Application News

Liquid Chromatograph Mass Spectrometer LCMS-8045

A Rapid, Simple and Sensitive Assay for Quantitative Determination of Melatonin in Human Plasma Using Shimadzu LCMS-8045

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User Benefits

◆ Rapid, simple and sensitive melatonin method with LLOQ of 5 pg/mL
◆ Low plasma volumes in sample extraction extends the life of mass spectrometer
◆ Validated method easy to transfer at customers laboratory

1. Introduction

Melatonin is an endogenous hormone produced by the pineal gland that regulates sleep-wake cycles and when provided exogenously has beneficial effects on sleep-onset latency. Melatonin is used orally for jet lag, insomnia, shift-work disorder, circadian rhythm disorders in the blind (evidence for efficacy) and benzodiazepine and nicotine withdrawal. Evidence indicates that melatonin is likely effective for treating circadian rhythm sleep disorders in blind children and adults. It has received FDA orphan drug status as an oral medication for this use[1]. The average maximal serum concentration (Cmax) is 405 pg/mL in the low dose group and the bioavailability of exogenous melatonin was highly variable, ranging from 1 to 74%[2].

Hence, it is necessary to develop a bioanalytical assay that have very low LLOQ concentration equivalent to 10 pg/mL. Several methods for determination of melatonin in biological matrices were reported up to date, most of these assays are at a sensitivity level higher than 20.00 pg/ml or use relatively large plasma volumes, which is a great concern for regulatory compliance of clinical studies for low-dose formulations. These methods fall short of the ideal target sensitivity required by the intended studies[2-3]. This motivated us to develop fast, simple, sensitive method using minimal sample volume to support regulatory studies.
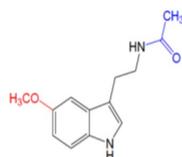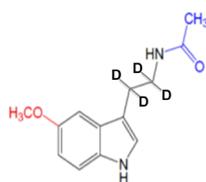

Fig. 1 Structure of Melatonin

Fig. 2 Structure of Melatonin-D4

2. Salient Features

• A rapid, simple and highly sensitive LCMS method for accurate measurement of melatonin in biological matrix.
• Simple sample extraction process minimizes the risk of sample processing errors.
• Shorter analysis run time (3.5 min) has efficiently enhanced sample throughput
• Less plasma volume (200 µL) avoided wastage of samples and increase the instrument life.
• Heated ESI along with New UF-Qarray™ ion guide technology contributes by increasing ion production and enhancing transmission, respectively. This ensures sensitive and selective quantification of melatonin at 5 pg/mL.
• The validated method is immediately deployable in the laboratory setting.

3. Method Validation

Melatonin LCMS method was validated as per US major guidelines. All method validation parameters evaluated meets the acceptance criteria. Summary of melatonin method validation results are given in Table 1.

Table 1 Method Validation Summary

Calibration curve range		5.00 to 10,000.00 pg/mL
Intraday precision and accuracy (For LLOQ-QC)-charcoal treated plasma	Accuracy (% Nominal)	117.17
	Precision (%RSD)	8.8
Intraday precision and accuracy (For LQC, MQC, HQC)- charcoal treated plasma	Accuracy (% Nominal)	96.25 to 102.61
	Precision (%RSD)	2.32 to 3.24
Global precision and accuracy (For LLOQ-QC)-charcoal treated plasma	Accuracy (% Nominal)	102.79
	Precision (%RSD)	18.15
Global precision and accuracy (For LQC, MQC, HQC)-charcoal treated plasma	Accuracy (% Nominal)	97.67 to 104.00
	Precision (%RSD)	2.70 to 3.42
Intraday precision and accuracy (For LQC, MQC, HQC)- untreated plasma	Accuracy (% Nominal)	101.19 to 102.95
	Precision (%RSD)	1.90 to 5.91
Global % recovery	Recovery (%)	79.23
	Precision (%RSD)	0.07
Matrix effect	Mean matrix Factor	LQC - 1.12 HQC - 1.05

Note: LLOQ QC- Lower Limit of Quantification Quality Control, LQC- Lower Quality Control, MQC_ Middle Quality Control and HQC- Higher Quality Control

%RSD: % of Relative Standard Deviation, same as CV (Coefficient of Variation)

4. Experimental

4.1. Sample preparation and analytical conditions

- Fifty microliters of internal standard (5 ng/mL of Melatonin-D4) was added to 200 μL of the pre-spiked calibration curve standards and quality control samples.

- To the blank and non-zero standard, 50 μL diluent was added to compensate the volume of internal standard and vortexed.

- Samples were further diluted in the ratio of 1:1 (v/v) with the extraction buffer and vortexed.

- 2.5 mL extraction solvent was added to the samples.

- Melatonin was extracted by vortexing the samples for 5 minutes at 2000 rpm followed by centrifugation for 15 minutes at 5 ℃.

- Two milliliters of clear supernatant were collected in a prelabelled RIA vials and evaporated under stream of nitrogen flow at 35 ℃.

- Evaporated samples were reconstituted in 300 μL of reconstitution solution, vortexed and filled in HPLC vials for injection.

4.2 Instrument parameters on LCMS-8045

Refer to Table 2 for analytical conditions and instrumental parameters. Refer to Table 3 for MRM transitions.

Table 2 Analytical conditions and instrument parameters

Parameter	HPLC
Column	Shim-packTM GIST C 18, 75 x 3 mm, 2 μm column (P/N: 227-30002-03)
Mobile Phase	A: 0.1% formic acid in water B: Methanol
Flow Rate	0.4 mL/min
Oven Temp	50 ℃
Injection volume	20 μl
Parameter	**MS**
Interface	ESI
Interface Voltage and temp	3 kV and 400 ℃
MS Mode	MRM, Positive
Heat Block Temp	500 ℃
DL Temp	200 ℃
CID Gas	230 kPa
Nebulizing Gas	3 L/min
Drying Gas	10 L/min
Heating Gas	10 L/min

Table 3 MRM transition and parameters of Melatonin on LCMS

Compound	MRM (m/z)	CE (V)
Melatonin	232.95→173.90	-26.8
Melatonin-D4	237.10→178.20	-27.1

Fig. 3 NexeraTM X2 with LCMS-8045 system

5. Result and Discussion

5.1. Method Development

Liquid chromatography

In the experiment, several mobile phase conditions were assessed in order to get shorter retention time, low background noise and excellent peak shape. Melatonin's signal responsiveness was enhanced by formic acid (0.1%). It was discovered that methanol produced superior elution capability than acetonitrile. To achieve adequate sensitivity and acceptable melatonin retention, a mobile phase composed of methanol and 0.1 % formic acid in water (50:50, v/v) was finally used. Melatonin and the IS had retention time of 1.0 min respectively, at the ideal LC conditions. Per sample, the overall analysis time was 3.5 minutes.

Mass spectrometry

The LC-MS/MS analysis in the study was carried out in both positive and negative ion ESI modes. Due to its higher signal-to-noise ratios, the positive ion mode was better suited for quantitative analysis. Melatonin formed a protonated ion of $[M + H]+$ at m/z 232.95 when it was readily ionized at the interface. Several significant fragment ions were seen in the $[M + H]+$ product ion scan spectrum at m/z 174.2, 173.9 and 216.2. After careful MS condition optimisation, the melatonin fragment ion at m/z 173.90 had a stronger signal response and lower noise level than the fragment ion at m/z 174.2 and 216.2. Thus, the final ion transition used for the measurement of melatonin was m/z 232.95→173.90. Melatonin D4 (IS) was detected using a mass transition of m/z 237.10→178.20, which showed comparable extraction.

Sample preparation

For sample preparation, liquid-liquid extraction (LLE) and protein precipitation were evaluated. After protein precipitation, a significant matrix impact of suppression was detected. Since liquid-liquid extraction is a more efficient way to prevent the matrix effect, it was chosen for the sample preparation in the current procedure. To find the sample's best extraction agent, ethyl acetate and diethyl ether were evaluated. Ethyl ether was finalized as an extraction solvent which showed higher analyte recovery. After extraction with ethyl ether the samples were centrifuged and 2mL of supernatant was collected in the RIA vials and evaporated to dryness under nitrogen stream. The dry residue was reconstituted with reconstitution solution and injected on LCMS-8045 system.
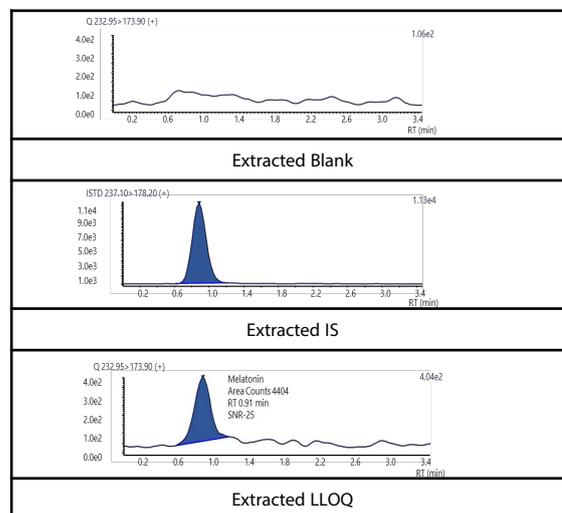

Fig. 4 Chromatograms of Melatonin (Ext Blank, Ext IS and Extracted LLOQ)

5.2. Method Validation

- **Selectivity**

  Selectivity of the method was assessed in different lots of blank human plasma. Interference from blank plasma was assessed for both melatonin and melatonin-D4. There was no evidence of interfering peaks from endogenous compounds at the retention times and MRM transition of melatonin and melatonin-D4 in all the tested six lots of blank matrix samples. The peak detected in the plasma was far less than 20% of the LLOQ. Selectivity results are presented in Table 4.

Table 4 Selectivity

| Plasma lot no. | Melatonin | | |
	Area in blank matrix	LLOQ area	% Interference
P4458	25	4483	0.56
P4791	254	4404	5.77
P5515	733	5080	14.43
P5516	94	5280	1.78
P5517	621	4655	13.34
P5519	550	4444	12.38

| Plasma lot no. | Melatonin-D4 | | |
	Area in blank matrix	LLOQ area	% Interference
P4458	633	1,27,161	0.5
P4791	2239	1,26,375	1.77
P5515	2549	1,29,585	1.97
P5516	1473	1,26,189	1.17
P5517	438	1,21,969	0.36
P5519	901	1,33,946	0.67

- **Linearity**

  The linearity was evaluated on three separate days with two sets of calibration curves per day. The standard curve was constructed to span an analytical measuring range of 5.0–10,000 pg/ml, demonstrating good reproducibility and linearity. The correlation coefficients of the generated calibration curves were greater than 0.99 (refer to Fig. 5). The LLOQ was confirmed to be 5.0 pg/ml, at which the accuracy was within 80% to 120% and the precision was ≤ 20%. Signal to noise ratio (s/n) at LLOQ (5.00 pg/mL) was found more than 20:1, across 3 PA batches. Representative chromatograms are shown in Fig. 4
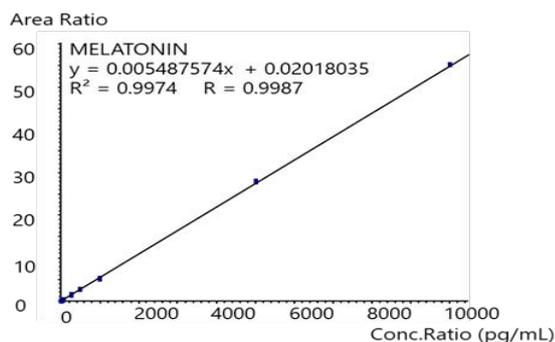
Area Ratio

MELATONIN
$y = 0.005487574x + 0.02018035$
$R^2 = 0.9974$ $R = 0.9987$

Fig. 5 Calibration curve

- **Intra-day precision and accuracy**

  Intraday precision and accuracy was evaluated by processing 6 replicates of QCs in a P&A batch. Results are summarized in Table 5

  The intra-day precision for melatonin was observed ≤15% at LLOQ QC, LQC, MQC and HQC. Intraday accuracy was observed within 80% - 115% at LQC, MQC and HQC level and 117.77% at LLOQ QC level.

Table 5 Intra-day accuracy and precision

Inter-day (n=6)			
Nominal Conc (pg/mL)	Observed Conc (pg/mL)	Accuracy (%)	Precision (%RSD)
LLOQ QC (5.00 pg/mL)	5.89	117.77	8.80
LQC (50.00 pg/mL)	48.12	96.25	3.24
MQC (760.00 pg/mL)	779.84	102.61	3.19
HQC (7500.00 pg/mL)	7616.29	101.55	2.32

- **Global precision and accuracy**

  Precision and accuracy experiments was conducted on 3 batches. Excellent repeatability and accuracy was observed with %RSD ≤ 15 % and % accuracy between 85 % to 115 % for LQC, MQC and HQC. At LLOQ QC, the %RSD was observed ≤ 20 % and % accuracy between 80 % to 120 %. Statistical results are presented in Table 6.

Table 6 Global Precision and accuracy

Inter-day (n=18)			
Nominal Conc (pg/mL)	Observed Conc (pg/mL)	Accuracy (%)	Precision (%RSD)
LLOQ QC (5.00 pg/mL)	5.14	102.79	18.15
LQC (50.00 pg/mL)	48.83	97.67	3.42
MQC (760.00 pg/mL)	786.76	103.52	2.70
HQC (7500.00 pg/mL)	7800.35	104.00	2.86

- **Treated vs untreated plasma analysis**

  In compliance with the regulations, method applicability was tested by comparing QC samples prepared using charcoal treated plasma against QC samples prepared using untreated plasma. This experiment will mimic the actual study sample analysis.

  Six replicates of treated and untreated QC samples at LQC, MQC and HQC levels were analyzed against a calibration curve prepared using untreated plasma. Precision and accuracy results for both treated and untreated QC samples were found within acceptance criteria and are presented in Table 7 and 8.

Table 7 Untreated Plasma Quality Control Samples

Intra-day (n=6)			
Nominal Conc (pg/mL)	Observed Conc (pg/mL)	Accuracy (%)	Precision (%RSD)
LQC (56.23 pg/mL)	56.90	101.19	5.91
MQC (766.23 pg/mL)	775.68	101.23	1.90
HQC (7506.23 pg/mL)	7727.64	102.95	2.54

Table 8 Treated Plasma Quality Control Samples

Intra-day (n=6)			
Nominal Conc (pg/mL)	Observed Conc (pg/mL)	Accuracy (%)	Precision (%RSD)
LQC (50.0 pg/mL)	47.44	94.87	4.17
MQC (760.0 pg/mL)	774.07	101.85	1.19
HQC (7500.0 pg/mL)	7057.76	94.10	10.38

- **Recovery**

  Recovery was determined by comparing the detector area response of melatonin at three distinct levels of extracted low, medium and high-quality control samples with detector area response obtained from the post extracted low, medium and high-quality control samples. The mean recovery at low, medium and high-quality control samples were 79.23%, 79.28% and 79.17% respectively. Global recovery of melatonin was found 79.23 % with %RSD of 0.07%. Recovery results are presented in in Table 9.

  The mean recovery for melatonin D4 (ISTD) was 72.23%.

Table 9 Global Recovery

QC level	Recovery
LQC (n=6)	79.23
MQC (n=6)	79.28
HQC (n=6)	79.17
Mean	**79.23**
SD	**0.05**
%RSD	**0.07**

- **Matrix effect**

  Matrix effect was studied at LQC and HQC levels. IS normalized matrix factor was found to be 1.12 and 1.05 respectively at LQC and HQC levels. Representative data of matrix effect is presented in Table 10. The results confirm the suitability of the method for quantitative estimation of melatonin in human plasma.

Table 10 Matrix effect

Melatonin	Aqueous sample	Post extracted sample	Matrix factor
LQC	0.27	0.28	1.04
	0.27	0.31	1.15
	0.26	0.28	1.08
	0.25	0.30	1.20
	0.24	0.28	1.17
	0.26	0.29	1.12
Mean			**1.12**
SD			**0.06**
%RSD			**5.34**

Melatonin	Aqueous sample	Post extracted sample	Matrix factor
HQC	38.58	42.60	1.10
	39.77	42.16	1.06
	37.72	40.79	1.08
	39.32	41.75	1.06
	39.86	40.97	1.03
	40.35	39.87	0.99
Mean			**1.05**
SD			**0.04**
%RSD			**3.88**

- **Carry-over effect**

  Carryover was evaluated by injecting extracted samples in the sequence of extracted blank, extracted highest calibrator, extracted blank and extracted lowest calibrator. No carryover was observed at the retention time and MRM transition of the melatonin and melatonin-D4 in the extracted blank sample following the highest standard calibrator.

6. Conclusion

Shimadzu LCMS-8045, along with special sample preparation, optimized chromatography provides a very selective and sensitive method for quantification of melatonin. Ultra-high speed and high-separation analysis was achieved on Nexera X2 UHPLC by using a simple mobile phase at a minimal isocratic flow rate of 0.4 mL/min. By providing these ready to use solutions, we partner with your labs to achieve desired results in your scientific endeavors.

7. References

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