Determination of an Endogenous Biomarker (Melatonin) at Picogram Levels in Human Plasma

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INTRODUCTION

Melatonin is a hormone secreted by the pineal gland in the brain. It helps to regulate other hormones and maintains the body's circadian rhythm. The circadian rhythm is an internal 24-hour "body clock" which plays a critical role in the sleep-wake cycle. When it is dark, the body produces more melatonin; when it is light, the production of melatonin decreases. Commercial kits are available for ELISA and RIA, but are either not sufficiently sensitive or are complex and expensive to run routinely.

A highly sensitive, robust, picogram level LC-MS/MS method for the determination of melatonin was developed. The method was fully validated to current regulatory standards and used to support clinical studies.

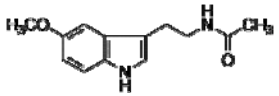

Figure 1: Structure of melatonin

OBJECTIVES

To develop and validate an automated method for the determination of picogram levels of endogenous melatonin in human plasma by LC-MS/MS.

The method must employ tight control over contamination and carry-over. It also required clean extracts to minimize the back-ground noise and sharp chromatography to achieve the desired lower limit of quantitation (LLOQ) with a low sample volume.

The target LLOQ for melatonin was 1.0 pg/mL.

Key characteristics of the method:

A solid phase extraction method utilising an ion-exchange sorbent was developed. The sample volume was 200 µL of human plasma. Plasma samples from different individuals were screened for the level of melatonin prior to preparing QCs. The surrogate matrix was prepared to mimic the study matrix and used for calibration standards and the LLOQ QC. A mixture of phosphate buffered saline and bovine serum albumin was used as a surrogate matrix.

Figure 2: Hamilton Microlab Star Robot

The extraction procedure was carried out using a Hamilton Microlab Star robot to prevent any possible contamination during extraction.

Analytical standards were prepared in a controlled environment to avoid contamination of the laboratory working area. Melatonin-d_4 was used as an internal standard.

HPLC separation was carried out on a Poroshell C18 sub 3 µm fused core column at a flow rate of 0.6 mL/min with a 4.5 minute gradient comprising 0.2% formic acid in in methanol/water/1M ammonium formate (10/90/0.2) and comprising 0.2% formic acid in methanol/water/1M ammonium formate (90/10/0.2). The typical injection volume was 10.0 µL.

Mass Spectrometry:

Instrument:	Waters Xevo TQ-S
Mode:	MRM
Interface:	APCI (positive)
Ions monitored:	233.1 → 174.1 (Melatonin)
	237.1 → 178.1 (Melatonin-D_4)
Dwell time:	100 ms
Temperature:	500°C

RESULTS

Optimum sensitivity

A lower limit of quantitation of 1.0 pg/mL for melatonin was achieved by limiting the dilution through extraction. SPE was utilised rather than simpler extraction methods to achieve this goal.

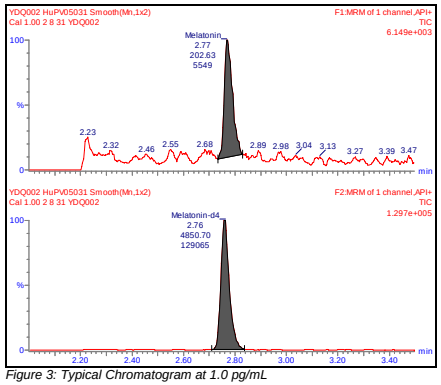

Figure 3: Typical Chromatogram at 1.0 pg/mL

Linearity

Calibration curves were generated over a 100-fold range by plotting the analyte to internal standard peak area ratios vs analyte concentration, utilising a linear regression with (1/x) weighting. Figure 4 represents the typical calibration line obtained during the validation study.

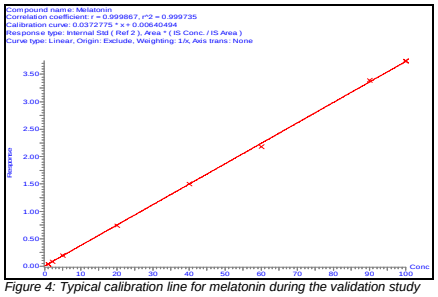

Figure 4: Typical calibration line for melatonin during the validation study

Run Date	Nominal Calibration Standard Concentration (pg/mL)							
	1.00	2.00	5.00	20.0	40.0	60.0	90.0	100
	Back-calculated concentration of Melatonin (pg/mL)							
26-Mar-2012	1.15	2.14	5.03	18.7	37.7	64.1	89.9	106
	0.84	-	-	-	-	-	-	93.9
29-Mar-2012	0.865	2.19	4.61	20.1	39.4	59.9	92	102
	1.12	-	-	-	-	-	-	96.7
29-Mar-2012	0.951	1.9	5.29	19.9	41.5	56.6	90.9	102
	1.04	-	-	-	-	-	-	99
02-Apr-2012	0.94	1.81	5.05	19.4	39.8	59.7	87.5	103
	1.18	-	-	-	-	-	-	101
03-Apr-2012	0.921	2.2	5.24	19.8	40.3	58.3	90.8	100
	0.949	-	-	-	-	-	-	100
03-Apr-2012	0.953	2.15	4.83	21.7	41.3	63	89.7	97.4
	0.898	-	-	-	-	-	-	97.2
03-Apr-2012	1.06	1.92	4.93	19.7	38.7	62	92.7	101
	1.01	-	-	-	-	-	-	96.5
Mean (pg/mL)	0.991	2.04	5.00	19.9	39.8	60.5	90.5	99.7
Standard Deviation (n-1)	0.106	0.162	0.235	0.915	1.36	2.66	1.7	3.16
Precision (CV%)	10.7	7.9	4.7	4.6	3.4	4.4	1.9	3.2
Bias (%)	-0.9	2.0	0.0	-0.5	-0.5	0.8	0.6	-0.3
n	14	7	7	7	7	7	7	14

Table 1: Back-calculated concentrations (ng/mL) of melatonin Calibration Standards

All coefficients of determination (r^2) of the calibration lines during validation were better than or equal to 0.9968.

Precision and Bias

Run Date	Nominal Quality Control sample Concentration (pg/mL)					
	1.00	3.00	10.0	80.0	100	250[a]
	Back-calculated concentration of Melatonin (pg/mL)					
Mean intra-run mean (pg/mL)	0.970	3.01	11.4	77.7	98.3	257
Mean Intra-run precision (CV%)	9.4	5.9	7.0	3.2	5.2	4.1
Mean Intra-run bias (%)	-3.0	0.2	13.7	-2.9	-1.7	2.8
Inter-run mean (pg/mL)	0.969	3.01	11.4	77.7	98.2	257
Inter-run standard Deviation (n-1)	0.140	0.174	1.24	2.67	6.73	11.4
Inter-run precision (CV%)	14.4	5.8	10.9	3.4	6.9	4.4
Inter-run bias (%)	-3.1	0.3	14.0	-2.9	-1.8	2.8
n	18	18	18	18	18	18

[a]. diltuion QC, diluted ten fold prior to extraction

Table 2: Intra-run and inter-run precision and bias of QC samples

For QC samples, the intra run CV ranged from 3.2% to 9.4% and the intra-run bias ranged from -3.0% to 13.7%. The inter run CV ranged from 3.4% to 14.4% and the intra-run bias ranged from -3.1% to 14.0%

Precision (CV%) and bias (%) for all quality controls were within acceptable limits. The signal to noise ratio was greater than five to one at the LLOQ.

Carry-over

No carry-over was observed, using Waters iClass Acquity LC system

Recovery

The mean recovery of melatonin at low, medium and high levels was 70%.

Matrix related modification of ionisation

Inter-individual matrix related modification of ionisation was investigated in plasma from six different subjects. Individual plasma samples were spiked with calibration standards. Each calibration curve should be of similar gradient to prove that matrix effects are not present following elimination of the effect of the endogenous melatonin.

Matrix ID	Calibration slope	Mean (CV%)	% difference from surrogate slope
Surrogate matrix	3.53E-02	-	
1	3.67E-02		
2	3.38E-02		
3	3.58E-02	3.46E-02	-2.0
4	3.42E-02	(3.8)	
5	3.40E-02		
6	3.33E-02		

Table 3: Matrix related modification of ionisation

The gradient of the six individual slopes had a CV of 3.8%.

Effect of lipaemia and haemolysis

The observed precision was 1.7% for both lipaemia and haemolysis. The observed bias was -6.7% and -3.4% for lipaemia and haemolysis, respectively.

Stability

Condition	Minimum stability
Freeze/thaw cycles	5 cycles
Room temperature stability	24 hours
Whole blood at RT	4 hours
Whole blood on ice	4 hours
Re-injection stability at 4°C	Up to 127 hours
Long term stability at -80°C	1 month*

***Additional long term stability for 3 month is planned to be conducted**

CONCLUSION

All three components of the method, analyte isolation, separation and detection were optimised for maximum selectivity and sensitivity to achieve a 1.0 pg/mL LLOQ for melatonin in human plasma. Melatonin was isolated from plasma using solid phase extraction methodology. A Hamilton Microlab Star robot used to automate the extraction procedure. Chromatographic separation was achieved with a sub 3 µm fused core column with a slow gradient. The Waters Xevo TQ-S LC-MS/MS system equipped with APCI interface was used to reach the lower limit of quantitation.

A sensitive, accurate and robust method for the determination of melatonin in human plasma was fully developed and validated at YBS. The method is currently being used to support a number of clinical studies.

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