Published in final edited form as:

J Mass Spectrom. 2012 March ; 47(3): 277–285. doi:10.1002/jms.2051.

Quantitation of Melatonin and N-acetylserotonin in Human Plasma by Nanoflow LC-MS/MS and Electrospray LC-MS/MS

Melissa D. Carter^{*}, M. Wade Calcutt^{*}, Beth A. Malow[‡], Kristie L. Rose^{*}, and David L. Hachey^{*,†,§}

^{*}Vanderbilt University, Department of Biochemistry, Nashville, TN 37235, USA

[†]Vanderbilt University, Department of Pharmacology, Nashville, TN 37232, USA

[‡]Vanderbilt University, Department of Neurology, Nashville, TN 37232, USA

Abstract

Melatonin (MEL) and its chemical precursor N-acetylserotonin (NAS) are believed to be potential biomarkers for sleep-related disorders. Measurement of these compounds, however, has proven to be difficult due to their low circulating levels, especially that of NAS. Few methods offer the sensitivity, specificity and dynamic range needed to monitor MEL and its precursors and metabolites in small blood samples, such as those obtained from pediatric patients. In support of our ongoing study to determine the safety, tolerability, and PK dosing strategies for MEL in treating insomnia in children with autism spectrum disorder, two highly sensitive LC-MS/MS assays were developed for the quantitation of MEL and precursor NAS at pg/mL levels in small volumes of human plasma. A validated electrospray ionization (ESI) method was used to quantitate high levels of MEL in PK studies and a validated nanospray (nESI) method was developed for quantitation of MEL and NAS at endogenous levels. In both assays plasma samples were processed by centrifugal membrane dialysis after addition of stable isotopic internal standards, and the components were separated by either conventional LC using a Waters SymmetryShield RP18 column (2.1×100 mm, 3.5 μm) or on a polyimide-coated, fused-silica capillary self-packed with 17 cm AquaC18 (3 μm, 125 Å). Quantitation was done using the SRM transitions m/z 233→174 and m/z 219→160 for MEL and NAS, respectively. The analytical response ratio vs. concentration curves were linear for MEL (nanoflow LC: 11.7–1165 pg/mL, LC: 1165–116500 pg/mL) and for NAS (nanoflow LC: 11.0–1095 pg/mL).

Keywords

Melatonin; N-acetylserotonin; Autism Spectrum Disorder; Nanoflow LC-MS/MS

INTRODUCTION

Melatonin (MEL) is a potent neurotransmitter and hormone produced primarily in the pineal gland that helps regulate the circadian rhythm and sleep.¹ This endogenous indoleamine is synthesized from tryptophan enzymatically through a number of key intermediates, including 5-hydroxytryptophan, serotonin and N-acetylserotonin (Scheme 1). MEL is commonly prescribed as a sedative, antioxidant, chronobiotic, antihypertensive and anxiolytic, but its popularity has been boosted chiefly by its efficacy in treating sleep-related disorders such as insomnia.² While a definitive mechanism of biological activity remains elusive, many of MELs medicinal properties have been linked to its affinity for G_i-coupled

[§]Corresponding author. Fax: +1 615 343 1268, david.l.hachey@vanderbilt.edu (D. L. Hachey).

MEL receptors, G_i-coupled opioid μ -receptors and GABA-B receptors, and its role in arginine metabolism.²⁻⁴

MEL's use in sleep regulation is particularly important in patients with autism spectrum disorder (ASD).⁵⁻⁹ About two-thirds of patients with ASD suffer from sleep disturbance with the most common clinical subtype being insomnia.⁵ In a recent meta-analysis of MEL treatment in ASD, 12 out of 18 studies reported an overall improvement rate of 84.2% (95% CI 81.4–88.9%) in sleep for the 349 participants, where improvement was qualified by fewer night wakings, earlier sleep onset and greater sleep duration.⁵ Generally, lower levels of MEL are observed in patients with ASD versus controls, but the reason is unclear.⁶⁻⁹ One report showed an association of a low urinary melatonin metabolite (6-sulfatoxymelatonin) and deeper levels of non-rapid eye movement sleep.¹⁰ Reduced production of MEL has been attributed to a range of factors including alterations in serotonergic or ASMT activity.^{7, 11, 12}

Studies on the endogenous production of MEL and its role in ASD have been difficult from an analytical perspective due to the low levels of MEL and related metabolites in human plasma. The most popular means of MEL quantitation is the use of antisera immunoassays like radioimmunoassay (RIA) or enzyme-linked immunoassay (ELISA).¹³ While these methods can achieve lower limits of quantitation (LLOQ) of 3 pg/mL in plasma, they require larger volumes of plasma (200–500 μ L), may exhibit crossreactivity with other indoleamines and saturate at upper limits of quantitation (ULOQ) of 1000 pg/mL.¹³ These limitations prove problematic for a thorough study of the MEL production pathway or in PK studies where the levels in plasma are much higher than the upper range of the immunoassay, and thus require dilution. Herein, we describe novel methods that incorporate not only the specificity of conventional LC-MS/MS, but also the increased sensitivity of nanoflow LC-MS/MS, in order to determine the pharmacokinetics of MEL in adults, as well as quantitate endogenous production of MEL and, for the first time, the plasma levels of NAS in children with ASD.

MATERIALS AND METHODS

Chemicals

Commercially available melatonin (MEL) (Natrol®, Chatworth, CA), N-acetyl-5-hydroxytryptamine (NAS), L-ascorbic acid, acetic acid and ammonium citrate were purchased from Sigma Aldrich (St. Louis, MO, USA). N-acetyl-5-methoxy-tryptamine- $\alpha,\alpha,\beta,\beta$ -d₄ (d₄-MEL) was obtained from C/D/N Isotopes, Inc. (Pointe-Claire, Quebec, Canada). D₇-N-acetylserotonin (d₇-NAS) was a gift from Dr. David Klein of the National Institute of Child Health and Human Development (received via the National Institute of Mental Health Chemical Synthesis and Drug Supply Program NIMH # A-906, Bethesda, MD, USA). Ultra pure LC acetonitrile, water and methanol solvents were ordered from JT Baker-VWR International, Inc. (Suwanee, GA, USA). Aqua 3 μ m, C18, 125 Å bulk packing material was purchased from Phenomenex (Torrance, CA, USA). Natrol melatonin, 2.5 mg liquid, was obtained from Botanical Laboratories (Ferndale, WA, USA).

Adult Pharmacokinetic Studies

Healthy adult volunteers were given three oral doses of 1 mg, 3 mg or 6 mg melatonin (Natrol®, Chatworth, CA) at approximately 8 A.M. and subjected to blood draws via an intravenous catheter at 10, 20, 30, 45, 60, 75, 90, 120, 150, 180, 240, 300, 360 and 480 minutes post-dose. The individual studies were separated by two days to allow for washout of the drug. Study protocol was approved by the institutional review board (IRB # 070126) of Vanderbilt University Medical Center.

MEL and NAS Endogenous Production Studies in Children with ASD

A cohort of 10 prepubescent (3–8 yrs old) children diagnosed with ASD, who were not taking medication other than those approved for allergy symptoms, were enrolled in a study measuring endogenous production of nighttime MEL and NAS. Children were admitted overnight to the Vanderbilt Clinical Research Center, and blood draws were taken at the child's natural bedtime and at 10, 20, 30, 45, 60, 75, 90, 120, 150, 180, 240, 300, 360 and 480 minutes post-bedtime (between 1900 and 0700 hours) via an intravenous catheter. Total blood drawn throughout the study was ≤ 5 mL, yielding ≤ 200 μ L plasma per blood draw. All protocols were approved by the institutional review board (IRB # 071269) of Vanderbilt University Medical Center.

Preparation of Human Plasma Samples

Patient blood samples were collected in the Vanderbilt Clinical Research Center following approved IRB protocols. Heparinized polypropylene tubes were used for blood collection. Tubes were chilled on ice immediately following collection. Plasma was harvested within 1 h of the blood draw by centrifuging samples at $5000 \times g$ for 3 min at 4°C. Samples were stored at -80°C until analysis.

Plasma Sample Processing

For all samples (calibration and clinical), 100 μ L of plasma was added to Amicon Ultra centrifugal filters (0.5 mL, 3kDa, UFC 500396) followed by 10 μ L of the appropriate working stock of MEL or NAS as well as 10 μ L of internal standard stock solutions of d₄-MEL or d₇-NAS. Samples that did not receive additions of d₀-MEL or d₀-NAS were referred to as “blank plasma”. Samples were diluted with 100 μ L of water/acetonitrile (4:1) to facilitate dialysis, and the tubes were lightly vortexed and centrifuged at $10,000 \times g$ for 2 h at 10°C. Afterwards, the clear filtrate was removed and dried under a stream of N₂ gas. Dry samples were redissolved in 100 μ L of 75 mM ammonium citrate and 12.5 mM ascorbic acid (pH 6) buffer, vortexed and transferred to 300 μ L silanized autosampler vial inserts.

Preparation of Stock Solutions

MEL, d₄-MEL, NAS and d₇-NAS were weighed at milligram quantities using a UMT2 microbalance (Mettler Toledo, Columbus, OH). Compounds were dissolved in 75 mM ammonium citrate and 12.5 mM ascorbic acid (pH 6) buffer. Stock concentrations were confirmed by UV/Vis (at 210 nm)-LC-MS/MS and stored at 5 μ M at -80°C . Stocks were thawed at room temperature prior to use, and working stock dilutions were made (0.05–500 nM, MEL and NAS; 20 nM d₄-MEL and d₇-NAS) by serial dilutions in 75 mM ammonium citrate and 12.5 mM ascorbic acid (pH 6) buffer. Blank plasma was obtained from volunteers and spiked with appropriate standards and used to construct QC and instrument calibration curves.

MEL Pharmacokinetics: Conventional Liquid Chromatography/Mass Spectrometry Analysis

Pharmacokinetic samples were analyzed for MEL on a Thermo Scientific TSQ Quantum Ultra triple-quadrupole instrument (Thermo Scientific, San Jose, CA, USA) equipped with an IonMax HESI source. The instrument was tuned and calibrated weekly over a mass range of m/z 182–997 using a tyrosine peptide mixture according to the manufacturer's autotune procedure. LC analysis was performed on a Waters Acquity UPLC system (Waters, Milford, MA, USA) using a SymmetryShield RP18 column (2.1 $\times$ 100 mm, 3.5 μ m, Waters, Milford, MA, USA). Column and autosampler temperatures were 50°C and 5°C, respectively. Mobile phases were 0.2% acetic acid in (A) water/acetonitrile (95:5) and in (B) water/acetonitrile (5:95). The following gradient conditions were used for a 20 μ L sample injection and

constant 300 μ L flow rate: 0–2 min 0% B; 2–7 min linear gradient 100% B; 7–8 min 100% B; 8–9 min linear gradient 0% B; 9–12 min 0% B. The following optimized instrument parameters were applied for the detection of analyte and internal standard: N₂ sheath gas 60 psi; N₂ auxiliary gas 20 psi; capillary temperature 300°C; spray voltage 4.5 kV; skimmer offset 10.0 V; capillary offset 35 V; tube lens offset 122 V; scan time 50 ms; and Q3 scan width 0.5 m/z . Quantitation was determined by MRM (d_0 -MEL m/z 233→174, collision energy 20 V; and d_4 -MEL m/z 237→178, collision energy 20 V) in positive ion mode.

Endogenous MEL and NAS: Nanoflow Liquid Chromatography/Mass Spectrometry Analysis

Endogenous production of MEL and NAS were determined on a Thermo TSQ Vantage triple quadrupole instrument (Thermo, San Jose, CA, USA). The instrument was tuned and calibrated bi-monthly over a mass range of m/z 182–997 using a tyrosine peptide mixture and manufacturer autotune procedure. Nano-LC elution was performed using a Waters Nanoacquity UPLC system (Waters, Milford, MA, USA). Samples (1 μ L) were injected onto self-packed, polyimide-coated, fused-silica capillary columns (360 μ m O.D. $\times$ 100 μ m I.D., Polymicro Technologies, Phoenix, AZ, USA) preceded by a trapping column fritted with a 2 mm Kasil frit (170 μ L potassium silicate and 30 μ L formamide) and packed with 5 cm of Aqua C18 (3 μ m, 125 Å) bulk packing material from Phenomenex (Torrance, CA, USA). The analytical column was packed with 17 cm Aqua C18 material and equipped with a 2 μ m emitter tip pulled using a P-2000 Laser Based Micropipette Puller (Sutter Instrument, Novato, CA, USA). The Aqua C18 material was suspended in methanol and columns were slurry-packed to desired capacity on a PicoView pressure loader (New Objective, Scientific Instruments Services, Ringoes, NJ, USA) under 1000 psi Helium. For each sample analyzed, 1 μ L of sample was injected into a 5 μ L loop using the Waters autosampler and then loaded onto the trapping column at 3 μ L/min for 4 min. Following sample loading, analytes were eluted with a gradient flow rate of 400 nL/min. The gradient consisted of: 0–1 min 0% B; 1–16 min linear gradient 40% B; 16–20 min linear gradient 90% B; 20–22 min 90% B; 22–23 min linear gradient 99% B; 23–27 min 99% B; 27–28 min linear gradient 90% B; 28–32 min 90% B; 32–38 min linear gradient 0% B. Mobile phases were 0.1% formic acid in (A) water and in (B) acetonitrile. The autosampler was kept at 5 °C. The following instrument parameters were applied for the detection of analyte and internal standard: capillary temperature 270°C; spray voltage 2.0 kV; scan time 250 ms; and Q3 scan width 0.5 m/z . Quantitation was determined by MRM (d_0 -MEL m/z 233→174, collision energy 20 V; d_4 -MEL m/z 237→178, collision energy 20 V; d_0 -NAS m/z 219→160, collision energy 13 V; and d_7 -NAS m/z 226→164, collision energy 20 V) in positive ion mode.

Data Acquisition and Processing

Data acquisition and quantitative spectral analysis were carried out utilizing Thermo Xcalibur v. 2.1.0 build 1139 and Thermo LCQuan v. 2.6.0 build 1128, respectively. Percent relative error was reported as $\%RE = [(C_e - C_t)/C_t] \times 100$ where C_e is the experimental concentration determined from the calibration curve slope, and C_t is theoretical concentration. Percent relative standard deviation $\%RSD = (SD/C_{avg}) \times 100$ was calculated as a measure of assay precision, where C_{avg} is the average concentration calculated, and SD is the standard deviation of C_{avg} . Peak area ratios of d_0/d_4 -MEL and d_0/d_7 -NAS were plotted to construct calibration curves of a series of six MEL and NAS plasma standards. Each standard was injected ($n=2$) and validated over the concentration range of 0.05–500 nM. The lower limit of quantitation (LLOQ) was defined as the lowest standards on the calibration curve with $\%RE$ and $\%RSD \leq 20\%$. Plasma quality controls (QCs) were made up at 0.2 and 5 nM for nanoflow LC-MS/MS and at 5 and 200 nM for electrospray LC-MS/MS and injected alongside calibration standards.

Pharmacokinetic Analysis

Pharmacokinetic parameters following oral dosing to humans were estimated using noncompartmental analysis methods in WinNonlin v. 5.2 (Pharsight, Palo Alto, CA, USA). Total body clearance, $Cl = [(Dose/BW)/AUC(0-inf)]$, was calculated where BW is body weight and AUC is the area under the plasma concentration-time curve given by the linear trapezoidal rule. Volume of distribution, $V_z = MRT \times Cl$, was calculated from the product of Cl and analyte mean residence time (MRT). For display purposes, plasma MEL from the adult study were also fit to the sum of three exponential terms using the numerical computation utility (Numwin) in SAAM II V1.1 (SAAM Institute, Univ. of Washington, Seattle, WA, USA).

RESULTS AND DISCUSSION

The circadian sleep-wake cycle is regulated by the suprachiasmatic nucleus (SCN) and triggered by light detection in the retina.¹⁴ When light begins to dim, the SCN initiates the secretion of MEL in the pineal gland and thus, sleep onset. This time point is known as dim light melatonin onset (DLMO) and is calculated as the first measurement above 10 pg/mL in serum from which MEL levels continue to increase.¹⁴ These levels peak between midnight and 5:00 AM and then decrease rapidly.¹³

Normal MEL patterns, as well as those induced by light, can vary by individual; therefore, deciphering a pattern due to age or disease often presents an analytical challenge. RIAs and ELISAs are very popular for clinical assays, but cross reactivity and nonspecific binding are still significant limitations.¹³ These methods also suffer from an ULOQ of ~350 – 1000 pg/mL, depending on the assay kit used, and cannot be easily adapted to high-dose pharmacokinetic studies unless multiple dilutions are used to bring the plasma concentration down to the working range of the assay kit. LC or capillary electrophoresis coupled to fluorescence detection^{15, 16} are common alternatives to RIA and ELISA, but MEL's co-elution with other indoleamines can limit the specificity of these methods¹³. GC-MS is sensitive and usually offers higher chromatographic resolution than LC, but MEL and its intermediates must be chemically derivatized prior to the analysis.¹⁷ We now report a validated assay for the reliable quantitation of MEL in small plasma volumes over a combined concentration range (nanoflow- and LC-MS/MS) of 11.65–116500 pg/mL with minimal sample processing.

Selectivity

Collision-induced dissociation of the precursor ions of NAS and MEL generated predominant product ions of m/z 160 and 174, respectively (Fig. 1). Sodium adducts were observed in the precursor ion spectra at m/z 255 for MEL and at m/z 241 for NAS. A small doubly charged species for NAS was seen at m/z 221. In-source fragmentation was not observed for either MEL or NAS. Quantitation by SRM was based on the following transitions: NAS, m/z 219→160, d_7 -NAS, m/z 226→164; MEL, m/z 233→174; d_4 -MEL, m/z 237→178. No chemical derivatization was required as NAS and MEL were retained, and their SRM provided a sensitive and specific means of detection. MEL and NAS were separated chromatographically by approximately 5 min in nanoflow LC, Figs 2E and 3B. The assay was generally free of any matrix effects (Supplemental 1).

Linearity

In the conventional LC-MS/MS system, the peak area ratios of d_0 -MEL/ d_4 -MEL standards were linearly proportional to the concentration of MEL over the nominal concentration range of 466–116500 pg/mL (Fig. 4A, Supplemental 6A and 7A) with a coefficient of determination of $r \geq 0.993$ and a slope of 0.0653 ± 0.040 . The nanoflow LC-MS/MS response

was also linear but over the nominal concentration range of 11.65–1165 pg/mL (Fig. 4B, Supplemental 6B and 7B) with a coefficient of determination of $r \geq 0.994$ and a slope of 6.03 ± 1.10 . NAS linearity (d_0 -NAS/ d_7 -NAS) was also determined using nanoflow LC-MS/MS over a range of 10.95–1095 pg/mL (Fig. 4C, Supplemental 6C and 7C) with a slope of 10.14 ± 0.88 and a coefficient of determination of $r \geq 0.9163$. Three validation experiments were analyzed on separate days in order to obtain the slopes, intercepts, coefficients of determination and back-calculated errors for MEL in both systems and for NAS in nanoflow LC-MS/MS (Supplemental 2).

Recovery

For the determination of extraction efficiency, plasma samples containing low-, mid- and high-levels of MEL or NAS were processed alongside equimolar samples prepared in aqueous buffer without plasma. All samples were processed in the same manner with the exception that isotope-labeled internal standards were not added to these samples until immediately before analysis by LC-MS/MS; recovery was calculated based on the ratio of peak areas, plasma vs. non-plasma. By this method, MEL average percent recovery from human plasma was 86.1, 71.2 and 75.1% at 1165, 11650 and 116500 pg/mL, respectively (Supplemental 3). For NAS, 123.4, 76.5 and 80.1% recovery was calculated at 1095, 10950 and 109500 pg/mL, respectively. While MEL is known to bind to proteins, the relationship between its protein binding and recovery from plasma is not fully understood.^{18, 19} In this work, it was found that the addition of least 10% (v/v) acetonitrile during the centrifugal filtration step significantly improved extraction efficiency. Without this addition, no MEL or NAS was observed in the sample. This supports other works reporting approximately 25% of plasma MEL as being protein bound.¹⁸ The group also reported nearly 100% recovery but only after a deproteinization step. Our addition of acetonitrile is suggestive of hydrophobic interactions between MEL and plasma proteins.

Precision and accuracy

The intrarun (within run) and interrune (between run) accuracy and precision for the determination of NAS using nanoflow LC-MS/MS and of MEL using nanoflow and conventional LC-MS/MS methods are reported in Tables 3, 4 and 5, respectively. Multiple QC samples were analyzed within single batch runs. The NAS nanoflow intrarun absolute %RE was ≤ 20.83 , ≤ 15.61 and $\leq 9.60\%$ at the low-, mid- and high-level QC concentrations, respectively (Table 1). Corresponding %RSD values were ≤ 6.14 , ≤ 9.45 and $\leq 11.61\%$. Interrune accuracy and precision were calculated from the average NAS concentration from each of the three interday batch runs. Absolute %RE for the interday runs was ≤ 15.80 , ≤ 8.36 and $\leq 3.38\%$, and the corresponding %RSD was ≤ 4.92 , ≤ 7.36 and $\leq 9.33\%$.

The MEL nanoflow intrarun absolute %RE was ≤ 18.42 , ≤ 27.63 and $\leq 4.89\%$ at the low-, mid- and high-level QC concentrations, respectively (Table 2). Corresponding %RSD values were ≤ 14.20 , ≤ 12.80 and $\leq 5.29\%$. Interrune accuracy and precision were calculated from the average MEL concentration from each of the three interday batch runs. Absolute %RE for the interday runs was ≤ 13.69 , ≤ 13.78 and $\leq 2.86\%$, and the corresponding %RSD was ≤ 8.30 , ≤ 8.53 and $\leq 4.47\%$.

For the MEL conventional LC-MS/MS validation, intrarun absolute %RE was ≤ 4.99 , ≤ 10.24 and $\leq 7.37\%$ at the low-, mid- and high-level QC concentrations, respectively (Table Supplemental 4). The corresponding %RSD was ≤ 3.56 , ≤ 7.83 and $\leq 0.92\%$. Interrune accuracy and precision were calculated by the interday absolute %RE values of ≤ 2.86 , ≤ 5.32 and $\leq 6.76\%$. The corresponding %RSD values were ≤ 2.55 , ≤ 2.85 and ≤ 4.20 .

Limits of quantitation and detection

The limits of quantitation for MEL and NAS were defined as the concentrations of analyte yielding %RSD and absolute %RE values $\leq 20\%$. The LLOQ for MEL using conventional LC-MS/MS was found to be 100 fmol on column (1165 pg/mL, 100 μ L plasma, 23.3 pg on column), and the ULOQ was 10 pmol on column (116500 pg/mL, 100 μ L plasma, 2.33 ng on column), Figs. 2A and 2B. MEL was often detected in plasma at levels as low as 100 pg/mL, but the %RSD of measurements below the LLOQ was $>20\%$. The LLOQ for MEL using nanoflow LC-MS/MS was determined to be 50 amol on column (11.65 pg/mL, 11.65 fg injected), and the ULOQ was 5.0 fmol on column (1165 pg/mL, 1.17 pg injected), Figs. 2D and 2E. With this system, MEL could be detected in plasma as low as 9 amol on column (2 pg/mL, 2 fg injected). The LLOQ for NAS measured with nanoflow LC-MS/MS was 50 amol on column (10.95 pg/mL, 10.95 fg injected); the corresponding ULOQ was 5.0 fmol on column (1095 pg/mL, 1.10 pg injected), Figs. 3A and 3B. These systems, therefore, enable the quantitative measurement of MEL and NAS over the range of four orders of magnitude at low sample volumes of human plasma.

Stability

To assess the effects of plasma incubation time on the analytical response ratio, blank plasma was spiked with 50 ng/mL MEL and NAS and allowed to stand for 0, 2, 3, 6 and 9 hr at 4, 22 and 37 °C prior to the addition of internal standard and centrifugal filtration. The average response ratios ($n=3$) obtained at successive time points were within $\pm 25\%$ of initial values for up to 9 hr at all temperatures (Supplemental 5). Effects of long-term storage were also assessed by measuring peak area ratios (d_0 -MEL/ d_4 -MEL and d_0 -NAS/ d_7 -NAS) after freeze-thaw cycles of 0, 12, 24 and 36 hr in plasma. The calculated concentrations of MEL and NAS were $\pm 10\%$ of the theoretical (data not shown).

Application of conventional LC-MS/MS method to a pharmacokinetic study in healthy adults

As part of a PK dose escalation study of MEL, plasma MEL was determined in healthy adults over the course of three days with the administration of 1, 3 and 6 mg of MEL orally. The subsequently measured plasma concentration of MEL is shown in Fig. 5 on a time scale of minutes post-dose. Data from two study participants are shown: Patient 1001 (a 28 yr old female, 72.6 kg), Fig 5A and Patient 1010 (a 36 yr old male, 86.2 kg), Fig 5B. The data were fit to the sum of three exponential terms using the numerical computation utility in SAAM II V1.1. Data were weighted using a fractional standard deviation of 0.1 (FSD=0.1), except for the terminal datum (FSD=0.5). Even at the lowest dose level, plasma concentrations of MEL were higher than the LOD at each time point up to 6 h post-dose, reaching an average $C_{\max}$ of 4000 pg/mL at the 1 mg dose, 15,300 pg/mL at the 3 mg dose and 63,100 pg/mL at the 6 mg dose (Table 3). The calculated PK parameters of this study are comparable to the values reported in a 1996 RIA study.²⁰ The previous group, however, reports intra- and interassay coefficients of variation ranges between 6–12% while the method described herein provides a more precise range of 0.07–7.83% (Supplemental 4). Full details of MEL PK in adults and children will be reported elsewhere.

Endogenous production of MEL and NAS in children with ASD by nanoflow LC-MS/MS

An overnight study of MEL and NAS endogenous production was conducted in children diagnosed with ASD. The subsequently measured plasma concentration of MEL and NAS is shown in Fig. 8 on a time scale of minutes following lights-out (defined as each child's natural bedtime). Two participants are shown: Patient 8011 (a 5 yr old male, 17.4 kg), Fig 6A, and Patient 8012 (a 4 yr old male, 17.5 kg), Fig 6B. MEL levels were found to peak 240 min after lights-out to 156 pg/mL (patient 8011) and to 310 pg/mL (patient 8012) after 360

min. These levels were well above the LLOQ of MEL, reaching LLOQ after approximately 30 min into each study and remaining elevated for the course of the 7 hr study. An RIA study of 43 ASD patients by Melke, et al. reported a mean of 73 pM (17 pg/mL) serum MEL between the morning hours of 9:00–11:00 AM.⁷ While the last time-point of the study reported herein was approximately 5:00 AM, MEL levels were still at or near peak concentrations. This differs considerably, perhaps due to study design or sampling times, with another RIA study by Kulman, et al. reporting an average serum MEL concentration of ≤ 50 pg/mL at 12:00 AM and 4:00 AM in 14 children with ASD.⁶

No reports of normal circulating levels of NAS in humans have been published, so this work represents the first such data. NAS is made by the pineal gland, as well as liver and peripheral organs. However, the pattern shown in this study suggests that plasma NAS originates mainly from neural tissues because it is coincident with the sleep cycle and plasma MEL. NAS levels were observed to peak earlier in plasma than those of MEL, but at much lower levels. Patient 8011 reached a peak of 15 pg/mL NAS at 150 min, and patient 8012 peaked at 24 pg/mL 180 min following lights out. While NAS detection was possible at the LOD levels of 5 pg/mL, endogenous NAS concentrations only exceeded the LLOQ during their peak production following DLMO. As this is the first method to monitor plasma NAS levels in children with ASD, few comparisons can be made. Melke and coworkers investigated the effects of acetylserotonin O-methyltransferase (ASMT) mutations on the enzymatic synthesis of MEL from its precursor NAS, but was unable to make a direct correlation to ASD onset.⁷ These results and the novel capability of this method to measure endogenous levels of MEL and its precursor NAS introduce the potential to begin mapping out the dynamics of the MEL production pathway in children with ASD.

CONCLUSIONS

The method presented herein was validated over the concentration ranges of 11.65–1165 pg/mL for MEL and 10.95–1095 pg/mL for NAS using nanoflow LC-MS/MS and 1165–116500 pg/mL for MEL using conventional LC-MS/MS, thus yielding an assay with not only the specificity to discern between MEL, NAS, and other plasma indoleamines, but also the sensitivity to accurately quantitate low pg/mL endogenous concentrations of MEL and NAS. With linearity over four orders of magnitude, this validated assay also enables a thorough study of relatively high levels of MEL in PK samples. The low volumes of plasma (100 μ L) required for analysis enable studies with children participants where permissible blood collection volumes are lower than in adult studies. Application of this method to understanding the MEL synthesis pathway in patients with ASD versus those of controls is currently under investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported in part by the NIH (1R01 HD059253-01A2), the Vanderbilt Institute for Clinical and Translational Research (UL1 RR024975-01) and the Vanderbilt Kennedy Center for Human Development. The authors would also like to thank Professor J. Scott Daniels and Dr. Ryan D. Morrison for assistance with PK modeling advice, Dr. David C. Klein for the kind gift of the d7-N-acetylserotonin standard, the Clinical Research Center Staff for arranging and carrying out patient recruitment and blood collection and Ms. Diane B. Fawkes, Ms. Deborah D. Wofford and Ms. Karen G. Adkins for their assistance in coordinating the PK participant studies.

References

1. Bubenik GA, Konturek SJ. Melatonin and aging: prospects for human treatment. *J Physiol Pharmacol.* 2011; 62:13. [PubMed: 21451205]
2. Wilhelmsen M, et al. Analgesic effects of melatonin: a review of current evidence from experimental and clinical studies. *J Pineal Res.* 2011
3. Ulugol A, et al. Antihyperalgesic, but not antiallodynic, effect of melatonin in nerve-injured neuropathic mice: possible involvements of the L-arginine-NO pathway and opioid system. *Life Sci.* 2006; 78:1592. [PubMed: 16107259]
4. Wang T, et al. Effects of melatonin on orphanin FQ/nociceptin-induced hyperalgesia in mice. *Brain Res.* 2006; 1085:43. [PubMed: 16566906]
5. Rossignol DA, Frye RE. Melatonin in autism spectrum disorders: a systematic review and meta-analysis. *Dev Med Child Neurol.* 2011; 53:783. [PubMed: 21518346]
6. Kulman G, et al. Evidence of pineal endocrine hypofunction in autistic children. *Neuro Endocrinol Lett.* 2000; 21:31. [PubMed: 11455326]
7. Melke J, et al. Abnormal melatonin synthesis in autism spectrum disorders. *Mol Psychiatry.* 2008; 13:90. [PubMed: 17505466]
8. Nir I, et al. Brief report: circadian melatonin, thyroid-stimulating hormone, prolactin, and cortisol levels in serum of young adults with autism. *J Autism Dev Disord.* 1995; 25:641. [PubMed: 8720032]
9. Tordjman S, et al. Nocturnal excretion of 6-sulphatoxymelatonin in children and adolescents with autistic disorder. *Biol Psychiatry.* 2005; 57:134. [PubMed: 15652871]
10. Leu RM, et al. Relation of melatonin to sleep architecture in children with autism. *J Autism Dev Disord.* 2011; 41:427. [PubMed: 20683768]
11. Anderson GM. Genetics of childhood disorders: XLV. Autism, part 4: serotonin in autism. *J Am Acad Child Adolesc Psychiatry.* 2002; 41:1513. [PubMed: 12447040]
12. Reddy KS. Cytogenetic abnormalities and fragile-X syndrome in Autism Spectrum Disorder. *BMC Med Genet.* 2005; 6:3. [PubMed: 15655077]
13. de Almeida EA, et al. Measurement of melatonin in body fluids: standards, protocols and procedures. *Childs Nerv Syst.* 2011; 27:879. [PubMed: 21104186]
14. Koch BC, et al. Circadian sleep-wake rhythm disturbances in end-stage renal disease. *Nat Rev Nephrol.* 2009; 5:407. [PubMed: 19468289]
15. Shear JB, et al. Determination of fluorogen-labeled neurotransmitters at the zeptomole level using two photon excited fluorescence with capillary electrophoresis. *Analytical Chemistry.* 1998; 70:3470.
16. Chen G, et al. Melatonin in Chinese medicinal herbs. *Life Sci.* 2003; 73:19. [PubMed: 12726883]
17. Simonin G, et al. Determination of melatonin in biological fluids in the presence of the melatonin agonist S 20098: comparison of immunological techniques and GC-MS methods. *J Pharm Biomed Anal.* 1999; 21:591. [PubMed: 10701426]
18. Rizzo V, et al. Determination of free and total (free plus protein-bound) melatonin in plasma and cerebrospinal fluid by high-performance liquid chromatography with fluorescence detection. *J Chromatogr B Analyt Technol Biomed Life Sci.* 2002; 774:17.
19. Gilad E, Zisapel N. High-affinity binding of melatonin to hemoglobin. *Biochem Mol Med.* 1995; 56:115. [PubMed: 8825074]
20. Cavallo A, Ritschel WA. Pharmacokinetics of melatonin in human sexual maturation. *J Clin Endocrinol Metab.* 1996; 81:1882. [PubMed: 8626852]

Figure 1. Chemical structures and electrospray product ion mass spectra of the precursor ions of A) NAS ($[M+H]^+$ m/z 219 and B) MEL ($[M+H]^+$ m/z 233). Ions were collisionally activated at a collision energy of 10 V. The dashed line in each chemical structure indicates the proposed site of collision-induced fragmentation.

Figure 2.

Peak signal intensity of conventional (A–C) and nanospray (D–F) ESI SRM chromatograms of extracted human plasma. Conventional ESI SRM chromatograms were acquired from plasma spiked with A) 100 fmol MEL on column (23.3 pg injected, 1165 pg/mL, corresponding to LLOQ), B) 10 pmol MEL on column, (2.33 ng injected, 116500 pg/mL, corresponding to the ULOQ) and C) no analyte. Nanospray ESI SRM chromatograms were acquired from plasma spiked with D) 50 amol MEL on column (11.65 fg injected, 11.65 pg/mL, corresponding to the LLOQ), E) 5 fmol MEL on column (1.17 pg injected, 1165 pg/mL, corresponding to the ULOQ) and F) no analyte. Detection was based on the SRM transition m/z 233→174; each sample was prepared from 100 μ L plasma volume. Analyte quantitation was calculated using the peak area response ratio d_0 -MEL/ d_4 -MEL. Signal intensity is indicated by vertical brackets.

Figure 3.

Peak signal intensity of nanospray ESI chromatograms of extracted human plasma spiked with A) 50 amol NAS on column (10.95 fg injected, 10.95 pg/mL, corresponding to the LLOQ), B) 5 fmol NAS on column (1.10 pg injected, 1095 pg/mL, corresponding to the ULOQ) and C) no analyte. Detection was based on the SRM transition m/z 219 $\rightarrow$ 160; each sample was prepared from 100 μ L plasma volume. Analyte quantitation was calculated using the peak area response ratio d_0 -NAS/ d_7 -NAS. Signal intensity is indicated by vertical brackets.

Figure 4.

SRM ion chromatogram calibration curves. Peak area ratios (d_0 -MEL/ d_4 -MEL) were plotted against analyte concentrations for a series of plasma standards over the nominal concentration ranges of A) 1165–116500 pg/mL in conventional ESI MS/MS and B) 11.65–1165 pg/mL in nanospray MS/MS. Peak area ratios for d_0 -NAS/ d_7 -NAS were plotted over the range C) 10.95–1095 pg/mL in nanospray MS/MS. The ranges of these calibration curves correspond to the observed analyte LLOQ and ULOQ.

Figure 5. Plasma concentration-time curves of MEL following oral administrations of MEL to two different adult human subjects A) Patient 1001 and B) Patient 1010 at dose levels of (---●---) 1 mg, (—■—) 3 mg or (···▲···) 6 mg.

Figure 6. Plasma concentration-time curves of overnight (●) MEL and (■) NAS endogenous production for two different children with ASD, Patients A) 8011 and B) 8012. Measurements are reported along an x-axis of minutes following lights-out.

Scheme 1.

Enzymatic production of MEL in human tissue. Gene names are indicated for each step.

Table 1

Nanospray ESI intrarun and interrune accuracy and precision for the determination of NAS in human plasma.

Standard conc. (pg/mL)	Day of Sampling	Calculated conc. (pg/mL)	% Error	Average calculated conc. (pg/mL)	Intraday % RSD	^a Average % Error	^b Interday % RSD
10.95	1	10.78	-1.56	10.45	4.56	-4.61	4.92
		10.11	-7.71				
	17	12.13	10.77	12.68	6.14	15.80	
		13.23	20.83				
109.5	23	9.16	-16.33	9.44	4.07	-13.84	
		9.71	-11.37				
	1	117.1	7.02	114.9	6.11	4.96	7.36
		113.1	3.45				
1095	17	114.6	4.67	118.7	9.45	8.36	
		110.7	1.13				
	23	126.6	15.61	110.2	6.53	0.64	
		105.1	-4.01				
1095	1	115.3	5.28	1085	8.64	-0.96	9.33
		1018	-7.00				
	17	1151	5.10	1058	7.73	-3.38	
		1116	1.91				
1095	23	1000	-8.66	1109	11.61	1.28	
		1018	-7.03				
		1200	9.60				

^a Defined as the [(avg. calculated conc. - theoretical conc.)/theoretical conc.] × 100%.

^b Defined as the %RSD of the average calculated concentrations on days 1, 17 and 23.

Table 2

Nanospray ESI intrarun and interrune accuracy and precision for the determination of MEL in human plasma.

Standard conc. (pg/mL)	Day of Sampling	Calculated conc. (pg/mL)	% Error	Average calculated conc. (pg/mL)	Intraday % RSD	^a Average % Error	^b Interday % RSD
11.65	1	12.94	11.07	13.12	1.86	12.58	8.30
	7	13.29	14.04				
		10.44	-10.42	11.61	14.20	-0.39	
		12.77	9.58				
116.5	17	12.69	8.92	13.25	8.85	13.69	
		13.80	18.42				
	1	111.6	-4.25	115.7	4.84	-0.72	8.53
		116.7	0.20				
		118.7	1.80				
	7	134.1	15.11	132.6	12.80	13.78	
		148.7	27.63				
		114.9	-1.41				
	17	98.6	-15.36	102.8	7.96	-11.80	
		106.9	-8.23				
	1	1222	4.89	1161	5.29	-0.34	4.47
		1134	-2.68				
		1127	-3.25				
	7	1196	2.69	1161	4.34	-0.37	
		1125	-3.43				
	17	1182	1.44	1198	3.79	2.86	
		1215	4.32				

^a Defined as the [(avg. calculated conc.- theoretical conc.)/theoretical conc.]×100%.

^b Defined as the %RSD of the average calculated concentrations on days 1, 7 and 17.

Pharmacokinetic parameters for MEL obtained from two different adult human subjects administered oral doses of 1, 3 and 6 mg.

Table 3

Patient 1001							
Actual dose given (mg)	C _{max} (µg/L)	T _{max} (min)	AUC _{last} (µg*min/L)	Half-life (min)	CL (L/min/kg)	V _z (L/kg)	
1	2.9	20	249.2	63.3	0.053	3.31	
3	10.0	10	555.5	38.7	0.074	3.56	
6	34.3	10	1593.0	37.4	0.052	3.31	
Patient 1010							
Actual dose given (mg)	C _{max} (µg/L)	T _{max} (min)	AUC _{last} (µg*min/L)	Half-life (min)	CL (L/min/kg)	V _z (L/kg)	
1	5.1	10	224.6	37.5	0.051	3.74	
3	20.5	10	759.6	68.4	0.032	3.7	
6	91.9	10	2694.6	31.6	0.025	1.67	