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Simultaneous determination of melatonin and 6-hydroxymelatonin in human overnight urine by LC-MS/MS

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ABSTRACT

For the quantification of the pineal hormone melatonin and its metabolite, 6-hydroxymelatonin, in human overnight urine, a single accurate method by liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been developed. Urine samples were deconjugated using β-glucuronidase/arylsulfatase from *Helix pomatia* before solid phase extraction (SPE) purification. Chromatographic separation was performed using a reverse phase C18 column with a 7-minute gradient elution. Water was used as matrix to prepare the calibration standards, and deuterated analogues of melatonin and 6-hydroxymelatonin were used as internal standards. This newly developed method was validated in terms of linearity, accuracy, repeatability, intermediate precision, recovery, matrix effect, and stability according to the guidelines of the European Medicines Agency. The method was successfully applied to the analysis of overnight urine samples from 12 healthy volunteers, showing significant correlations of urinary melatonin and 6-hydroxymelatonin excretion rates with age. The urinary 6-hydroxymelatonin to melatonin ratio was also established and will be assessed in further studies as a potential endogenous metric of CYP1A2 activity.

1. Introduction

Cytochrome P450 1A2 (CYP1A2) accounts for around 15% of the total CYP content in the liver [1]. This isoenzyme in known to be responsible for the metabolism of drugs from various therapeutic areas including theophylline, clozapine, olanzapine and tizanidine. The activity of CYP1A2 is affected by a variety of environmental factors (e.g. tobacco, cruciferous vegetables, charcoal-grilled meat, drug-drug interactions) and genetic polymorphisms resulting in up to 60-fold inter-individual variability [2]. Exogenous probe drugs commonly used to phenotype CYP1A2 activity include caffeine, theophylline and tizanidine [3]. Phenotyping with exogenous substances is, however, burdensome for caregivers and patients. It implies delays before sampling since probe drug exposure depends on absorption and is not without risks (e.g. allergy, dosage error) [4].

Melatonin is a hormone endogenously released from the pineal gland and mainly biotransformed through CYP1A2 enzyme into 6-hydroxymelatonin, which is further largely conjugated into 6-sulfatoxymelatonin and 6-hydroxymelatonin glucuronide [5]. Compared to placebo, Skene et al. reported that a single dose of fluvoxamine 100 mg could significantly reduce urinary elimination of 6-sulphatoxymelatonin between 12 am – 9 am (overnight collection), and increase plasma melatonin concentrations in eight healthy male participants ($P < 0.01$) [6]. Two other studies reported also a significant increase in nocturnal melatonin levels following fluvoxamine intake in healthy volunteers [7,8]. These results encourage the acquisition of additional data on the use of melatonin and its metabolite as endogenous CYP1A2 biomarkers.

Quantification of endogenous melatonin and 6-hydroxymelatonin in urine samples can be very challenging due to low concentrations (pg/mL-ng/mL) and complexity of this biological matrix, requiring highly sensitive and selective methods for accurate detection [9,10]. Quantifying endogenous compounds is also complex due to their presence in

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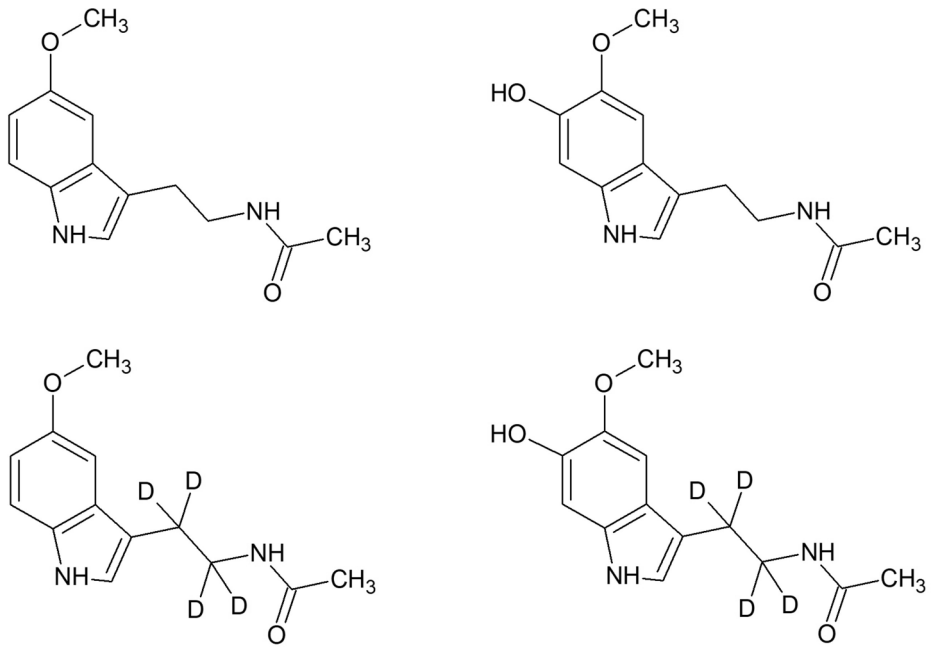

Fig. 1. Structures for melatonin (top left), melatonin-D4 (bottom left), 6-hydroxymelatonin (top right) and 6-hydroxymelatonin-D4 (bottom right).

biological matrices [11]. In addition, since the diurnal excretion of melatonin and 6-hydroxymelatonin is minor compared to nighttime excretion, it appears more convenient to use overnight urine for quantification of these compounds [12,13]. In most instances, melatonin and 6-hydroxymelatonin are measured through conventional immunoassays, mainly enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay (RIA) [9]. Despite high sensitivity, these methods may show cross-reactivity with structurally related compounds. In contrast, liquid chromatography-tandem mass spectrometry (LC-MS/MS) is often recognized as one of the most appropriate and convenient method for the specific and sensitive analysis of small amounts of analytes in biological fluids [14]. Solid phase extraction (SPE) is commonly used to clean up and concentrate samples prior to LC-MS analyses [15]. Regarding phase II metabolites, measurement of conjugated compounds would require the synthesis of 6-sulfatoxymelatonin and 6-hydroxymelatonin glucuronide, which can be challenging and expensive [16]. In contrast, deconjugation with β-glucuronidase and arylsulfatase allows measurement of total concentrations (conjugated and unconjugated) [16]. Hydrolysis would also improve the detection of the unconjugated form 6-hydroxymelatonin. In addition, this step is required to overcome the variability caused by the polymorphic glucuronosyltransferase and sulfotransferases enzymes [17,18].

To the best of our knowledge, a method for the quantification of both endogenous melatonin and 6-hydroxymelatonin after enzymatic deconjugation in human urine collected overnight has never been reported using a SPE-LC-MS/MS method.

In the present study, a single LC-MS/MS assay was developed for the simultaneous quantification of melatonin and 6-hydroxymelatonin in nighttime urine using stable isotope-labeled internal standards for each analyte and SPE pre-treatment. The validated method was finally applied in the analysis of overnight urine samples from twelve healthy volunteers.

2. Material and methods

2.1. Chemicals and reagents

Melatonin (Fig. 1-top left), 6-hydroxymelatonin (Fig. 1-top right), sodium acetate and ammonium acetate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Melatonin-D4 (Fig. 1-bottom left) and 6-hydroxymelatonin-D4 (Fig. 1-bottom right) were obtained from Alsachim (Illkirch-Graffenstade, France). *β*-Glucuronidase/Arylsulfatase (β-GA) from *Helix pomatia* was acquired from Roche (Mannheim, Germany). Oasis MAX 3 cc (60 mg) extraction catridges were purchased from Waters (Milford, MA, USA). Acetonitrile (ACN) ULC/MS grade and formic acid were from Biosolve (Valkenswaard, The Netherlands).

2.2. Instrumentation

LC-MS/MS analyses were carried out using an Agilent 1290 Infinity series LC system from Agilent (Paolo Alto, USA) coupled to a 6500 QTrap® triple quadrupole linear ion trap mass spectrometer from AB Sciex equipped with an electrospray ionization (Darmstadt, Germany). Separation was performed with a Kinetex® C18 column (50 × 2.1 mm, 2.6 μm) from Phenomenex (Brechbühler, Switzerland). Analyst software, version 1.6.2. was used for system control, data acquisition and quantification. A linear gradient was applied with a mobile phase composed of A: water containing 0.1% formic acid and B: acetonitrile containing 0.1% formic acid. Gradient elution was performed at a 600 μL/min flow rate as follows: 0–1.0 min 2% B, 1.0–3.5 from 2% to 29% B, 3.5–3.6 from 29% to 95% B, 3.6–4.6 min 95% B, 4.6–4.7 from 95% to 2% B and 4.7–7.0 2% B. The injection volume was set at 20 μL. Detection of analytes was obtained in positive mode detection using multiple reaction monitoring. Values of QTrap parameters are as follows: curtain gas = 40 psi, collision gas = high, IonSpray voltage = 4500 kV, temperature = 550 °C, ion source gas 1 = 60 psi, ion source gas 2 = 60 psi. The optimized collision energy was + 21 V for all analytes, the optimized declustering potential (DP) was 21 V for melatonin and melatonin-D4, and 26 V for 6-hydroxymelatonin and 6-hydroxymelatoin-D4, and the optimized cell exit potential (CXP) was 12 V for melatonin and melatonin-D4, and 18 V for 6-hydroxymelatonin and 6-hydroxymelatoin-D4. The transitions monitored for each analyte (precursor ion > product-fragment ions) were: melatonin 233.1 > 174.1, melatonin-D4 237.2 > 178.1, 6-hydroxymelatonin 249.1 > 190.1, 6-hydroxymelatonin-D4 253.1 > 193.1.

2.3. Preparation of stock solutions, calibration standard solutions and quality controls samples

Stock solution of melatonin and 6-hydroxymelatonin were prepared

in methanol at 0.2 and 10 μg/mL respectively. Regarding the internal standards (IS), stock solution was prepared at 30 ng/mL of melatonin-D4 and 1400 ng/mL of 6-hydroxymelatonin-D4 in methanol. More specifically, for each of the three non-consecutive days, seven calibration standard concentrations were prepared in duplicate in 2 mL of water: 7.5, 15, 25, 50, 125, 250 and 500 pg/mL for melatonin, and 375, 750, 1250, 2500, 6250, 12 500 and 25 000 pg/mL for 6-hydroxymelatonin.

The quality control (QC) samples were prepared in quadruplicate and separated into two complementary groups similar to the report by Wang et al. [19]. The first group includes LLOQ and low concentrations and was prepared in 2 mL of water to give the following concentrations: 7.5 and 22.5 pg/ml of melatonin, and 375 and 1125 pg/mL of 6-hydroxymelatonin. The second group of QC samples was prepared in 2 mL of urine: 200 and 400 pg/mL of melatonin, and 10 000 and 20 000 pg/mL of 6-hydroxymelatonin, representing medium and high concentrations. Baseline levels of endogenous melatonin and 6-hydroxymelatonin of the latter group were systematically established at each analysis.

2.4. Sample extraction procedure

To 2 mL of water or urine samples, 10 μL of the IS stock solution was added. Two hundred μL of 1 M sodium acetate buffer (pH 5.5) and 40 μL of the β-GA solution were also added to all samples before being vortexed and incubated for 2 h at 37 °C for rapid hydrolysis of the glucuronide and sulfate linkage.

After incubation, 1.75 mL of 25 mM ammonium acetate buffer (pH 7) was added, samples were vortexed and centrifuged at 3000 g for 5 min. Analytes extraction was then performed by SPE using Oasis MAX cartridges. MAX cartridges were conditioned with methanol (3 mL) and equilibrated with water (3 mL). Samples are passed through the MAX cartridges followed by washing with 3 mL 25 mM ammonium acetate buffer (pH 7) and 3 mL 10% methanol. All analytes, including the IS, were finally eluted by 100% methanol (3 mL). The eluates were evaporated to dryness under a stream of nitrogen. The residue were then dissolved in 100 μL of water, vortexed and centrifuged again before injection into the LC-MS/MS system.

2.5. Method validation

The method was fully validated according to the guidelines of the European Medicines Agency (EMA) by assessing its selectivity, carry-over, linearity, matrix effect, recovery, accuracy, precision and stability [20].

2.5.1. Selectivity and Carry-over

The selectivity of the method was assessed by verifying the absence of interfering peaks at compound retention time (RT) in water and in urine from six different volunteers collected between 12 pm and 2 pm (minor excretion of melatonin and 6-hydroxymelatonin during daytime) for melatonin, 6-hydroxymelatonin and the two IS.

The carry-over effect was determined for each analyte by assaying the blank injected just after the analysis of the highest calibration standard solution.

2.5.2. Extraction recovery and matrix effect

Extraction recovery was assessed in quadruplicate using QC samples at the four different concentrations (LLOQ, low, medium and high) spiked with IS by comparing the peak area ratios of the analytes before and after the extraction procedure.

Matrix effect was determined in quadruplicate using the second group of QC (medium and high) spiked with IS by comparing the differences between peak area ratios of the analytes in urine samples after extraction with peak area ratios in water.

2.5.3. Linearity, accuracy, precision

For each of the three non-consecutive days, the linearity of the assay was assessed by duplicate analyses of calibrators (n = 2) with concentrations ranging from 7.5 to 500 pg/mL for melatonin and 375 to 25 000 pg/mL for 6-hydroxymelatonin. Quantitation was performed by plotting the ratios of the peak area of melatonin and 6-hydroxymelatonin to their respective IS versus concentration.

Accuracy and precision (repeatability and intermediate precision) were evaluated during the three days of validation by two distinct groups of QC samples (see Section 2.3) at four concentration levels in four replicates spiked with IS. Accuracy was determined as percentage of the ratio between mean concentrations obtained from experimental measurements and theoretical concentrations for the first group of QC. For the second group of QC, the basal concentration of the urine must first be subtracted from the observed concentrations in order to measure accuracy. To estimate precision of the measurements, variances of repeatability (intra-day variances) and intermediate precision (sum of intra-day and inter-day variances) were calculated and expressed as relative standard deviation (RSD).

2.5.4. Stability

Stability experiments were conducted on the second group of QC samples (three replicates) containing melatonin and 6-hydroxymelatonin in the urine. The QC samples were exposed to different conditions: at room temperature for 14 h, at +4 °C for 24 h, and at −80 °C for 4 months. The mean of the concentrations of each QC sample after all stability studies were measured using a calibration curve prepared with freshly spiked calibration standards, and the observed concentrations were compared with nominal concentrations.

2.6. Application of method to clinical samples and statistical analysis

The method was applied to urine samples from 12 healthy volunteers thoroughly collected overnight (between 9 pm and 9 am) on three different days (day 1, day 4 ± 1 and day 30 ± 2) under baseline conditions (NCT04420611). This study was approved by the Geneva Research Ethics Committee and was performed in accordance with the Declaration of Helsinki ethical principles.

Study participants were asked to refrain from alcohol consumption and methylxanthine-containing beverages and foods 24 h before the overnight urine collection.

GraphPad Prism 8 was used for data analysis. Measures of associations were established using Spearman's rank correlation. A probability level ($P < 0.05$) was considered statistically significant.

3. Results and discussion

3.1. Sample preparation

SPE was chosen for sample clean-up because it is easy to automate, removes a number of interfering components from the biological matrix and improves sensitivity thanks to the concentration factor [21]. Mixed-mode SPE cartridges were tested, including strong cation exchange, strong anion exchange, weak cation exchange and weak anion exchange. Reversed-phase (Phenyl), normal-phase (CN) and hydrophilic–lipophilic balance (HLB) sorbents were also tested (data not shown).

In this study, the SPE method using the column MAX, a mixed-mode ion exchange (strong anion exchange) cartridge, produced the best results for the isolation of melatonin and its metabolite from urine. Slightly colored and clear extracts were achieved for most samples. Puris et al. obtained relatively low recoveries for both melatonin and 6-hydroxymelatonin using the column MAX [22]. They used 5% ammonium hydroxide in water during the wash step. However, during the method development we observed that 6-hydroxymelatonin was unstable in basic media. Thus, we optimized the extraction procedure by replacing the ammonium hydroxide wash step with a neutral acetate buffer. At pH 7, melatonin and 6-hydroxymelatonin are essentially neutral and retained on the MAX column only through reversed-phase

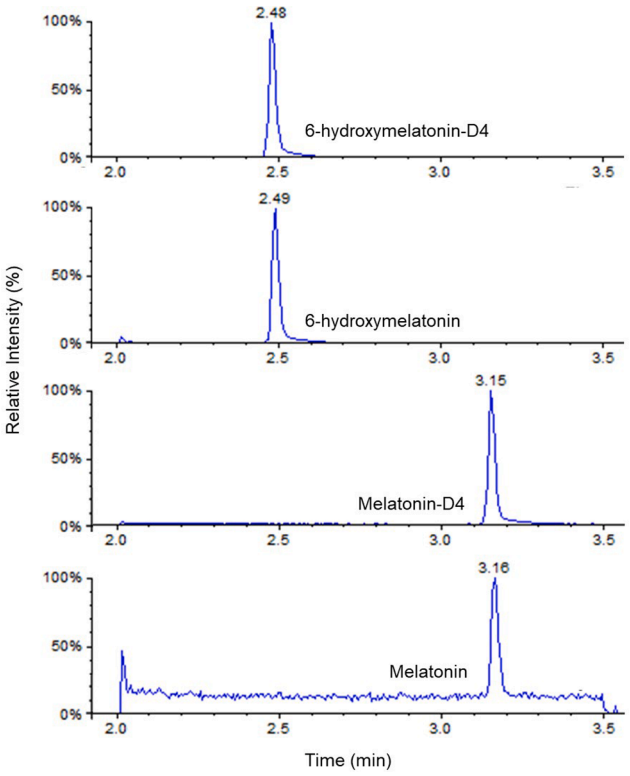

Fig. 2. Representative chromatograms for the LC–MS/MS analysis of melatonin and 6-hydroxymelatonin at their LLOQ concentrations and their IS.

interactions. Consequently, the compounds of interest were efficiently eluted using 100% methanol, while the interfering acidic compounds were retained in the column. This method allowed suitable extraction of both melatonin and its metabolite within acceptable and detectable levels, as described in Section 3.3.2.

Regarding enzymatic hydrolysis, we used the β-glucuronidase/arylsulfatase solution from *Helix pomatia* since 6-hydroxymelatonin is both, sulphated and glucuronidated [5,23]. In addition, this solution was shown to be effective in hydrolysing the conjugated forms of 6-hydroxymelatonin in a previous report [23]. Monitoring of the deconjugation process is often not performed because the conjugated probes required to estimate the deconjugation rate of the phase II metabolites are not available [16]. To optimize the hydrolysis conditions in this study, in particular the hydrolysis time, we evaluated the time required to reach a plateau in the formation of free 6-hydroxymelatonin after incubation of a urine sample with the enzymes, as previously performed by Härtter et al. [23]. Complete hydrolysis indicated by a plateau phase was reached after 2 h (data not shown). We chose all the other conditions (pH, buffer, volume of the β-glucuronidase/arylsulfatase solution, temperature of incubation etc.) according to the supplier instructions.

3.2. Chromatography

The positive ion mode (ESI+) was used to assess both melatonin and 6-hydroxymelatonin, as well as their respective IS.

As previously described, the product ion scan spectra showed a high abundance fragment ions at *m/z* 174.1 and 190.1 for melatonin and 6-hydroxymelatonin, respectively [22]. Regarding IS, fragment ions were observed at 178.1 for melatonin-D4 and 193.1 for 6-hydroxymelatonin-D4.

Under the optimized LC conditions, the retention times of melatonin and melatonin-D4 were 3.16 and 3.15 min, respectively and those of 6-hydroxymelatonin and 6-hydroxymelatonin-D4 of 2.49 and 2.48 min, respectively. Typical LC-MS/MS chromatograms of the four compounds can be seen in Fig. 2.

3.3. Method validation

3.3.1. Selectivity and carry-over

No interference was observed when analysing blank samples extracted from water at the respective retention times of the four compounds.

Regarding selectivity in human urine from six different sources, no significant interferences were observed for melatonin-D4 and 6-hydroxymelatonin-D4. It was however not possible to fully evaluate selectivity in human urine for melatonin and 6-hydroxymelatonin because of the constant occurrence of these compounds at endogenous levels, even during daytime. This is a limitation of such bioanalytical methods as explained by Wang et al. [19]. At least, though, it was possible to see that no co-eluting peaks were present at these retention times.

A significant carry-over for melatonin and 6-hydroxymelatonin was observed when analysing a blank sample directly after the highest calibration standard. Indeed, the signals were equal to about 58% and 30% for melatonin and 6-hydroxymelatonin, respectively, of the signal of the LLOQ. Nevertheless, when 20 μL of 100% acetonitrile was injected just after the highest calibration standard, no significant residual signal was found on the blank sample. Therefore, as a precautionary measure, an acetonitrile sample was always injected following the analysis of clinical samples in order to eliminate any potential carry-over effect.

3.3.2. Extraction recovery and matrix effect

Interfering substances and the resulting risks of matrix effects were corrected by using deutered IS. The matrix effect and recovery of the analytes can be visualized in Table 1.

The mean IS-normalized matrix effects were 100–109 % (RSD ≤ 5%) for both compounds for medium and high concentrations indicating that there was no significant matrix effect as the RSD is not greater than 15%.

Total recoveries for both substances at all concentration levels were satisfactorily in the interval 94–102% (RSD ≤ 8%) showing that the method is reliable.

3.3.3. Linearity, accuracy, precision

Because endogenous melatonin and 6-hydroxymelatonin are unavoidably present in urine, calibration standard solutions were spiked into water to establish the calibration curve. For each compound, a

Table 1
Internal standard–normalized matrix effect and extraction recovery in human urine of melatonin and 6-hydroxymelatonin.

Compound	QC level	Concentration (pg/mL)	IS-normalized matrix effect % (RSD %)	IS-normalized recovery % (RSD %)
Melatonin	LLOQ	7.5	NA	94 (4)
	Low	22.5	NA	99 (8)
	Medium	200	104 (4)	95 (5)
	High	400	109 (3)	94 (2)
6-hydroxymelatonin	LLOQ	375	NA	99 (4)
	Low	1125	NA	99 (3)
	Medium	10 000	100 (3)	102 (3)
	High	20 000	103 (5)	96 (2)

Table 2
Accuracy, repeatability, and intermediate precision in human urine.

Compound	QC level	Concentration (pg/mL)	Accuracy (%)	Precision	
				Repeatability (RSD%)	**Intermediate precision (RSD%)**
Melatonin	LLOQ	7.5	92.4	10.4	10.4
	Low	22.5	96.4	7.4	7.4
	Medium	200	104.6	3.4	4.8
	High	400	103.9	3.8	7.3
6-hydroxy-melatonin	LLOQ	375	97.9	5.8	14.8
	Low	1125	102.6	4.2	6.1
	Medium	10 000	95.0	6.0	10.4
	High	20 000	94.0	7.9	10.4

Table 3
Stability of melatonin and 6-hydroxymelatonin in human urine. Data are reported as deviations (%) from the nominal concentrations.

Compound	QC level	Concentration (pg/mL)	Room temperature for 14 h (%)	+4°C for 24 h (%)	−80 °C for 4 months (%)
Melatonin	Medium	200	12.5	2.8	−8.9
	High	400	10.8	−1.3	4.5
6-hydroxy-melatonin	Medium	10 000	−13.4	−5.3	−14.3
	High	20 000	−14.4	−11.5	−3.5

Table 4
Age, urinary 6-hydroxymelatonin and melatonin excretion rates, and urinary 6-hydroxymelatonin/melatonin metabolic ratio measured in the urine collected from twelve healthy volunteers between 9 pm and 9 am (overnight). Results are displayed as the mean of the three study sessions ± standard deviation (SD).

Subject Identification	Age	Nocturnal 6-hydroxymelatonin excretion (ng/12 h) (mean ± SD)	Nocturnal melatonin excretion (ng/12 h) (mean ± SD)	6-hydroxymelatonin/melatonin ratio (mean ± SD)
1	61	1028 ± 208	8 ± 2	136 ± 16
2	30	1665 ± 464	18 ± 3	93 ± 17
3	43	3308 ± 1597	29 ± 17	122 ± 16
4	30	3946 ± 1951	30 ± 13	130 ± 25
5	29	2664 ± 1586	40 ± 21	66 ± 13
6	29	1624 ± 679	31 ± 13	55 ± 17
7	26	4097 ± 2286	15 ± 3	270 ± 138
8	25	5559 ± 1835	25 ± 4	226 ± 83
9	30	1443 ± 303	8 ± 3	195 ± 113
10	24	8017 ± 2143	75 ± 7	106 ± 22
11	26	10618 ± 5220	48 ± 24	219 ± 43
12	29	2810 ± 537	16 ± 1	178 ± 27

seven-point calibration curve was constructed by plotting the peak area ratio analyte/IS against the analyte concentration. The curves were fitted using linear regression model using a $1/x^2$ weighting factor. For each of the three non-consecutive days, the correlation coefficients for melatonin and 6-hydroxymelatonin of the calibration curves were over 0.991 demonstrating a linear relationship.

Accuracy and precision can be seen in Table 2.

The accuracy data were found to be within the acceptance criteria range for melatonin and 6-hydroxymelatonin (85–115% of the theoretical value): 92.4–104.6% and 94.0–102.6%, respectively.

Precision values for all QC samples of both analytes were also within the acceptance range, in accordance with the guidelines (under 15%). The following performances were obtained in terms of repeatability: 3.4–10.4% for melatonin and 4.2–7.9% for 6-hydroxymelatonin. Intermediate precision for melatonin and 6-hydroxymelatonin were within 4.8–10.4% and 6.1–14.8%, respectively.

3.3.4. Stability

The established stabilities for melatonin and 6-hydroxymelatonin in human urine are summarized in Table 3.

Both analytes were stable for 14 h at room temperature, for 24 h at + 4 °C and for 4 months at −80 °C without significant degradation of the compounds (±15% of nominal concentrations).

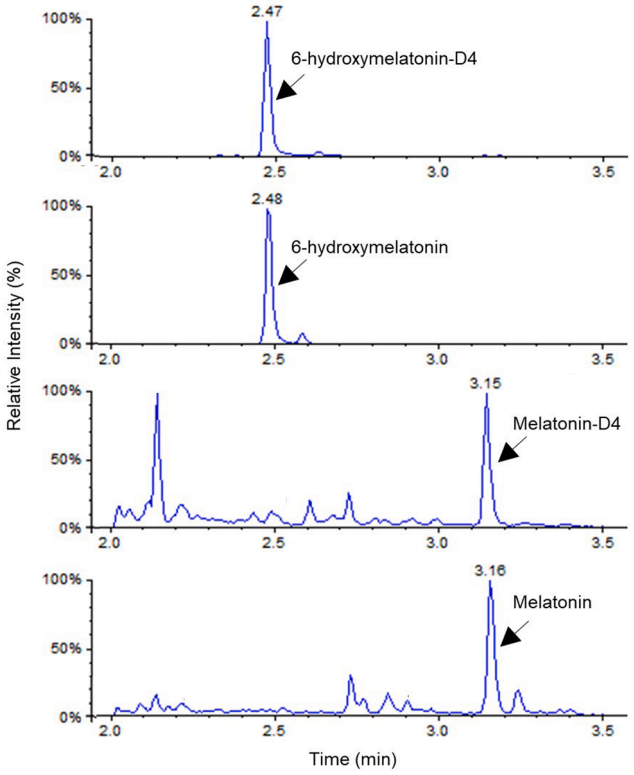

Fig. 3. Representative chromatograms for the LC–MS/MS analysis of melatonin, 6-hydroxymelatonin and their IS in a healthy human urine sample.

3.3.5. Clinical application

To test our method, 6 women and 6 men were enrolled in the study. The median age was 29 (range 24–61). The concentrations of both melatonin and 6-hydroxymelatonin were measured in urine collected from 9 pm to 9 am. To adjust for variations in urinary concentration due to altered water contents, we measured the urinary excretion rates (calculated by multiplying the analyte concentration by the volume of urine collected over 12 h) of melatonin and 6-hydroxymelatonin [24].

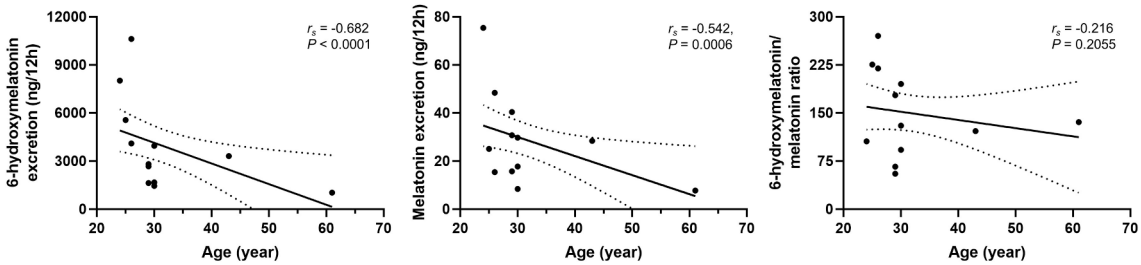

Fig. 4. Relationships between age and 6-hydroxymelatonin excretion rate, melatonin excretion rate, and 6-hydroxymelatonin/melatonin ratio. The dotted lines indicate the 95% confidence interval. r_s = Spearman correlation coefficient.

To some extent, the use of the 6-hydroxymelatonin to melatonin ratio can also offset urine dilution and correct for intra- and inter-individual variability. Results are described in Table 4.

Fig. 3 shows a representative chromatogram of an extracted urine sample. Both analytes and their ISs were detectable in regions free of interfering peaks.

The mean ± SD melatonin and 6-hydroxymelatonin excretion rates over a period of 12 h were 29 ± 21 and 3898 ± 3286 ng/12 h, respectively and were significantly negatively correlated with age (r_s = -0.542 and −0.682, respectively, $P < 0.001$) despite the small sample size and limited age diversity of the volunteers (Fig. 4). It has already been reported in many studies that melatonin secretion declines with age, leading to a simultaneous decrease in urinary 6-hydroxymelatonin concentrations [12,25,26]. In line with our results, von Bahr et al. measured a nocturnal urinary melatonin excretion rate over a period of 14 h of 42 ± 9 ng/14 h (reported as 0.18 ± 0.04 nmol/14 h) in seven healthy volunteers aged between 25 and 44 years old [8].

The mean urinary metabolic ratio of 6-hydroxymelatonin/melatonin was 150 ± 83. Age-related variations were corrected by using the 6-hydroxymelatonin/melatonin ratio, since this metric did not correlate with age (r_s = -0.216, P = 0.210) (Fig. 4).

These findings demonstrate the ability of our LC-MS/MS methodology to quantify melatonin and 6-hydroxymelatonin in human urine and to reproduce the results concerning the negative correlation obtained between the urinary excretion of these compounds and age.

4. Conclusion

In conclusion, this study describes a straightforward, fast, and reliable LC–MS/MS method for the simultaneous determination of melatonin and its metabolite, 6-hydroxmelatonin, in human urine collected overnight. The sample preparation involved enzymatic hydrolysis of glucuronide and sulfate conjugates, followed by SPE. The present methodology satisfied the required international validation criteria for both tested compounds and could be readily applied for clinical and research purposes. In particular, the ability of the urinary 6-hydroxymelatonin/melatonin ratio to phenotype the activity of the CYP1A2 isoenzyme will be assessed in future studies.

CRediT authorship contribution statement

G. Magliocco: Conceptualization, Investigation, Methodology, Validation, Writing – original draft. **F. Le Bloc'h:** Methodology, Validation, Writing – review & editing. **A. Thomas:** Supervision, Writing – review & editing. **J. Desmeules:** Resources, Supervision, Writing – review & editing. **Y. Daali:** Conceptualization, Methodology, Resources, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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