Research Paper

Comparative study of stable isotope-labeled internal standard substances for the LC-MS/MS determination of the urinary excretion of melatonin

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Abstract Melatonin (MEL) is mostly metabolized by cytochrome P450 1A2 (CYP1A2) to 6-hydroxylated melatonin (6-O-MEL) and then excreted in urine as a sulfate conjugate (6-S-MEL) or glucuronide conjugate. Therefore, the MEL secretion and CYP1A2 activity can be evaluated on the basis of the total excreted amount of 6-O-MEL, which can be obtained via deconjugation. Two internal standard methods can be used for the quantification of the total excreted amount of 6-O-MEL via liquid chromatography/tandem mass spectrometry: adding stable isotope-labeled 6-O-MEL (6-O-MEL-$^2H_4$) after deconjugation or adding stable isotope-labeled 6-S-MEL (6-S-MEL-$^2H_4$) before deconjugation. This study compares these two internal standard methods. Adding 6-O-MEL-$^2H_4$ after deconjugation increased the deconjugation rate of 6-O-MEL with increasing the amount of deconjugating enzyme, of which 5920 units (400 μL) were required to reach a plateau. In contrast, when adding 6-S-MEL-$^2H_4$ before deconjugation, the deconjugation rate of 6-O-MEL did not change upon increasing the deconjugating enzyme amount from 1480 to 5920 units; therefore, 1480 units (100 μL) of deconjugating enzyme were sufficient for the determination. Urine obtained from healthy subjects (n=18, 0.07−9.00 μg) was measured using both internal standards, yielding a linear correlation coefficient of 0.9976 in their relationship. The method of adding 6-S-MEL-$^2H_4$ before deconjugation can automatically correct the quantitative value of the deconjugation rate; therefore, only a small amount of deconjugating enzyme is required, providing a cost-effective method for the quantification of the total excreted amount of 6-O-MEL in urine.

Key words: deconjugating enzyme, melatonin, LC-MS/MS, stable isotope, internal standards

Introduction

Melatonin (MEL), an endogenous hormone secreted by the pineal gland, is involved in sleep regulation and exhibits antioxidant, anti-inflammatory, and immunomodulatory effects[1−3]. MEL secretion depends on light exposure, with secretion increasing at night to promote sleep and decreasing during the day. This secretion process plays an important role in maintaining circadian rhythms. In fact, it has been suggested that disruption of the MEL secretion rhythms may lead to sleep disorders, cognitive decline, and cardiac disease[4−6].

MEL is mainly metabolized to 6-hydroxymelatonin (6-O-MEL) by cytochrome P450 1A2 (CYP1A2) in the liver after secretion and then excreted in urine as the sulfate conjugate 6-sulfatoxy melatonin (6-S-MEL) and the glucuronate conjugate 6-hydroxymelatonin glucuronide (6-G-MEL)[7] in respective percentages of 70−90% and 10−30%[8,9]. Since the total excreted amount of 6-O-MEL in urine reflects the amount of MEL secreted, MEL secretion could be evaluated by quantifying the excreted amount of 6-O-MEL[10]. Moreover, the measurement of the total excreted amount of 6-O-MEL

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could be used to evaluate the activity of CYP1A2, which shows large inter- and intraindividual variability[11,12]. Therefore, the development of an accurate and simple method to measure the total excreted amount of 6-O-MEL is highly desirable.

Although 6-S-MEL can be measured directly via liquid chromatography/tandem mass spectrometry (LC-MS/MS) as an indicator of the total excreted amount of 6-O-MEL[13], this method is not accurate because 6-G-MEL cannot be quantified. In contrast, hydrolysis of 6-S-MEL and 6-G-MEL in urine to 6-O-MEL via deconjugation reaction enables the measurement of all conjugates and, in turn, the accurate determination of the total excreted amount of 6-O-MEL in urine via gas chromatography–mass spectrometry or LC-MS/MS[14–17]. Although both analytical techniques can detect small amounts of 6-O-MEL, LC-MS/MS is particularly useful because it has good sensitivity and does not require derivatization[16,17]. Furthermore, the use of a stable isotope-labeled compound as an internal standard increases the accuracy of the measurements by automatically compensating the losses due to preprocessing.

The internal standard used in LC-MS/MS measurements based on a deconjugation reaction is a stable isotope-labeled deconjugated substance, which is added after deconjugation, or a stable isotope-labeled conjugated substance added before deconjugation. Generally, the former method is commonly used[16,17] because both sulfate and glucuronate conjugates can be applied. Meanwhile, the addition of a stable isotope-labeled conjugated substance before deconjugation allows to automatically correct the measured value even if the deconjugation reaction is not 100% because the conjugated substance is used as the internal standard. In the case of MEL, two methods can be used: adding the stable isotope-labeled 6-O-MEL (6-O-MEL-$^2H_4$) after deconjugation or adding the stable isotope-labeled 6-S-MEL (6-S-MEL-$^2H_4$) before deconjugation. However, it is still unclear which of the two approaches would be better suited for the accurate determination of the total excreted amount of 6-O-MEL.

Motivated by this background, we recently developed a method for the measurement of the total excreted amount of 6-O-MEL based on the addition of 6-O-MEL-$^2H_4$ after deconjugation[17]. 6-O-MEL-$^2H_4$ can be used as an internal standard regardless of the type of conjugate because it is added after deconjugation. However, since the deconjugation rate affects the quantitative value, it was necessary to add excess deconjugating enzyme. Furthermore, adding 6-S-MEL-$^2H_4$ before deconjugation would allow to perform the quantification even for deconjugation reaction below 100% because the internal standard is also a conjugate, and the amount of enzyme could be reduced. In this study, we aimed to clarify whether the addition of 6-O-MEL-$^2H_4$ or 6-S-MEL-$^2H_4$ as an internal standard would afford equivalent results and to reduce the amount of enzyme used in the determination of the total excreted amount of 6-O-MEL.

Materials and Methods

Chemicals and materials

N-[2-(6-Hydroxy-5-methoxy-1*H*-indol-3-yl)ethyl]acetamide (6-O-MEL), *N*-[2-[5-methoxy-6-(sulfooxy)-1*H*-indol-3-yl]ethyl]acetamide sodium (6-S-MEL), 6-O-MEL-$^2H_4$, and 6-S-MEL-$^2H_4$ were purchased from Toronto Research Chemicals (Toronto, ON, Canada). The deconjugating enzyme, β-glucuronidase/arylsulfatase from *Helix pomatia* (Roche-Glu/Sul), was purchased from Roche Diagnostics GmbH (Mannheim, Germany). All other reagents were purchased from Kanto Chemical (Tokyo, Japan).

Preparation of standards

Standard solutions of 6-O-MEL and 6-O-MEL-$^2H_4$ were prepared in methanol at concentrations of 24.5 and 67.0 ng/mL, respectively. 6-S-MEL and 6-S-MEL-$^2H_4$ were prepared in methanol at concentrations of 86.0 and 78.0 ng/mL, respectively.

Deconjugation reaction

Urine samples were thawed and centrifuged at 3000 rpm for 15 min, and the supernatant (0.2–1 mL) was aliquoted. Then, distilled water was added to reach a volume of 1 mL, and 1 mL of 0.5 M acetate buffer (pH 4.0) was added. If 6-S-MEL-$^2H_4$ was used as the internal standard, it was added at this point. For the deconjugation reaction, 74–7400 units of Roche-Glu/Sul were added as the deconjugating enzyme, and incubation was performed at 37°C for 60 min. After incubation, the reaction was stopped by rapid cooling under ice-cold conditions. If 6-O-MEL-$^2H_4$ was used as the internal standard, it was added at this point (Fig. 1).

Extraction and sample preparation

Extraction from the urine sample was performed by solid-phase extraction using Oasis HLB cartridges (Waters, Milforld, MA, USA). After deconjugation, the urine sample

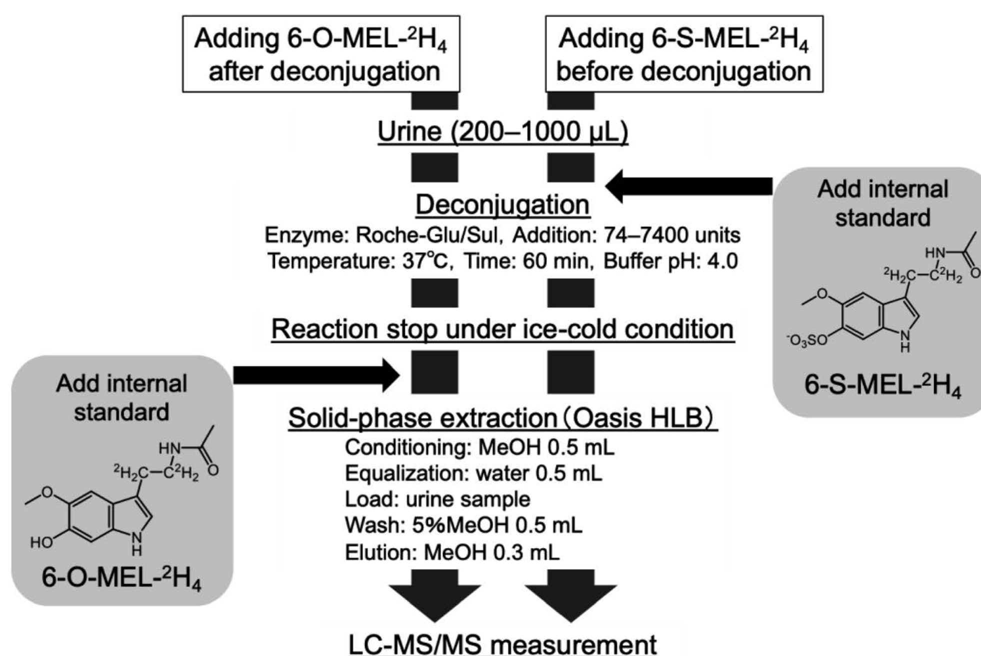

Fig. 1. Urine sample preparation procedure.
The order of addition of 6-O-MEL-$^2H_4$ and 6-S-MEL-$^2H_4$ is shown.

was injected into an Oasis HLB cartridge (1 cc Vac Cartridge, 30 mg sorbent) pre-equilibrated with methanol (0.5 mL) and distilled water (0.5 mL). The samples were then washed with 5% methanol solution (0.5 mL) and eluted with methanol (300 μL). After extraction, 10 mM ammonium acetate/0.2% formic acid solution (130 μL) was added, and then 5 μL was injected into the LC-MS/MS system.

LC-MS/MS conditions

LC-MS/MS analyses were performed using an ACQUITY UPLC H-class and Xevo TQD triple quadrupole mass spectrometer (Waters, Milford, MA, USA). Chromatographic separations were conducted with an ACQUITY UPLC BEH C18 column (2.1 mm×100 mm, 1.7 μm) and an ACQUITY UPLC BEH C18 VanGuard precolumn (2.1 mm×5 mm, 1.7 μm). The sample manager temperature was 15°C, the column temperature was 35°C, and the flow rate was 0.2 mL/min. The mobile phase was 10 mM ammonium acetate solution/0.2% formic acid solution and methanol. The gradient was set to 10% (0 min), 90% (10–13 min), and 10% (13.1–18 min) of methanol. Electrospray ionization was used as the ionization method, and the sauce temperature was set at 500°C. Positive ion mode of multiple reaction monitoring was selected for quantification. Precursor and product ions of 6-O-MEL and 6-O-MEL-$^2H_4$ were monitored at *m/z* 249.1→190.1 and *m/z* 253.1→193.1 with a collision energy of 12 eV.

Partial validation of the method

The calibration curve was prepared using five concentrations of 6-S-MEL (1.72, 2.58, 4.30, 8.60, and 17.20 ng), adding 3.90 ng of 6-S-MEL-$^2H_4$ as the internal standard. After adding the standard solution to 1 mL of water, deconjugation reaction was performed to obtain 6-O-MEL for measurement. This calibration curve was repeated six times to confirm linearity. The accuracy and precision were evaluated using stored urine from healthy subjects. In the method adding 6-S-MEL-$^2H_4$ as the internal standard, 15 urine samples were divided into three groups. One group (n=5) was not spiked, one group (n=5) was spiked with 13.01 ng/mL of 6-S-MEL, and the other group (n=5) was spiked with 26.01 ng/mL of 6-S-MEL. Each of these samples was measured after 1 day for intraday accuracy and precision and after 6 days for interday accuracy and precision.

Urine sample collection for comparison of the two internal standard methods

To compare the two internal standard methods, 2-h urine samples (10:00–12:00) from nine subjects (22–41 years, one female and eight males) and 8-h urine samples (6:00–14:00, 14:00–22:00, 22:00–6:00) from three subjects (22–

46 years, three males) were used. After collection, the urine volume was measured and the samples were stored at −20°C until measurement. This study was approved by the Human Subjects Review Board of the Tokyo University of Pharmacy and Life Sciences (approval number: 18-02), and informed consent was obtained from all subjects.

Results and Discussion

LC-MS/MS method development

In the measurement of total urinary excretion by LC-MS/MS, a stable isotope-labeled deconjugated substance is generally added after deconjugation[16,17]. Since the quantitative value may be lower than the true value if the deconjugating reaction does not proceed to 100%, an excess amount of enzyme is normally required to ensure that the deconjugating reaction reaches completion. Meanwhile, when a stable isotope-labeled conjugated substance is added before deconjugation, the quantitative value is corrected by the internal standard. Therefore, the measurement can be sufficiently accurate even though the deconjugation reaction is not 100% and the amount of enzyme is not enough for the deconjugation reaction.

In the case of 6-O-MEL, the stable isotope-labeled compounds of both conjugates excreted in urine, i.e., the sulfate conjugate 6-S-MEL and the glucuronic acid conjugate 6-G-MEL[7], are potential candidates as the internal standard. In general, the reactivity of the sulfate conjugate toward deconjugating enzymes is lower than that of the glucuronide conjugate. For example, Azuma et al. reported that the glucuronide conjugate of acetaminophen is more readily deconjugated than the corresponding sulfate conjugate during the reaction using β-glucuronidase/arylsulfatase[18]. Furthermore, Azuma et al. quantified the conjugates of 19 drugs after deconjugation reactions. This suggests that if the deconjugation of 6-S-MEL to 6-O-MEL proceeds, the deconjugation of 6-G-MEL to 6-O-MEL also progresses simultaneously. Therefore, the stable isotope-labeled compound 6-S-MEL-$^2H_4$ is suitable as an internal standard. If β-glucuronidase/arylsulfatase is present in insufficient quantity, the deconjugation reactions of the glucuronate and sulfate conjugates do not proceed. Therefore, the concentration of glucuronide conjugates also can be calculated by using an internal standard for sulfate conjugates and correcting for the deconjugation-reaction efficiency. In this study, the use of 6-S-MEL-$^2H_4$ as an internal standard for LC-MS/MS determination based on the deconjugation reaction was investigated and compared with a previously reported method using 6-O-MEL-$^2H_4$ as an internal standard[17] (Fig. 1).

Optimization of the deconjugation reaction

First, the amount of deconjugating enzyme Roche-Glu/Sul required when using 6-O-MEL-$^2H_4$ and 6-S-MEL-$^2H_4$ as internal standards was evaluated. The temperature and time of the reaction followed our previously reported method[17]. Enzyme additions that maximized the deconjugation rate of 6-O-MEL were examined in the range of 74−7400 units using 8-h urine samples from three healthy subjects. In the method of adding 6-O-MEL-$^2H_4$ after deconjugation, the deconjugation rate of 6-O-MEL reached a plateau when 5920 units (400 μL) of deconjugating enzyme were added. Furthermore, the lowest standard deviation (S.D.) value of 6.53% was achieved for this amount of enzyme, which was set as the required enzyme amount for the reaction (Fig. 2(a)). This result is consistent with our previous report[17]. In the case of the method of adding 6-S-MEL-$^2H_4$ before deconju-

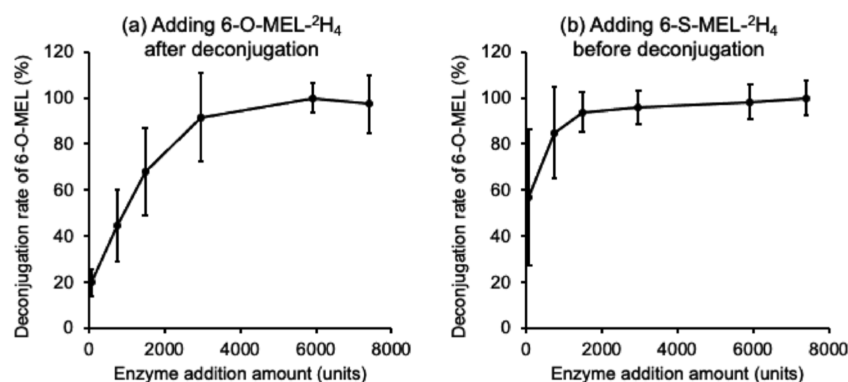

Fig. 2. Comparison of the enzyme addition amount.
(a) 6-O-MEL-$^2H_4$ was used as an internal standard and (b) 6-S-MEL-$^2H_4$ was used as an internal standard.

gation, the deconjugation rate of 6-O-MEL reached a plateau at 93.77% when 1480 units (100 μL) were added (Fig. 2(b)). The S.D. was 8.49% at 1480 units (100 μL) and 19.86% at 740 units (50 μL), indicating that the necessary amount of enzyme was 1480 units (100 μL). The LC-MS/MS analysis of the deconjugation reaction with the 1480 units of 6-S-MEL-$^2H_4$ afforded single and sharp peaks of 6-O-MEL and 6-O-MEL-$^2H_4$ in the urine sample at a retention time of 5.6 min, and no influence of endogenous substances was observed (Fig. 3).

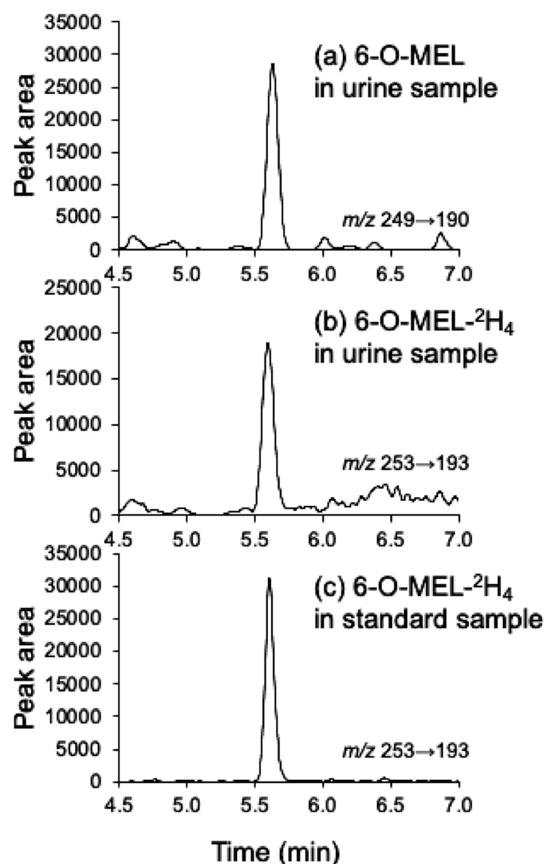

Fig. 3. LC-MS/MS chromatograms of 6-O-MEL and 6-O-MEL-$^2H_4$ in urine samples and 6-O-MEL-$^2H_4$ in the standard sample.

(a) Chromatogram of 6-O-MEL in the urine sample obtained after deconjugation reaction, (b) chromatogram of 6-O-MEL-$^2H_4$ in the urine sample obtained after adding 6-S-MEL-$^2H_4$ and performing deconjugation reaction, and (c) chromatogram of 6-O-MEL-$^2H_4$ in the standard sample obtained after deconjugating 6-S-MEL-$^2H_4$.

Partial validation of the method

To verify whether the method of adding 6-S-MEL-$^2H_4$ before deconjugation was comparable to that of adding 6-O-MEL-$^2H_4$ after deconjugation, partial validation of the former was performed by constructing a calibration curve and confirming the accuracy and precision.

The calibration curve yielded a correlation coefficient of 0.99949 ± 0.00066, a slope of 0.6721 ± 0.0258, and an intercept of −0.031±0.087 (Table 1). These values were comparable to those previously reported for the method of adding 6-O-MEL-$^2H_4$ after deconjugation (correlation coefficient: 0.99971 ± 0.00020, slope: 0.437 ± 0.023, intercept: −0.012 ± 0.027)[17], confirming that calibration curves with intercepts close to zero and high linearity can be obtained for both methods.

The accuracy and precision of the method of adding 6-S-MEL-$^2H_4$ before deconjugation were evaluated by preparing urine samples at three different concentrations. As a result, the relative error (R.E.) ranged from −5.92% to 3.01% and the relative standard deviation (R.S.D.) was within 9.78% (Table 2), which were in accord with those

Table 1. Linearity of the calibration curve of the method of adding 6-S-MEL-$^2H_4$ as the internal standard before deconjugation

Concentration (ng)	Correlation coefficient (Mean±S.D.)	Slope (Mean±S.D.)	Intercept (Mean±S.D.)
1.30−13.01	0.99949 ± 0.00066	0.6721 ± 0.0258	−0.031 ± 0.087

S.D.: Standard deviation.

Table 2. Accuracy and precision in the method adding 6-S-MEL-$^2H_4$ as the internal standard before deconjugation

	Added (ng/mL)	Expected (ng/mL)	Found (Mean±S.D., ng/mL)	R.E. (%)	R.S.D. (%)
Intraday	0		11.93 ± 0.32	—	2.68
(*n*=5)	13.01	24.94	25.69 ± 1.06	3.01	4.11
	26.01	37.95	37.66 ± 1.00	−0.76	2.65
Interday	0		12.21 ± 1.20	—	9.78
(*n*=6)	13.01	25.22	24.54 ± 1.67	−2.69	6.79
	26.01	38.22	35.96 ± 2.29	−5.92	6.36

S.D.: Standard deviation, R.E.: relative error, R.S.D.: relative standard deviation.

corresponding to the method of adding 6-O-MEL-$^{2}H_4$ after deconjugation (R.E.: −3.60% to −0.47%, R.S.D. within 6.80%)[17]. Therefore, the method could be performed with high accuracy and precision using either of the internal standards.

Comparison of the two internal standard methods

Finally, the feasibility of using both methods, i.e., adding 6-O-MEL-$^{2}H_4$ after deconjugation and adding 6-S-MEL-$^{2}H_4$ before deconjugation, to analyze urine samples was examined using 2-h urine samples from nine healthy subjects and 8-h urine samples from three healthy subjects (n=18). The excreted amount of 6-O-MEL measured using each method ranged from 0.07 to 9.00 μg; the correlation coefficient of the graph obtained by plotting the measured values of both methods was 0.9976. The obtained equation has a correlation coefficient and slope close to 1 and an intercept close to 0, which is considered a good result. This result demonstrates that the two methods afford equivalent results in the measurement of actual urine samples (Fig. 4).

Conclusion

This study demonstrates that similar quantitative results can be obtained by adding either 6-O-MEL-$^{2}H_4$ after deconjugation or 6-S-MEL-$^{2}H_4$ before deconjugation as internal standards. The latter method is an alternative to the conventional method of adding 6-O-MEL-$^{2}H_4$ after deconjugation. Therefore, adding 6-S-MEL-$^{2}H_4$ before deconjugation is a cost-effective method because it allows reducing the amount of deconjugating enzyme, providing a new approach for the evaluation of MEL secretion and CYP1A2 activity in the future.

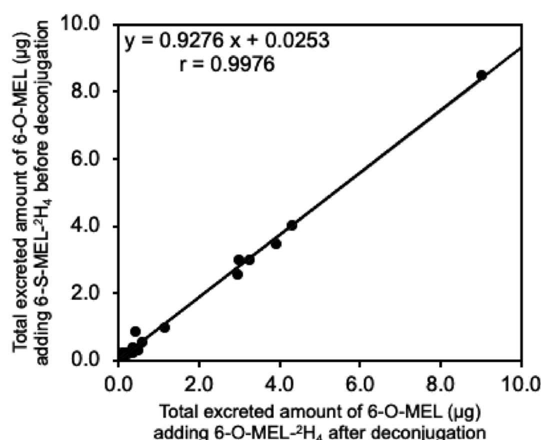

Fig. 4. Comparison of two measurement methods in healthy human urine samples.
The relationship of the total excreted amount of 6-O-MEL between adding 6-O-MEL-$^{2}H_4$ after deconjugation and adding 6-S-MEL-$^{2}H_4$ before deconjugation showed good linearity with a correlation coefficient of 0.9976.

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Conflict of 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.

References

1) Vasey C, McBride J, Penta K: Circadian rhythm dysregulation and restoration: the role of melatonin. *Nutrients* 13: 3480, 2021.
2) Chitimus DM, Popescu MR, Voiculescu SE, Panaitescu AM, Pavel B, et al: Melatonin's impact on antioxidative and anti-inflammatory reprogramming in homeostasis and disease. *Biomolecules* 10: 1211, 2020.
3) Haldar C, Ahmad R: Photoimmunomodulation and melatonin. *J Photochem Photobiol B* 98: 107–117, 2010.
4) Xie Z, Chen F, Li WA, Geng X, Li C, et al: A review of sleep disorders and melatonin. *Neurol Res* 39: 559–565, 2017.
5) Prodhan AHMSU, Cavestro C, Kamal MA, Islam MA: Melatonin and melatonin's impact on antioxidative and anti-inflammatory reprogramming in homeostasis and disease. *CNS Neurol Disord Drug Targets* 20: 736–754, 2021.
6) Prado NJ, Muñoz EM, Farias Altamirano LE, Aguiar F, Ponce Zumino AZ, et al: Reperfusion arrhythmias increase after superior cervical ganglionectomy due to conduction disorders and changes in repolarization. *Int J Mol Sci* 21: 1804, 2020.
7) Ma X, Idle JR, Krausz KW, Gonzalez FJ: Metabolism of melatonin by human cytochromes p450. *Drug Metab Dispos* 33: 489–494, 2005.
8) Webb SM, Puig-Domingo M: Role of melatonin in health and disease. *Clin Endocrinol (Oxf)* 42: 221–234, 1995.
9) Macchi MM, Bruce JN: Human pineal physiology and functional significance of melatonin. *Front Neuroendocrinol* 25: 177–195, 2004.

10) Tetsuo M, Markey SP, Kopin IJ: Measurement of 6-hydroxymelatonin in human urine and its diurnal variations. *Life Sci* 27: 105–109, 1980.
11) Härtter S, Ursing C, Morita S, Tybring G, von Bahr C, et al: Orally given melatonin may serve as a probe drug for cytochrome P450 1A2 activity in vivo: A pilot study. *Clin Pharmacol Ther* 70: 10–16, 2001.
12) Magliocco G, Desmeules J, Samer CF, Thomas A, Daali Y: Evaluation of CYP1A2 activity: Relationship between the endogenous urinary 6-hydroxymelatonin to melatonin ratio and paraxanthine to caffeine ratio in dried blood spots. *Clin Transl Sci* 15: 1482–1491, 2022.
13) van Faassen M, van der Veen A, van Ockenburg S, de Jong H, de Vries EGE, et al: Mass spectrometric quantification of urinary 6-sulfatoxymelatonin: Age-dependent excretion and biological variation. *Clin Chem Lab Med* 59: 187–195, 2020.
14) Tetsuo M, Markey SP, Colburn RW, Kopin IJ: Quantitative analysis of 6-hydroxymelatonin in human urine by gas chromatography—negative chemical ionization mass spectrometry. *Anal Biochem* 110: 208–215, 1981.
15) Francis PL, Leone AM, Young IM, Stovell P, Silman RE: Gas chromatographic-mass spectrometric assay for 6-hydroxymelatonin sulfate and 6-hydroxymelatonin glucuronide in urine. *Clin Chem* 33: 453–457, 1987.
16) Magliocco G, Le Bloc'h F, Thomas A, Desmeules J, Daali Y: Simultaneous determination of melatonin and 6-hydroxymelatonin in human overnight urine by LC-MS/MS. *J Chromatogr B Analyt Technol Biomed Life Sci* 1181: 122938, 2021.
17) Ohki S, Kunimatsu M, Ogawa S, Takano H, Furihata T, et al: Development and validation of an LC-MS/MS- based method for quantifying urinary endogenous 6-hydroxymelatonin. *Chem Pharm Bull (Tokyo)* 70: 375–382, 2022.
18) Azuma T, Ishida M, Hisamatsu K, Yunoki A, Otomo K, et al: A method for evaluating the pharmaceutical deconjugation potential in river water environments. *Chemosphere* 180: 476–482, 2017.