Free Cortisol and Creatinine in Urine of Healthy Children

P. V. Bertrand, B. T. Rudd, P. H. Weller, and A. J. Day

Cortisol and creatinine were measured in two consecutive overnight urine collections from 103 healthy school children, ages seven to 18.5 years. Mean cortisol and creatinine concentrations were respectively 194 nmol/L and 12.7 mmol/L. The data were statistically analyzed to simultaneously assess any effects of sex, age, weight, day of collection, and urine volume. Mean urine volume for boys exceeded that for girls, increased with body weight, and was greater on the second day of collection than on the first. Cortisol concentration was independent of sex, age, and weight, but decreased with urine volume. Boys excreted more cortisol than did girls, and the amount increased with urine volume. Creatinine concentration increased with weight, decreased with urine volume. Total creatinine increased with weight, was greater for boys than girls, and increased with urine volume. The cortisol/creatinine ratio was valueless as an index of adrenocortical status.

Additional Keyphrases: reference interval • adrenocortical function assessment • pediatric chemistry • sex-, weight-, and agerelated effects • steroids

The primary objective of this study was to obtain reference data on the ranges for cortisol and creatinine excretion by a large group of healthy volunteer school children. Our secondary objective was to assess the relation, if any, between age, sex, weight, urine volume, and day of urine collection on results for concentration and total excretion of creatinine and of cortisol and for the ratio of cortisol to creatinine.

Establishment of a normal reference interval for children was needed for the assessment of adrenocortical function in a group of steroid-treated asthmatic children currently under investigation, and we found sparse evidence in the literature that urinary free cortisol is independent of age, sex, body weight, urine volume, and creatinine excretion in a large normal group.

Non-conjugated (free) cortisol measured in urine is a valuable indication of adrenocortical function that reflects daily cortisol secretion (1). It is also a valuable indication of certain pathological conditions (2).

Materials and Methods

Procedures

Urinary free cortisol was measured by a competitive protein-binding assay (CPBA) procedure as described for serum cortisol (3). Extraction of the cortisol from urine into dichloromethane was followed by solvent evaporation and reconstitution of the residue in assay buffer. The interassay CV was 20%, the limits of detection were equivalent to \( \geq 30 \) nmol/L, and the assay performed well within the limits accepted by the United Kingdom National External Quality Assessment Scheme for urinary cortisol.

Urinary creatinine was measured by an automated procedure, with alkaline picrate used for color development (4), for which the interassay CV was 5% during 12 months.

Children Studied

The co-operation of headmasters and their pupils from two independent coeducational boarding schools in the West Midlands was obtained, with permission given for the children to participate in the study. There were 70 boys and 33 girls.

Our conclusions may have some bias towards children of a high social class, because the study involved only children in boarding schools, but it would have been much more difficult to collect data in controlled circumstances otherwise.

Under the supervision of teachers, all the children were asked to empty their bladders before retiring and to collect all subsequent urine samples, including those voided on waking the next morning. The procedure was repeated the next day. On each occasion, the samples were promptly delivered to the laboratory, the volume was measured, and 50-mL aliquots were stored at \(-20\) °C for subsequent analysis.

In all cases, age, weight, and sex were recorded, and a brief medical enquiry revealed no overt disease.

Statistical Analysis

The repeated-measures analysis program 2V of the Biomedical Data Processing Package (5) was used to carry out several analyses. First, we determined the effects on urine volume of sex as a grouping factor and linear and quadratic effects of age and weight as covariates and the two days of collection as repeated measures. We then determined the effects of these factors, and also of urine volume, on the following dependent variables: creatinine and cortisol concentrations, total creatinine, total cortisol, and the ratio of cortisol to creatinine.

Many program runs were carried out in the analysis of each dependent variable, so that the effect of any one factor could be precisely established by both its inclusion and exclusion in various repeated-measures models. The procedures appropriate for such analyses are described by Fleiss (6) and the levels of statistical significance reported in this paper are, where appropriate, those of his approximation 4. This approximation is "the one that makes it hardest to find significant effects for the time factor" and "is the safest and most conservative of all, because it is valid in all cases." The SD reported in the results is that appropriate to a prediction interval for a further single observation. Logarithmic transformations were made of the cortisol measurements to produce residuals that were approximately normally distributed. To indicate the percentage of the total variation explained by significant factors, the coefficient of determination, uncorrected for degrees of freedom, is reported where

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relevant. This was determined by considering the relationship between the variables as a straightforward multiple-regression problem and reporting the value given by the "Minitab" statistical computing package (7).

The central 95% ranges (2.5 to 97.5 percentile) and the median for overnight samples are given for urine volume, total excretion of cortisol, and creatinine per kilogram body weight for both boys and girls less than 12 years of age and for 12 years of age and above. These data facilitate comparison of our data on overnight samples with published data on daily samples. Pubertal staging was not carried out on these children as it was considered unethical for such normal volunteers, so 12 years has been taken as the age separating these children into approximately pre-pubertal and adolescent groups (8, p 74).

Results

Table 1 summarizes the mean, standard deviation, and range of the variables measured in the 103 children (33 girls, 70 boys) and the number of observations available on each day excluding the few missing values. The age range of the boys, 7 to 16.5 years, differed somewhat from that of the girls, 10 to 18.5 years, so the results reported are only applicable within the relevant age ranges.

Statistical Analysis of Urine Volume

Figure 1a illustrates the relation of urine volume to the weight of each child. Overall, the mean urine volume was 271 mL (SD 152 mL). In the analysis of urine volume significant effects were found due to sex (P <0.0001), weight (P <0.01), and the different days of collection (P <0.025). The regression model fitted to data from 97 children was:

\[ \text{volume (mL)} = 165.4 + 2.42 \text{ wt (kg)} + s + d \pm \text{SD} \]

where s is an effect due to sex, which is +31.4 mL for boys and -67.0 mL for girls, and d is an effect due to the day of collection, which is -25.7 for day 1 and +25.7 for day 2, and SD = 136. The percentage of the variation explained by this regression (i.e., the coefficient of determination) was 15%. That explained by days of collection alone was 3%, that by sex alone was 8%, that by weight alone was 3%, and that by sex and weight together was 12%. The SD has two components, one attributable to the variation between children, with SD = 62 mL, and the other to variation between different days of collection by the same child, with SD = 121 mL. Figure 1a shows the mean regression on weight, ignoring the sex effect and the day effect, together with two SD about this line. The differences between the two sexes and the two days of collection are illustrated by the use of different symbols in Figure 1a. The difference between the

<table>
<thead>
<tr>
<th>Table 1. Mean, Standard Deviation, and Range of Variables Measured in 103 Children (33 Girls, 70 Boys)</th>
</tr>
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<tbody>
<tr>
<td><strong>No.</strong></td>
</tr>
<tr>
<td>observations</td>
</tr>
<tr>
<td><strong>Age of boys, y</strong></td>
</tr>
<tr>
<td><strong>Body weight of girls, kg</strong></td>
</tr>
<tr>
<td><strong>Body weight of boys, kg</strong></td>
</tr>
<tr>
<td><strong>Urine vol., mL, day 1</strong></td>
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<tr>
<td><strong>Urine vol., mL, day 2</strong></td>
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<tr>
<td><strong>Creatinine, mmol/L, day 1</strong></td>
</tr>
<tr>
<td><strong>Creatinine, mmol/L, day 2</strong></td>
</tr>
<tr>
<td><strong>Cortisol, nmol/L, day 1</strong></td>
</tr>
<tr>
<td><strong>Cortisol, nmol/L, day 2</strong></td>
</tr>
</tbody>
</table>

Fig. 1. a. Urine volume for children of different weights. b. Creatinine concentration for children of different weights. c. Cortisol concentration (analyzed on a logarithmic scale) for children of different ages

©, males on day one; ☑, males on day two; ◄, females on day one; □, females on day two; ---, mean regression; —— two standard deviations above and below the mean regression.
two days of collection was similar in the two sexes, as was confirmed by a nonsignificant interaction between sex and day of collection in the statistical analysis.

One of the urine volumes recorded was extremely large (1320 mL). This occurred in a 13-year-old boy, weight 44 kg, on the second day of collection. Concentrations of creatinine (3.18 mmol/L) and cortisol (80 mmol/L) were in no sense outliers, nor were the excretions of total creatinine or total cortisol. The boy probably was drinking excessively, but the influence of this observation was investigated and its deletion was not found to substantially alter the significance of the difference in average excretion between the two days of collection nor any other results. Therefore it was included in all analyses reported.

The central 95 percent ranges and the medians of the urine volumes of the overnight samples for all children and for children categorized by sex and by age group are given in Table 2.

Statistical Analysis of Creatinine

Creatinine concentration, all the data, is shown plotted against weight in Figure 1b. Overall, the mean creatinine concentration was 12.7 (SD 6.0) mmol/L. Significant effects were due to weight (P <0.0001) and urine volume (P <0.0001). The regression equation for pooled data on 96 children was:

creatinine (mmol/L) = 8.38 + 0.219 \times \text{wt (kg)} - 0.0187 \times \text{volume (mL)} \pm \text{SD}

where SD = 4.54 and the coefficient of determination was 42%. In separate regression models the coefficient of determination was 18% for weight alone and 16% for urine volume alone.

Urine volume was then excluded as a possible regressor. Variable and significant effects were found due to weight (P <0.0001) and due to the difference between the two days of urine collection (P <0.01). The fitted regression equation was:

creatinine (mmol/L) = 4.39 + 0.194 \times \text{wt (kg)} + 0.87d \pm \text{SD}

where d = +1 for day 1 and d = −1 for day 2, and SD = 5.43 and the coefficient of determination was 18%. The SD has two components, one due to variation between children, with SD = 3.55, and the other due to variation between days of collection, with SD = 4.11. The mean regression on weight, ignoring the difference between the two days of collection, and two standard deviations about it are shown in Figure 1b.

In the analysis of the total creatinine excreted, significant effects were found due to sex (P <0.002), the linear effect of weight (P <0.0001), and urine volume (P <0.0001). The regression equation for pooled data was

Total creatinine (mmol) = −0.297 + 0.305 \text{s} + 0.0576 \times \text{wt (kg)} + 0.00332 \times \text{volume (mL)} \pm \text{SD}

where \text{s} is +1 for boys and −1 for girls and SD = 1.046. The SD has two components, one due to variation between children, with SD = 0.618 mmol, and the other due to variation between days of observation, with SD = 0.844 mmol.

When urine volume was excluded as a covariate in the analysis of the data on total creatinine, significant effects were found related to sex (P <0.0001) and weight (P <0.0001), with SD = 1.124. Table 2 gives the central 95% ranges and the medians of creatinine per kilogram body weight per overnight sample for all children and for children categorized by sex and by age group.

Statistical Analysis of Data on Cortisol

Overall, the mean cortisol concentration was 194 (SD 72) mmol/L. Preliminary analysis showed that the data on urine cortisol concentration were best analyzed on a logarithmic scale because the residuals were thereby more nearly normally distributed. The positively skewed cortisol concentrations may be seen in Figure 1c, where the data are plotted vs the age of each child. Our observations on the two sexes and on the two days of collection are indicated in Figure 1c by different symbols.

In the analysis of the logarithm of cortisol concentration, urine volume was the only covariate found to have a significant effect (P <0.01). Cortisol concentration decreased by 9.2% (95% confidence interval from 4.4% to 14.8%) with each 100 mL increase in urine volume. Nine percent of the variation was explained by this regression on urine volume.

The logarithm of cortisol concentration was then analyzed, excluding urine volume as a covariate, and no significant effect was found due to sex, days of collection, or to linear or quadratic effects of age or weight. Overall, the geometric mean cortisol concentration was 181.3 nmol/L. Values corresponding to ±2 SD of scatter on the logarithmic scale give a range, appropriate to 95% of normal children, of 83 to 396 nmol/L. These values are illustrated by the horizontal lines in Figure 1c. The variance corresponding to this scatter has two components, 39% of it being ascribable to variation between children and 61% of it being ascribable to differences between collections from the same child.

When the logarithm of total cortisol was analyzed, the value for the geometric mean for total cortisol was 43.3 nmol, with the values corresponding to ±2 SD being 18.2 to 101.6 nmol. The significant results from the analysis were that total cortisol increased by 24% (95% confidence interval from 17% to 32%, P <0.0001) with each 100-mL increase in urine volume, and boys produced 17% (95% confidence interval from 1% to 35%, P <0.05) more total cortisol than girls after adjusting for the effect of volume of urine. When the data for total cortisol were analyzed with no adjustment for urine volume, the boys produced 41% (95% confidence

**Table 2. Central 95% Ranges and Medians of Urine Volume, Creatinine Excretion per kg, and Total Excretion of Cortisol in Overnight Specimens for All Children and for Children Categorized by Sex and by Age Group**

<table>
<thead>
<tr>
<th>Age Group</th>
<th>Urine volume, mL</th>
<th>Creatinine, μmol/kg per spec.</th>
<th>Cortisol, nmol/specimen</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5% Median</td>
<td>97.5%</td>
<td>2.5% Median</td>
<td>97.5%</td>
</tr>
<tr>
<td>All children</td>
<td>90</td>
<td>235</td>
<td>700</td>
</tr>
<tr>
<td>Boys &lt;12 years</td>
<td>103</td>
<td>240</td>
<td>670</td>
</tr>
<tr>
<td>Boys ≥12 years</td>
<td>96</td>
<td>270</td>
<td>780</td>
</tr>
<tr>
<td>Girls &lt;12 years</td>
<td>55</td>
<td>170</td>
<td>300</td>
</tr>
<tr>
<td>Girls ≥12 years</td>
<td>58</td>
<td>180</td>
<td>560</td>
</tr>
</tbody>
</table>

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interval from 19% to 67%, \( P = 0.0001 \) more total cortisol than girls and the SD was 21% greater than when urine volume was included as a covariate. Table 2 gives the central 95% ranges and the medians of total excretion of cortisol per overnight sample for all children and for children categorized by sex and by age group.

The Ratio of Cortisol to Creatinine

This ratio was calculated for available data (203 observations), and the mean was 20.1 with \( SD = 28.1 \), the range being 5.2 to 368. Its CV was 139%, which may be contrasted with a CV of 47% for creatinine concentration and a CV of 37% for cortisol concentration. Statistical analyses corresponding to those for creatinine and cortisol were carried out on both untransformed and log transformed data, but no significant effects emerged.

Discussion

Urinary free cortisol is advocated as a measure of adrenocortical function, particularly in cases of hyperadrenocorticalism (2, 9). In adults, the most popular procedure for assessing adrenocortical status is to collect a 24-h specimen of urine and calculate the cortisol concentration on the basis of the volume of urine voided. In children, 24-h samples have been proposed with appropriate correction for differences in cortisol concentration based on the changing body weight (10, 11). In the present study the time span of the overnight collection of urine has varied between the children, depending precisely on when they retired at night and awoke in the morning. The urine collection, however, covers the time of maximum cortisol secretion by the adrenal and takes into account the diurnal rhythm of serum cortisol: low values throughout the day, tending to a minimum between midnight and 0300 h, then rising to a peak on waking (2). The overnight collection in this study, which is relatively easy for children, contains that fraction of excreted free cortisol produced before waking, which is the period of enhanced cortisol secretion.

The ratio of cortisol to creatinine has been advocated for random samples for diabetics on the assumption that creatinine excretion remains constant for a given age or weight (12). In this study we did several analyses of the ratio of cortisol to creatinine, finding that this ratio had a much larger CV than that for either variable considered alone. In these analyses the errors in the two variables were compounded, so that significant effects observed in the variables when considered alone were not observed in the ratio of the two. This has been commented upon by others (13), who also remarked that it is inappropriate to use the ratio of cortisol to creatinine because of the significant effect of weight on urinary creatinine but not on urinary cortisol—which we confirm. Thus we did not find the ratio of cortisol to creatinine to be a useful index in these studies.

We thought that the supervised collection of two consecutive overnight urine samples would be a useful procedure to adopt to assess the constancy or otherwise of urinary cortisol excretion by a healthy group of children and adolescents in relation to the constancy of creatinine excretion as an index of adequate urine collections. Urine volume, on average 271 mL, was found to increase with weight (2.42 mL per kilogram, Figure 1a), to be on average 98 mL greater in boys than in girls of the same weight, and to be on average 51 mL greater on the second day of collection than on the first. The variation in the data was considerable, and the multiple regression on weight, sex, and days of collection accounted for 15% of it, so that the effects reported could only be detected in a large group such as we have studied. The relation between urine volume and weight was expected, that between volume and sex was unexpected, and that between volume and days of collection was surprising. The difference related to day of collection was most likely a consequence of the children being under psychological stress on the first day, which may have prevented their uninhibited voiding of urine, whereas on the second day they would tend to be more relaxed and less inhibited.

The mean urine volume in all the overnight samples of 271 mL is rather less than a third of the means of the normal ranges of 650–1000 mL/24 h and 800–1400 mL/24 h for five- to eight-year-olds and eight- to 14-year-olds reported in daily samples, and it is consistent with the observation that the ratio of day to night urine is greater than 2:1 and often more than 3:1 (14, p 576). In both age groups we find that both the 2.5 percentile and the 97.5 percentile are greater in boys than are the corresponding percentiles for girls, which is consistent with our observation with regard to the mean values reported above and with the information (8, 14) that, during 24 h, men tend to produce a larger volume of urine than women do.

Our analyses of the data on cortisol and creatinine have revealed the high statistical significance of the influence of urine volume on their interpretation. Inclusion of urine volume as a covariate resulted in a smaller SD about the regression line, both when concentrations and total amounts were considered. Concentrations of both creatinine and cortisol decreased with an increase in urine volume, and both total creatinine and total cortisol significantly increased with an increase in urine volume. Clearly, for both creatinine and cortisol, the concentration is not inversely proportional to urine volume, because if it were the product of the two, which is the total amount, would then have been constant. Rather, when the total of each analyte excreted was examined, it was found they increased with urine volume. Thus, for both creatinine and cortisol, the total excreted increased with urine volume but by less than we could account for by a proportionate relationship to volume. The concentration of cortisol and creatinine also decreased with an increase in urine volume, but not by as much as would be accounted for by a simple dilution effect. The change in cortisol concentration with urine volume would not have been detected at the 5% level of significance in a sample of fewer than 25 children because the effect would be masked by the variation in the data. These observations suggest that the renal control of urine flow importantly affects the measured output of both creatinine and cortisol.

The relation of urine volume to creatinine concentration clearly explained the difference in concentration between the two collections that was observed when urine volume was excluded as a covariate. No such difference between the two days of collection was observed with respect to cortisol, probably because an increase of 100 mL in urine volume resulted in a more significant \( P < 0.0001 \) decrease (about 15%) in the concentration of creatinine than was true for the concentration of cortisol \( P < 0.05, \sim 9\% \).

Further, as is well documented (15), creatinine excretion is statistically highly correlated with body weight. Of the variation in creatinine concentration, 18% was accounted for by body weight alone, 16% by urine volume alone, and 42% by the two considered simultaneously. The apparent anomaly of addition in this latter observation is accounted for by the intercorrelations between the variables. That this
can occur has been demonstrated theoretically in a paper recently submitted for publication by one of us (P.V.B.).

Overall, for the children the central 95% range of creatinine output per kilogram per overnight specimen was 16–151 \( \mu \text{mol/kg} \) per day. The mean time span of an overnight urine collection was about 10 h, so our equivalent daily output per kilogram would be 38–362 \( \mu \text{mol/kg} \) per day. These ranges are consistent with, but wider than, the reported (16) normal values of 71–194 \( \mu \text{mol/kg} \) per day for children and 71–265 \( \mu \text{mol/kg} \) per day for adolescents. Our wider normal range is to be expected, for results for short collections of urine are likely to vary more than those for longer periods. However, the increase in variability is not very great, so that the measurement of creatinine in overnight samples is satisfactory.

Creatinine is often used as an index of adequate urine collection, but it is a dubious one. If it is used, appropriate adjustment for body weight should be made. The pooled CV for both creatinine concentration and cortisol concentration was around 42%, with the ratio of the standard deviation of variation between children to that between days of collection being about 86% for creatinine and 64% for cortisol. Thus the variation between days of collection in the same child was greater than that between children for both creatinine and cortisol concentration.

The excretion of total creatinine and total cortisol differed between the sexes: greater for boys than for girls. Probably this simply reflects fluid intake or, conceivably, unrecognized differences in renal function. Complete specimens were probably obtained from all the children, because they showed willingness to cooperate and were closely supervised. It is pertinent to note that the difference between the sexes would not have been statistically significant at the 5% level for total creatinine in a sample of fewer than 38 children and for total cortisol in a sample fewer than 25. Nevertheless, in the analysis of concentration of cortisol there were no regression effects of age, sex, weight, or days of collection when urine volume was included or excluded as a covariate.

The range of measurements of concentration of cortisol appropriate to 95% of normal children was from 83 to 396 nmol/L and is a reasonable norm for children of both sexes of various ages and weights on two consecutive days. When allowance is made for diurnal variation, these data from overnight collections are consistent with values for daily concentration of cortisol in children reported by others (13). Overall, for the children the central 95% range of the total excretion of cortisol in overnight samples was 14–118 nmol, the median 44 nmol. This will correspond to approximately half the daily excretion, because the peak production of cortisol occurs in the early morning (2), so our data would correspond to about 28–236 nmol/day and a median of 88 nmol/d. This is a little higher than, but considerably overlaps with, published normal ranges of 5.5–74.5 nmol/day for young children and 13.8–151.8 nmol/day for adolescents (reported in 17, p 148). When our 95% ranges in Table 2 are examined, the ranges and medians in the four sex-by-age groups show reasonable consistency with each other and with the result described earlier that, on average, boys produce 41% more cortisol than girls do, a result particularly apparent when the medians are compared. Indeed, this explains to some extent why our range exceeds that reported in ref. 17. There are more than twice as many boys as girls in our study group, and boys excrete more cortisol than girls. We thus conclude that measurement of cortisol in overnight urine samples is of value in assessing adrenocortical function in children, and these data provide a valuable reference set.

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