Application Note

Clinical Research

Quick and Routine Research Quantification of Melatonin in Plasma with the Agilent Ultivo LC/TQ

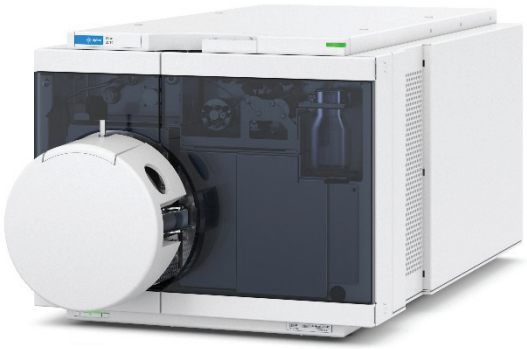

Figure 1. Ultivo LC/TQ with standard ESI ion source.

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Abstract

This Application Note demonstrates the research quantification of melatonin (MEL) with an Agilent 1260 Infinity II Prime LC system coupled to an Agilent Ultivo triple quadrupole LC/MS with an ESI source. The optimized LC/MS method achieves a lower limit of quantification (LLOQ) of 10 pg/mL melatonin-D4 (MEL-D4) spiked into plasma. This sensitive, fast research method can quantify low levels of endogenous MEL from a complex human plasma extract prepared with a simple protein-precipitation procedure.

Introduction

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 radioimmunoassay, and have been developed to meet the bio-analytical requirements for sensitive and specific quantification of MEL in plasma.

This Application Note demonstrates that a 1260 Infinity II Prime LC system with an Ultivo LC/TQ equipped with an ESI source achieves the required analytical sensitivity for precise quantification of MEL in human plasma.

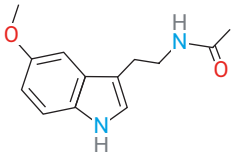


Figure 2. Melatonin.

Experimental

Reagents and chemicals

All reagents and solvents were HPLC or LC/MS grade. Acetonitrile and methanol were purchased from Honeywell (Morristown, NJ, USA). Ultrapure water was produced with a Milli-Q Integral system equipped with an LC-Pak Polisher and a 0.22-µm point-of-use membrane filter cartridge (EMD Millipore, Billerica, MA, USA). LC/MS-grade formic acid was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Ammonium fluoride and LC/MS grade ammonium formate were purchased from Millipore Sigma (St. Louis, MO, USA). MEL (≥98 %) and MEL-D4 (≥98 % MEL, ≥99 % deuterated forms D1–D4) were purchased from Cayman Chemical (Ann Arbor, MI). NIST SRM 1950 human plasma was purchased from Millipore Sigma. For recovery studies only, citrated human plasma was also purchased from Millipore Sigma.

For LLOQ determination of MEL-D4 in plasma, 13 calibration levels with concentrations ranging from 10 pg/mL to 100 ng/mL were prepared in plasma extracts. Following manufacturer recommendations for deuterated MEL (D1-D4), this standard stock mixture was first quantitated against a precise quantity of unlabeled MEL through a standard curve relating peak intensity ratios of the quantifier transitions (237.1 → 178.1 versus 233.1 → 174.1). We did not observe significant amounts of the D0 form in MEL-D4. This was evidenced by the lack of significant unlabeled MEL MRM signal (<0.1 %) when analyzing the 100 ng/mL MEL-D4 level in neat solvent.

Sample preparation

For all experiments, protein precipitation of plasma was as follows: EDTA (0.5 M, 50 µL) was added to a 200 µL aliquot of plasma on ice, and vortexed briefly. Acetonitrile (750 µL) was added, vortexed for 30 seconds, incubated on ice for 20 minutes, and centrifuged at 16,000 × g for 10 minutes at 4 °C to pellet proteins. The organic supernatants (800 µL) were transferred to 2-mL amber LC/MS vials, and dried by a vacuum concentrator. The extracts were reconstituted with 100 µL of methanol, bath-sonicated for five minutes, and transferred to 250-µL glass autosampler vial inserts for LC/MS analysis. Alternatively, reconstituted plasma was pooled to prepare the calibration curve samples. MEL is a light-sensitive compound, and care was taken to minimize light exposure.

For recovery evaluation, four replicates of plasma were spiked with MT-D4 at 1 ng per mL of plasma prior to extraction (prespiked). These replicates were compared to four replicates of post spiked plasma for recovery calculations.

For determination of endogenous MEL, an aliquot of plasma was prespiked with MT-D4 at 10 ng per mL of plasma to adjust for losses through sample preparation and enable accurate quantification.

Instrumentation

Agilent 1260 Infinity II Prime LC:

- Agilent 1260 Infinity II Prime flexible pump (G7104C)
- Agilent 1260 Infinity II multisampler with cooler (G7167A)
- Agilent 1260 Infinity II multicolumn thermostat (G7116A)
- Agilent Ultivo triple quadrupole LC/MS system
- Agilent electrospray ionization source (G1948B)

Method

The LC/MS conditions and parameters are provided in the following tables.

Software

Agilent MassHunter LC/MS Data Acquisition for Ultivo 1.1 software, including MassHunter Optimizer and MassHunter Source Optimizer software were used to operate the Ultivo-ESI LC/MS system and optimize parameters. MRM data were quantitated using Agilent MassHunter Quantitative Analysis Software with the Quant-My-Way feature (version B.09).

Results and discussion

Method optimization

MassHunter Optimizer software was used to optimize MRM transitions for MEL and MEL-D4 (Table 1).

1260 Infinity II Prime LC System	
Column	Agilent InfinityLab Poroshell 120 EC-C18, 2.1 × 50 mm, 1.9 µm (p/n699675-902)
Column temperature	35 °C
Injection volume	3 µL with stacked injection program
Autosampler temperature	4 °C
Needle wash	10 seconds in flush port (50:50 methanol/H_2O)
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 program	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
Stop time	6.5 minutes
Observed pressure range	350 to 570 bar

Ultivo triple quadrupole LC/MS	
Ion source	ESI
Polarity	Positive
Gas temperature	350 °C
Gas flow	13 L/min
Nebulizer pressure	60 psi
Capillary voltage	2,000 V (+)
Scan type	MRM
Q1/Q2 resolution	Unit (0.7 amu)
Cycle time	564 ms
Total number of MRMs	4
Dwell time per MRM	140 ms

Table 1. Optimized MRM transition parameters.

Compound	Type	Precursor (*m/z*)	Product (*m/z*)	Fragmentor (V)	CE (V)
MEL	Quantifier	233.1	174.1	87	12
MEL	Qualifier	233.1	159.0	87	32
MEL-D4	Quantifier	237.2	178.1	87	12
MEL-D4	Qualifier	237.2	163.1	87	32

Optimized fragmentor and collision energy (CE) voltages were compared for MEL with previously obtained values using MassHunter Optimizer software with an Agilent 6470 triple quadrupole LC/MS (Figure 3). 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 illustrating that MRM methods can confidently be migrated across Agilent LC/TQ instruments.

Method 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 (Figure 4). 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 that the plasma samples were concentrated two-fold, the original concentrations in plasma were also calculated.

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

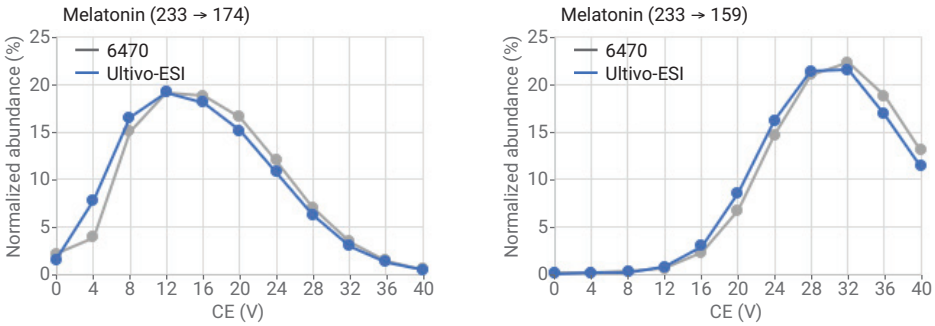


Figure 3. Comparison of ion breakdown profiles and optimized fragmentor and CE voltages for a 6470 triple quadrupole LC/MS and Ultivo-ESI, obtained with MassHunter Optimizer software.

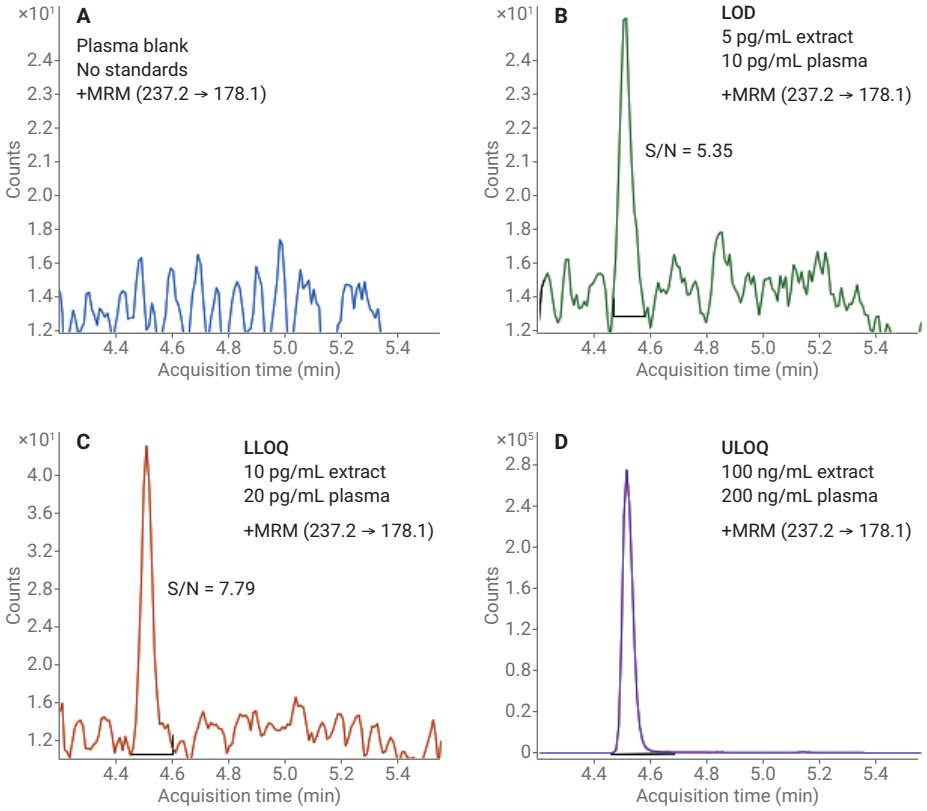
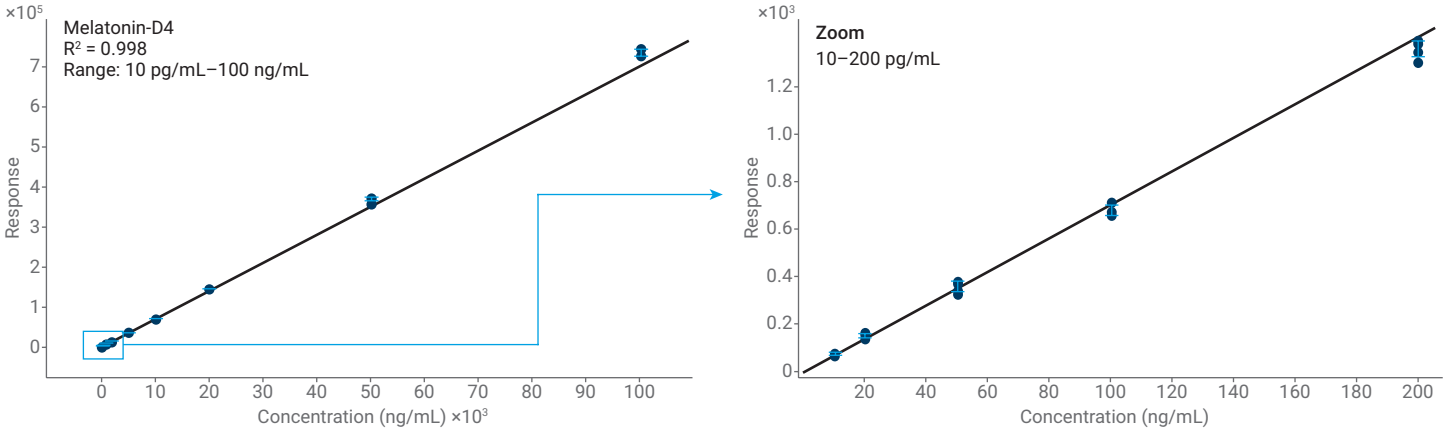


Figure 4. Representative quantifier (*m/z* 237.2 → 178.1) MRM chromatograms for MT-D4 spiked into plasma extract.

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 (Figure 5). Excellent retention time precision was observed (RSD% = 0.07 %) for the 78 injections.

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 postspiked at 1 ng per mL of plasma, and four replicates of each were evaluated. Average recovery was 95.0 % (±6.0 %, 1 SD).



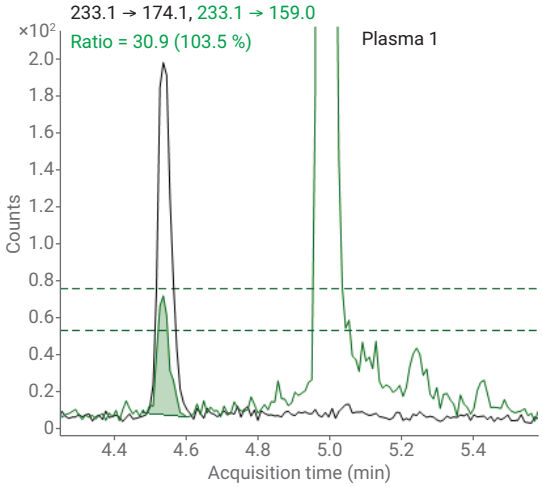
Melatonin-D4	Concentration												
	10	20	50	100	200	500	1,000	2,000	5,000	10,000	20,000	50,000	100,000
%Accuracy (average, n = 6)	99.5	102.3	100.0	95.6	96.2	96.9	99.1	99.0	99.6	99.9	102.7	104.8	104.3
Reproducibility (%RSD, n = 6)	6.5	7.2	5.8	3.0	2.4	1.4	1.1	1.1	0.7	0.6	0.9	1.5	1.2

Figure 5. Calibration curve for MEL-D4 spiked into plasma extract. Average accuracies and precision (%RSD) for each level are provided in the table.

The NIST SRM 1950 plasma is pooled plasma intended to represent normal human plasma, and should have a MEL concentration representative of an averaged human population. Endogenous MEL was calculated from the external calibration curve of MEL-D4 spiked into plasma extract. To account for losses in the sample preparation, the calculated concentrations were corrected with the observed-versus-expected MRM peak areas ratio from prespiked MEL-D4. A dilution factor of 0.5 was applied to determine the final concentration of MEL in plasma. Five extraction replicates were analyzed (Figure 6).

Conclusion

This Application Note demonstrates that the Ultivo-ESI inherits the outstanding performance of the standard Ultivo system. It achieves the required analytical sensitivity for low ppt level research quantification of melatonin in plasma. The system is also an economical and fit-for-purpose instrument for quantifying low levels of endogenous melatonin from plasma with minimal sample preparation.



Extraction replicate	RT (min)	% expected qualifier ratio	Calculated concentration (pg per mL extract)	Observed/expected Mel-D4 ratio	Dilution factor	Final concentration (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
					Avg. ±SD	40.8 ±3.0
					RSD%	7.3 %

Figure 6. Endogenous MEL in human plasma. The table provides metrics and calculations for MEL from five extraction replicates. Shown above is a typical MRM chromatogram for endogenous MEL in plasma.

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