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Development and Validation of an Ultrasensitive LC-MS/MS Method for the Quantification of Melatonin in Human Saliva

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KEYWORDS: melatonin, LC-MS/MS, method validation, circadian rhythms, ultrahigh sensitivity

ABSTRACT

A growing body of literature describes the potential effects of circadian disruption on human health. Indeed, psychiatric diseases, metabolic syndrome, and cancers may be linked to disturbance of the circadian rhythm. Currently, the best practice to assess circadian rhythm is the measurement of melatonin levels. Our goal was thus to develop and validate a highly sensitive LC-MS/MS method to follow salivary melatonin levels throughout the day and night. Our method reached a lower limit of the measuring interval (LLMI) of 0.8 pg/mL. To our knowledge, it is the most sensitive method allowing quantitation of melatonin in saliva. Saliva, obtained from passive drooling or salivette, was extracted by an efficient and quick liquid—liquid extraction with no further cleanup needed. The method was validated according to the European Medicines Agency (EMA) guidelines and provided excellent results regarding accuracy, precision, linearity, selectivity, and specificity. Comparison between

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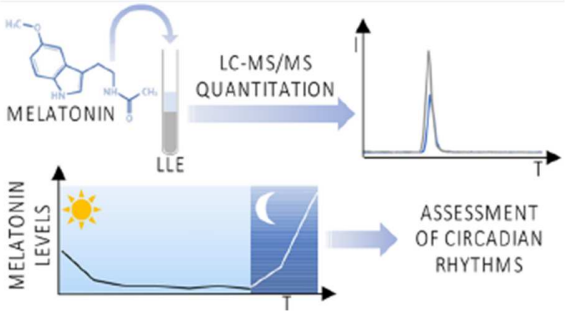
radioimmunoassay and our method was performed and showed differences at low levels, most likely due to cross-reactivity with other indols. To assess daytime melatonin levels in humans, salivary melatonin levels of ten volunteers were monitored throughout the day and showed lower daytime levels than reported in previous studies.



1. Introduction

Melatonin is a hormone produced enzymatically in the pineal gland.[1,2] It is synthesized from the amino acid tryptophan that is circulating in the blood.[2] It is believed to be one of the first compounds produced in accordance with the biological clock to coordinate fundamental events in life due to its widespread presence in animals, plants, and bacteria.

Melatonin concentrations can be monitored in three cases. First, melatonin measurement is used for the diagnosis of circadian rhythm sleep wake disorder.[5,6] Salivary and urine melatonin are recommended for routine use as they are more feasible measurements than plasma melatonin measurement.[5] Second, some evidence support the possibility that melatonin measurement could be used to confirm a total pinealectomy.[7] This surgery is performed in case of parenchyma tumor, which is a rare condition. Third, it also provides information about potential disturbance and misalignment of circadian rhythms, which is thought to be involved in a growing number of diseases.[8,9] Indeed, disturbance of circadian rhythms may have a role in the pathogenesis of psychiatric disorders such as major depression, bipolar disorders, schizophrenia, anxiety disorder, and even attention deficit and hyperactivity disorder. Patients suffering from psychiatric disorders with less robust circadian rhythm may display a greater severity of their symptoms.[10,11] Other diseases such as metabolic syndrome and cancer could be linked to disturbance of circadian rhythms.[8]

Saliva represents a convenient matrix for melatonin quantification as its collection is less

invasive than blood. Melatonin saliva concentration is 3 times lower than in blood;[12] it corresponds to the biologically active fraction of the hormone in plasma.[8] For this reason, from an analytical point of view, highly sensitive methods are required for an accurate quantification of melatonin in saliva, especially at daytime. Currently, salivary melatonin can be measured with different methods. The most common ones are immunological assays: enzyme-linked immunosorbent assays (ELISAs) and radioimmunoassays (RIAs). All of the commercially available immunoassays kits are reported in the work of Kennaway.[13] Those assays claim an interesting sensitivity (lower limit of the measuring interval (LLMI) lower than 1 pg/mL) but may lack specificity, especially at basal levels, due to cross reactivity with metabolites of the melatonin synthesis pathway. Some authors have obtained a very low sensitivity (up to 4.64×10^{-4} pg/mL) by quantifying derivatized melatonin by an HPLC-fluorescence detector in diverse media. However, such methods were not suitable for samples containing mixtures of indoles.[14,15] Several groups have developed LC-MS/MS, LC-MS, and GC-MS assaysto quantify melatonin in saliva as reported in Table 1.[16–25] However, none of these assays could reach the sensitivity of the immunoassays while keeping the high specificity and accuracy recovery of mass spectrometry. The aim of this work was to develop a LC-MS/MS method reaching the high sensitivity of immunoassays (<1 pg/mL) while keeping the high specificity of the mass spectrometry. High sensitivity is indeed required to measure daytime concentrations of less than 5 pg/mL.

Table 1. *Previously Developed MS-Based Methods and Their LOD/LLMI*

authors	method	LOD	LLMI
Eriksson et al.[16] (2003)	LC-MS/MS	1.05 pg/mL	3 pg/mL
Motoyama et al.[17] (2004)	LC-MS	2.5 pg/mL	
Jensen et al.[18] (2011)	LC-MS/MS	<1 pg/mL	
Dermanowski et al.[19] (2022)	LC-MS/MS		20 pg/mL (plasma) 5 pg/mL (saliva)
Shin et al.[20] (2021)	LC-MS/MS	0.0989 pg/mL	0.52 pg/mL
McBride et al.[22] (2019)	LC-MS/MS		
Van Faassen et al.[23] (2017)	LC-MS/MS		4.0 pmol/L
Simonin et al.[24] (1999)	GC/MS		1 pg/mL
Fourtillan et al.[25] (1994)	GC/MS		0.2 pg/mL

2. MATERIALS AND METHODS

2.1. CHEMICALS AND REAGENTS.

Methanol (MeOH), acetonitrile, and water (LC-MS grade) were purchased from Biosolve (Dieuze, France). Ethyl acetate (HPLC grade) was obtained from Chem-Lab (Zedelgem, Belgium). Ethanol (HPLC grade)

was purchased from VWR Chemicals (Radnor, PA, USA). Ammonium formate (HPLC grade) and HCl were obtained from Fischer Chemicals (Pittsburgh, PA, USA). $MgCl_2$ $6H_2O$, $CaCl_2$ $6H_2O$, K_2HPO_4-$2H_2O$, K_2CO_3, NaCl, and KCl were all purchased from Merck KGaA (Darmstadt, Germany).

Melatonin (lot number: 1380105; purity: 0.996 mg/mg) was purchased from USP (Rockville, MD, USA), and melatonin-d_4 (*N* -acetyl-5-methoxytryptamine-α, α, β, β - d_4) labeled internal standard (IS) was obtained from Medical Isotopes Inc. (Pelham, NH, USA).

For calibrator matrices comparison, a *melatonin direct Serum/ Plasma/Saliva RIA* kit was purchased from IBL (RE29301, IBL International GmbH, Hamburg, Germany).

6PLUS1Multilevel Saliva Calibrator Set Cortisol, Cortisone was purchased from Chromsystems (Grafelfing, Germany) and was used as melatonin free human saliva for the validation sample preparation.

The vacuum evaporator used for the sample preparation was a Christ RVC 2-25 CD concentrator from Grosseron (Coueron, France).

2.2. **Preparation of Solutions.**

Stock solutions of melatonin (5 mg/mL) and melatonin-d_4 IS (1 mg/mL) were prepared in MeOH and stored at -20 °C in Certan capillary bottles. Working and spiking solutions were prepared in H_2O, 50% MeOH (v/v). Spiking solution at 1 ng/mL was used to prepare the calibrators. IS spiking solution at 125 pg/mL was prepared in H_2O, 50% MeOH (v/v) on the same day of the analysis, and 100 μL was added to each sample prior to sample preparation.

2.3. **Sample Preparation.**

Saliva samples, collected by salivette, were thawed and centrifuged for 5 min at 4000 rpm. 500 μL of saliva sample, quality controls, or calibrator was transferred to a glass tube, spiked with 100 μL of IS spiking solution, and homogenized using a reciprocating shaker for 10 min; 1 mL of ethyl acetate was added for liquid/liquid extraction and shaken for 10 min at medium speed, followed by centrifugation at 4000 rpm for 10 min. The maximum volume of the supernatants (approximately 1 mL) was transferred to new glass tubes, washed by adding 1 mL of LC-MS grade water, and vortexed for 30 s. Then, samples were centrifuged again for 10 min at 4000 rpm, and the organic layer was transferred to glass tubes for evaporation to dryness under vacuum at 25 °C for 45 min.

Extracts were reconstituted with 50 μL of H_2O, 50% MeOH (v/v) and transferred to 1.5 mL vials for

injection.

Plastic consumables must be strictly avoided during sample preparation, because of interference release. In this work, consumable plastics such as plastic caps were used after a thorough wash by sonication in ethanol for 15 min to avoid the release of interferences.

Matrix factors were calculated by dividing the IS area under the curve (AUC) of the saliva samples by the IS AUC of the reference. Eight human saliva samples were processed and reconstituted with 50 μL of 100 pg/mL IS in H_2O, 50% MeOH (v/v). The reference was prepared by processing 500μL of H_2O and reconstituting with 50 μL of 100 pg/mL IS in H_2O, 50% MeOH (v/v). Mean matrix factor was calculated by dividing the mean of the IS areas under the curve of the saliva samples by the IS AUC of the reference.

Recovery rate was calculated by dividing the area ratio of the observer sample by the area ratio of the reference. The observer sample was produced by processing 500 μL of saline solution spiked with 25 μL of 40 pg/mL of native melatonin. g of $MgCl_2^{6}H_2O$, 0.15 g of $CaCl_2^{6}H_2O$, 0.76 g of K_2HPO_4-2H_2O, 0.53 g of Saline solution was prepared according to the work of Özer et al.:[26] 0.17 K_2CO_3, 0.33 g of NaCl, and 0.75 g of KCl were dissolved in 1 L of water, and the solution pH was adjusted to 6.8 ± 0.1 with 1% HCl. After evaporation, reconstitution was realized with 25 μL of 40 pg/mL IS, 50% MeOH and 25 pL of H2O, 50% MeOH. Regarding the reference, 25 μL of 40 pg/mL IS and 25 μL of 40 pg/mL of native melatonin were directly charged in a vial containing an insert.

2.4.LC-MS/MS Conditions.

Samples were analyzed on a Nexera X2 UPLC from Shimadzu (Shimadzu Corporation, Kyoto, Japan) coupled to a QTrap6500 mass spectrometer (Triple Quadrupole and Linear Trap analyzers) from Sciex (CA, USA) fitted with an lonDriveTurbo V ion source and using electrospray ionization in positive mode (ESI+). Chromatographie separation was achieved on a Kinetex C18 100 Å core- shell column (100 X 3 mm, 2.6 μm) from Phenomenex (Torrance, CA, USA), maintained at 35 °C, using 10 mM $HCOONH_4$ in water (mobile phase A) and 10 mM $HCOONH_4$ in a mixture of acetonitrile and 10% methanol (mobile phase B). Mobile phases were optimized by flow injection analysis (FIA) in order to select the ones which gave the best ionization of native and labeled melatonin. Thirty microliters of the final sample extract was injected. A gradient method at 0.7 mL/min flow rate was applied as follows: start at 20% B, stay for 0.2 min; increase to 95% B over 2 min and held for 1.9 min; then decrease to 20% B over 0.1 min and maintain for 1.5 min, for a total run time of 5.6 min. Once the gradient optimized, human saliva samples obtained from passive drooling were pooled, extracted, and injected in order to evaluate potential

interferences.

Two MRM transitions were monitored for native and labeled melatonin. All the MS parameters were optimized to reach the required sensitivity in the range of low pg/mL level. MRM transitions and MS compound parameters are reported in Table 2.

Table 2. *MS Compound Parameters Obtained after Optimization by Q1 Scan, Product Ion Experiments, and MRM Experiments*

ID	precursor ion (m/z)	product ion (m/z)	declustering potential (V)	entrance potential (V)	collision energy (V)	exit potential (V)
melatonin 1	233.1	174.1	111	10	24	12
melatonin 2	233.1	159.2	111	10	34	12
melatonin-d_4 1	237.1	178.2	167	10	21	13
melatonin-d_4 2	237.1	163.1	167	10	43	13

2.5. CALIBRATORS MATRICES COMPARISON.

Four potential calibrator matrices were tested to choose the most suitable one: prepared in LC-MS grade water, saline solution, commercial depleted saliva, and a surrogate matrix provided by IBL. For all matrices, the first calibration point (100 pg/mL) was prepared using 100 μL of the spiking solution in 900 μL of blank matrix, and then, seven points were prepared by serial dilution. Calibration levels were 100, 50, 25, 12.5, 6.25, 3.13, 1.56, and 0.78 pg/mL. The calibrators were processed with the same sample preparation as the regular samples.

Sixty-five human saliva samples were sampled and analyzed. Their concentrations (0.93 to 43.34 pg/mL) were calculated with each calibration curve. Passing-Bablok regressions were then performed to compare the concentrations calculated with each calibration curve.

2.6. METHOD VALIDATION.

2.6.1. ***Calibration Curve***. The calibration curve was prepared in water by serial dilution. The highest calibrator was prepared by spiking 100 μL of a solution of 1 ng/mL melatonin in 900 μL of LC-MS/MS grade H_2O. The following calibrators were prepared by diluting 500 μL of the previous calibrator with 500 μL of LC-MS/MS grade H_2O. One calibration curve was prepared per day for 3 days. Linearity of the calibration curve was assessed by performing linear regression.

2.6.2. ***Validation Samples***. Validation samples were prepared in commercial depleted saliva

from Chromsystem at four levels, 0.8 (LLMI), 2.4, 40.0, and 80.0 pg/mL, and underwent the same sample preparation as regular samples.

2.6.3. ***Inter- and Intrarun Precision and Accuracy Recovery***. The method was validated according to the European Medicines Agency (EMA) guidelines. Validation samples were analyzed in quintuplets for 3 days. Anew solvent-based calibration curve was prepared every day.

2.6.4. ***Calculations***. Inter- and intrarun precisions were calculated by dividing the standard deviation by the mean, while inter- and intrarun accuracy recoveries were calculated by dividing the mean by the target concentration.

2.6.5. ***Measurement Uncertainty***. For this validation, 4 different uncertainties were calculated: uncertainty of the bias, combined uncertainty, expanded uncertainty, and related uncertainty. Uncertainty of the bias was calculated by dividing the standard deviation by the square root of *n*, where *n* stands for the number of replicates. Combined uncertainty was calculated using the formula:

$$\sqrt{\frac{\mathrm{SD}}{\sqrt{n}} + \sqrt{\frac{\text{average concentration} - \text{expected concentration}}{\sqrt{3}}}} \qquad (1)$$

Expanded uncertainty was assessed by multiplying the combined uncertainty by the factor *K* (*K* = 2). Related uncertainty was calculated by dividing the combined uncertainty by the expected concentration.

As there were no data available on the biological variation of melatonin, we arbitrarily set the MU goal as lower than 15%.

2.6.6. ***Linearity***. A highly concentrated sample was diluted to obtain 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100% of its initial concentration. The obtained concentrations were then multiplied by their corresponding dilution factor. Dilution integrity was verified if the obtained concentrations were encompassed between 85% and 115% of the undiluted sample concentration. The experiment was realized in duplicate.

2.6.7. ***Specificity***. Saliva samples (*n* = 11) were collected by passive drooling and salivettes. Volunteers were instructed to refrain from eating or drinking 15 min prior to collection. Every sample was then processed and analyzed.

2.7. **SELECTIVITY.**

Saliva samples from 48 volunteers were collected with salivettes and processed. All of the samples were pooled and evaporated together. The pool was then reconstituted with 50 μL of H_2O, 50% MeOH and directly injected. Transitions of 8 metabolites of the melatonin synthesis pathways, namely, *N*-acetyl serotonin, 6-hydroxymelatonine, 5- methoxytryptophol, 5-methoxytryptamine, 5-methoxyindol acetic acid, 5-hydroxytryptophol, serotonin, and 5-hydroxyindol acetic acid, obtained from the work of Eugster et al., were monitored to assess the selectivity of the liquid chromatography method.[27]

2.7.1. ***Standard Stability***. Two solutions of standard were produced in H_2O, 50% MeOH (v/v) in order to reach the concentrations of 100 pg/mL and 1 ng/mL. Each solution was aliquoted in five vials, which were stored at -20 °C for different periods of time: 0, 1, 2, 3, and 4 weeks. After storage, solutions were directly injected. Accuracy recoveries should range between 90% and 110%.

2.7.2. ***Long-Term Reproducibility***. Internal QCs were produced by pooling large volumes of human saliva obtained from volunteers in a 200 mL glass bottle. After the assessment of its concentration, the main pool was then split in three equal 50 mL subpools. Two of the subpools were spiked to obtain concentrations close to 20 and 45 pg/mL while the last remained at the original concentration. The three subpools were then aliquoted and stored in a -20 °C freezer. Multiple sets of subpools were used through the year. At each run, one sample of each of them was launched. Precisions of each subpool were calculated as explained above.

2.8. **DAYTIME SALIVA SAMPLES ANALYSIS.**

Ten volunteers were asked to provide saliva from salivettes at different times of the day. The first sampling was performed immediately upon awakening, followed by samplings every two hours until the normal bedtime of the volunteers.

2.9. CARRYOVER.

Highly concentrated melatonin standard solutions (5 and 10 ng/mL) were injected prior to five solvent

injections.

2.10. Comparison with RIA.

Human saliva samples (n = 37) were collected and quantified first by the *melatonin direct Serum/Plasma/Saliva RIA* kit (IBL International, Hamburg, Germany) and then by our LC-MS/MS method. Results were then compared using a Passing-Bablok regression.

3. RESULTS AND DISCUSSION

3.1. Sample Preparation.

Liquid—liquid extraction was chosen due to its ease and rapidity. The optimized sample preparation showed a percentage recovery above 74% while the average matrix factor for the eight samples was 79% ± 19.4%. The high dispersion of matrix factors was expected as saliva composition can highly vary depending on the food and drink intake.

3.2. Liquid Chromatography.

The KINETEX (2.6 /μm, C18 100 Å) LC Column (100 X 3 mm) gave the best peak shape and was thus chosen in order to obtain the highest sensitivity.

The buffer composition containing 45% acetonitrile, 5% methanol, and 10 mM ammonium formate yielded the highest signal intensity. It was thus chosen as the mobile phase. Ammonium formate at a final concentration of 10 mM was essential for obtaining a signal with ultrahigh sensitivity in the method, whereas the addition of formic acid diminished signal intensity.

No interference at retention time was noticeable on the chromatogram. The optimized injection volume was 30 μL, and optimized solvent injection was H_2O, 50% MeOH (v/v).

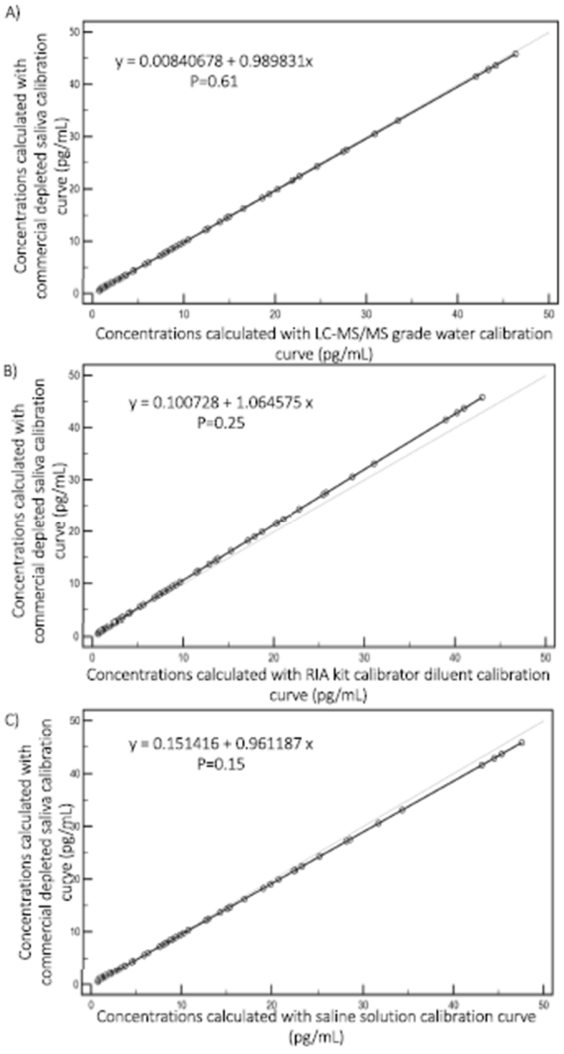
3.3. Mass Spectrometry.

For both native and labeled compounds, the molecular ions were chosen as precursor ions. The two most sensitive transitions for native and labeled melatonin were selected and are reported in Table 2.

Regarding the ion spray voltage, the value giving the highest intensity was 2000 V. Source temperature was set to 600 °C.

Dwell times were calculated to obtain a description of the peak with 20 points.

Figure 1. *Passing-Bablok regressions comparing the calculated concentration of 65 patients obtained with calibrations curves realized in different matrices. (A) Passing-Bablok regression comparing concentrations obtained by a calibration curve prepared in commercially depleted saliva and by one prepared in LC-MS/MS grade water. (B) Passing-Bablok regression comparing concentrations obtained by a calibration curve prepared in commercially depleted saliva and by one prepared in RIA kit diluent. (C) Passing-Bablok regression comparing concentrations obtained by a calibration curve prepared in commercially depleted saliva and by one prepared in saline solution.*



3.4. CALIBRATOR MATRICES COMPARISON.

Results and plot of the Passing-Bablok regression are reported in Figure 1. The comparison between the depleted saliva calibration curve and water calibration curve showed no bias. Indeed, the equation: [depleted saliva calibration curve] = 0.0 (0.0061 to 0.0096) + 0.99 (0.9897 to 0.9900) [water calibration

curve] was obtained by the Passing-Bablok regression. Other comparisons yielded comparable outcomes; consequently, all calibration curves provided statistically comparable outcomes for the same sample. The less complex type of calibrator, LC-MS grade water, was thus selected as the calibrator.

3.5. **METHOD VALIDATION.**

Regarding the calibration curve, the equation of the linear regression was $y = 0.105x - 0.008$ ($r = 0.99999$). The calibration curve is represented in Figure 2.

LLMI (0.8 pg/mL) was selected as the smallest concentration providing a coefficient of variation (CV) below 20% and an accuracy recovery between 80% and 120%. Signal-to-noise ratios obtained for LLMI ranged between 10.8 and 12.3. Regarding LLMI validation samples, intra- and inter-run accuracy recoveries ranged between 96% and 103% while intra- and inter-run precision ranged between 2.4% and 4.3%. For the validation samples at 2.4, 40, and 80 pg/mL intra- and inter-run precision were encompassed between 0.9% and 4.5%. Regarding intra- and inter-run accuracy recoveries, they were comprised between 98.0% and 112.6%. Values are reported in Table 3.

Concerning the measurement of uncertainty, the results are reported in Table 4. Related uncertainty did not exceed 10.3% for any of the values.

Linearity was assessed as excellent. Indeed, all the multiplied concentrations of diluted samples fell between 102.70% and 114.62%. Thus, highly concentrated samples can be diluted up to ten times to reach a concentration in the range of the calibration curve.

Regarding the specificity, no interferences were spotted at retention time. Indeed, the peak corresponding to the melatonin

displayed good peak shape and symmetry. Plus, melatonin and the different monitored metabolites were chromatographically completely separated as represented in Figure 3. Thus the selectivity of the method was proved.

After analysis for standard stability assessment, accuracy recoveries ranged between 93% and 108% for both concentrations. No clear variation or trend was observed from week to week. Concerning the reproducibility of the method, none of the three batches of each level of subpool (≈2, ≈20, and ≈40 pg/mL) that was produced through the year yielded a calculated CV above 10%. The method was thus qualified as highly robust and reproducible.

According to our analysis of daytime saliva samples, during the daytime (11-19 h), all healthy individuals

displayed melatonin levels under 0.8 pg/mL. Melatonin levels increased at the end of the evening and remained high until the early morning. The higher level of melatonin obtained for all of the volunteers ranged between 10.44 and 46.43 pg/mL. Obtained profiles are reported in Figure 4.

***Figure 2**. Linear regression of the calibration curve.*

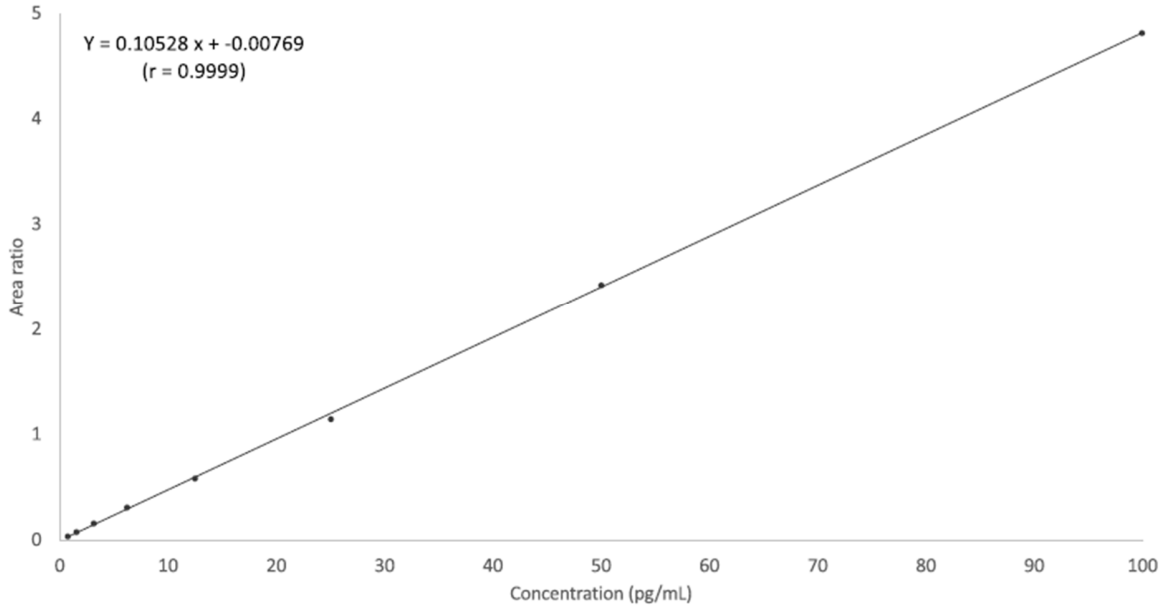


***Figure 3**. Chromatogram obtained from MRM experiment realized with a highly preconcentrated saliva sample. All the metabolites from the melatonin synthesis pathway were monitored.*

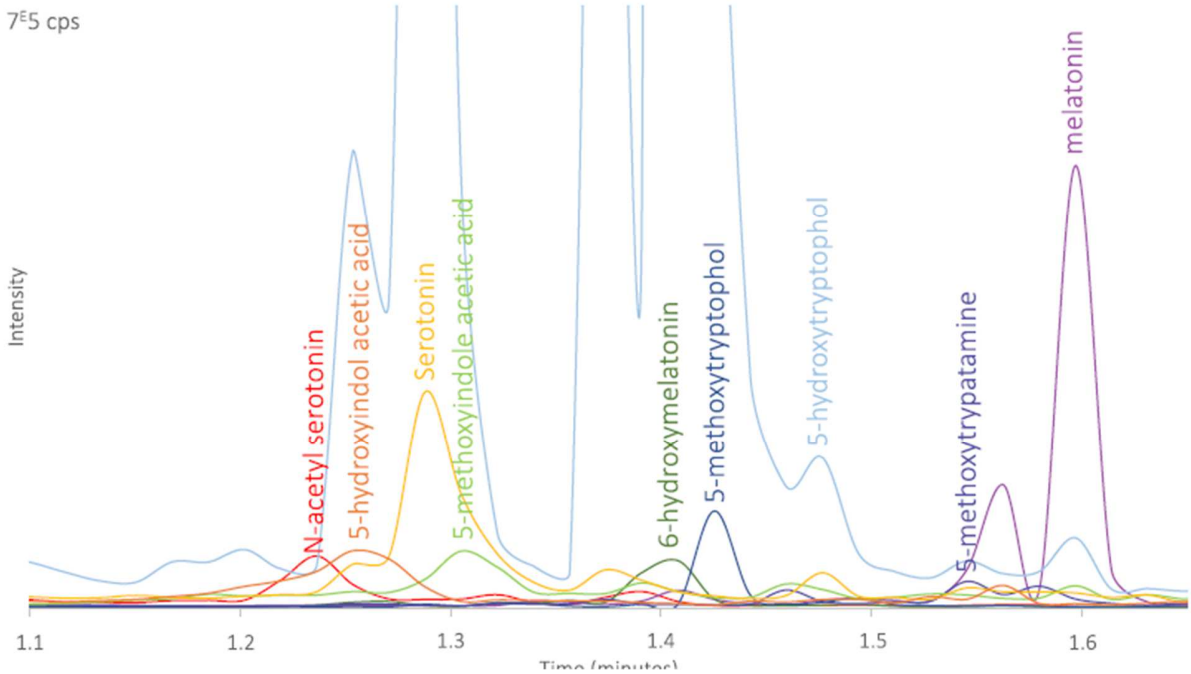


Table 3. *Calculated Inter- and Intraprecisions and Accuracy Recoveries for LLMI-Spiked, Depleted Saliva*

	day	calculated concentration (pg/mL)	intrarun precisions (%)	intrarun accuracy (%)	inter-run precision (%)	inter-run accuracy (%)
QC LLOQ (0.8 pg/mL)	1	0.79	2.43	96.42	4.28	98.42
		0.78				
		0.78				
		0.75				
		0.75				
	2	0.8	3.05	103		
		0.86				
		0.81				
		0.81				
		0.84				
	3	0.74	2.82	96		
		0.75				
		0.78				
		0.79				
		0.78				
QC low (2.4 pg/mL)	1	2.45	3.98	98	4.45	102.06
		2.44				
		2.28				
		2.24				
		2.35				
	2	2.49	1.81	101.67		
		2.46				
		2.46				
		2.41				
		2.38				
	3	2.56	2.69	106.5		
		2.64				
		2.45				
		2.58				
		2.55				
QC mid (40 pg/mL)	1	40.78	4.42	104.11	4.31	108.67
		41.95				
		44.20				
		39.20				
		42.08				
	2	44.23	1.93	109.4		
		43.80				
		44.40				
		42.30				
		44.07				
	3	43.96	2.08	112.56		
		45.99				
		44.09				
		45.68				
		45.40				
QC high (80 pg/mL)	1	86.50	3.49	102.55	4.38	107.48
		82.66				
		81.86				
		80.03				
		79.13				
	2	85.50	0.94	107.41		
		86.71				
		85.05				
		86.86				
		85.52				
	3	90.50	1.24	112.48		
		88.59				
		90.23				
		89.18				
		91.43				

Figure 4. *Daytime levels of salivary melatonin profile.*

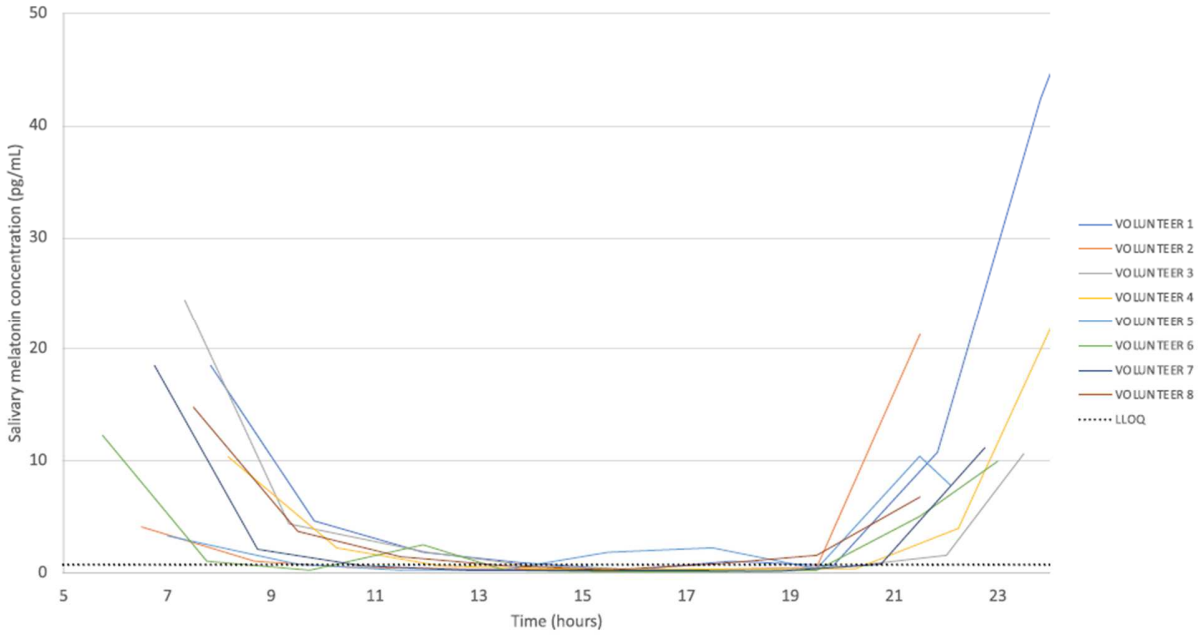
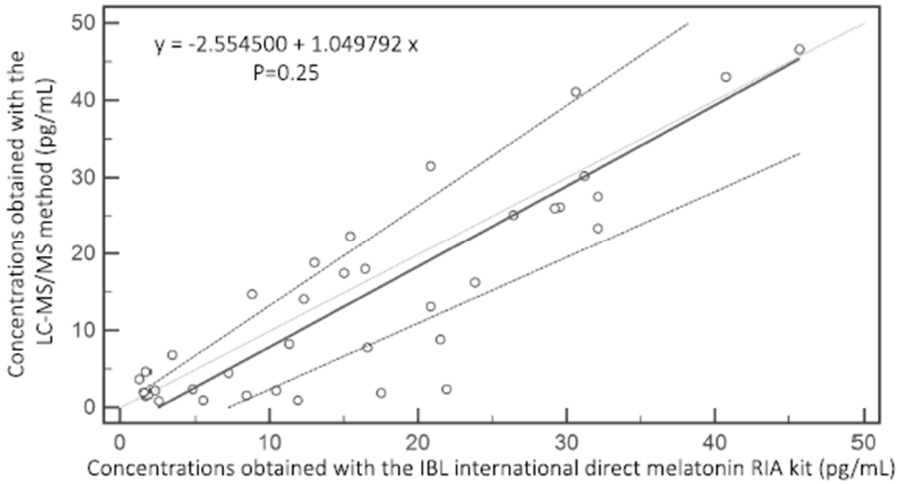


Figure 5*. Passing Bablok regression comparing the results obtained by the RIA method and LC-MS/MS method for 37 human plasma samples.*



Carryover was assessed as null as no deviation of the noise was found in the blanks after the injections of highly concentrated standards.

Passing-Bablok regression obtained from the comparison with the RIA, reported in Figure 5, displayed no significant deviation of the linearity. The equation regression was [LC-MS/MS results] = -2.56 (-6.17 to 0.32) + 1.05 (0.86 to 1.30) [RIA results]. An important scatter was observed and confirmed by the large

confidence interval of the slope. In addition, some samples below 10 pg/mL were substantially overquantified by the RIA compared to the LC-MS/MS method.

3.6. Discussion.

In this study, we developed a trace-level LC-MS/MS method for the determination of salivary melatonin. Our aim was to be able to monitor melatonin levels throughout the day, when they are reported to be the lowest. As mentioned above, our study showed salivary melatonin levels below 0.8 pg/mL in all subjects during the day. In contrast, previous studies using immunoassays to measure salivary melatonin have reported daytime levels ranging from 1 to 10 pg/mL.[13,28-30] The immunoassays maybe the most widely used method for melatonin determination, but they suffer from a lack of specificity. In fact, cross-reactivity is a well-known problem with these assays.

Table 4. *Calculated Uncertainty for Every Levels*

expected value (pg/mL)	bias uncertainty (pg/mL)	combined uncertainty (pg/mL)	expanded uncertainty (pg/mL)	related uncertainty (%)
0.8	0.01	0.01	0.02	2.8
2.4	0.03	0.04	0.08	3.3
40	0.48	2.06	4.13	10.3
80	0.97	3.47	6.95	8.7

For melatonin determination, indolic and naphthalenic compounds have been reported to be cross-reactive for most of the immunoassays.[24,29,31] For example, the metabolites of the melatonin synthesis pathway, especially *N* - acetyl serotonin, 5-methoxytryptophol, and 5-methoxytrypt-amine, are well-known to interfere with the determination of melatonin by immunoassay. Indeed, those molecules display, respectively, 1.2%, 1.2%, and 2.5% cross-reactivity with melatonin. As displayed in Figure 3, 5-methoxy-tryptamine seems to be extremely low in saliva, even highly concentrated. This finding corresponds to the results obtained by Eugster et al., which states that the metabolite is undeterminable in plasma.[27] However, *N*-acetyl serotonin and 5-methoxytryptophol peaks were seen during our selectivity experiment even if our sample preparation and LC-MS/MS method were not optimized for these compounds; thus, their concentration in saliva must not be negligible. Therefore, at low concentration, melatonin levels may be overquantitated by immunoassays due to the presence of both of those metabolites. This can be confirmed by our results obtained by comparing our method with the *Melatonin direct Serum/Plasma/Saliva RIA* kit from IBL. It is clear, especially at lowlevels, that the RIA

we used overquantified compared to our LC-MS/MS method.

4. CONCLUSION

In conclusion, we present a highly sensitive and selective LC- MS/MS method for quantitation of salivary melatonin that is compatible with the low concentrations observed during the day. Our results show that immunoassays lack specificity, especially at basal levels. Therefore, it will be essential to establish new reference ranges in further studies.

Complete contact information is available at: https://pubs.acs.org/10.1021/jasms.3c00021

Notes

The authors declare no competing financial interest.

ABBREVIATIONS

LC-MS/MS, Liquid chromatography coupled to tandem mass spectrometry

IS Labeled internal standard

EMA, European Medicines Agency

RIA, Radioimmunoassay

AUC, Area under the curve

CV, Coefficient of variation

ELISA, Enzyme linked immunosorbent assay

LLMI, Lower limit of the measuring interval

HPLC, High performance liquid chromatography

ESI+, Electrospray ionization in positive mode

MRM, Multiple reaction monitoring

MS, Mass spectrometry

FIA, Flow injection analysis

REFERENCES

(1) *Rajaratnam, S. M. W.; Arendt, J. Health in a 24-h Society* ***2001****,358, 999—1005.*

(2) *Claustrat, B.; Leston, J. Melatonin: Physiological Effects in Humans. Neurochirurgie* ***2015****, 61 (2—3), 77—84.*

(3) *Hardeland, R. Melatonin and 5-Methoxytryptamine in Non- Metazoans. Reprod. Nutr. Dev.* ***1999****, 39 (3), 399—408.*

(4) *Van Tassel, D. L.; Roberts, N.; Lewy, A.; O'Neill, S. D. Melatonin in Plant Organs. J. Pineal Res.* ***2001****, 31 (1), 8—15.*

(5) *Pavlova, M. K.; Latreille, V. Sleep Disorders. Am. J. Med.* ***2019****,132 (3), 292—299.*

(6) *Riemann, D.; Baglioni, C.; Bassetti, C.; Bjorvatn, B.; Dolenc Groselj, L.; Ellis, J. G.; Espie, C. A.; Garcia-Borreguero, D.; Gjerstad, M.; Goncalves, M.; Hertenstein, E.; Jansson-Frojmark, M.; Jennum, P. J.; Leger, D.; Nissen, C.; Parrino, L.; Paunio, T.; Pevernagie, D.; Verbraecken, J.; WeeB, H. G.; Wichniak, A.; Zavalko, I.; Arnardottir, E. S.; Deleanu, O. C.; Strazisar, B.; Zoetmulder, M.; Spiegelhalder, K. European Guideline for the Diagnosis and Treatment of Insomnia. J. Sleep Res.* ***2017****, 26 (6), 675—700.*

(7) *Vorkapic, P.; Waldhauser, F.; Bruckner, R.; Biegelmayer, C.; Schmidbauer, M.; Pendl, G. Serum Melatonin Levels: A New Neurodiagnostic Tool in Pineal Region Tumor? Neurosurgery* ***1987****, 21 (6), 817—824.*

(8) *Van Faassen, M.; Bischoff, R.; Kema, I. P. Relationship between Plasma and Salivary Melatonin and Cortisol Investigated by LC-MS/ MS. Clin. Chem. Lab. Med.* ***2017****, 55 (9), 1340—1348.*

(9) *Reid, K. J. Assessment of Circadian Rhythms. Neurol. Clin.* ***2019****, 37 (3), 505—526.*

(10) *Novakova, M.; Paclt, I.; Ptàcek, R.; Kuzelova, H.; Hajek, I.; Sumova, A. Salivary Melatonin Rhythm as a Marker of the Circadian System in Healthy Children and Those with Attention-Deficit/ Hyperactivity Disorder. Chronobiol. Int.* ***2011****, 28 (7), 630—637.*

(11) *Melo, M. C. A.; Abreu, R. L. C.; LinharesNeto, V. B.; de Bruin, P. F. C.; de Bruin, V. M. S. Chronotype and Circadian Rhythm in Bipolar Disorder: A Systematic Review. Sleep Med. Rev.* ***2017****, 34, 46—58.*

(12) *Voultsios, A.; Kennaway, D. J.; Dawson, D. Salivary Melatonin as a Circadian Phase Marker: Validation and Comparison to Plasma Melatonin. J. Biol. Rhythms* ***1997****, 12 (5), 457—466.*

(13) *Kennaway, D. J. Measuring Melatonin by Immunoassay. J. Pineal Res.* ***2020****, 69 (1), 0—2.*

(14) *Rizzo, V.; Porta, C.; Moroni, M.; Scoglio, E.; Moratti, R. 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 Anal. Technol. Biomed. Life Sci.* ***2002****, 774 (1), 17—24.*

(15) *Hirano, J.; Hamase, K.; Fukuda, H.; Tomita, T.; Zaitsu, K. Novel Stable Fluorophore, 6-Methoxy-4-Quinolone, with Strong Fluorescence in Wide PH Range of Aqueous Media, and Its Application as a Fluorescent Labeling Reagent. J. Chromatogr. A* ***2004****, 1059 (1—2), 225—231.*

(16) *Eriksson, K.; Ostin, A.; Levin, J. O. Quantification of Melatonin in Human Saliva by Liquid Chromatography-Tandem Mass Spectrom- etry Using Stable Isotope Dilution. J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* ***2003****, 794 (1), 115—123.*

(17) *Motoyama, A.; Kanda, T.; Namba, R. Direct Determination of Endogenous Melatonin in Human Saliva by Column-Switching Semi- Microcolumn Liquid Chromatography/Mass Spectrometry with onLine Analyte Enrichment. Rapid Commun. Mass Spectrom.* ***2004****, 18 (12), 1250-1258.*

(18) *Jensen, M. A.; Hansen, A. M.; Abrahamsson, P.; N0rgaard, A. W. Development and Evaluation of a Liquid Chromatography Tandem Mass Spectrometry Method for Simultaneous Determination of Salivary Melatonin, Cortisol and Testosterone. J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* ***2011****, 879 (25), 2527-2532.*

(19) *Dermanowski, M. M.; Hejduk, A.; Kuczynska, J.; Wichniak, A.; Urbanska, A.; Mierzejewski, P. Assessment of Dim Light Melatonin Onset Based on Plasma and Saliva Samples. Chronobiol. Int.* ***2022****, 39 (5), 626-635.*

(20) *Shin, S.; Oh, H.; Park, H. R.; Joo, E. Y.; Lee, S. Y. A Sensitive and Specific Liquid Chromatography-Tandem Mass Spectrometry Assay for Simultaneous Quantification of Salivary Melatonin and Cortisol: Development and Comparison with Immunoassays. Ann. Lab. Med.* ***2021****, 41 (1), 108-113.*

(21) *Flanagan, S. C.; Cobice, D.; Richardson, P.; Sittlington, J. J.; Saunders, K. J. Elevated Melatonin Levels Found in Young Myopic Adults Are Not Attributable to a Shift in Circadian Phase. Investig. Ophthalmol. Vis. Sci.* ***2020****, 61 (8), 45.*

(22) *McBride, E. M.; Lawrence, R. J.; McGee, K.; Mach, P. M.; Demond, P. S.; Busch, M. W.; Ramsay, J. W.; Hussey, E. K.; Glaros, T.; Dhummakupt, E. S. Rapid Liquid Chromatography Tandem Mass Spectrometry Method for Targeted Quantitation of Human Performance Metabolites in Saliva. J. Chromatogr. A* ***2019****, 1601, 205-213.*

(23) *Van Faassen, M.; Bischoff, R.; Kema, I. P. Relationship between Plasma and Salivary Melatonin and Cortisol Investigated by LC-MS/ MS. Clin. Chem. Lab. Med.* ***2017****, 55 (9), 1340-1348.*

(24) *Simonin, G.; Bru, L.; Lelievre, E.; Jeanniot, J. P.; Bromet, N.; Walther, B.; Boursier-Neyret, C. 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 (3), 591-601.*

(25) *Fourtillan, J. B.; Gobin, P.; Faye, B.; Girault, J. A Highly Sensitive Assay of Melatonin at the Femtogram Level in Human Plasma by Gas Chromatography/Negative Ion Chemical Ionization Mass Spectrom- etry. Biol. Mass Spectrom.* ***1994****, 23 (8), 499-509.*

(26) *Ozer, E. T.; Gucer, S. Determination of Some Phthalate Acid Esters in Artificial Saliva by Gas Chromatography-Mass Spectrometry after Activated Carbon Enrichment. Talanta* ***2011****, 84 (2), 362-367.*

(27) *Eugster, P. J.; Dunand, M.; Grund, B.; Ivanyuk, A.; Fogarasi Szabo, N.; Bardinet, C.; Abid, K.; Buclin, T.; Grouzmann, E.; Chtioui, H. Quantification of Serotonin and Eight of Its Metabolites in Plasma of Healthy Volunteers by Mass Spectrometry. Clin. Chim. Acta* ***2022****, 535, 19-26.*

(28) *Bonmati-Carrion, M. A.; Middleton, B.; Revell, V.; Skene, D. J.; Rol, M. A.; Madrid, J. A. Circadian Phase Asessment by Ambulatory Monitoring in Humans: Correlation with Dim Light Melatonin Onset. Chronobiol. Int.* ***2014****, 31 (1), 37-51.*

(29) *Kennaway, D. J. A Critical Review ofMelatoninAssays: Past and Present. J. Pineal Res.* ***2019****, 67 (1), e12572.*

(30) *Kennaway, D. J. Measuring Morning Melatonin Levels with Plasma Melatonin ELISA Kits Is a Poor Choice on Two*

Levels. J. Pineal Res. ***2022****, DOI: 10.1111/jpi.12773.*

(31) *De Almeida, E. A.; Di Mascio, P.; Harumi, T.; Spence, D. W.; Moscovitch, A.; Hardeland, R.; Cardinali, D. P.; Brown, G. M.; Pandi- Perumal, S. R. Measurement of Melatonin in Body Fluids: Standards, Protocols and Procedures. Child's Nerv. Syst.* ***2011****, 2 7 (6), 879-891.*