MODERNIZING USP MELATONIN ASSAY AND IMPURITIES METHODS FOR INCREASED THROUGHPUT AND REDUCED SOLVENT WASTE

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INTRODUCTION

- Melatonin is a neurohormone that plays an important role in the sleep cycle in vertebrates [1], therefore, it is often used for alleviating sleep-related disorders such as insomnia, anxiety, and jet lag. Melatonin is readily available for purchase over the counter as dietary supplements.
- The incidents of Pediatric Melatonin Ingestion (reported to Poison Control Centers) increased 530% from 2012 to 2021. These incidents were associated with 287 intensive care unit admissions and 2 deaths [2]. Recent studies also revealed that as high as 478% [3] and 347% [4] of the label value were found in a Melatonin dietary supplement and a Melatonin gummy, respectively. These led to recent media coverage of the quality of melatonin dietary supplements on the market.
- **The scope** of this work is to modernize a US Pharmacopoeia (USP) Melatonin assay and procedure on a Arc™ HPLC System with a Waters 2998 PDA Detector using an XBridge™ BEH™ C18 (2.5 µm, 4.6 mm X 75 mm) Column.

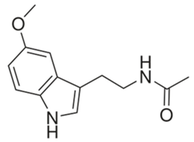


Figure 1. *Chemical structure of Melatonin (N-acety-5-methoxytryptamine).*

EXPERIMENTAL

LC conditions

System: Arc HPLC System with a 2998 PDA Detector
Sample Loop: 50 µL (Standard)
Column: XBridge BEH C18 Column, 130Å, 2.5 µm, 4.6 mm X 75 mm (p/n 186006038)
Column pre-heater: No (By-passed)
Vial: 2 mL glass screw neck vial (p/n 186000273) with screw neck cap (p/n 186000305)
Temp: 30 °C
Sample Manager purge solvent: Acetonitrile and buffer (22:78 v/v)
Sample Manager wash solvent: Acetonitrile and water (22:78 v/v)
Seal wash solvent: Methanol and water (1:1 v/v)
Injection volume: 2.0 µL
UV wavelength: 222 nm (resolution 4.8 nm) compensated by reference (310-410 nm)
Software: Empower™ 3 CDS

For Assay:
Mobile phase (isocratic): Acetonitrile and buffer (22:78 v/v)
Run time: 5.0 min
For Impurities:
Mobile phase A: Acetonitrile
Mobile phase B: Buffer (0.5 g/L monobasic potassium phosphate in water, pH 3.5)
Gradient elution program: See Table 1.
Run time: 13.0 min

Standards and samples:

USP Melatonin RS, 5-Methoxytryptamine (5-MT) were purchased from Sigma-Aldrich (Allentown, PA). Dietary supplements were purchased from online stores. These products include 5 different brands in 4 dose forms (tablet, capsule, softgel, and liquid) and at melatonin contents ranging from 1 mg to 10 mg/serving size (see Table 2 for sample information).

Standard solution: 0.1 mg/mL of USP Melatonin RS in mobile phase (acetonitrile and buffer 22/78 v/v).

System suitability solution: 0.1 mg/mL of USP Melatonin RS and 0.02 mg/mL 5-MT (Melatonin related compound A) in mobile phase (acetonitrile and buffer 22/78 v/v).

Buffer: 0.5 g/L of monobasic potassium phosphate in water. Adjusted with phosphoric acid to a pH of 3.5, and filtered.

Table 1. Gradient elution program for impurities.

Time (min)	Flow rate (mL/min)	MP A (%)	MP B (%)	Curve
Initial	1.00	22	78	Initial
3.50	1.00	22	78	6
7.50	1.00	80	20	6
8.50	1.00	80	20	6
8.75	1.00	22	78	6
13.00	1.00	22	78	6

Sample Preparation:

The tablets were ground to a fine powder before use. The contents of capsules (fine powder) and softgel (semi-solid) were used directly. Appropriate amounts of samples (recorded in 0.0001 g) that made the final solutions of melatonin in a range of 0.04 – 0.1 mg/L were weighed and added into 25 mL volumetric flasks. The samples were kept at room temperature for 30 min, then mixed, and aliquots (about 1.5 mL) were taken and centrifuged at 2000 rcf for 5 min. Aliquots (about 1 mL) of clear supernatant were transferred to LC vials for LC analysis.

Table 2*. Sample information.*

Sample Code	Product description	Dose form	Label claim (mg/serving size)	Other ingredients
A	Children's Sleep Liquid with Melatonin, Natural Berry Flavor with other natural flavors	Liquid	1	Glycerine, natural flavor
B	Ultra strength sleep multi-benefit blend	Softgel	10	Rice Bran Oil, Gelatin, Glycerin, Water, Sunflower Lecithin, Beeswax, Natural Flavor, Vegetable Juice, Titanium Dioxide, Maltodextrin, Carmine
C	Sleep with Melatonin, natural orange flavor	Chewable tablet	5	Fructose, Sugar, Natural Flavor, Modified Cellulose, Organic Acids, Honey, Gum Arabic, Magnesium Stearate, Silicon Dioxide, Stevia Leaf Extract, Natural Colors, Guar Gum.
D	Melatonin plus Chamomile and Lavender	Tablet	2	Microcrystalline Cellulose, Croscarmellose sodium, Calcium Phosphate, Maltodextrin, Corn Starch, Magnesium Stearate, Silicon Dioxide, Polyvinyl Alcohol, Polyethylene Glycol, Titanium Dioxide, talc, Food Colors.
E	Melatonin	Capsules	5	Microcrystalline Cellulose, Magnesium Stearate
F	Melatonin, Raspberry Flavor with other natural flavors	Chewable tablet	3	Sugar, Natural Flavors, Stearic Acid, Vegetable Magnesium Stearate, Fruit Powders, Vegetable Cellulose.

RESULTS

1) Method adjustment and optimization

The column particle size, column length, and mobile phase composition were modified within the USP guideline. The flow rate was optimized based on the investigation of the effects of flow rate on the separation efficiency and resolution (see Table 3).

Table 3*. Effects of flow rate on the separation of Melatonin and its related compounds (5-MT).*

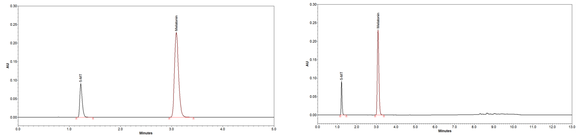
Flow rate (mL/min)	RT (min)		Plate Count Number		RRT (5-MT/ Melatonin)	Resolution (5-MT/ Melatonin)	Pressure (psi)
	5-MT	Melatonin	5-MT	Melatonin			
1.00	1.225	3.100	3058	7202	0.40	16.2	2500
1.20	1.030	2.605	2798	6672	0.40	15.6	3000
1.40	0.886	2.248	2791	6574	0.39	15.6	3530
1.60	0.784	1.988	2774	6555	0.39	15.6	4070
1.80	0.705	1.792	2629	6278	0.39	15.2	4600
2.00	0.636	1.618	2430	5929	0.39	14.7	5120

2) System suitability

Under the optimized conditions, the USP system suitability requirements were met for both the assay and the impurities testing (see Table 4). Chromatograms of Melatonin system suitability solution for the Melatonin assay (under isocratic elution) and impurities testing (under gradient elution) are shown in Figure 1.

Table 4*. USP System Suitability requirements and Arc HPLC System performance.*

Parameters	USP System Suitability requirements	Arc HPLC System Performance
RRT	5-MT: 0.4; Melatonin: 1.0	5-MT: 0.4; Melatonin: 1.0
Resolution	Not less than 4	16
RSD	Not more than 2.0%	0.11%



A B

Figure 1. *Chromatograms of the system suitability solution under the isocratic elution condition for Melatonin assay (A), and under the gradient elution condition for impurities testing (B).*

3) Melatonin assay analytical performance

The Melatonin assay specificity, linearity, sensitivity, accuracy and precision were investigated.

The specificity was confirmed by the retention time and the UV/Vis spectrum. A UV/Vis spectral library was created in the Empower CDS from standards under the same LC conditions and was used to confirm the peak I.D. in the sample analysis (using the PDA Library Match). Peak purity (at Inflection Points on both sides of peaks) was also checked for signs of potential interference (co-elution).

Figure 2 shows a typical calibration plot for melatonin assay. Excellent linearity (R^2: 0.999995, line through zero) was obtained. This excellent linearity validates the single point calibration approach as recommended by the USP Melatonin Monograph for assay.

The limit of quantification (LOQ) was estimated using a low concentration melatonin solution (0.25 µg/mL) following the US FDA Q2(R1) Validation of Analytical Procedure.[5] LOQ of 0.5 µg/mL was obtained by the approach using 10 times the standard deviation of melatonin peak divided by the slope of calibration curve.

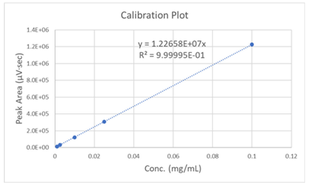


Figure 2. *Calibration plot (UV peak area vs concentration) for melatonin from duplicated injections of 5 concentration levels in the range of 0.001 to 0.1 mg/mL. (Slope: 1.227x10^7; R^2: 0.999995).*

The accuracy of this analysis method was evaluated by spiking experiments using dietary supplement samples. Table 5 is a summary of the accuracy and precision results. Excellent accuracy (98.2 to 106.6%) and repeatability (RSD ≤ 5.8%) were demonstrated.

Table 5*. Accuracy and repeatability result*

Sample	Native Level (mg/ serving size)	Spiking Level (rel. to native level)	Mean Recovery (n=3)	RSD
A	1.28	77% - 94%	106.60%	5.10%
B	10.67	76% - 79%	102.30%	1.00%
E	6.06	47% - 66%	98.20%	5.80%
Melatonin QC	0.1 mg/mL	N/A	100.50%	0.80%

4) Determination of Melatonin in dietary supplements

Table 6 shows sample analysis results for 6 supplements. The determined melatonin contents were 3% to 42% higher than their label claims. The repeatability (RSD) values were less than 4.0%.

Table 6*. Sample analysis results and comparison to Label Claims.*

Sample Code	Label claim (mg/serving size)	Measured value (mg/serving size)	RSD (n≥3)	Difference from label claim (%)
A	1	1.26	0.4%	26%
B	10	10.76	0.7%	8%
C	5	6.29	2.5%	26%
D	2	2.83	1.7%	42%
E	5	6.40	4.0%	28%
F	3	3.08	1.5%	3%

CONCLUSION

- **USP Melatonin assay and testing procedures were optimized using an Arc HPLC System and an XBridge BEH C18 Column. The separation of Melatonin and the related compound A met all USP system suitability requirements, i.e., the relative retention times, resolution, and relative standard deviation.**
- **The optimized LC conditions using a 2.5 µm particle XBridge BEH C18 Column offers short run time and reduced solvent consumption, which shortens the turnaround time and reduces operating costs.**
- **The optimized USP Melatonin assay demonstrated excellent analytical performance. Six supplement products that contained melatonin from 1 to 10 mg per serving in liquid, tablet, softgel, and capsule forms were successfully analyzed.**
- **This modernized USP Melatonin assay and impurities testing procedure are suitable to be used for the rapid and economical analysis of melatonin in supplements.**

For additional details and references info, please scan the QR Code on the right to download the relevant application notes at Waters.com.

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