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## Research Article

Martijn van Faassen, Anna van der Veen, Sonja van Ockenburg, Helma de Jong, Elisabeth G. E. de Vries and Ido P. Kema\*

# Mass spectrometric quantification of urinary 6-sulfatoxymelatonin: age-dependent excretion and biological variation

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## Abstract

**Objectives:** Regulators of circadian rhythm, including melatonin, influence fundamental biological processes. Measuring the melatonin metabolite 6-sulfatoxymelatonin in urine can estimate melatonin production. 6-sulfatoxymelatonin is mainly analyzed by immunoassays, but these methods are hampered by cross-reactivity and poor reproducibility when used to analyze small molecules. Therefore, we validated a high-throughput liquid chromatography with tandem mass spectrometry (LC–MS/MS) method to quantify 6-sulfatoxymelatonin in urine. We evaluated age-dependent 24-h excretion of 6-sulfatoxymelatonin into urine and the biological variation of urinary excretion in healthy individuals.

**Methods:** The online solid phase extraction method combined with LC–MS/MS was validated according to international guidelines, and used to measure the excretion of 6-sulfatoxymelatonin into urine of 240 healthy individuals. Biological variation of 6-sulfatoxymelatonin excretion was examined in 10 healthy individuals.

**Results:** Urinary 6-sulfatoxymelatonin results were well within the validation criteria (interassay coefficient of variation: <5.4%, quantification limit: 0.2 nmol/L). There

was an age-related decrease in 6-sulfatoxymelatonin excretion into 24-h urine [ $F(5, 234)=13.9$ ;  $p<0.001$ ]. Within-subject variation of 6-sulfatoxymelatonin was 39.2% in day urine, 15.1% in night urine, and 12.2% in 24-h urine. Between-subject variation was 39.1% in day urine, 37.9% in night urine, and 36.8% in 24-h urine.

**Conclusions:** This MS-based method enables straightforward, reproducible, and sensitive quantification of 6-sulfatoxymelatonin in urine. Urinary 6-sulfatoxymelatonin levels decreased with age. Biological variation of 6-sulfatoxymelatonin excretion into urine was high between subjects and lower within subjects, indicating that repeated measurements of 6-sulfatoxymelatonin in 24-h urine are needed in future studies.

**Keywords:** biological variation; melatonin; tandem mass spectrometry; 6-sulfatoxymelatonin; urine.

## Introduction

Disturbed circadian rhythm and sleep is increasingly associated with the development of neurodegenerative, cardiovascular, and metabolic disorders [1, 2]. Melatonin is an important circadian regulator that signals the length of day and night. It also has antioxidant properties and modulates antioxidant enzymes and the immune system [3]. There is increasing interest in the mediating role of melatonin, since melatonin levels have been associated with breast and prostate cancer [4–6], myocardial infarction, and type 2 diabetes [7, 8]. Reliable measurement of melatonin levels is important to substantiate these studies and elucidate the role of melatonin.

In humans, melatonin is metabolized by the liver to 6-hydroxymelatonin, 60–80% of which conjugates to sulfate to form 6-sulfatoxymelatonin, and ~10–20% of which conjugates to glucuronide. A small portion is excreted as 6-hydroxymelatonin [9, 10]. Measuring 6-sulfatoxymelatonin excretion into urine collected over 24 h (24-h urine) gives a reliable estimate of total daily

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melatonin production [11–13]. This is useful for field-based studies, where frequent sampling in blood or saliva is not practical [14]. To confirm the aforementioned association of urinary 6-sulfatoxymelatonin, the biological variation must also be determined. However, no data on the biological variation of 6-sulfatoxymelatonin excretion into urine is available. This information is relevant for the design and interpretation of epidemiological studies. Biological variation is an important element of the random measurement error [15]. Random measurement error can influence the association between a variable and outcome of interest, and can cause regression dilution bias [15, 16]. Analytical variation is important when calculating the biological variation. To minimize analytical variation, measurements should be highly reproducible and free of interferences. Reliable methods are needed to achieve this.

Small molecules are preferably quantified by liquid chromatography in combination with tandem mass spectrometry (LC–MS/MS) because quantification of small molecules by immunoassays can be seriously flawed [17–20]. The purpose of the current study was to validate a reproducible, highly automated isotope dilution LC–MS/MS method for quantifying 6-sulfatoxymelatonin. We used this method to investigate the excretion of 6-sulfatoxymelatonin into urine in a large healthy population. We also determined biological variation of urinary 6-sulfatoxymelatonin excretion.

## Materials and methods

### Reagents

We obtained ULC–MS-grade methanol, acetonitrile, isopropanol, ammonium acetate, and formic acid from Biosolve BV (Valkenswaard, The Netherlands). Phosphate buffered saline (PBS; 0.01 M Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, 0.138 M NaCl, 27 mM KCl, pH 7.4) was prepared by the Department of Pharmacy of the UMCG. 6-sulfatoxymelatonin sodium salt was purchased from Toronto Research Chemicals Inc. (Toronto, Canada), and the D<sub>4</sub>-labeled internal standard (IS) for 6-sulfatoxymelatonin was purchased from CacheSyn Inc. (Mississauga, Canada). Ultrapure water was produced using an in-house purification system (Merck Millipore, MA, USA).

### Standard solutions and samples

Working standard solutions containing 6-sulfatoxymelatonin as well as IS solutions were serially diluted from stock solutions with PBS.

Before analysis, 100 µL aliquots of urine were pipetted into a 2.0 mL 96-deepwell plate (Greiner Bio-One, Kremsmünster, Austria). Subsequently, 50 µL IS and 850 µL PBS were added to all wells, and 50 µL was injected into the online SPE LC–MS/MS system.

### Analyses and quantification

A Symbiosis™ Pharma system (Spark Holland, Emmen, The Netherlands) was used for online solid phase extraction (SPE) and liquid chromatography as previously described [21]. Chromatography was performed using an Agilent Pursuit XRS Diphenyl column (2.0 × 150 mm, 3.0 µm) at 25 °C. The binary gradient system was composed of 10 mM ammonium acetate, 0.1% formic acid (FA) in water (eluent A), and 0.1% FA in acetonitrile (eluent B). A linear gradient elution was performed, starting at 85% A, 15% B, and in 2.5 min to 60% A and 40% B. Then in 0.5 min to 25% A, 75% B. After that, the column was flushed for 1 min. Finally, the column was equilibrated for 1.5 min at the starting gradient (85% A, 15% B) resulting in a total chromatographic run-time of 5.5 min, which includes the online SPE extraction.

Mass spectrometry was performed with an XEVO TQ system (Waters, Milford, MA, USA) equipped with an electrospray source in positive ion mode. 6-sulfatoxymelatonin was analyzed in selected reaction monitoring mode (SRM). An overview of the SRM transitions, collision energies, and retention times for 6-sulfatoxymelatonin and its stable isotope is given in Supplemental Data, Table 1.

## Method validation

The method was validated based on bioanalytical method validation guidelines from the European Medicines Agency [22].

Imprecision was determined by analyzing three urine pools with low, medium, or high 6-sulfatoxymelatonin concentrations 20 times on one day, and in replicate on 12 different days. The lower limit of quantification (LLOQ) for 6-sulfatoxymelatonin was determined by diluting a low-concentration urine sample with 6-sulfatoxymelatonin-free urine, and analyzing the dilutions on six different days. The LLOQ was <20% precision and a signal to noise ratio of >10.

Recovery was assessed by spiking 6-sulfatoxymelatonin concentrations in three different urine pools containing endogenous 6-sulfatoxymelatonin at three different levels. These samples were analyzed on five different days. The recovery percentage was calculated as follows:  $([\text{final concentration} - \text{initial concentration}] / \text{added concentration}) \times 100\%$ . Recovery between 85 and 115% was considered acceptable.

Results of the LC–MS/MS method were compared with those of a commercial ELISA (catalogue number RE54031, IBL International, Hamburg, Germany) by analyzing 40 residual 24-h urine samples collected during routine patient care. The ELISA was performed according to the vendor's instructions: 10 µL urine, standard, and control samples were diluted with 500 µL assay buffer, and 50 µL of these diluted samples were pipetted into the microtiter plate wells. Then, 50 µL of freshly prepared enzyme

conjugate was pipetted into each well, followed by 50  $\mu\text{L}$  of antibody solution. The plate was covered with adhesive foil and incubated for 2 h on a plate shaker. The plate was then washed four times and 100  $\mu\text{L}$  substrate was added. Plates were then incubated for 30 min on a shaker. The reaction was stopped by adding 100  $\mu\text{L}$  of stop solution. The plate was briefly mixed and the optical density was measured at 450 nm. Results were evaluated using Passing-Bablok and Bland-Altman regression.

## Excretion study

We used archived 24-h urine samples taken from healthy participants ( $n=240$ ) in a previous study [23]. Participants were randomly selected from the Lifelines Cohort Study [24]. Participants aged 20–80 years were included, and stratified for 20 males and 20 females per age-decade group. Participants were normotensive, had a normal blood count, and were not medicated. The BMI of participants was between 21 and 30  $\text{kg}/\text{m}^2$ . For 24-h urine, the first sample was collected at 10 pm and then all voided urine was collected until 10 pm the next day. The collected urine was mixed and aliquots were stored at  $-80^\circ\text{C}$  (collection of urine between 2009 and 2012) until analysis for 6-sulfatoxymelatonin by LC-MS/MS (in 2014). Long-term stability of 6-sulfatoxymelatonin in urine was previously shown to be at least 15 years at  $-20^\circ\text{C}$  [25]. The 24-h excretion of 6-sulfatoxymelatonin (in nmol) was calculated as follows: 6-sulfatoxymelatonin concentration in 24-h urine ( $\text{nmol}/\text{L}$ )  $\times$  volume of day urine (L).

## Biological variation study

Biological variation comprises the random variation of an analyte in one individual, the within-subject variation ( $\text{CV}_i$ ), the overall variation in different individuals, the between-subject variation ( $\text{CV}_g$ ), and the analytical variation ( $\text{CV}_a$ ). Biological variation was determined using urine samples collected during a previous longitudinal prospective observational study [26]. Ten adults between 18 and 65 years of age, who were free of medication and somatic and mental disease collected urine for 63 consecutive days. Urine was collected in two separate containers: a “night container” and a “day container”. Participants were instructed to use the “night container” from the moment they went to bed until the first morning void. The “day container” contained all urine produced after the first morning void until the last void before going to bed. On each Wednesday, the biological variation of 6-sulfatoxymelatonin excretion in day, night,

and 24-h urine samples was estimated. Nine samples from each participant were analyzed in duplicate in the same analytical run. The 24-h excretion of 6-sulfatoxymelatonin (in nmol) into urine was computed by combining the results of day and night urine as follows: (6-sulfatoxymelatonin concentration of night urine [ $\text{nmol}/\text{L}$ ]  $\times$  volume of night urine [L]) + (6-sulfatoxymelatonin concentration of day urine [ $\text{nmol}/\text{L}$ ]  $\times$  volume of day urine [L]). Excretion of 6-sulfatoxymelatonin (in nmol) into day urine and night urine was computed as follows: 6-sulfatoxymelatonin concentration of day or night urine  $\times$  volume of day or night urine.  $\text{CV}_a$ ,  $\text{CV}_i$ ,  $\text{CV}_g$ , reference change value (RCV), index of individuality (II), and homeostatic setting points for day urine, night urine, and 24-h urine were calculated as previously described [27, 28]. Quality specifications for analytical imprecision, bias and total error, expressed in percentages were calculated from the biological variation data for the 24 h urinary excretion of 6-sulfatoxymelatonin as follows: analytical imprecision  $< 0.5 \text{ CV}_i$ ; bias  $< 0.25 (\text{CV}_i^2 + \text{CV}_g^2) \times 0.5$ ; total error  $< k \times 0.5 \times \text{CV}_i + 0.25 (\text{CV}_i^2 + \text{CV}_g^2) \times 0.5$ , being  $k=1.65$  at  $\alpha=0.05$  [27].

## Statistical analysis

Statistical analyses were performed using SPSS version 23.0 for Windows (IBM Corporation, Chicago, IL, USA) and RStudio (Boston, MA, USA). Normally distributed data were expressed as mean  $\pm$  standard deviation (SD), and non-normally distributed data as median (interquartile range). A p-value of  $<0.05$  (two-sided) was considered statistically significant. Differences in 6-sulfatoxymelatonin excretion between men and women were assessed by an independent-samples t-test, and differences between age decades were assessed by ANOVA with post-hoc Bonferroni correction. Non-parametric data were logarithmically transformed before analysis.

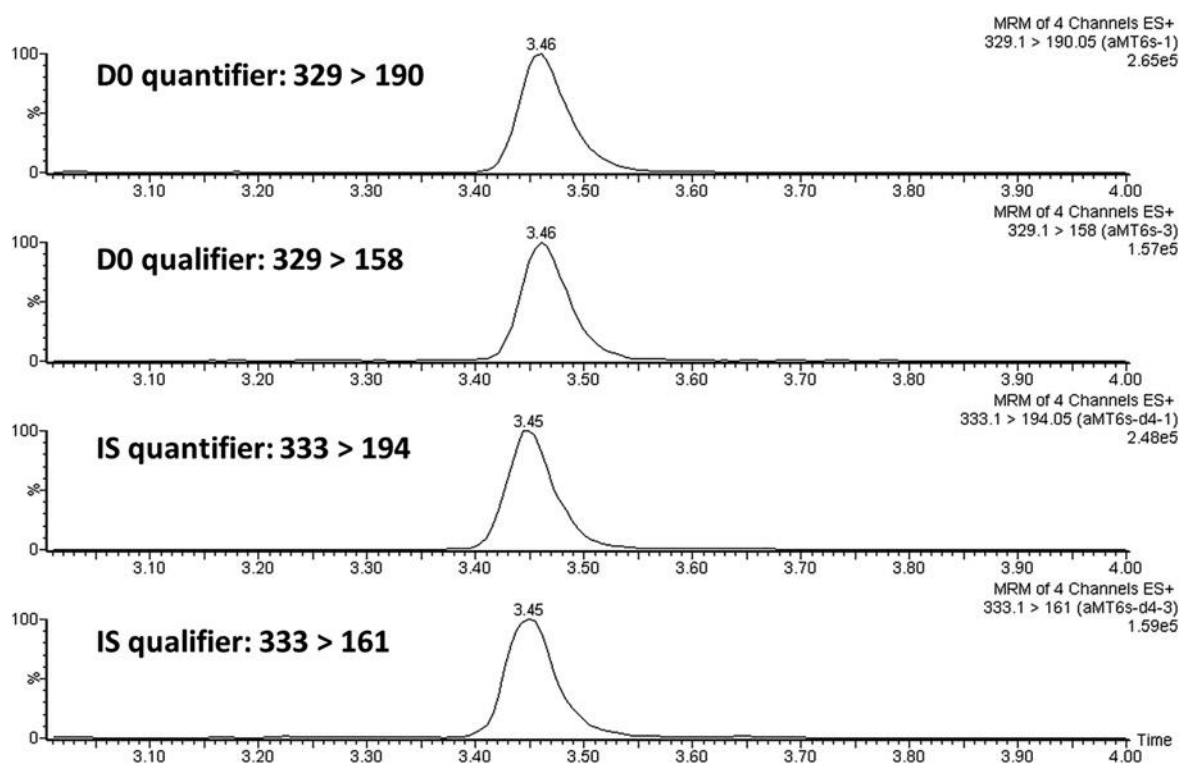
## Results

### Method validation

The total analysis time for one sample was 6 min, which includes online SPE sample clean-up. 6-Sulfatoxymelatonin was eluted in 3.54 min. Representative chromatograms for a standard solution and urine sample are shown in Figure 1.

Intraassay imprecision was  $<2.5\%$  and interassay imprecision was  $<5.4\%$  at the evaluated concentrations (Supplemental Data, Table 2). The LLOQ was 0.2  $\text{nmol}/\text{L}$ .





**Figure 1:** Representative chromatogram of 6-sulfatoxymelatonin measurements in urine. Traces of 6-sulfatoxymelatonin are shown. Y-axis shows the relative intensity, where the X-axis shows the retention time in min. 6-sulfatoxymelatonin elutes at a retention time of 3.46 minutes. The concentration of 6-sulfatoxymelatonin in this urine sample was 27.5 nmol/L.

Recovery for 6-sulfatoxymelatonin, as determined by a spike in concentration at three different levels, ranged from 90 to 115% (Table 2). No significant ion suppression was present in the region where 6-sulfatoxymelatonin eluted.

We compared the LC-MS/MS method with a commercial ELISA kit (IBL) using the Passing-Bablok equation. For ELISA, this was  $0.47 \times \text{LC-MS/MS} - 3.2$ ;  $\rho=0.846$ . When the same calibrators were used in both assays, the equation was  $\text{ELISA} = 0.74 \times \text{LC-MS/MS} - 4.4$ ;  $\rho=0.838$  (Supplemental Data, Figure 1).

## Excretion study

There was no difference in 6-sulfatoxymelatonin excretion between males and females [ $t(238)=0.90$ ,  $p=0.37$ ], so further data analyses were performed on the whole group ( $n=240$ ). There was an age effect on 24-h 6-sulfatoxymelatonin excretion; excretion decreased with age [ $F(5, 234)=13.9$ ;  $p<0.001$ ; Supplemental Data, Table 1; Figure 2]. The strength of the relationship as indexed by  $\omega$  was 0.54. 6-sulfatoxymelatonin output differed between age decades

**Table 1:** Urinary 6-sulfatoxymelatonin excretion over 24 h in healthy individuals divided by age decade,  $n=40$  individuals per decade.

| Decade                                       | 20–29        | 30–39        | 40–49        | 50–59      | 60–69      | 70–79       |
|--|--------------|--------------|--------------|------------|------------|-------------|
| 6-Sulfatoxymelatonin, nmol/24 h <sup>a</sup> | 47.7 [30–71] | 48.7 [28–62] | 32.8 [21–48] | 23 [16–36] | 21 [12–30] | 19 [9.2–30] |
| % Change to 20–29 decade                     |              | +2           | –31          | –52        | –56        | –60         |
| One-way ANOVA <sup>b</sup>                   |              |              |              |            |            |             |
| 20–29  | -            | -            | -            | -          | -          | -           |
| 30–39  | 1.0          | -            | -            | -          | -          | -           |
| 40–49  | 0.87         | 1.0          | -            | -          | -          | -           |
| 50–59  | 0.003        | 0.009        | 1.0          | -          | -          | -           |
| 60–69  | <0.001       | <0.001       | 0.013        | 1.0        | -          | -           |
| 70–79  | <0.001       | <0.001       | <0.001       | 0.14       | 1.0        | -           |

<sup>a</sup>Values are represented as median [interquartile range].

<sup>b</sup>Post-hoc analysis with Bonferroni correction.

**Table 2:** Biological variation of 6-sulfatoxymelatonin excretion into urine.

|                   | CV <sub>a</sub> | CV <sub>i</sub> <sup>a</sup> | CV <sub>g</sub> | II   | RCV (%) | RCV log normal |     |      | Samples required <sup>b</sup> |
|-------------------|-----------------|------------------------------|-----------------|------|---------|----------------|-----|------|-------------------------------|
|                   |                 |                              |                 |      |         | +              | -   | Mean |                               |
| Day urine, nmol   | 3.1             | 39.2 (28–49)                 | 39.1            | 0.91 | 109     | 201            | -63 | 132  | 59                            |
| Night urine, nmol | 1.7             | 15.1 (12–18)                 | 37.9            | 0.40 | 42      | 53             | -34 | 43   | 9                             |
| 24-h urine, nmol  | 1.6             | 12.2 (8.6–16)                | 36.8            | 0.33 | 34      | 42             | -28 | 35   | 6                             |

<sup>a</sup>CV<sub>i</sub> with 95% confidence interval; <sup>b</sup>To estimate homeostatic setting point with 10% accuracy with 95% confidence. CV<sub>a</sub>, analytical variation; CV<sub>i</sub>, within-subject variation; CV<sub>g</sub>, between-subject variation; II, index of individuality; RCV, reference change value; 24 h, 24 h.

20–29 and 50–59, 60–69 and 70–79, 30–39 and 50–59, 60–69 and 70–79, 40–49 and 60–69, and 40–49 and 70–79 (Figure 2). Individuals who were 70–79 years old excreted 60% less 6-sulfatoxymelatonin on average than those who were 20–29 years old did (Table 1).

## Biological variation study

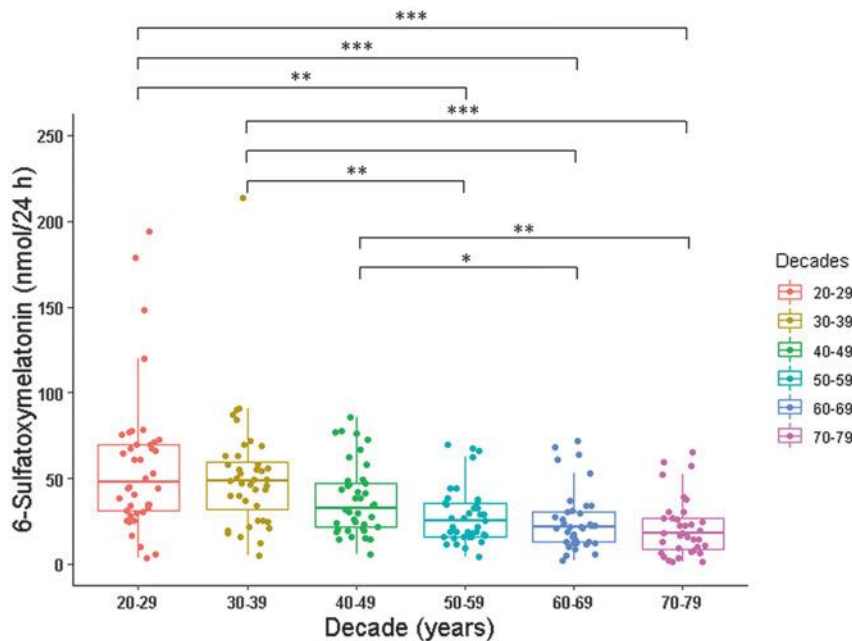
One out of the 10 participants in the biological variation study (ID 3) was identified as an outlier based on results from the Cochran and Dixon-Reed tests (Figure 3). Three males (average age, 27 years) and six females (average age, 34 years) were included (Supplemental Data, Table 3).

The average CV<sub>a</sub> of the LC–MS/MS method was 3.1% (range, 1.6–4.7%) for day urine, 1.7% (range, 1.4–2.4%) for night urine, and 1.6% (range, 1.1–2.2%) for 24-h urine (Table 2). The CV<sub>a</sub> was higher in day urine as 6-sulfatoxymelatonin concentrations are lower during the day. The CV<sub>i</sub> for 6-sulfatoxymelatonin was highest in day urine (average 39.2%; 95% confidence interval [CI], 28–49).

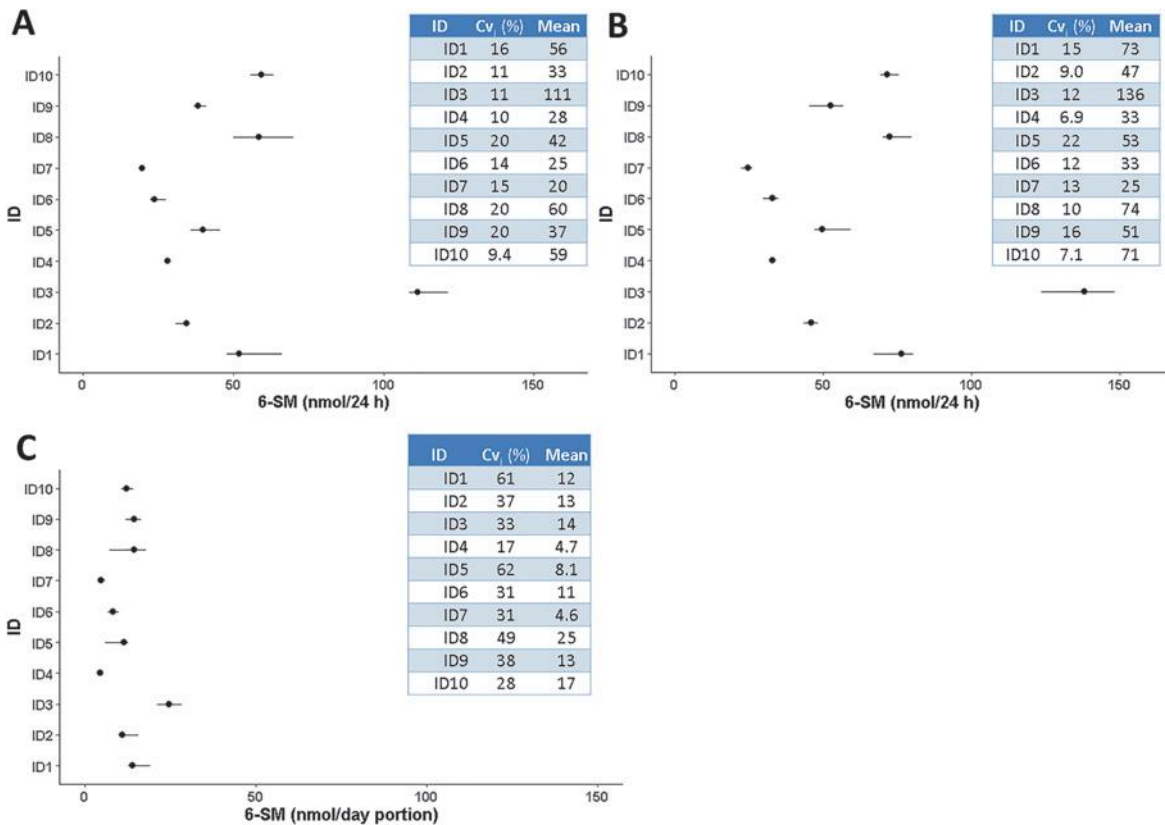
The CV<sub>i</sub> was 15.1% for night urine (95% CI, 11.8–18.3), and 12.2% for 24-h urine (95% CI, 8.6–15.9). The CV<sub>g</sub> was 39.1% in day urine, 37.9% in night urine, and 36.8% in 24-h urine. The RCV (the relative change of serial measurements) was 109% for day urine, 42% for night urine, and 34% for 24-h urine. Furthermore, the II was <1.0 for day urine, night urine, and 24-h urine, suggesting that population-based reference intervals are not valid for measuring urinary 6-sulfatoxymelatonin. An average of nine samples of night urine, 59 samples of day urine, and six samples of 24-h urine were needed to determine the individual homeostatic set point with 10% accuracy (Table 2). The analytical performance requirements for imprecision, accuracy and total allowable error for the 24-h excretion of 6-sulfatoxymelatonin in urine were as follows: 6.1, 9.7, and 19.8%, respectively.

## Discussion

This study shows that online SPE in combination with LC–MS/MS can rapidly detect 6-sulfatoxymelatonin in urine



**Figure 2:** Box plot of 6-sulfatoxymelatonin excretion in 24-h urine samples from healthy individuals aged between 20 and 80 years, divided by decades (n = 40 individuals per decade). One-way ANOVA with posthoc analysis with Bonferroni correction; \*p<0.05; \*\* p<0.01; \*\*\*p<0.001. Abbreviation: h, hour.



**Figure 3:** A: 6-sulfatoxymelatonin excretion into night urine; B: 6-sulfatoxymelatonin excretion into 24-h urine; C: 6-sulfatoxymelatonin excretion into day urine portion for each individual. On the y-axis, the individual participants (ID1–ID10) are shown. Median (solid dot) and interquartile range (horizontal line) are shown for each participant. Inserted tables show the CV<sub>i</sub> (%) and mean value for 6-sulfatoxymelatonin for each participant. ID3 was identified as an outlier, determined by results from either Cochran (CV<sub>i</sub>) or DixonReed (CV<sub>g</sub>) tests and was omitted from further analyses. Abbreviations: 6-SM, 6-sulfatoxymelatonin; 24 h, 24 hour; CV<sub>i</sub>, within-subject variation; CV<sub>g</sub>, between-subject variation.

samples in a sensitive and reproducible manner. This is the first attempt to quantify the biological variation of urinary 6-sulfatoxymelatonin excretion, and high between-subject variation was revealed. Excretion of 6-sulfatoxymelatonin into 24-h urine had the lowest within-subject variation.

There is a clear need for this reproducible and potentially traceable analysis method, given the rising interest in melatonin [29]. The most frequently used method for measuring 6-sulfatoxymelatonin is the radioimmunoassay or enzyme-linked immunoassay [30]. Although immunoassays are easy to use and provide valuable information, LC–MS/MS is the preferred method for analyzing small molecules [18, 31]. This novel LC–MS/MS method performed very well in our validation study. Compared with the previous study that used LC–MS/MS to detect 6-sulfatoxymelatonin in urine, our method showed improved interassay precision and a lower LOQ [32]. Our method fulfilled the analytical performance requirements for imprecision derived from the biological variation, as

our interassay imprecision was below the desired imprecision of 6.1% at all concentrations tested.

Our study showed an age-related decline of 6-sulfatoxymelatonin excretion into 24-h urine. Individuals aged 40–49 years excreted 31% less 6-sulfatoxymelatonin than individuals who were 20–29 or 30–39 years old. This was a gradual age-related decline (Supplemental Data, Figure 2). The oldest participants (70–79 years old) excreted 60% less 6-sulfatoxymelatonin into their urine than those aged 20–29 years. Other studies have also shown a gradual age-related decline, although this was less than the difference reported in the present study [33, 34]. There are two potential explanations for this disparity between studies. The first is different definitions of the healthy population. The two previous studies did not have clear criteria for selecting healthy individuals. In contrast, we performed a physical examination and used a questionnaire when selecting our healthy participants [24]. We also had clearly defined inclusion criteria: only individuals who were

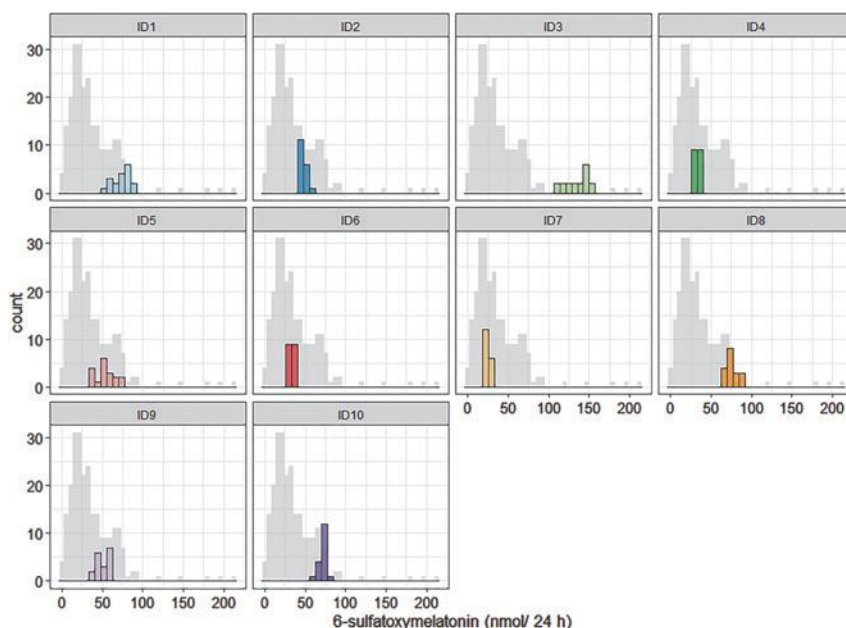
normotensive, unmedicated, and had a BMI of 21–30 kg/m<sup>2</sup> were included. Second, previous studies used immunoassays, which have higher analytical variation and interferences, which can influence the outcome [33, 34]. We analyzed if the 6-sulfatoxymelatonin excretion correlated with the length of day (collection date of urine was used for this), to see if the excretion pattern was affected by seasonal changes. No correlation was found between length of day and 6-sulfatoxymelatonin excretion (data not shown). Our study substantiates previous reports of extreme variability between individuals of the same age (Figure 2) [33, 34]. However, as we only used single 24 h urine per individual no firm conclusions can be drawn from our study about the decline of urinary 6-sulfatoxymelatonin with age. Longitudinal studies with multiple samples per individual are needed to clarify whether the observed decline of urinary 6-sulfatoxymelatonin excretion with age is causal or not.

Biological variation is the natural fluctuation of body-fluid constituents, and is used to diagnose and follow-up disease as well as to set analytical specifications in the clinical laboratory [35]. Our findings have revealed a large between-subject variation in urinary 6-sulfatoxymelatonin excretion. However, there was less biological variation within the same subject.

This is the first study to investigate the within-subject and between-subject biological variation of urinary 6-sulfatoxymelatonin excretion. This means that we cannot compare our findings with those of previous studies, as these studies did not correct for analytical variation, which is a prerequisite for estimating biological variation. Previous studies have reported a 13.7–17.4% intra-individual

variation for 6-sulfatoxymelatonin excretion in 24-h urine, which is similar to the within-subject variation of 12.2% reported in the current study [12, 36–38]. Notably, 6-sulfatoxymelatonin excretion into 24-h urine had a lower within-subject variation than 6-sulfatoxymelatonin excretion into night urine. Many studies have only used urine from the first morning void to represent 6-sulfatoxymelatonin excretion, as this is more convenient for the participant. Previous research has shown that this reflects approximately 80% of total 6-sulfatoxymelatonin excretion, which agrees with our finding that average 6-sulfatoxymelatonin excretion is similar in night urine and 24-h urine (47.2 vs. 59.5 nmol) [12, 39]. However, we revealed higher variability in results obtained using night urine samples. Using urine from the first morning void likely introduces even more variation, as possible urine voids during the night are missed. Collecting all urine over 24 h is a more reliable estimate of 6-sulfatoxymelatonin excretion. Figure 4 illustrates the high between-subject variation for 6-sulfatoxymelatonin excretion into 24-h urine. This finding emphasizes that epidemiological studies using 6-sulfatoxymelatonin excretion as a variable should use 24-h urine, take repeated measurements, and apply a precise analysis method. This will improve estimates of the true values. Increasing the sample size will only make estimates more precisely incorrect [15].

Caution is advised when drawing conclusions from single measurements of 6-sulfatoxymelatonin excretion. This could explain the contradicting results of two meta-analyses examining the association of 6-sulfatoxymelatonin



**Figure 4:** Histogram of the within-subject variation of 6-sulfatoxymelatonin excretion into 24-h urine in ten individuals from the biological variation study (ID1 – ID10) against 6-sulfatoxymelatonin excretion into 24-h urine of 240 healthy individuals from the excretion study plotted in the background (grey shaded area). Abbreviation: h, hour.



excretion and breast cancer [40, 41]. Although within-subject variation is much lower than between-subject variation in measurements of 6-sulfatoxymelatonin excretion into 24-h urine, there is still considerable difference in within-subject variation between the participants. For example, some participants show only 7% variation, whereas others show around 20% variation (Figures 3B and 4). To what extent this variation is caused by factors like variation in sleeping behavior or light exposure remains to be established. Within-subject variation may be lower under controlled laboratory conditions [42].

Future prospective cohort studies investigating the role of 6-sulfatoxymelatonin should use well-validated, precise methods to improve estimates. In addition, subjects should be their own control, and urine should be collected over 24 h at six different time points to accurately determine an individual's setting point.

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