Waters™

Nota de aplicación

Modernizing USP Melatonin Monograph Assay and Impurities Methods (II) – Analytical performance and Analysis of Dietary Supplements

Jinchuan Yang, Paul D. Rainville

Waters Corporation

Abstract

The USP Melatonin procedures for assay and impurities were previously optimized on an Arc™ HPLC™ System coupled with a Waters™ 2998 PDA Detector and a XBridge™ BEH™ C_{18} (2.5 µm, 4.6 mm x 75 mm) Column. In this note, the analytical characteristics of the modernized USP Melatonin assay were investigated. Excellent performance in specificity, linearity, sensitivity, accuracy, and precision (repeatability) have been demonstrated. This assay was applied to six representative dietary supplements, and the results showed 3% to 42% overage in melatonin content. This modernized USP Melatonin assay is suitable for use for the rapid and economical analysis of melatonin in supplements.

Benefits

- Rapid and economical analysis of melatonin in dietary supplements
- Reliable and accurate determination of melatonin in supplements of various forms

Introduction

In response to a recent public concern on the increased ingestion of melatonin in children,[1,2] we demonstrated a successful modernization of the US Pharmacopoeia (USP) Melatonin Monograph assay and impurities procedures[3] on a Waters Arc HPLC System coupled with a the 2998 PDA Detector and a XBridge BEH C_{18} (2.5 µm, 4.6 mm x 75 mm) Column with optimized LC conditions.[4] In this application note, we will further demonstrate the analytical performance and the applicability of the USP assay procedure using representative dietary supplements in various forms (*i.e.*, liquid, softgel, tablet, and capsule) and at various melatonin contents (1–10 mg/serving).

Experimental

Melatonin (USP Melatonin RS) and 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 four dose forms (tablet, capsule, softgel, and liquid) and at melatonin contents ranging from 1 mg to 10 mg/serving size (see Table 1 for 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.

Table 1. Sample information.

Standard and Mobile Phase Preparation

Standard solution: 0.1 mg/mL of USP Melatonin RS in methanol.

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

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. The shells of capsules and softgel were cleaned and weighed to obtain the actual serving size in content (by removing the mass of shells from the gross weight of capsule or softgel). The average serving size obtained from ten pills was used in analysis. 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. For spiking experiments, aliquots of melatonin standard solutions (weighed in 0.0001 g) were spiked into the flasks. 15 mL of methanol (LC grade, Fisher Scientific) were added to the volumetric flasks and then vortexed for at least 1 minutes. Additional methanol was then added to the 25 mL mark. 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 five minutes. Aliquots (about 1 mL) of clear supernatant were transferred to LC vials for LC analysis. Samples were quantified using a single point calibration as recommended by the USP Melatonin Monograph.

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)
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
Mobile phase (isocratic):	Acetonitrile and buffer (22:78 v/v)
Run time:	5.0 min
UV detection:	UV absorbance (wavelength 222 nm, resolution 4.8 nm) compensated by reference (310–410 nm)
Software:	Empower 3 CDS

Results and Discussion

Analytical Performance

We have shown in our previous note titled "Modernizing USP Melatonin Monograph assay and impurities methods for increased throughput and reduced solvent waste" that HPLC analysis under optimized conditions has satisfied the USP System suitability requirements, which include the relative retention times, the resolution, and the repeatability of melatonin and its related compound A (5-MT) for both the assay (under an isocratic elution) and the impurities (under a gradient elution).[4] In this note, we focused on the analytical characteristics of the assay procedure for the determination of melatonin in dietary supplements. Specifically, the analytical specificity, linearity, sensitivity, accuracy, and precision (repeatability) were investigated.

The analytical specificity (or peak identity) was confirmed by the retention time and the UV/Vis spectrum. A UV/Vis spectrum 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). There was no interference found for the assay of melatonin in the samples analyzed in this study.

Figure 1 is the UV response (peak area) versus melatonin concentration plot. The data set includes results from duplicated injections of five concentration levels in a range of 0.001 to 0.1 mg/mL. A line through zero was fitted by the least square regression (no weighing) with a coefficient of determination (R^2) larger than 0.999. The relative errors of all data points against the calibration line were within ±5% (data not shown). This excellent linearity validates the single point calibration approach as recommended by the USP Melatonin Monograph for assay.

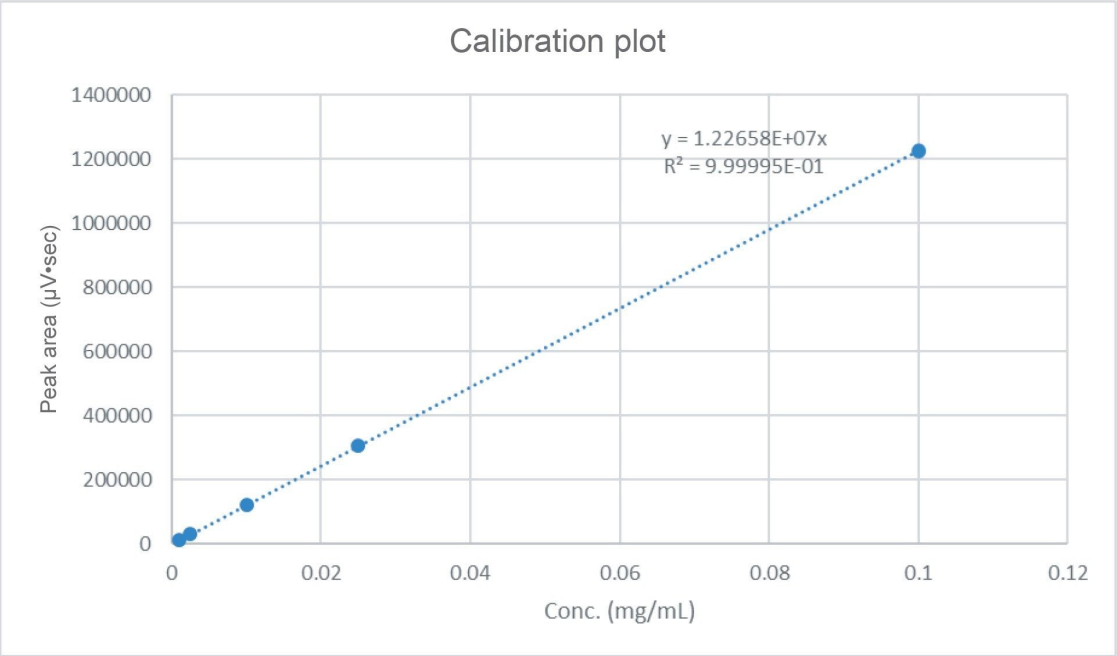

Figure 1. Calibration plot (UV peak area vs concentration) for melatonin from duplicated injections of 5 concentration levels in the range of 0.001 to 0.1 mg/mL.(Slope: 1.227x10^7; R^2: 0.999995).

The 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 ten times the standard deviation of melatonin peak divided by the slope of calibration curve. This LOQ is about the same as those LOQ values reported by other LC-UV based studies after injection volume adjustment.[6–8]

The accuracy of this analysis method was evaluated by spiking experiments using dietary supplement samples.

Three supplements in liquid, softgel, and tablet forms that contained melatonin at different concentrations (1 to 10 mg/serving size) were spiked at various levels relative to their melatonin native levels (see Table 2). Recovery results were calculated by comparing the determined spiked amounts against the theoretical spiked amounts. The mean recoveries were from 98.2 to 106.6%, and the repeatability (relative standard deviation, RSD) were less than 5.8% (n=3). The results from a melatonin QC solution (0.1 mg/mL melatonin) also demonstrated excellent accuracy and repeatability (mean accuracy of 100.5% with RSD of 0.8%, n=3).

Sample	Native level (mg/serving size)	Spiking level (rel. to native level)	Mean recovery (n=3)	RSD
A	1.26	77%–94%	106.6%	5.1%
B	10.76	76%–79%	102.3%	1.0%
E	6.40	47%–66%	98.2%	5.8%
Melatonin QC	0.1 mg/mL	N/A	100.5%	0.8%

Table 2. Accuracy and repeatability result summary.

Sample Analysis

Figure 2 shows HPLC-UV chromatograms of six supplements. Table 3 details sample analysis results for these six supplements. The determined melatonin contents were 3% to 42% higher than label claims. The repeatability (RSD) values were less than 4.0%. During dietary supplement manufacturing, many ingredients are added at levels higher than label claims. The observed overages in melatonin content are not uncommon compared to other ingredients in dietary supplements.[9] Previous studies reported extremely high melatonin content (four to five times higher than label claims) in dietary supplements. In this study, we did not find any product that contained such a high melatonin content.[7,8]

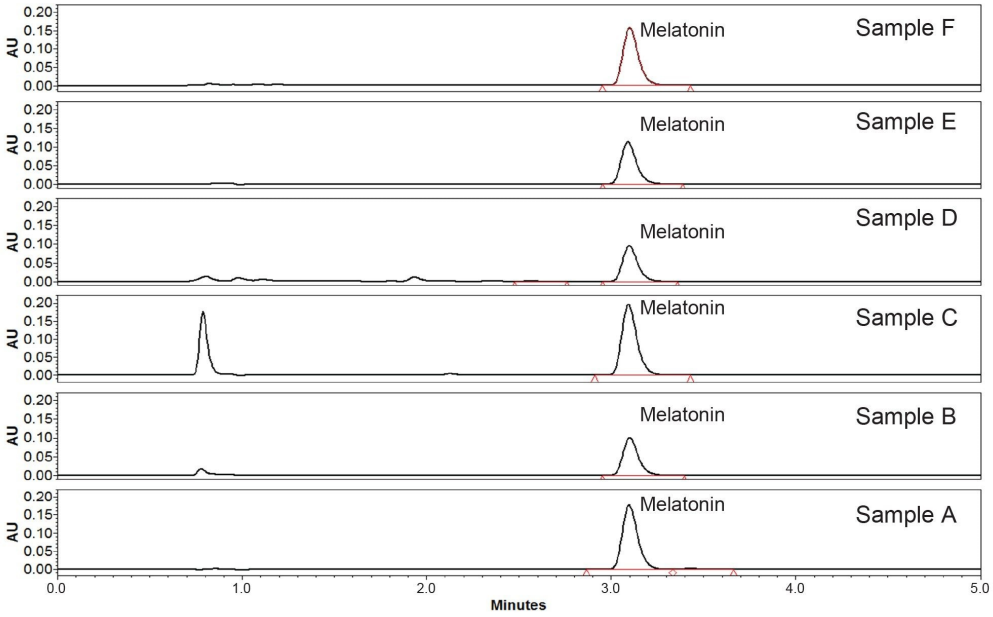

Figure 2. HPLC-UV chromatograms of 6 dietary supplements obtained under the assay conditions (see Experimental).

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%

Table 3. Sample analysis results and comparison to Label Claims.

These samples contained various other ingredients, such as natural flavors and natural colors (see Table 1). These ingredients can interfere with the USP Melatonin impurities test, therefore, no impurities test was carried out for these samples.

Conclusion

To address the recent public concern on the melatonin content in dietary supplements, we modernized the USP Melatonin procedures using an Arc HPLC System and an XBridge BEH C_{18} Column (130 Å, 2.5 µm, 4.6 mm x 75 mm). The optimized USP Melatonin assay procedure demonstrated excellent analytical performance in specificity, linearity, sensitivity, accuracy, and precision. Six supplement products that contained melatonin from 1 to 10 mg per serving in liquid, tablet, softgel, and capsule forms were successfully analyzed. The results showed that these products had melatonin overages ranged from 3% to 42%. This modernized USP Melatonin assay procedure is suitable to be used for the rapid and economical analysis of melatonin in supplements.

References

1. Stobbe, M. Melatonin poisoning reports are up in kids, study says. Associated Press. June 2, 2022. https://apnews.com/article/covid-science-health-poisoning-716dbfe4eb49fc4cc224d388d91e8dbf < https://apnews.com/article/covid-science-health-poisoning-716dbfe4eb49fc4cc224d388d91e8dbf> . Accessed on May 12, 2023.
2. LaMotte, S. Potentially dangerous dose of melatonin and CBD found in gummies sold for sleep. CNN. April 25, 2023. https://edition.cnn.com/2023/04/25/health/melatonin-gummies-wellness/index.html < https://edition.cnn.com/2023/04/25/health/melatonin-gummies-wellness/index.html> . Accessed on May 12, 2023.
3. Melatonin Monograph. United States Pharmacopeia, Docid: GUID-454646BE-F1DF-458C-9011-1FBBCCEFE5BC_4_en-US, 2023.
4. Yang, J.; Rainville, P. Modernizing USP Melatonin Monograph Assay and Impurities Methods for Increased Throughput and Reduced Solvent Waste. Waters Corporation Application Note, 720007978, 2023.
5. US FDA. Q2(R1) Validation of Analytical Procedures: Text and Methodology Guidance for Industry. Sept. 2021. https://www.fda.gov/regulatory-information/search-fda-guidance-documents/q2r1-validation-analytical-procedures-text-and-methodology-guidance-industry <https://www.fda.gov/regulatory-information/search-fda-guidance-documents/q2r1-validation-analytical-procedures-text-and-methodology-guidance-industry> .

Accessed on June 2nd, 2023.

6. Cerezo, A.B.; Leal, A.; Álvarez-Fernández, M.A.; Hornedo-Ortega, R.; Troncoso, A.M.; García-Parrilla, M.C.; Quality control and determination of melatonin in food supplements, *J. Food Compost. Anal*., 2016; 45: 80-86. doi: 10.1016/j.jfca.2015.09.013.

7. Lauren A.E. Erland, Praveen K. Saxena. Melatonin natural health products and supplements: presence of serotonin and significant variability of melatonin content. *J Clin Sleep Med*. 2017 13(2):275-281. doi: 10.5664/jcsm.6462.

8. Cohen P.A., Avula B., Wang Y-H, Katragunta K., Khan I. Quantity of Melatonin and CBD in Melatonin Gummies Sold in the US. *JAMA*. 2023; 329(16): 1401-1402. doi:10.1001/jama.2023.2296.

9. Andrews, K.W.; Gusev, P.A.; McNeal, M.; Savarala, S.; Dang, P.T.V.; Oh, L.; Atkinson, R.; Pehrsson, P.R.; Dwyer, J.T.; Saldanha, L.G.; Betz, J.M.; Costello, R.B.; Douglass, L.W. Dietary Supplement Ingredient Database (DSID) and the Application of Analytically Based Estimates of Ingredient Amount to Intake Calculations, *J Nutr.*, 2018; 148(2): 1413S-1421S, doi: 10.1093/jn/nxy092.

Featured Products

Arc HPLC System </nextgen/es/es/products/chromatography/chromatography-systems/arc-hplc-system.html>

2998 Photodiode Array (PDA) Detector <https://www.waters.com/1001362>

Empower Chromatography Data System <https://www.waters.com/10190669>

720008009, July 2023