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Application Note

Modernizing USP Melatonin Monograph Assay and Impurities Methods for Increased Throughput and Reduced Solvent Waste

Jinchuan Yang, Paul D. Rainville

Waters Corporation

Abstract

Melatonin is a neurohormone that can regulate the sleep cycle in humans. It is readily available as dietary supplements and is often used for alleviating sleep-related disorders. Recent data has shown a dramatic increase in the number of incidents of pediatric Melatonin ingestion. Melatonin contents significantly higher than their labeled values in dietary supplement products have also been reported. To assist quality control of the Melatonin product manufacturing process, a modernized version of a USP Melatonin Monograph procedure has been developed on a Waters Arc™ HPLC System using an XBridge BEH™ C_{18} Column (2.5 µm, 4.6 mm x 75 mm). The liquid chromatographic conditions were appropriately adjusted within the USP <621> guidelines, and the resulting separation performance meets the USP system suitability requirements, with the benefit of shorter run times and reduced solvent waste.

Benefits

- Assay of Melatonin in a five minute isocratic run
- Quantification of impurities in a 13-minute gradient elution program
- Fulfillment of all system suitability requirements

Introduction

Melatonin (N-acetyl-5-methoxytryptamine) is a neurohormone that is produced by the mammalian pineal gland. Since Melatonin plays an important role in the sleep cycle in vertebrates, it is often used for alleviating sleep-related disorders such as insomnia, anxiety, and jet lag.[1] Melatonin is readily available for purchase over the counter as dietary supplements in a variety of forms, including flavored liquid, chewable tablets, softgels, capsules, and gummies. Recently, it has been reported that the number of incidents of pediatric Melatonin ingestion increased 530% from 2012 to 2021 and these incidents were associated with 287 intensive care unit admissions and two deaths.[2] Recent studies also revealed that Melatonin content in some dietary supplements was found much higher than the label claim values. As high as 478% and 347% of the label value were found in a dietary supplement and a Melatonin gummy, respectively.[3,4] This has led to a real concern about the quality and safety of Melatonin-containing dietary supplements. Improved quality control is required for the dietary supplement manufacturers to ensure accurate label claims. The US Pharmacopoeia (USP) Melatonin Monograph provides industrial standards for the Melatonin assay and impurities.[5] The goal of this application note is to modernize the USP methods on a Waters Arc HPLC System with a Waters 2998 PDA Detector using an XBridge BEH C_{18} (2.5 µm, 4.6 mm x 75 mm) Column.

Experimental

The standard and sample solution preparation and the Liquid Chromatographic (LC) conditions recommended by US Pharmacopoeia were adopted with minor adjustments.[5] USP Melatonin RS, 5-Methoxytryptamine (5-MT) were purchased from Sigma-Aldrich (Allentown, PA).

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

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).

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

LC Conditions

System:	Arc HPLC System with a 2998 PDA Detector
Sample loop:	50 µL (Standard)
Column:	XBridge BEH C_{18} Column, 130 Å, 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)
Temperature:	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
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

Table 1

Time (min)	Flow rate (mL/min)	Mobile phase A (%)	Mobile phase 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

Results and Discussion

USP Method Modernization

The USP Melatonin Monograph specifies a procedure for the assay and impurities of Melatonin. These methods were implemented on an Arc HPLC System with minor adjustments that were made under the USP allowable adjustment guidelines.[6] A 2.5 µm particle size XBridge BEH C_{18} Column was used for its higher separation efficiency per unit length, instead of a 5 µm particle column as in the USP Monograph. The column length was adjusted to 75 mm to keep the L/dp (column length to particle size ratio) the same as that in the USP monograph. The mobile phase of acetonitrile and buffer at 25:75 (v/v) was recommended by the USP Monograph, however, a small adjustment of the mobile phase composition to 22:78 (acetonitrile and buffer) was found necessary to satisfy the system suitability requirement on RRT (relative retention time) of the related compound A (5-MT) and Melatonin of 0.4 and 1, respectively. This small adjustment in composition was within the limit for adjustments under the USP guideline (less or equal than 10% absolute, or within 30% relative for the minor component of the mobile phase). The scaled flow rate for the 2.5 µm particle column was 2.0 mL/min based on the USP recommended flow rate of 1.0 mL/min for a 5 µm column (with the same column I.D.). However, based on our investigation of the separation performance (see Table 2), a flow rate of 1.0 mL/min was selected for the assay because of its optimal separation efficiency (plate count number) in the 1.0–2.0 mL/min range. Please note that the pre-heating tubing inside the column heater was by-passed when connecting the column to the injector. This helped with the separation efficiency by reducing the extra-column band spreading for this 75 mm length column (4.6 mm x 75 mm). Figure 1 and 2 show chromatograms of the Melatonin and the related compound A (5-MT) under the isocratic elution conditions (for assay) and the gradient elution conditions (for impurities). The run time was five minutes in the isocratic elution and 13 minutes in the gradient elution, respectively. These run times were about half of the run times in the USP methods. These short run times and the associated reduced solvent wastes would shorten the analysis turnaround time and reduce the operating cost.

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

Table 2. Effects of flow rate on the separation of Melatonin and its related compound A (5-MT).

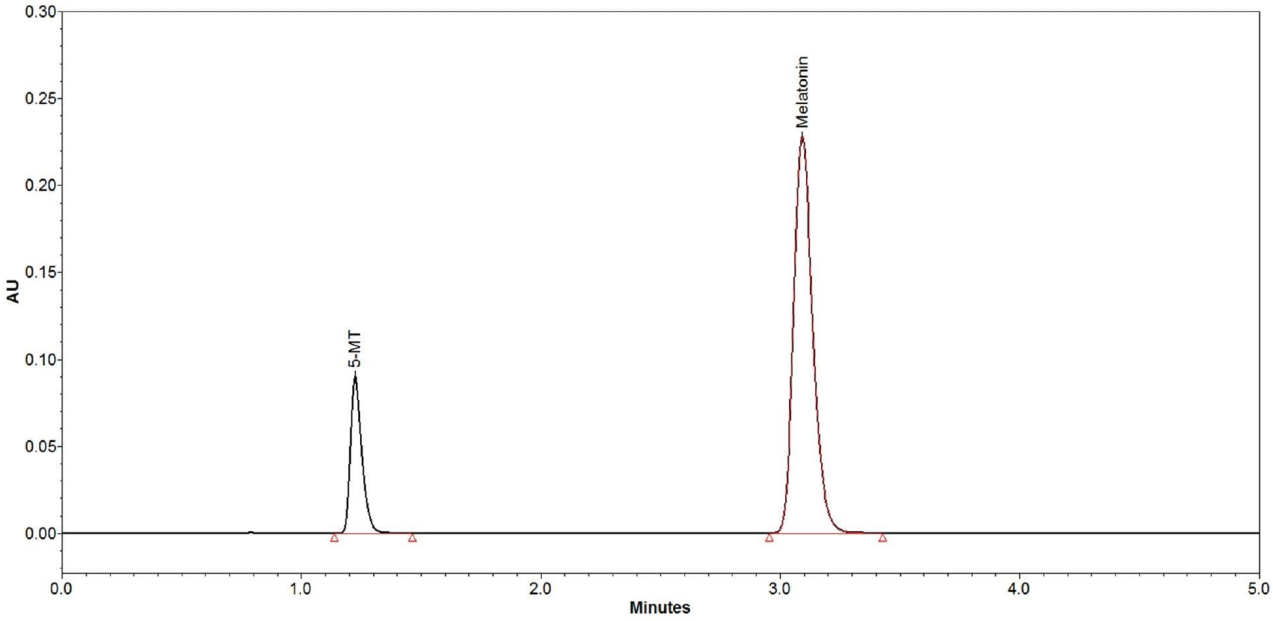

Figure 1. Chromatogram of the system suitability solution (0.1 mg/mL Melatonin and 0.02 mg/mL 5-MT in mobile phase) under the isocratic elution condition (for Melatonin assay).

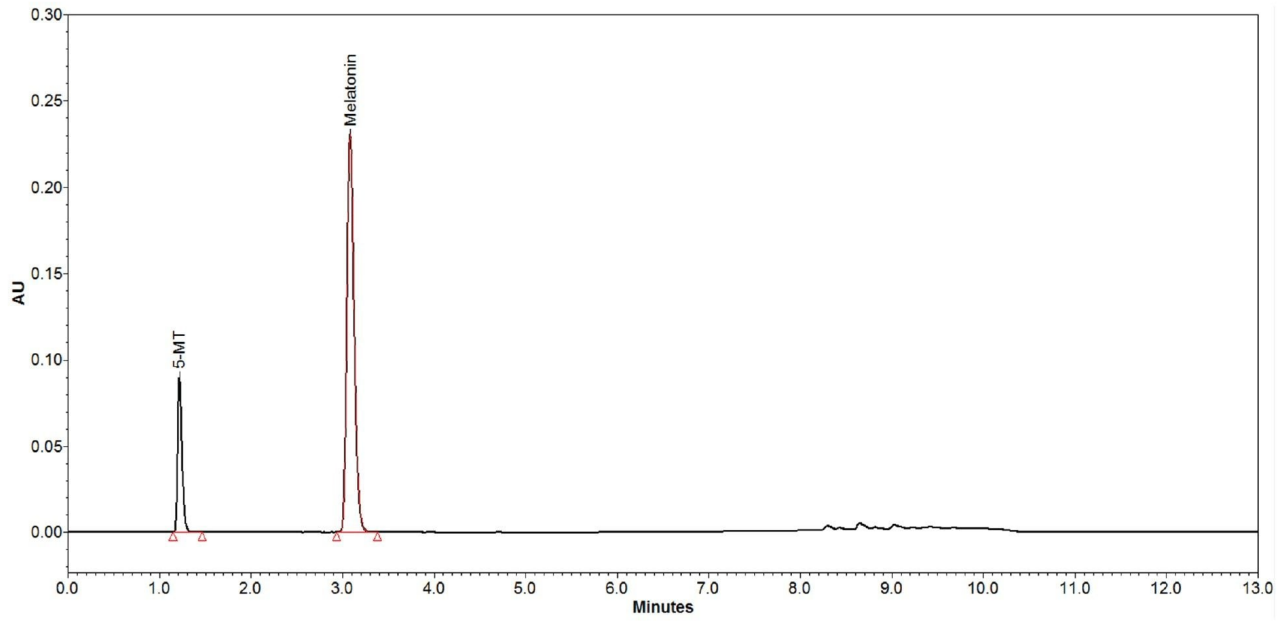

Figure 2. Chromatogram of the system suitability solution (0.1 mg/mL Melatonin and 0.02 mg/mL 5-MT in mobile phase) under the gradient elution condition (for impurities).

System Suitability Performance

The USP Melatonin Monograph specifies system suitability requirements for the assay and the related compounds. These requirements include the RRT of the related compound A and Melatonin, resolution between the related compound A and Melatonin, and relative standard deviation (RSD) on melatonin (see Table 3). Table 4 shows the system suitability performance and repeatability results for both Melatonin and its related compound A (5-MT) on the Arc HPLC System with an XBridge BEH C_{18} Column (2.5 µm, 4.6 mm x 75 mm). RSD of 0.11% was obtained for the Melatonin peak area. The RRT values, the resolution, and the repeatability all met the USP system suitability requirements (See Table 3).

Parameters	USP system suitability requirements	Arc HPLC 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%

Table 3. USP System Suitability requirements and Arc HPLC performance.

	Melatonin related compound (5-MT)				Melatonin			
Inj #	RT (min)	Peak area (µV·sec)	USP plate number	RRT	RT (min)	Peak area (µV·sec)	USP plate number	Resolution
1	1.224	303580	3090	0.40	3.089	1264991	7221	16.15
2	1.225	303567	3081	0.40	3.091	1268360	7257	16.18
3	1.225	302870	3075	0.40	3.091	1267848	7199	16.18
4	1.226	303539	3070	0.40	3.098	1268049	7247	16.23
5	1.227	303544	3090	0.40	3.101	1268199	7217	16.20
Mean RSD	1.2254	303420	3081	0.40	3.094	1267489	7228	16.19
RSD (%)	0.09	0.10	0.29	0.08	0.17	0.11	0.32	0.18

Table 4. System suitability performance and repeatability of the separation on Arc HPLC System with XBridge BEH C_{18} 2.5 µm 4.6 mm x 75 mm Column.

Conclusion

This application note demonstrates a modernization of the USP Melatonin Monograph procedure using a Waters Arc HPLC System and an XBridge BEH C_{18} Column (130 Å, 2.5 µm, 4.6 mm x 75 mm). The LC conditions were appropriately adjusted under the USP guidelines. The separation of Melatonin and the related compound A met all USP system suitability requirements, including the relative retention times, resolution, and relative standard deviation. The use of a 2.5 µm particle XBridge BEH C_{18} Column (4.6 mm x 75 mm) offers short run time and reduced solvent consumption, which shortens the turnaround time and reduces operating costs.

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