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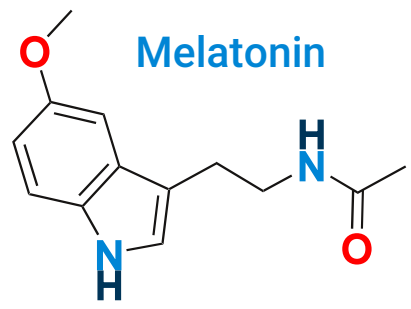
Catch Them Sleeping: Quick and Routine Quantification of Melatonin in Plasma with Ultivo LC/TQ

Mark J. Sartain and Aaron Boice

Agilent Technologies, Inc., Santa Clara, CA, USA

Introduction

Melatonin (MEL) is an endogenous hormone and potent neurotransmitter that helps regulate circadian rhythm and sleep. It is commonly prescribed as a treatment for sleep disorders such as insomnia. Studies on pharmacokinetics and endogenous MEL production require analytical techniques capable of measuring low circulating levels of MEL, which can vary by individual and time of day. Due to their greater specificity, LC/MS/MS methodologies have gained popularity over conventional radioimmunoassays and have been developed to meet the bio-analytical requirements for sensitive and specific quantification of MEL in plasma.



In this work we demonstrate that a triple quadrupole LC/MS equipped with a standard ESI source and an optimized MRM method achieves the required analytical sensitivity for precise quantification of MEL in human plasma.

Experimental

Reagents and Sample Preparation

MEL was extracted from human plasma with a simple acetonitrile-based protein precipitation procedure. For lower limit of quantitation (LLOQ) determination of MEL-D4 in plasma, levels with concentrations ranging from 10 pg/mL to 100 ng/mL were prepared in plasma extracts. For recovery evaluation, plasma was spiked with MEL-D4 prior to extraction and compared to post-spiked plasma. For determination of endogenous MEL, plasma was pre-spiked with MEL-D4 to adjust for losses through sample preparation and enable accurate quantification.

Experimental

LC/MS Method

Major UHPLC and MS parameters are as follows:

Agilent 1260 Infinity II Prime LC System	
Column	Agilent InfinityLab Poroshell 120 EC-C18, 2.1x50mm, 1.9 µm (p/n 699675-902)
Column temp.	35 ºC
Injection vol.	3 µL (stacked injection program)
Mobile phase	A = 5 mM ammonium formate, 0.2 mM ammonium fluoride, 0.1 % formic acid in water B = 5 mM ammonium formate, 0.2 mM ammonium fluoride, 0.1 % formic acid in methanol
Flow rate	400 µL/min
Gradient	Time %A %B 0.00 95 5 3.00 55 45 3.10 2 98 4.10 2 98 4.20 95 5 6.50 95 5

Agilent Ultivo Triple Quadrupole LC/MS	
Ion source	ESI
Polarity	Positive
Drying gas (nitrogen), Temp	13 L/min, 350 ºC
Nebulizer gas (nitrogen)	60 psi
Capillary voltage	2,000 V
Scan type	MRM
Cycle time	564 ms
Total number of MRMs	4
Dwell time per MRM	140 ms

Ultivo LC/TQ with ESI Ion Source

Method Optimization

Agilent MassHunter Optimizer software was used to optimize MRM transitions for MEL and MEL-D4. Optimized fragmentor and collision energy (CE) voltages were compared for MEL with previously obtained values using Optimizer software with an Agilent 6470 LC/TQ mass spectrometer (Fig 1). The same top two product ions (*m/z* 174 and 159) were selected in both cases, and results demonstrated remarkably similar ion breakdown profiles and optimized parameters. This is a typical example that illustrates MRM methods can be confidently migrated across Agilent LC/TQ instruments.

Parameter	6470	Ultivo-ESI
Fragmentor V	90	87
CE (233 → 174)	12	12
CE (233 → 159)	32	32

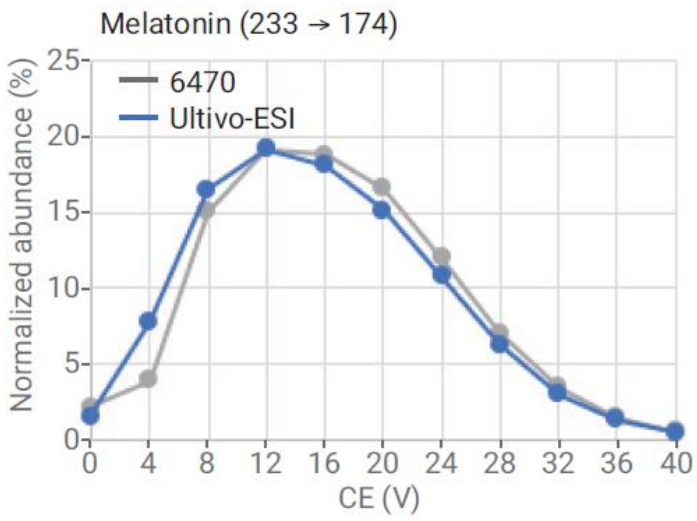


Figure 1. Comparison of ion breakdown profiles and optimized fragmentor and CE voltages for both the 6470 and Ultivo LC/TQ systems obtained with MassHunter Optimizer software.

Analytical Sensitivity, Precision, and Linearity

Due to the presence of endogenous MEL in plasma, MEL-D4 spiked into plasma extract was used to determine the limit of detection (LOD), LLOQ, and the upper limit of quantitation (ULOQ) of MEL (Fig 2). The LOD was defined as the lowest concentration significantly different from the blank with a signal-to-noise ratio (S/N) greater than three. Given the plasma samples were concentrated two-fold, the original concentrations in plasma were also calculated.

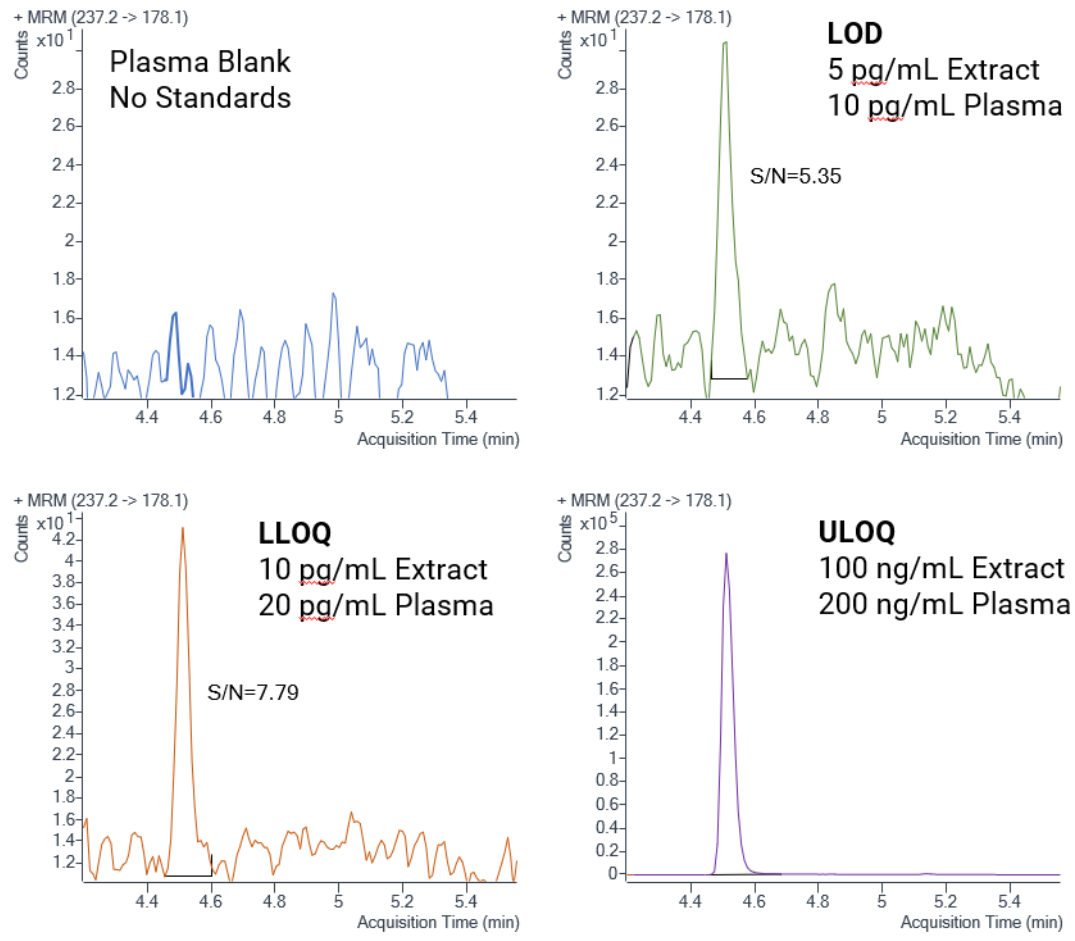
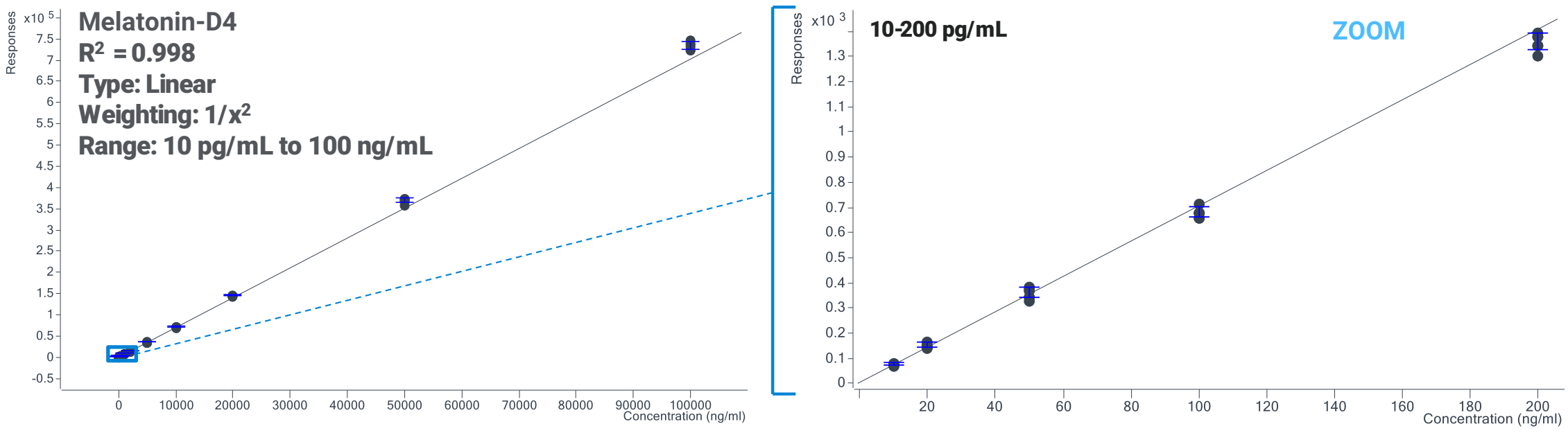


Figure 2. Representative quantifier (*m/z* 237.2 → 178.1) MRM chromatograms for MEL-D4 spiked into plasma extract.

The precision and accuracy of measurements were evaluated at 13 concentrations ranging from the LLOQ at 10 pg/mL to the ULOQ at 100 ng/mL, and were calculated from six replicate injections at each level. Excellent assay precision (RSD% <10 %) as well as average accuracy (95 to 105 %) were obtained for all levels. The correlation coefficient (R^2) for the calibration curve was 0.998 over four orders of dynamic range (Fig 3). Excellent retention time precision was observed (RSD% = 0.07 %) for the 78 injections.

Results and Discussion



Melatonin-D4	Concentration (pg/mL)												
	10	20	50	100	200	500	1,000	2,000	5,000	10,000	20,000	50,000	100,000
%Accuracy (average, n= 6)	99.5	102.3	100.0	95.6	96.2	96.9	99.1	99.0	99.6	99.9	102.7	104.8	104.3
Reproducibility (%RSD, n= 6)	6.5	7.2	5.8	3.0	2.4	1.4	1.1	1.1	0.7	0.6	0.9	1.5	1.2

Figure 3. Calibration curve for MEL-D4 spiked into plasma extract. Average accuracies and precision (%RSD) for each level are provided in the table.

Quantification of Endogenous MEL

A simple protein precipitation procedure was evaluated for extraction recovery of MEL from plasma. MEL-D4 was used as a surrogate for MEL for recovery % calculations. MEL-D4 was pre- and post-spiked at 1 ng per mL of plasma, and four replicates of each were evaluated. Average recovery was 95.0 % (±6.0 %, 1 SD).

Endogenous MEL was calculated from the external calibration curve of MEL-D4 spiked into plasma extract (Fig 4 and Table 1). To account for losses in the sample preparation, the calculated concentrations were corrected with the observed-versus-expected MRM peak areas ratio from pre-spiked MEL-D4.

Extraction Replicate	RT (min)	% Expected Qualifier Ratio	Calculated Conc. (pg per ml extract)	Observed/ Expected Mel-D4 Ratio	Dil. Factor	Final Conc. (pg per ml plasma)
Plasma 1	4.535	103.5	71.2	0.80	0.5	44.5
Plasma 2	4.535	98.0	73.9	0.96	0.5	38.5
Plasma 3	4.535	96.9	75.9	0.96	0.5	39.5
Plasma 4	4.535	108.0	76.5	1.01	0.5	37.9
Plasma 5	4.535	95.4	75.6	0.87	0.5	43.4
					Average ± SD	40.8 ± 3.0
					RSD%	7.3%

Table 1. Metrics and calculations for endogenous MEL from five plasma extraction replicates.

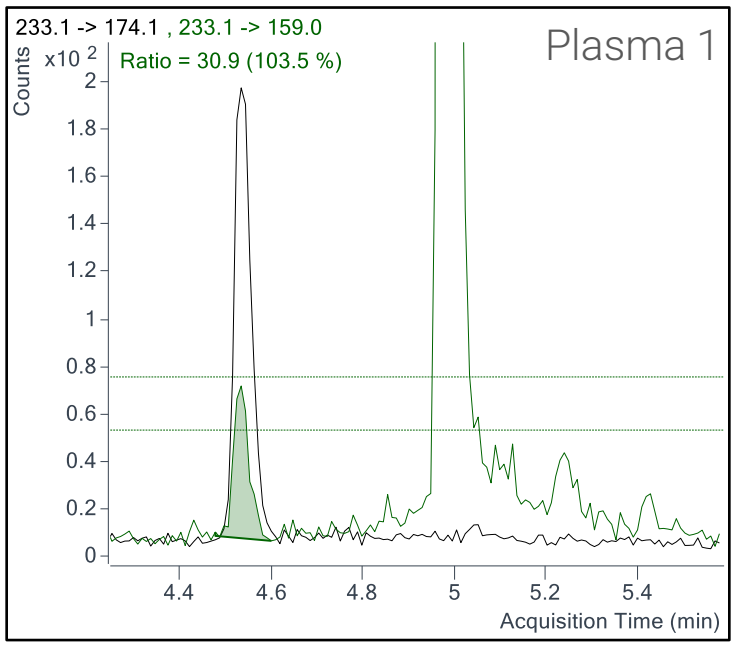


Figure 4. Typical MRM chromatograms for endogenous MEL in human plasma.

Conclusions

A targeted MRM method for melatonin was developed on the new Ultivo with ESI ion source that provides:

- Analytical sensitivity required for low ppt-level research quantification in plasma
- Minimal sample preparation requirements
- An economical and fit-for-purpose instrument

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