APPLICATIONS

HPLC-UV Analysis of Melatonin

Method Status: Scientifically Valid per cGMPs for Dietary Supplements

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Introduction

The verification of label claim data in nutraceutical formulations has come under scrutiny by the FDA. Manufacturers and contract testing labs alike are looking for accurate and scientifically valid methods that are suitable for use with different formulations. The complex nature of nutraceuticals and botanicals often requires long analysis and difficult sample cleanup steps to resolve matrix interferences.

There were two primary goals of this project: 1) to optimize our pre-exisiting method to reduce total analysis time using newer high efficiency HPLC technologies and 2) demonstrate the suitability for analysis of two commercially available formulations of Melatonin.

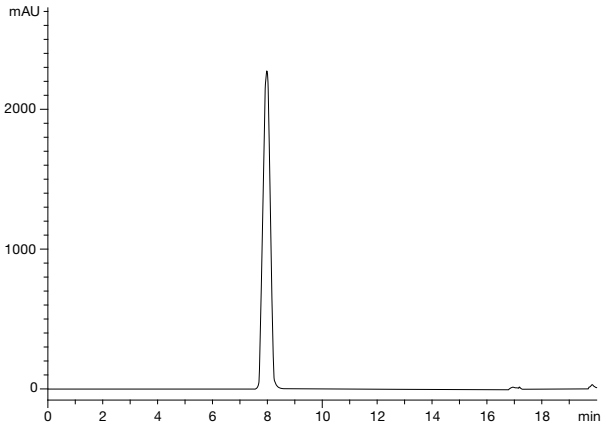
Therapeutic Use Overview

Melatonin is a neurohormone naturally occurring in several animals and plants. Melatonin levels typically cycle in a daily pattern playing a role in the natural circadian rhythms of several biological functions. Melatonin containing dietary supplements have been available in the US since the mid 1990s and are used as sleep aids.

Experimental

HPLC analysis was performed using an Agilent® 1100 LC System (Agilent Technologies Inc., Palo Alto, CA, USA). The system was optimized in order to reduce dead volume and improve performance including increasing the UV scan rate, changing the injector needle seat, re-plumbing the system with red PEEKsil™ Tubing (SGE), and using a semi-micro flow cell. The fully porous Luna® 5 µm C18(2) 150 x 4.6 mm, Luna 2.5 µm C18(2) HST 100 x 2.0 mm, and the Kinetex® Core-Shell Technology 5 µm C18 50 x 4.6 mm HPLC columns were from Phenomenex, Torrance, CA. All chromatographic conditions are specified on their corresponding chromatograms in **Figures 1 through 8**.

Figure 1.
Melatonin Reference Standard (0.5 mg/mL) Using Luna® 5 µm C18(2) Column

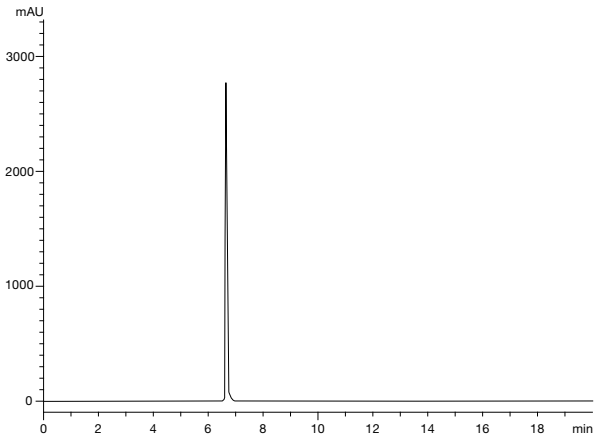


Column: Luna 5 µm C18(2)
Dimension: 150 x 4.6 mm
Part No.: 00F-4252-E0
Mobile Phase: A: 0.25 % Phosphoric acid in Water
B: Acetonitrile
Flow Rate: 1.5 mL/min

Gradient: Time (min)	% B
0	20
15	20
15.5	90
18	90
18.5	20
20	20

Injection Volume: 20 µL
Temperature: 25 °C
Detection: UV @ 225 nm
Back Pressure: 133 bar

Figure 2.
Melatonin Reference Standard (0.5 mg/mL) Using Luna 2.5 µm C18(2) HST Column

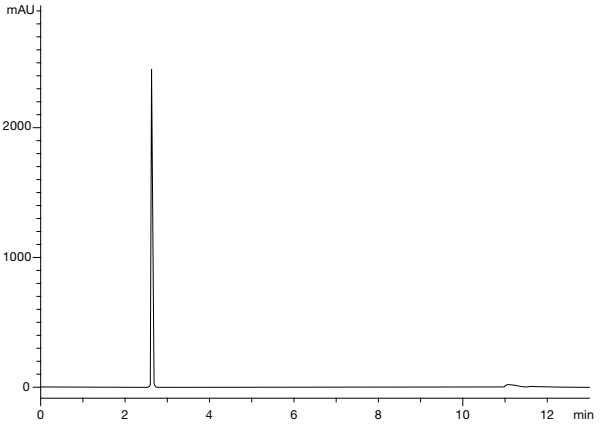


Column: Luna 2.5 µm C18(2) HST
Dimension: 100 x 2.0 mm
Part No.: 00D-4446-B0
Mobile Phase: A: Water
B: Acetonitrile
Flow Rate: 0.5 mL/min

Gradient: Time (min)	% B
0	10
1	10
9	40
10	75
13	75
14	10
18	10

Injection Volume: 3 µL
Temperature: 50 °C
Detection: UV @ 225 nm
Back Pressure: 303 bar

Figure 3.
Melatonin Reference Standard (0.5 mg/mL) Using Kinetex 5 µm C18 Column



Column: Kinetex 5 µm C18
Dimension: 50 x 4.6 mm
Part No.: 00B-4601-E0
Mobile Phase: A: 0.25 % Phosphoric acid in Water
B: Acetonitrile
Flow Rate: 1.5 mL/min

Gradient: Time (min)	% B
0	10
7	70
8	90
10	90
10.1	10
13	10

Injection Volume: 3 µL
Temperature: 30 °C
Detection: UV @ 225 nm
Back Pressure: 133 bar

For additional technical notes, visit www.phenomenex.com

APPLICATIONS

Figure 4.
Representative Calibration Curve for Melatonin from 3 to 300 µg/mL

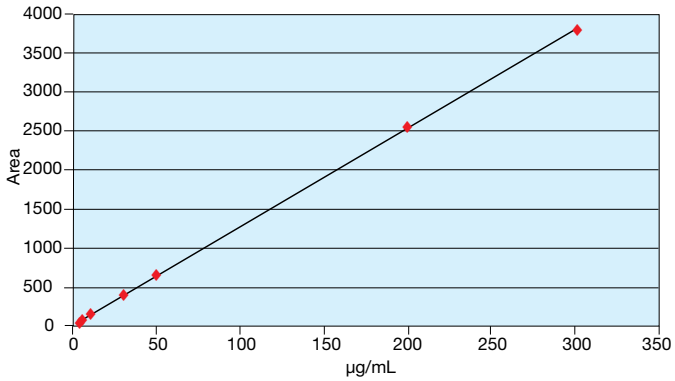


Table 1.
Calibration Curve from Kinetex® 5 µm C18 Core-Shell Technology Column Based on ChromaDex® Reference Materials

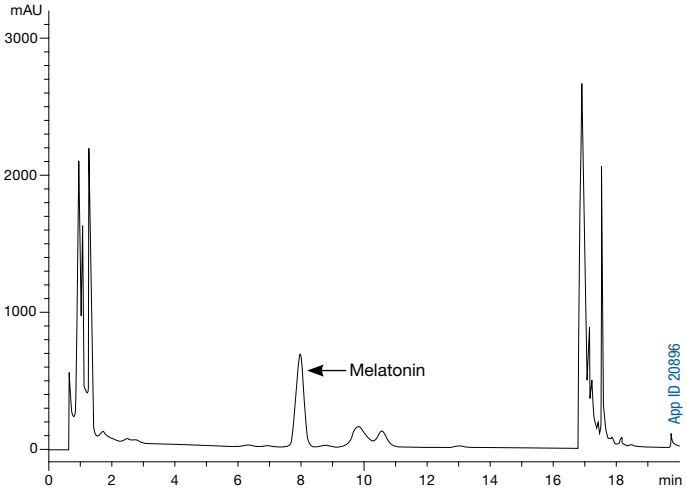
Compound	R^2	LOQ (µg/mL)	
Sample 1	50.57	199.86	298.99
Sample 2	50.72	202.63	299.65
Sample 3	50.45	199.39	299.55
Average	50.58	200.63	299.40
STDEV	0.14	1.75	0.36
% CV	0.27	0.87	0.12

Table 2.
Determination of Method Accuracy

	50 µg/mL	200 µg/mL	300 µg/mL
Sample 1	50.57	199.86	298.99
Sample 2	50.72	202.63	299.65
Sample 3	50.45	199.39	299.55
Average	50.58	200.63	299.40
STDEV	0.14	1.75	0.36
% CV	0.27	0.87	0.12

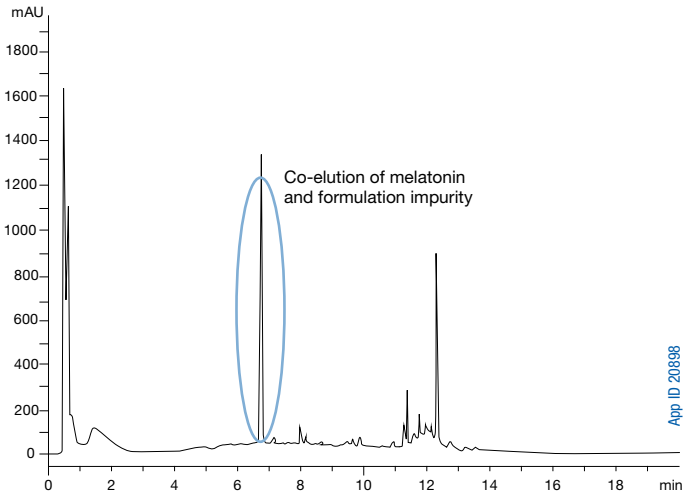
The commercial melatonin formulation gelcaps were dissolved whole in acetonitrile/water (50:50) while the tablet formulation was dissolved whole in methanol/water (50:50). Each sample was then sonicated for 10 minutes and was filtered using Phenex™ 0.45 µm PTFE syringe filters and then transferred to a Verex™ autosampler vial. Analysis of gelcaps and tablets are depicted in **Figures 5 through 7**.

Figure 5.
Analysis of Commercial Gelcap Formulation Using Luna® 5 µm C18(2) Column



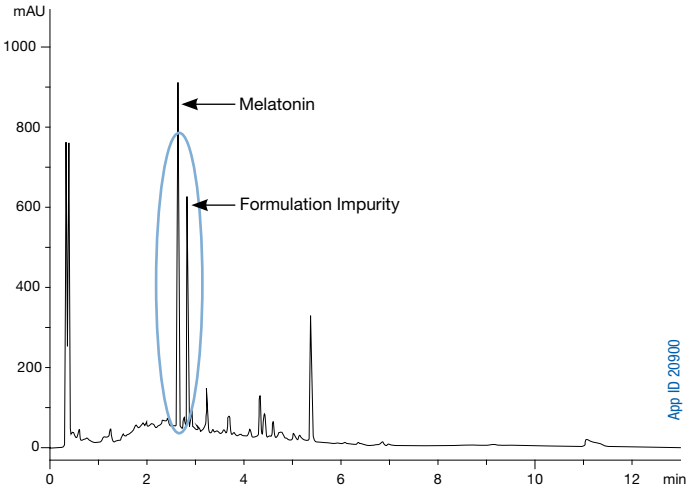
Running conditions can be found in **Figure 1**

Figure 6.
Analysis of Commercial Gelcap Formulation Using Luna 2.5 µm C18(2) HST Column



Running conditions can be found in **Figure 2**

Figure 7.
Analysis of Commercial Gelcap Formulation Using Kinetex® 5 µm Core-Shell Technology Column



Running conditions can be found in **Figure 3**

Table 3.
Experimental Results as Compared to Label Claims

	Label Claim Melatonin (mg)	**Experimental Results** Melatonin (mg) n = 3	**RSD (%)**
Gelcap	1	1.52	1.01
Dry capsule	3	2.90	5.43

Results and Discussions

The original method supplied by ChromaDex®, Inc. required more than 8 minutes using the Luna® 5 µm C18(2) column and more than 6 minutes using the Luna 2.5 µm C18(2) HST column to analyze melatonin. In addition to longer run times, co-elution of an impurity was observed using the Luna 2.5 µm C18(2) HST column during the analysis of a gelcap. This separation is based primarily on the efficiency of the column rather than the selectivity of the C18 stationary phase, making it difficult for traditional particle technologies to resolve the impurity and melatonin.

Kinetex Core-Shell Technology columns allow scientists to achieve substantially higher chromatographic efficiencies at much lower pressures than the equivalent fully porous material, making it ideal for this separation. The Kinetex Core-Shell Technology enhances the performance of any existing HPLC platform, including UHPLC systems. For those labs that have older HPLC systems without high pressure capabilities, the Kinetex 2.6 µm and 5 µm materials can substantially improve the useable lifetime of these systems.

The increased efficiency of the Kinetex Core-Shell Technology 5 µm allowed the method to be shortened by more than 50 % as compared to both of the Luna methods with 75 % less backpressure. Not only was the analysis time reduced, resolution between melatonin and an impurity in the gelcap were also resolved using the Kinetex Core-Shell HPLC Column. The gelcap impurity had previously co-eluted with the melatonin peak using the Luna 2.5 µm C18(2) HST column making the quantitation of melatonin difficult.

When analyzing botanicals and nutraceuticals, the separation of standards can often be misleading since the plant extract can contain many other endogenous components that could lead to poor results. To demonstrate specificity of the new method, melatonin reference materials were run using the new method and no co-elutions were observed (**Figure 3**).

Having demonstrated that the new method provided equivalent results, we performed experiments to determine linearity, accuracy, range, and limit of quantitation (LOQ) using the Kinetex Core-Shell Technology column. Methods were shown to be linear over a range of 3 to 300 µg/mL (**Figure 4**). The LOQ was determined to be 0.5 µg/mL and produced a Signal to Noise ratio of 20:1 (**Table 1**). Accuracy and precision were determined at the 50 µg/mL level and found to be less than 1 % CV (**Table 2**).

The final experiment was to analyze commercially available formulations and determine if the results we obtained were similar to label claims. To ensure that we properly tested our new assay, we attempted to choose difficult formulations such as gelcap pills and dry capsules.

Analysis of a formulated product obtained results that were slightly higher than label claim for the gelcaps and slightly lower than label claim for the dry capsules (**Table 3**). Chromatographic separation for each formulation is depicted in **Figures 5 through 7**. The increased sensitivity of the Kinetex HPLC column as compared to the Luna HST column resulted in separation of an impurity peak that had previously co-eluted with the melatonin. The ability to resolve the melatonin and impurity yields more accurate results in terms of quantitation

Conclusion

Analysis time was reduced by over 50 % and back pressures were reduced by 75 % by adapting the current methods to a Kinetex Core-Shell Technology column. We performed experiments to determine that the methodology was suitable for nutraceutical formulations. Final results indicate that this method is robust, sensitive, and ready for routine samples analysis.

Analysis of melatonin reference materials provided results that were consistent with the supplied certificate of analysis. Analysis of formulated products results in values that were slightly higher than label claims for gelcaps and slightly lower than label claims for dry capsules. Providing standardized reference methods for analysis is the first step to ensuring quality in nutraceutical products.

Scientifically Valid

Section 21CFR111.320 of cGMPs for Dietary Supplements requires you to "identify and use an appropriate scientifically valid method for each established specification for which testing or examination is required to determine whether the specification is met". The FDA does not elaborate on what is considered a scientifically valid method in the cGMPs. ChromaDex has defined scientifically valid as a method that meets minimum linearity, precision, sensitivity and range requirements. These requirements are outlined in an FDA laboratory document, ORA LABORATORY PROCEDURE Food and Drug Administration, ORA-LAB.5.4.5. This laboratory guidance document defines minimal performance attributes for selected methods of analysis and has been applied by ChromaDex to the selection of methods that are fit for purpose in the dietary supplements industry. According to the above definition, the method detailed in this document is considered scientifically valid as application to the cGMP requirements. Product specific, full method validations according to AOAC guidelines can be applied to customer samples upon request, to further document method performance in specific samples and matrices.

APPLICATIONS

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ChromaDex Ordering Information

Phytochemical Reference Standards

Description	Quantity	Part No.
Melatonin (P)	10 mg	ASB-00013170-010
Melatonin (P)	50 mg	ASB-00013170-050
Melatonin (P)	100 mg	ASB-00013170-100
Melatonin (SH)	50 mg	ASB-00013171-050
Melatonin (SH)	250 mg	ASB-00013171-250

Phenomenex Ordering Information

Kinetex® Core-Shell HPLC Columns

5 µm Columns (mm)	SecurityGuard™ ULTRA Cartridges*					SecurityGuard ULTRA Cartridges*	
	50 x 2.1	3/pk	50 x 4.6	100 x 4.6	150 x 4.6	250 x 4.6	3/pk
XB-C18	00B-4605-AN	AJ0-8782	00B-4605-E0	00D-4605-E0	00F-4605-E0	00G-4605-E0	AJ0-8768
C18	00B-4601-AN	AJ0-8782	00B-4601-E0	00D-4601-E0	00F-4601-E0	00G-4601-E0	AJ0-8768
PFP	00B-4602-AN	AJ0-8787	00B-4602-E0	00D-4602-E0	00F-4602-E0	00G-4602-E0	AJ0-8773
Phenyl-Hexyl	00B-4603-AN	AJ0-8788	00B-4603-E0	00D-4603-E0	00F-4603-E0	00G-4603-E0	AJ0-8774
		for 2.1 mm ID					for 4.6 mm ID

* SecurityGuard ULTRA cartridges require holder, Part No. AJ0-9000.

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