Endocrine, *vol. 15, no. 2, 199–202, July 2001* 0969–711X/01/15:199–202/$11.00

Long-Term Stability of 6-Hydroxymelatonin Sulfate in 24-h Urine Samples Stored at –20°C

Barbara Griefahn,[1] Thomas Remer,[2] Meinolf Blaszkewicz,[1] and Peter Bröde[1]

[1]Institute for Occupational Physiology at the University of Dortmund, Dortmund, Federal Republic of Germany; and [2]Research Institute of Child Nutrition Dortmund, Dortmund, Federal Republic of Germany

The purpose of the present feasibility study was to determine whether the concentration of 6-hydroxymelatonin sulfate (6-OHMS) remains stable in urine samples stored over at least 15 yr. To test this, 117 twenty-four-hour urine samples were analyzed, which were obtained from healthy children ages 8 to 9 yr within the periods of 1985–1987, 1991–1993, and 1997–1999. 6-OHMS concentrations were determined by enzyme-linked immunosorbent assay. The statistical analyses clearly indicate that the concentration of 6-OHMS remains stable for at least 15 yr if the urine is stored at –20°C.

Key Words: Longitudinal study; urinary 6-hydroxymelatonin sulfate excretion; long-term stability.

Introduction

The concentration of many substances in the urine as well as in other even deep-frozen biologic materials alters with time *(1,2)*. Concerning 6-hydroxymelatonin sulfate (6-OHMS), which is the major enzymatic metabolite of melatonin excreted in the urine, storage at –20°C is recommended. Whether and to what extent its concentration is altered with time is not yet known.

In 1985, the Research Institute of Child Nutrition Dortmund began the (still ongoing) longitudinal Dortmund Nutritional and Anthropometric Longitudinally Designed Study (DONALD) to investigate the interrelations among nutrition, growth, and metabolic and endocrine changes during childhood and adolescence. The repeatedly performed medical examinations include the collection of urine over 24 h.

In cooperation with the Institute for Occupational Physiology at the University of Dortmund, plans have been made to analyze the urinary concentrations of 6-OHMS of these samples for the estimation of the individual courses of melatonin production during childhood and adolescence. The use of 6-OHMS as an indicator of melatonin production is justified by the high correlation reported by investigators such as Cook et al. *(3)* between creatinine-corrected 6-OHMS and nocturnal plasma melatonin output and peak melatonin values. This, however, presupposes first a feasibility study—i.e., to prove whether the urinary concentration of 6-OHMS remains stable over at least 15 yr.

However, this is not a trivial problem because melatonin production changes with age. It increases significantly several months after birth, then increases steeply, reaches a peak at the age of 1–3 yr, and decreases thereafter by about 80% until adolescence *(4,5)*. Assuming that the age-related average melatonin production did not change within the last decades, the average urinary excretion of 6-OHMS must be the same at present as at the beginning of DONALD 15 yr ago. The present study was undertaken to test this.

Materials and Methods

DONALD was approved by the institutional review board of the Research Institute of Child Nutrition Dortmund, and parental consent and children's assent were obtained before entry into the study. Children and adolescents (3–18 yr) were repeatedly submitted to medical examinations, anthropometric measurements, and the collection of urine over 24 h. To ensure compliance in the urine collection, the children and their parents were carefully instructed in the collection procedure and also received written guidance. Additionally, a dietitian (visiting the families at home) explicitly asked about the child's compliance and discussed the completeness of the urine collection in detail with the family. Aliquots of the 24-h urine samples were stored at –20°C.

To investigate the stability of urinary 6-OHMS concentrations over 15 yr, urine samples of 117 children (59 boys, 58 girls) belonging to three groups defined by the sampling period were analyzed. Each group consisted of 37–40 randomly selected children who were 8 or 9 yr of age within the periods of 1985–1987, 1991–1993, and 1997–1999, respectively. Samples reported to contain incomplete micturitions were not included.

Some personal characteristics of the children as determined in the three groups are presented in Table 1. Body weight was measured to the nearest 0.1 kg, and body height to the nearest 0.1 cm, by using an electronic scale and an

Received April 24, 2001; Revised May 17, 2001; Accepted June 11, 2001.
Author to whom all correspondence and reprint requests should be addressed: Prof. Barbara Griefahn, Institute for Occupational Physiology at the University of Dortmund, Ardeystr. 67, D-44139 Dortmund, Federal Republic of Germany. E-mail: griefahn@arb-phys.uni-dortmund.de

Table 1
AMs, SDs, and Results of Kruskal-Wallis Test for Some Variables in Three Sampling Periods[a]

	Sampling period				
	1985–1987 (n = 37, 18f, 19m)	1991–1993 (n = 40, 20f, 20m)	1997–1999 (n = 40, 20f, 20m)	Kruskal-Wallis test	
	AM ± SD	AM ± SD	AM ± SD	χ^2 (2 df)	p Value
Age (yr)	8.3 ± 0.5	8.5 ± 0.5	8.4 ± 0.5	3.19	0.2034
Body height (cm)	134.2 ± 6.8	134.2 ± 6.0	133.2 ± 6.3	1.37	0.5047
Body weight (kg)	29.8 ± 5.2	29.6 ± 6. 5	28.9 ± 5.0	0.61	0.7368
6-OHMS concentration (µg/L)	53.8 ± 31.4	46.5 ± 34.7	49.7 ± 23.7	3.71	0.1562
CV (%) of between-kit imprecision	11.4 ± 7.1	6.2 ± 6.0	4.9 ± 3.8	19.74	0.0001*
24-h Excretion of 6-OHMS (µg)	30.3 ± 11.2	31.9 ± 17.7	32.6 ± 14.2	0.35	0.8378
Urine volume (mL)	641 ± 216	778 ± 263	714 ± 259	5.69	0.0582

[a]AM, arithmetic mean; f, female; m, male; 6-OHMS; 6-hydroxymelatonin sulfate. *$p < 0.01$.

electronic stadiometer, respectively. Urinary creatinine concentration was determined by the Jaffé method utilizing a Beckman-2 creatinine analyzer (Beckman).

Analysis of 6-OHMS

The urinary concentrations of 6-OHMS were determined by use of an enzyme-linked immunosorbent assay (ELISA) (IBL, Hamburg, Germany). The assay procedure follows the basic principle of a competitive ELISA. The visualization is carried out by an enzymatic reaction of horseradish peroxidase and tetramethylbenzidine as substrate. The optical density at 450 nm (reference wavelength of 620 nm) is read with a microtiter plate reader (SLT-340 ATC; Tecan, Crailsheim, Germany). The samples stored at –20°C were thawed immediately before the analysis, and the measurements were carried out strictly according to the instructions of the kit provider. The detection limit is about 1 µg/L of urine. The lowest and highest single measurements are 9.0 and 187 µg/L, respectively. The coefficients of variation (CVs) for between-day imprecision for a standard and two control materials (15.6, 17, and 54 µg/L) are determined to 3–7% (n = 6). The crossreactivity of the 6-OHMS antigen of this ELISA against melatonin and 6-hydroxymelatonin is 0.002 and 0.001%.

All samples were analyzed in doubled duplicates. The first replicates were measured on the same microtiter plate as recommended by IBL. This procedure was repeated to provide 2 × 2 concentration values. From both means a final mean concentration value and the CV describing between-kit imprecision were calculated, which were used for further statistical operations.

Statistical Analyses

The 24-h excretions of 6-OHMS were computed by multiplying the 6-OHMS concentrations by the urine volumes. The differences of the variables' means in the three sampling periods were tested for significance nonparametrically by applying the Kruskal-Wallis test. Spearman rank correlation coefficients were computed for the associations of the 6-OHMS concentrations and 6-OHMS 24-h excretions with age, body height, body weight, daily creatinine excretion, and between-kit imprecision. Differences between boys and girls were assessed by the Wilcoxon two-sample test.

The statistical analysis of equivalence of the 6-OHMS concentrations and 6-OHMS 24-h excretions in the three sampling periods was carried out following the approach of Wiens and Iglewicz *(6,7)*. The test statistic

$$Z_{\min} = \min_{ij} \left[-(|AM_i - AM_j| - \delta_0) / (SD_i^2/n_i + SD_j^2/n_j)^{0.5}\right]$$

was computed for the log-transformed data in which δ_0 = log(1.25), and i and j = the sampling period groups. Thus, a commonly applied criterion for bioequivalence was used; that is, for all pairwise comparisons, the ratio of the geometric means had to be between 0.8 and 1.25. The values of $Z_{\min}$ were compared to the standard normal critical values, yielding a conservative test *(7)*, and to critical values developed by Wiens and Iglewicz *(6)*, resulting in a more powerful test.

Results

The individual characteristics were similarly distributed in the three groups representing different sampling periods. There were no statistically significant differences regarding age, body height, and body weight (Table 1).

Overall means ± SDs of 6-OHMS concentration and 6-OHMS 24-h excretion were 49.9 ± 30.1 µg/L and 31.6 ± 14.6 µg/d, respectively. Both did not vary systematically among the three groups. However, the means of between-kit imprecision increased with the samples' age, resulting in a statistically significant difference for the CV.

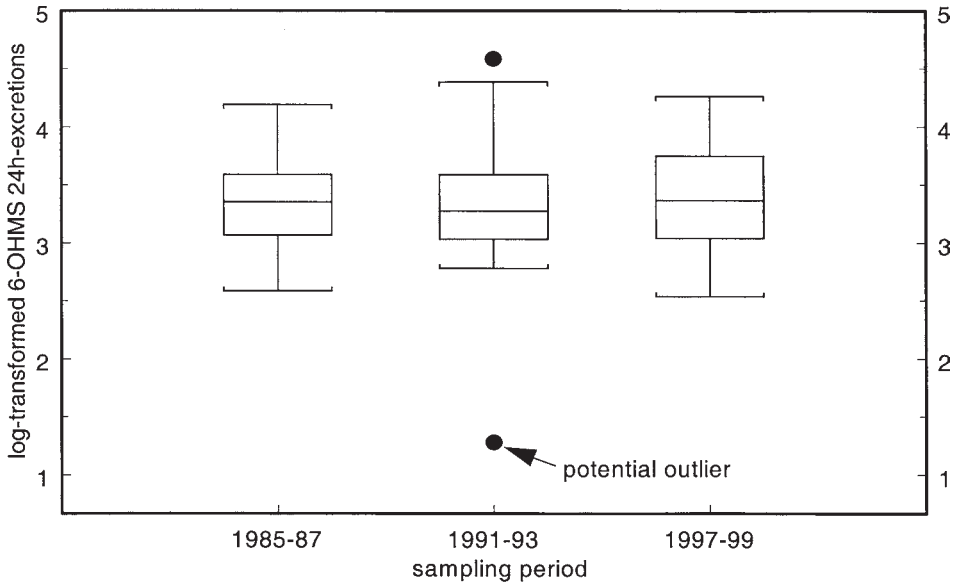

Fig. 1. Box plots showing medians, quartiles, minima, maxima, and outliers of log-transformed 6-OHMS excretions in the three sampling periods.

Table 2
AMs, SDs, and Results of Equivalence Tests
for Log-Transformed 6-OHMS Concentrations and 6-OHMS 24-h Excretions in Three Sampling Periods[a]

	Sampling period					
	1985–1987 (n = 37, 18f, 19m)	1991–1993 (n = 40, 20f, 20m)	1997–1999 (n = 40, 20f, 20m)	Test of equivalence[b]		
	AM ± SD	AM ± SD	AM	Z_{min}[c]	p Value[d]	p Value[e]
Log 6-OHMS concentration (µg/L)	3.8458 ± 0.5275	3.6455 ± 0.6026	3.7890 ± 0.5122	0.1768	0.4299	>0.05
Log 24-h excretion of 6-OHMS (µg)	3.3464 ± 0.3601	3.3348 ± 0.5336	3.3951 ± 0.4229	1.5132	0.0651$^+$	<0.05*
Analyses omitting one potential outlier[f]						
Log 6-OHMS concentration (µg/L)	3.8458 ± 0.5275	3.6815 ± 0.5652	3.7890 ± 0.5122	0.4691	0.3195	>0.05
Log 24-h excretion of 6-OHMS (µg)	3.3464 ± 0.3601	3.3876 ± 0.4219	3.3951 ± 0.4229	1.9535	0.0254*	<0.01**

[a] AM, arithmetic mean; f, female; m, male; 6-OHMS, 6-hydroxymelatonin sulfate.
[b] For all pairwise comparisons the ratio of the geometric means is between 0.8 and 1.25.
[c] $Z_{min} = min_{ij}\ [-(|AM_i - AM_j| - \delta_0) / (SD_i^2/n_i + SD_j^2/n_j)^{0.5}]$ in which $\delta_0 = \log(1.25)$ and i and j = the sampling period groups.
[d] Conservative test, comparison to standard normal values *(7)*; $^+p < 0.1$, $*p < 0.05$.
[e] More powerful test *(6)*; $*p < 0.05$; $**p < 0.01$.
[f] Sampling period 1991–1993, male, 6-OHMS concentration = 9.4 µg/L, 6-OHMS 24-h excretion = 3.6 µg.

No significant differences between boys and girls were observed for 6-OHMS concentration, 6-OHMS 24-h excretion, and the CV ($p > 0.2$) of the between-kit imprecision.

Figure 1 shows the box plots of the log-transformed 6-OHMS 24-h excretions in the three sampling periods. The tests of equivalence showed no significant results regarding 6-OHMS concentration (Table 2), but for the 6-OHMS 24-h excretion, equivalence could be demonstrated ($p < 0.05$). Equivalence was even more pronounced by omitting a potentially outlying data point of a boy with the lowest 6-OHMS concentration.

Discussion

The purpose of the present feasibility study was to determine whether the concentration of 6-OHMS remains stable in urine over at least 15 yr. One hundred seventeen 24-h urine samples taken from children ages 8 to 9 yr within the periods of 1985–1987, 1991–1993, and 1997–1999 were analyzed. The statistical analyses clearly indicate that the concentration of 6-OHMS remains stable for at least 15 yr if the urine is stored at –20°C. The differences concerning the CV of the between-kit imprecision are probably related to

the fact that the deviating sample group was analyzed by a person who carried out the ELISA method for the first time.

Overall, these results show that retrospective estimates of melatonin production are reasonably accurate, e.g., to investigate in depth the relationships among physiologic, metabolic, endocrine, and nutritional parameters. Because the participants in the present study were studied repeatedly over years, it is now possible to investigate the individual course of melatonin production during childhood. These melatonin patterns might deviate considerably from the curve determined in the cross-sectional study conducted by Waldhauser et al. *(5)* with 367 participants ages 3 d to 90 yr. In particular, the role of melatonin in determining sexual development in humans remains enigmatic *(8)*. It is therefore of great importance to study melatonin secretion during puberty longitudinally.

References

1. Froom, P., Bieganiec, B., Ehrenrich, Z., and Barak, M. (2000). *Clin. Chem.* **46,** 1384–1386.
2. Schultz, C. J., Dalton, R. N., Turner, C., Neil, H. A., and Dunger, D. B. (2000). *Diabetes Med.* **17,** 7–14.
3. Cook, M. R., Graham, C., Kavet, R., Stevens, R. G., Davis, S., and Kheifets, J. (2000). *J. Pineal. Res.* **28,** 41–47.
4. Kennaway, D. J., Lushington, K., Dawson, D., Lack, L., van den Heuvel, C., and Rogers, N. (1999). *J. Pineal. Res.* **27,** 210–220.
5. Waldhauser, F., Weiszenbacher, G., Tatzer, E., Gisinger, B., Waldhauser, M., Schemper, M., and Frisch, H. (1988). *J. Clin. Endocrinol. Metab.* **66,** 648–652.
6. Wiens, B. L. and Iglewicz, B. (1999). *J. Biopharm. Stat.* **9,** 465–483.
7. Wiens, B. L. and Iglewicz, B. (2000). *Controlled Clin. Trials* **21,** 121–137.
8. Reiter, R. J. (1998). *Ann. Med.* **30,** 103–108.