Short Communication

Reproducibility over 5 Years of Measurements of 6-Sulphatoxymelatonin in Urine Samples from Postmenopausal Women

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Abstract

To assess the appropriateness of a single measurement of urinary 6-sulfatoxymelatonin (aMT6S) as a marker for long-term exposure to endogenous melatonin in epidemiological studies, we examined the reproducibility of aMT6S in first morning urine voids collected from 40 postmenopausal women. Urine specimens were collected on three different occasions, and the mean time between the first and the third urine sample was 5.1 years. Urinary aMT6S levels were measured by radioimmunoassay and adjusted for creatinine. The intraclass correlation for aMT6S adjusted for creatinine was 0.56 (95% confidence interval, 0.39–0.73). The classification of aMT6S concentrations in first morning voids from postmenopausal women appears to be sufficiently reproducible to justify its use as a marker for long-term exposure to melatonin in epidemiological studies.

Introduction

Melatonin[2] is the main hormonal product of the pineal gland and has a major physiological role in the control of circadian rhythmicity (1). It has been proposed that circulating levels of the pineal hormone melatonin may be associated with risk for several chronic diseases, including breast cancer. A few case-control studies have used estimates of the concentration of aMT6S, the major metabolite of melatonin, in a single urine specimen to assess the relationship between endogenous melatonin levels and breast cancer risk (2), but no prospective studies have yet been published. The measurement of excreted aMT6S in overnight or 24-h urine samples as an index of melatonin production is very attractive: the collection of urine is noninvasive and the measurement obtained has been found to be a reliable estimate of plasma melatonin during the periods over which the urine was collected (3–6). Studies have found that intraindividual levels of aMT6S are relatively stable on a day-to-day basis (7, 8), as well as in samples collected over periods of 3–6 months (9), but none, to our knowledge, has investigated reproducibility over a longer period. This study assesses the reproducibility of urinary aMT6S levels over a ~5-year period in first morning voids from a random sample of 40 postmenopausal women participating in a Dutch prospective cohort study.

Materials and Methods

Subjects and Urine Collections. The DOM (Diagnostisch Onderzoek Mammacarcinoom) project is a prospective cohort study of 27,718 women born between 1911 and 1945 and now living in Utrecht and vicinity, the details of which are described elsewhere (10). Women who responded to the first invitation to participate in this study between 1975 and 1986 were then invited for regular screening examinations. Women were asked to bring a first morning urine sample on the day of their examination. Upon their arrival at the laboratory, urine samples were stored at −20°C in 250 ml plastic polypropylene jars with a screw cap, without preserving agents. Before laboratory analysis, samples were split into 3-ml aliquots. At each visit, women completed a detailed lifestyle questionnaire and anthropometric measurements were taken. BMI, defined as weight in kilograms divided by height in meters squared, was calculated from these measurements.

Previous work in the DOM cohort showed that a sample size of between 40 and 45 provided sufficient power to examine the reproducibility of urinary hormone excretions (11). Forty-four women were selected from the 27,718 women participating in the DOM cohort. The women were postmenopausal at recruitment and did not use exogenous hormones. Forty-three of these women had provided urine samples on at least three different occasions over a period of ~5 years; one in each of the first and second annual rounds and a third sample in either the third, fourth, or fifth rounds of the study. Of these forty-three participants, three women were excluded because of incomplete results from the aMT6S assays. Thus, the results from 120 urine specimens from 40 women were analyzed.

Hormone Assays. Hormone assays were performed by Stockgrand Ltd. at the School of Biomedical and Life Sciences (University of Surrey, Guildford, United Kingdom). The 120 3-ml specimens were analyzed in four batches, with all samples from any one individual in the same batch.

aMT6S was assayed by radioimmunoassay using a ^{125}I-labeled tracer (3, 4). Diluted urine samples were incubated with a specific antiserum to aMT6S raised in sheep before the addition of trace amounts of ^{125}I-aMT6S. The free and antibody-bound fractions of aMT6S were separated using a

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[2] The abbreviations used are: melatonin, *N*-acetyl-5-methoxytryptamine; aMT6S, 6-sulfatoxymelatonin; DOM, Diagnostisch Onderzoek Mammacarcinoom; BMI, body mass index; ICC, intraclass correlation coefficient.

Table 1 Age, body mass index, urinary aMT6S, creatinine, and creatinine-adjusted urinary aMT6S over time ($n = 40$)

	t1 Mean (CI)[a]	t2 Mean (CI)	Difference t2-t1 (%)	t3 Mean (CI)	Difference t3-t1 (%)	P^b
Age (years)	57.6 (56.0–59.1)	58.6 (57.1–60.2)	1.0 (1.8)	62.7 (61.1–64.2)	5.1 (8.8)	NA
BMI (kg/m^2)	25.7 (24.6–26.9)	25.6 (14.4–26.8)	−0.1 (0.4)	26.3 (25.2–27.5)	0.6 (2.3)	0.035[c]
aMT6S[d] (mg/l)	11.00 (8.28–14.62)	8.09 (6.08–10.75)	−2.9 (−26.5)	7.04 (5.30–9.36)	−4.0 (−36.0)	0.005
Creatinine[d] (mg/l)	818.6 (732.3–915.1)	788.6 (705.4–881.5)	−30.0 (−3.7)	727.0 (650.3–812.7)	−91.6 (−11.2)	0.144
Adjusted aMT6S[d] (ng/mg creatinine)	13.44 (10.49–17.22)	10.25 (8.00–13.14)	−3.2 (−23.7)	9.69 (7.56–12.41)	−3.8 (−27.9)	0.013

[a] CI, confidence interval; t1, timepoint 1; t2, timepoint 2; t3, timepoint 3.
[b] Test for heterogeneity using Greenhouse-Geisser's ϵ as the correction factor for repeated measures.
[c] If Box's more conservative ϵ is used, this relationship becomes nonsignificant at the 5% level, $P = 0.059$.
[d] Geometric means and 95% CI.

dextran-coated charcoal suspension. The free aMT6S fraction was precipitated with charcoal by centrifugation and the radioactivity counted in a gamma counter. A standard curve was fitted from standards made up in charcoal-stripped urine. The lower limit of detection for aMT6S was 0.2 ng/ml. Assays were run in duplicate, and the duplicate results were averaged for the analyses. The within-sample coefficient of variation for aMT6S was 2.7%. The assays included in-house quality controls and the interassay coefficients of variation for aMT6S were 1.6, 4.0, and 4.0% for low (mean value of 3.1 ng/ml), medium (mean value of 21.4 ng/ml), and high (mean value of 41.4 ng/ml) control samples, respectively.

Total urinary output was unknown, therefore creatinine levels were measured for each sample and urinary metabolite levels are additionally presented as standardized values (aMT6S values divided by creatinine concentration in each sample; Refs. 5, 6). Urinary creatinine levels were assayed using a COBAS MIRA Chemistry Systems kit (kinetic, Jaffé method).

Statistical Analyses. Statistical analyses were performed using the Stata 7 statistical software package (12). The hormonal values were logarithmically transformed for statistical analyses to approximately normalize their frequency distributions and geometric means and confidence intervals were calculated. Repeated measures ANOVAs were conducted to assess whether heterogeneity between measurements of aMT6S, the dependent variable, and age and BMI, as independent variables, at the three time points was significant. One-way ANOVAs were conducted to estimate components of variance associated with variation between women and variability among time points within women. The reproducibility of hormone measurements was assessed by computing ICCs (13).

Results

The mean concentrations of aMT6S varied significantly between time points, both before and after adjustment for creatinine ($P = 0.005$ and $P = 0.013$, respectively), with the highest levels being observed at t1 and the lowest at t3 (Table 1). A similar trend was observed for creatinine, with mean creatinine being highest at t1 and lowest at t3, although this variation was not statistically significant ($P = 0.144$). Women were on average 1.0 year older at t2 and 5.1 years older at t3 than at t1. BMI varied slightly between time points ($P = 0.035$), with the mean BMI highest at t3.

ICCs. Table 2 shows between-women and within-women variances and ICCs over time. The ICC for aMT6S was 0.54 (95% CI, 0.37–0.72), the ICC for creatinine over time was 0.42 (95% CI, 0.22–0.61), and the ICC for aMT6S changed little after adjustment for creatinine (ICC = 0.56, 95% CI, 0.39–0.73).

Table 2 Variance components and ICCs of urinary aMT6S, creatinine, and creatinine-adjusted urinary aMT6S over time ($n = 40$)

Hormone	Variance between-subjects	Variance within-subjects	ICC	(95% CI)[a]
aMT6S (mg/l)	0.47	0.40	0.54	(0.37–0.72)
Creatinine (mg/l)	0.05	0.08	0.42	(0.22–0.61)
aMT6S (ng/mg creatinine)	0.36	0.29	0.56	(0.39–0.73)

[a] CI, confidence interval; ICC, intraclass correlation coefficient.

Discussion

For urinary aMT6S levels to be a useful indicator of long-term melatonin, excretion of aMT6S must not only be highly correlated with endogenous melatonin production during the period over which the urine was collected but must also be representative of long-term aMT6S excretion. The few studies to date that have assessed the reproducibility over time of measurements of aMT6S in urine samples (7–9) have found that intraindividual aMT6S levels in nocturnal urine samples were highly correlated from day-to-day [$r = 0.85–0.92$, $P < 0.001$ for both (7, 8)], as well as over periods of 3–6 months ($r = 0.75$, $P < 0.0001$; Ref. 9). This is the first study, however, to report on the reproducibility of aMT6S measurements over a longer time period. We found that the concentration of aMT6S taken from first morning urine specimens, either unadjusted or adjusted for creatinine, was moderately stable over a period of 5 years in postmenopausal women.

The ICCs found in this study for early morning urinary aMT6S levels are similar to those for a number of other endogenous biomarkers commonly used in epidemiology. For example, other intraclass correlations in postmenopausal women were: 0.74 and 0.68 for estrone and estradiol, respectively, over 3 years (14); 0.56 for estradiol over 8 years (15); 0.76 for serum cholesterol over 2 years (16); and 0.60 for systolic blood pressure over 2 years (16).

The measurement of aMT6S in first morning urine voids provides a fairly reproducible estimate of aMT6S levels over 5 years and, thus, is a valid method of assessing long-term exposure to endogenous melatonin in epidemiological studies of risk factors for diseases such as breast cancer.

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References

1. Brzezinski, A. Melatonin in humans. N. Engl. J. Med., *336:* 186–195, 1997.

2. Bartsch, C., Bartsch, H., Karenovics, A., Franz, H., Peiker, G., and Mecke, D. Nocturnal urinary 6-sulphatoxymelatonin excretion is decreased in primary breast cancer patients compared to age-matched controls and shows negative correlation with tumor-size. J. Pineal. Res., *23:* 53–58, 1997.

3. Arendt, J., Bojkowski, C., Franey, C., Wright, J., and Marks, V. Immunoassay of 6-hydroxymelatonin sulfate in human plasma and urine: abolition of the urinary 24-hour rhythm with atenolol. J. Clin. Endocrinol. Metab., *60:* 1166–1173, 1985.

4. Aldhous, M. E., and Arendt, J. Radioimmunoassay for 6-sulphatoxymelatonin in urine using an iodinated tracer. Ann. Clin. Biochem., *25* (Pt. 3)*:* 298–303, 1988.

5. Graham, C., Cook, M. R., Kavet, R., Sastre, A., and Smith, D. K. Prediction of nocturnal plasma melatonin from morning urinary measures. J. Pineal. Res., *24:* 230–238, 1998.

6. Cook, M. R., Graham, C., Kavet, R., Stevens, R. G., Davis, S., and Kheifets, L. Morning urinary assessment of nocturnal melatonin secretion in older women. J. Pineal. Res., *28:* 41–47, 2000.

7. Levallois, P., Dumont, M., Touitou, Y., *et al*. Effects of electric and magnetic fields from high-power lines on female urinary excretion of 6-sulfatoxymelatonin. Am. J. Epidemiol., *154:* 601–609, 2001.

8. Stevens, R. G., Davis, S., Mirick, D. K., Kheifets, L., and Kaune, W. Alcohol consumption and urinary concentration of 6-sulfatoxymelatonin in healthy women. Epidemiology, *11:* 660–665, 2000.

9. Davis, S., Kaune, W. T., Mirick, D. K., Chen, C., and Stevens, R. G. Residential magnetic fields, light-at-night, and nocturnal urinary 6-sulfatoxymelatonin concentration in women. Am. J. Epidemiol., *154:* 591–600, 2001.

10. de Waard, F., Collette, H. J., Rombach, J. J., Baanders-van Halewijn, E. A., and Honing, C. The DOM project for the early detection of breast cancer, Utrecht, the Netherlands. J. Chronic. Dis., *37:* 1–44, 1984.

11. Rinaldi, S., Moret, C. N., Kaaks, R., Biessy, C., Kurzer, M. S., Déchaud, H., Peeters, P. H. M., and van Noord, P. A. H. Reproducibility over time of measurements of androgens, estrogens and hydroxy estrogens in urine samples from post-menopausal women. Eur. J. Epidemiol., in press, 2003.

12. Stata 7.0. Statistical Software. Release 7.0. College Station, TX: Stata Corporation, 2000.

13. Armstrong, B. K., White, E., and Saracci, R. Principles of Exposure Measurement in Epidemiology. Oxford: Oxford University Press, 1995.

14. Hankinson, S. E., Manson, J. E., Spiegelman, D., Willett, W. C., Longcope, C., and Speizer, F. E. Reproducibility of plasma hormone levels in postmenopausal women over a 2–3-year period. Cancer Epidemiol. Biomark. Prev., *4:* 649–654, 1995.

15. Thomas, H. V., Key, T. J., Allen, D. S., Moore, J. W., Dowsett, M., Fentiman, I. S., and Wang, D. Y. A prospective study of endogenous serum hormone concentrations and breast cancer risk in post-menopausal women on the island of Guernsey. Br. J. Cancer, *76:* 401–405, 1997.

16. Cauley, J. A., Gutai, J. P., Kuller, L. H., and Powell, J. G. Reliability and interrelations among serum sex hormones in postmenopausal women. Am. J. Epidemiol., *133:* 50–57, 1991.

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