interference but with reduced sensitivity. Ultimately, 602/674 nm was chosen as the optimal solution.

3.2. HPLC Method Validation

The validation of the HPLC method was performed according to the International Conference on Harmonization of Technical Requirements for the Registration of Medicinal Products for Human Use (ICH Q2) guidelines [48]. Specificity, linearity, repeatability, intermediate precision, accuracy, and robustness were tested.

3.2.1. Specificity

To examine specificity, it was necessary to prepare at least one blank and one standard sample and evaluate the chromatograms. In the present experimental conditions, water (final diluent of analytes) and a solution obtained from the extraction of a chocolate substrate were tested as blanks for the quantification of the formulation. For the in vitro digestion experiments, three blank solutions (at SSF, SGF, and SIF diluents) were also tested as blank. The absence of peaks in the blank chromatograms at the same elution times (tr) as those of the two analytes, as shown in Figure 2, leads to the conclusion that the method exhibits specificity.

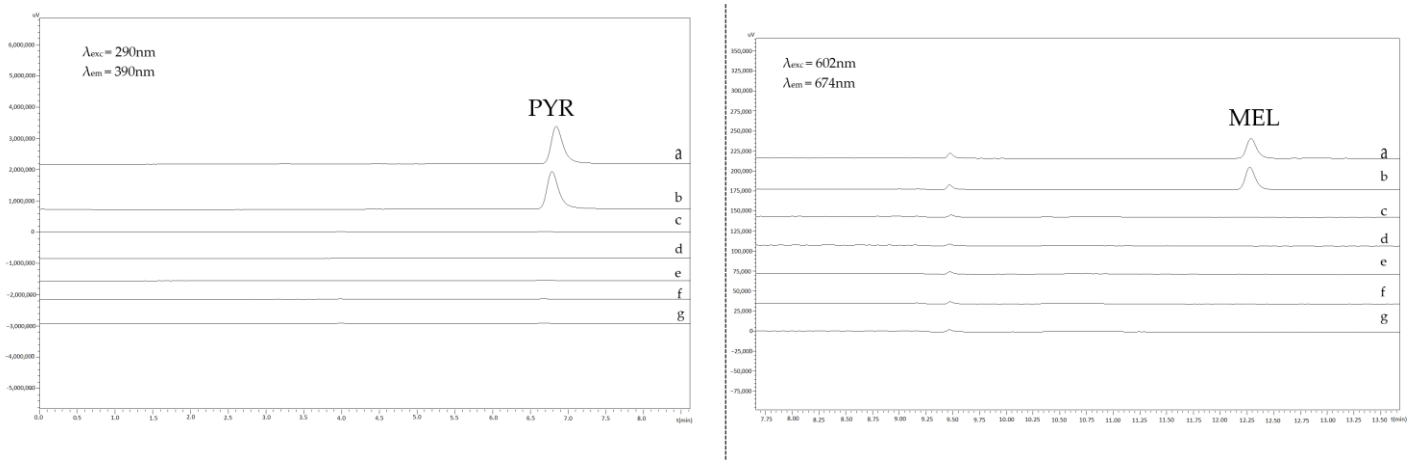

Figure 2. Typical chromatograms of (a) standard mix solution of MEL (right) and PYR (left), (b) sample extracted from chocolate, (c) blank diluent, (d) blank of chocolate, (e) SSF blank, (f) SGF blank, and (g) SIF blank.

3.2.2. Linearity, Limit of Detection (LOD), and Limit of Quantitation (LOQ)

Linearity was investigated in seven different concentration ranges. Each standard was analyzed in triplicate. The good linearity of the method was also estimated by the % y-intercept values (intercept value × 100/AUC at 100% response) for each analyte, which should be <2%. The results of linear regression, correlation coefficient, limits of detection

(LODs), and quantitation (LOQs) for MEL and PYR were also determined (Table 2). The signal-to-noise ratio technique was used to calculate the LOD and LOQ values [49].

Table 2. Linear regression analysis data of the analytes and LOD and LOQ values.

Analytes	Concentration Range (ng/mL)	Linear Regression	%y Intercept	Correlation Coefficient	LOD (ng/mL)	LOQ (ng/mL)
PYR-HCl	0.3–9.6	y = 1387575.43 ± 36854.28 x + 17874.82 ± 202851.86	0.1	0.999	0.09	0.29
MEL	3.2–96	y = 3155.3 ± 113.7 x + 5886.3± 6257.71	1.5	0.999	0.92	3.04

3.2.3. Precision

Precision was studied for both repeatability and intermedia precision. Within-day repeatability was tested at three concentration levels (low, medium, and high), with five replicates for low concentration and three for the others. For intermedia precision, three levels of concentration (low, medium, and high) were also tested, with three replicates on three consecutive days. The results are shown in Table 3.

Table 3. Repeatability and intermediate precision results.

Analytes	Repeatability		Intermediate Precision				
	Concentration (ng/mL)	%RSD	Concentration (ng/mL)	%RSD			
				1st Day	2nd Day	3rd Day	Total
PYR	0.32 (n = 5)	0.57	0.32 (n = 5)	0.57	0.77	0.96	1.32
	4.8 (n = 3)	1.47	4.8 (n = 3)	1.47	1.97	1.84	1.88
	9.6 (n = 3)	0.30	9.6 (n = 3)	0.30	0.46	1.45	1.40
MEL	3.2 (n = 5)	1.66	3.2 (n = 5)	1.66	1.08	1.19	1.26
	48 (n = 3)	1.55	48 (n = 3)	1.55	1.55	0.87	1.59
	96 (n = 3)	1.06	96 (n = 3)	1.06	0.89	0.61	1.01

3.2.4. Accuracy

The precision of an analytical method is expressed as % recovery of the analyte in the sample. In the present study, five standard solutions of both APIs, with known concentrations, were prepared and re-calculated, based on linear regression (Table 4).

Table 4. The % recovery values of pyridoxine and melatonin.

Concertation PYR-HCl (ng/mL)	Found PYR-HCl (ng/mL)	Recovery (%) ±sd (n = 3)	Concertation MEL (ng/mL)	Found MEL (ng/mL)	Recovery (%) ±sd (n = 3)
0.32	0.324	101.3 ± 1.7	32	32.31	101.0 ± 1.9
1.92	1.916	99.8 ± 1.2	19.2	19.34	100.7 ± 1.4
5.76	5.70	99.0 ± 0.9	57.6	57.89	100.5 ± 0.8
7.68	7.70	100.3 ± 0.6	76.8	76.02	99.0 ± 0.9
9.6	9.55	99.5 ± 1.1	96	96.04	100.0 ± 0.6

The % recovery values were satisfactory in all cases, and the method was deemed adequate and within the permitted limits.

3.2.5. Robustness

A method can be considered robust if small changes in column temperature, flow rate, $\lambda_{exc/em}$, and mobile phase composition have minimal effect (expressed as %RSD) on the tailing factors (Tfs) and peak areas under the curve (AUC) for both analytes (Table 5).

Table 5. Robustness investigation.

Parameters	%RSD			
	PYR-HCl		MEL	
	AUC	Tf	AUC	Tf
Mobile phase A:B (100:0, 99:1, 98:2)	1.01	1.19	0.98	1.33
Flow Rate mL/min (1.20, 1.25, 1.15)	3.09	0.86	2.51	0.66
Column T (°C) (44, 45, 46)	0.56	1.86	1.14	0.18
$\lambda_{exc/em}$ (391/291, 390/290, 289/389 and 601/673, 602/674, 603/675)	0.74	0.36	0.97	0.60

According to the results of Table 5, small changes in the chromatographic analysis conditions do not affect the robustness of the method, as the %RSD values are <2. The only exception, was the effect of the flow rate on AUC values, as expected.

3.3. Sample Extraction Procedure

In an attempt to design a liquid–solid extraction process for chocolate, it was necessary to find solvents that could quantitatively extract MEL and PYR from the substrate. It was also particularly important that the proposed solvents were selective, so as not to dissolve sugars (e.g., water) or fatty components of the couverture. Thus, acetonitrile and methanol were selected as the most suitable (Supplementary Table S1), while their concentration ratio was determined after investigation. Specifically for the solubility of PYR, MEL in acetonitrile, due to lack of bibliographic references, was tested in a sample of twice the concentration of the two substances than they are found in the formulation.

The sample, after being subjected to ultrasound for 19 min, was quantified (in relation to a corresponding standard solution in methanol solvent), and it was found that both active ingredients dissolved sufficiently in acetonitrile.

Another factor that contributes to the quantitative recovery of the actives and needs to be investigated was the time of processing the sample in the ultrasonic bath. Finally, for greater clarification of the extract, the freezing time played an important role since it helps in the precipitation of fatty components [50]. Since the factors under investigation were many, they were studied using the mixture D-Optimal methodology of experimental design (Design-Expert 11) [51]. According to this technique, a mixture of (A) methanol and (B) acetonitrile (total volume 50 mL) was cross-corelated with the factors (C) stirring/sonication time and (D) freezing time. The number of experiments proposed was 18, and the limits of the exploration values are presented in Table 6.

The proposed model (Table 7) was evaluated by analysis of variance (ANOVA) and was found to be overall significant ($p < 0.05$ and $F > 3.2$). The Adeq. Precision, which measures the signal-to-noise ratio, was greater than 4, whereas the Predicted R^2 values were in reasonable agreement with the Adjusted R^2.

Table 6. Constrains, units, mixture components' data, process factors, and responses.

Mixture Components					
Component	**Name**	**Units**	**Type**	**Minimum**	**Maximum**
A	MeOH	mL	Mixture	0	25
B	ACN	mL	Mixture	25	50
				Total =	50.0
Process Factors					
Factor	**Name**	**Units**	**Type**	**Minimum**	**Maximum**
C	Sonic/stirring	min	Numeric	20.0	60.0
D	Freezing	h	Numeric	0.5	12.0
Responses					
Response	**Name**	**Units**	**Observations**		
R1	B6	% Recovery	18		
R2	Melatonin	% Recovery	18		

Table 7. Statistical results of the proposed model with ANOVA.

	PYR		**MEL**		
Source	**F-Value**	***p*-Value**	**F-Value**	***p*-Value**	
Model	8.27	0.0021	3.55	0.0423	
Linear Mixture	5.87	0.0295	3.20	0.0952	
AB (A–B)	18.80	0.0007	7.23	0.0176	
	PYR	MEL		PYR	MEL
Std. Dev.	2.87	3.26	R^2	0.6391	0.4323
Mean	97.77	98.51	Adjusted R^2	0.5618	0.3106
C.V. %	2.94	3.31	Predicted R^2	0.4321	0.1735
			Adeq. precision	7.8434	4.8899

Based on the results, only factors A and B play a decisive role in the effectiveness of the method and not the other two. Of course, these observations are valid, provided that the sample has been subjected to at least the minimum prescribed freezing and sonication time. At this point, it should also be explained that the clarification of the sample, with the help of freezing, aims mainly to protect the column and is therefore necessary regardless of the objectives of the model.

Figure 3 presents, in three dimensions, the effect of the mixture of the two solvents on the two responses (% B6 Recovery and % Melatonin Recovery) as a function of factor C. According to the diagram, three combinations of methanol–acetonitrile mixture yield the best results.

To find the best solution of the model, a mathematical approach based on the desirability function was applied. According to the results, the model suggests the use of a solvent mixture of 0.2 mL of MeOH and 49.8 mL of ACN, with an optimal sonication time of 38 min and a freezing time of 8 h (Figure 4).

The proposed method was applied to five chocolate samples. In parallel, three blank samples were analyzed in order to check the selectivity in the chocolate formulation as well. The % recoveries were found to be 97.4% (%RSD = 2.97) for pyridoxine and 100.0% (%RSD = 1.59) for melatonin. No peaks were found in the blank samples at the elution times of the APIs, and therefore the proposed method can be considered to be specific.

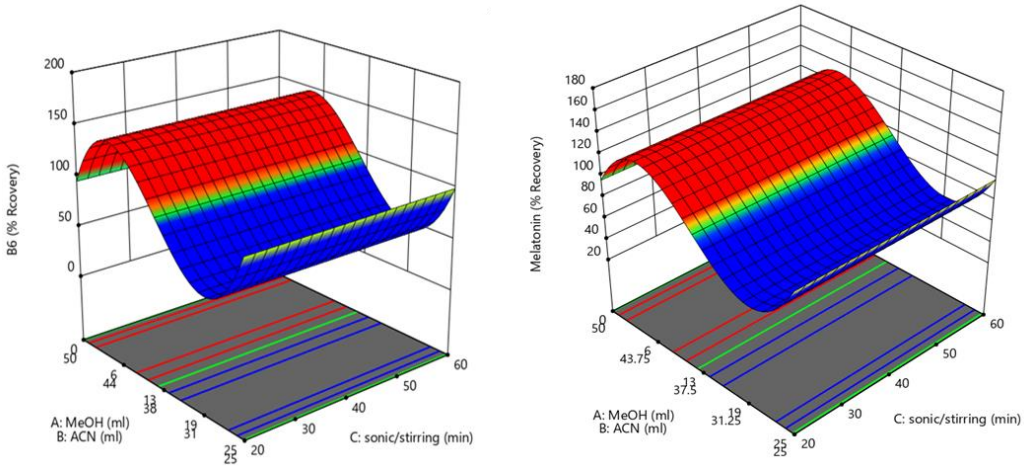

Figure 3. Effect of mixture composition and sonication time on % recovery values of responses.

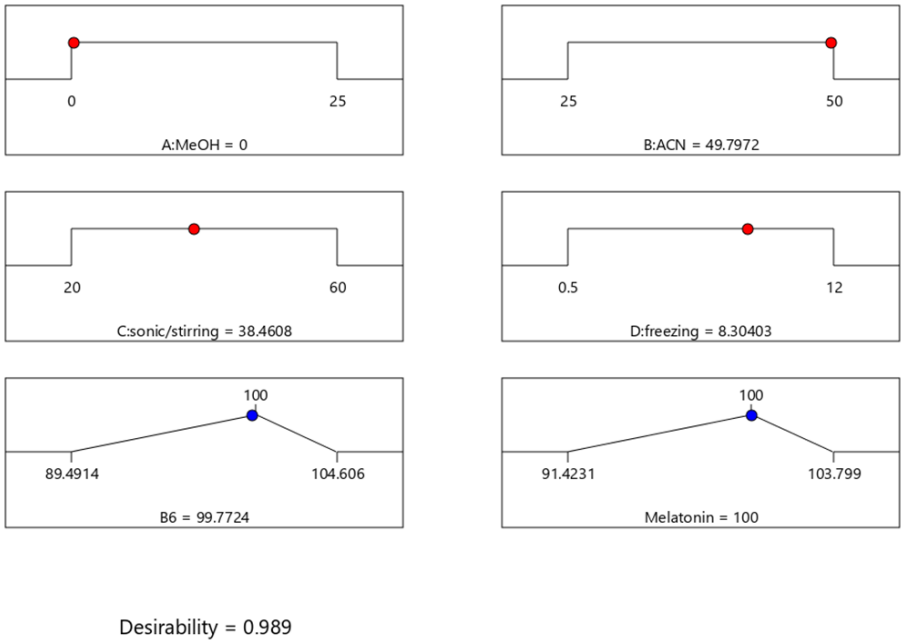

Figure 4. Suggested optimal values of factor A, MeOH (mL); B, ACN (mL); C, sonication time (min); and D, freezing time (min) and predicted % recovery values of B6 and melatonin.

3.4. Short-Term Stability Test

In order to evaluate the compatibility of the two active ingredients with the substrate, a basic stability study was conducted over a short period of time (seven days). In more detail, the quantitative determination of the two active ingredients was carried out at time 0 on the 1st, 3rd, 5th, and 7th day, in a chocolate preparation stored at 2 °C.

The quantitative determination of the two substances, their retention time, and the presence/absence of other additional peaks were used as evaluation criteria. The recovery values of the substances showed a deviation of <3%, while no additional peaks were present in the chromatogram.

3.5. In Vitro Analysis of Digestion for Chocolate-Based Drug Formulations

This research highlighted the suitability of chocolate as an effective carrier for the oral administration of melatonin and vitamin B6 (pyridoxine-HCl). The release behavior of these compounds from the chocolate matrix was analyzed by measuring the amount of drugs released in simulated gastrointestinal fluids at different digestion stages. The

in vitro digestion model that was used mimicked three phases: oral, gastric, and small-intestinal [22]. The small-intestine phase received particular attention, as it represents the primary absorption site for most compounds, including melatonin [20] and vitamin B6 [52]. Drug release during each phase was quantified and expressed as a cumulative percentage of the initial drug content in the chocolate-based formulations.

As shown in Figure 5, melatonin release exhibited a sharp increase during the intestinal phase, tripling compared to the final gastric phase and achieving a cumulative release of about 50% by the end of this phase. In contrast, vitamin B6 showed a steady release across all phases, eventually reaching a plateau at approximately 70% after one hour in the intestinal phase. This behavior is likely due to the enzymatic activity of pancreatin, which breaks down triglycerides into more soluble fatty acids, facilitating the dissolution of the lipid matrix and enhancing the release of both compounds [53].

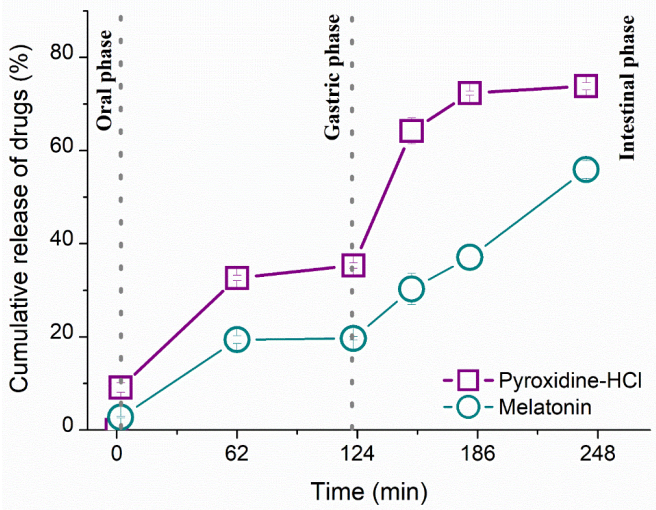

Figure 5. Release profiles of melatonin and pyridoxine-HCl from chocolate dosage forms during in vitro simulated gastrointestinal conditions. The dotted lines indicate the end of each phase and the beginning of the subsequent phase. Error bars represent the standard deviation (n = 3).

During the intestinal phase, extensive disintegration of chocolate particles was observed, resulting in a significant increase in melatonin release. This effect was attributed to the neutral pH of the simulated intestinal fluid (SIF) and the presence of bile salts, as melatonin demonstrates greater solubility at neutral pH levels [54]. The proportion of drugs retained in the sediment was quantified as 27.68 $\pm$ 0.95% for vitamin B6 and 44.67 $\pm$ 0.84% for melatonin, suggesting that the theoretical amount of both drugs was effectively incorporated into the dosage forms.

4. Conclusions

A flexible chromatographic HPLC-FLD method suitable for the quantitative determination of melatonin and pyridoxine was developed and validated. The method was applied for the determination of both substances in a chocolate pharmaceutical preparation in routine analyses tests. The extraction process of the drugs was designed using the D-Optimal experimental design methodology. The proposed procedure, due to its high sensitivity, was successfully applied to study the digestion of the product in the gastrointestinal tract. Similarly, it could be applied to the next step of a study of a new product, the step known as permeability and bioequivalence testing, in order to obtain approval from the National Medicines Agency. The developed chocolate composition can serve as a springboard for the synthesis of future pharmaceutical products in "favorite" substrates, with the aim of achieving patient compliance.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/chemistry7010014/s1, Table S1: Physicochemical properties of melatonin and pyridoxine-HCl; Table S2: Gradient elution program of mobile phase.

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