Paediatric reference range for overnight urinary cortisol corrected for creatinine

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Published in:
Clinical Chemistry and Laboratory Medicine

DOI:
10.1515/cclm-2021-0371

Publication date:
2021

Document version:
Final published version

Citation for published version (APA):

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Download date: 14. Sep. 2023
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Abstract

Objectives: Systemic activity of inhaled corticosteroids (ICS) may be assessed via urinary cortisol measurement. Overnight urinary free cortisol corrected for creatinine (OUFCC) has been extensively reported in adult studies. However, a paediatric mass spectrometric (MS) reference range for OUFCC is not established. MS methods for OUFCC avoid cross-reactivity with other steroid hormones and are thus preferable to immunoassays. The aim of the present study was to define an MS OUFCC normative range in children.

Methods: This was a cross-sectional study of healthy pre-pubertal children from 5 to 11 years. Children collected urine from 10 pm or bedtime, whichever was earlier, until 8 am. Urinary free cortisol was measured via a liquid chromatography tandem mass spectrometry (LC-MS/MS) assay (Acquity UPLC with Xevo TQ-S Mass Spectrometer [Waters]) with in-house reagents. Urinary creatinine was measured using a commercial assay (Roche).

Results: Complete urine collections were obtained from 72 males and 70 females, mean age (SD) 8.6 (1.9) (range 5.0–11.8) years. The OUFCC 95% prediction interval was 1.7–19.8 nmol/mmol. Geometric mean OUFCC was 5.7; range 1.1–24.8 nmol/mmol.

Conclusions: The obtained normative LC-MS/MS OUFCC reference data facilitate the use of mass spectrometry OUFCC assays in assessment of systemic activity of endogenous and exogenous corticosteroids in children.

Introduction

Endogenous cortisol is regulated by the hypothalamic-pituitary-adrenal axis (HPA-axis) and secreted in a diurnal pattern with low nocturnal and high morning serum blood levels [1]. Therefore, serum must be sampled frequently over 24 h to give a valid picture of the secretory profile [1]. For decades stimulatory (Synacthen) tests of the HPA-axis have been used to assess potential cortisol suppression due to exogenous corticosteroids. However, such tests require hospitalization and invasive procedures and in addition may be non-physiological [1]. Thus, non-invasive outpatient methods such as the assessment of spontaneous urinary cortisol excretion over 24 h have been developed [1, 2]. However, 24 h urine collection is inconvenient and may consequently predispose to collection errors [3]. Therefore, the more convenient method of overnight urine sampling was evaluated with the result that overnight urinary free cortisol corrected for creatinine (OUFCC) is now a well-established means by which to assess the systemic activity of exogenous corticosteroids [1–10]. However, whilst the effects of several different treatments upon OUFCC have been assessed in paediatric research protocols, in the absence of a reference range, interpretation of any differences is substantially compromised in this context, as is interpretation of OUFCC in the clinical management of children on exogenous corticosteroids or in other cases of suspected cortisol suppression. Therefore, the aim of the present study was to establish a reference range for OUFCC in children.

Materials and methods

Patients

Inclusion criteria for this cross-sectional study were healthy pre-pubertal subjects, aged 5–11 years. Exclusion criteria were any chronic or acute conditions and ingestion of any medication within 1 week of urine sampling.
Study design

Oral and written information about the study rationale were given to children and parents separately. Firstly, parents were informed of the study at parent-teacher conferences arranged by the school (Randers Realskole) and were asked for their written consent allowing their children’s participation. Thereafter, children whose parents had consented were informed about the study procedures in their classrooms and were asked for their assent to participate in the study.

Parents completed a questionnaire about their child consisting of five questions: age, gender, any illness during the past week (yes/no), any chronic diseases (yes/no) and pubertal symptoms or signs (yes/no) [11]. Height (cm, one decimal) (Charder HM200P Transportable Stadiometer; one estimation) and weight (kg, one decimal) (electronic weight) were recorded.

Overnight urinary collection was performed as previously described [1, 12]. On a Friday morning during October 2018 plastic bottles for urine collection, funnels and written urine sampling guidelines were delivered to the children in their classrooms. The children commenced collection the same night or on the following Saturday night at 10 pm or bedtime, whichever was earlier. They were instructed to empty their bladder before going to bed (or no later than at 10 pm). Urine passed thereafter until 8 am in the morning (including any urine passed during the night) was collected. At 8 am the children emptied their bladder a final time, with that void also contributing to the overnight collection. The urine collection guidelines specified that if the collection was not completed in accordance with guidelines the parents should mark the urine can FIPO (Danish abbreviation of “Not collected in accord with guidelines”). Until the urine bottles were collected at the children’s classroom the following Monday morning they were stored in a refrigerator.

Ethics

The study did not require authorization from Ethics Committee.

Cortisol assay

Urinary free cortisol was measured via a liquid chromatography tandem mass spectrometry (LC-MS/MS) assay after solid phase extraction with in-house reagents.

Using a Hamilton STARlet workstation, 250 μL urine, calibrator or control was mixed with 250 μL internal standard (200 nmol/L 13C3-cortisol from IsoSciences in methanol:water 10:90) and 500 μL formic acid 1.5% in water. Next 400 μL of the sample mixture was loaded on Oasis® PRIME HLB 96-well plate 30 mg (Waters) and after washing with 1,000 μL methanol:water 5:95 the cortisol was eluted with 400 μL methanol and diluted with 400 μL formic acid 0.1% in water.

The analyses were conducted on a Waters Acquity ultra performance liquid chromatograph (UPLC) with Xevo TQ-S tandem mass spectrometer operated in electrospray positive mode. The chromatographic separation was achieved with a Waters BEH C18 (100 × 2.1 mm, 1.7 μm) column. The mobile phases consisted of 0.1% of ammonium hydroxide (25%) in water (mobile phase A) and methanol (mobile phase B).

Gradient elution was as follows: Initially 50% B; 0–2 min linear gradient to 68% B; 2–3 min 100% B, 3–4 min 50% B. Two microlitre sample was injected at a flowrate of 0.35 mL/min. The multiple reaction monitoring (MRM) transitions used for cortisol was 363.27>121.00 as quantifier and 363.27>97.20 as qualifier. For 13C3-cortisol 366.27>124.00 was used. The analysis was calibrated by in-house prepared calibrators (25–1,000 nmol/mL), and the intermediate precision for urinary internal controls at three levels determined over 26 runs were 4.4% (target 10.8 nmol/mL), 3.2% (target 74.3 nmol/mL) and 2.4% (target 486 nmol/mL). The lower limit of quantification (LOQ) was chosen at 10 nmol/L since the method in general is used for revealing patients with high urinary cortisol secretion. However, the intermediate precision at 10.8 nmol/mL was 4.4% meaning that the LOQ is probably substantially lower. Quality was further assured by monthly participation with satisfactory results in the external quality control program for steroid hormones from the United Kingdom National External Quality Assessment Service (UKNEQAS). For the distribution in the preceding 3 months the mean bias was 4.9%.

Creatinine assay

Urinary creatinine was measured using Creatinine plus version 2 assay from Roche on a Cobas c702 module. Measuring range was 0.1–135 mmol/L and the intermediate precision for urinary internal controls at two levels determined over 115 runs were 3.7% (target 6.3 mmol/L) and 2.7% (target 13.3 mmol/L).

Sample size

Approximately 50% of the population was to be male and 50% female to allow for analyses within each gender. A sample size of 120 subjects was thus planned, which was estimated to yield a 95% prediction interval with a width approximately 1.2% larger than that of the smallest achievable prediction interval, whilst analyses of each gender (60 in each group) would yield a 95% prediction interval with an expected width approximately 2.5% larger than the smallest achievable [13]. In our experience 25–50% of invited children would refuse to participate in normative studies [14, 15]. Therefore, accounting for potential inclusion/exclusion criteria failures and the expected rate of refusals to participate, 200 children were invited aiming at similar numbers of children of each age (5 years old, 6 years old, etc.).

Statistics

An analysis of variance (ANOVA) model with a fixed intercept term and a random subject effect was used to model the log-transformed data. The estimated intercept and corresponding 95% prediction interval were back-transformed to the original scale in creating 95% prediction intervals, which were used as the basis for the proposed normative ranges. Data were analysed both for the overall population and stratified for gender, and with and without correction. Urine bottles marked “FIPO” led to the exclusion of the data from the primary statistical analysis.

A further analysis of covariance (ANCOVA) model with fixed term for gender and weight as a continuous covariate was applied to the log-transformed data to test for any differences between boys and girls with a ratio calculated by transforming the difference between the natural log least square means back to the original scale. Results were compared to those from an ANOVA model without adjusting for weight.
Results

A total of 200 children were invited to participate. Twenty-five (12.5%) children had commenced puberty or had a chronic condition, so they were excluded. A total of 175 children complied with all inclusion and exclusion criteria and 148 (84.6%) (136 Caucasians and 12 Asians) consented/assented to study participation. Six of the 148 children (4.1%) delivered urine cans marked FIPO (“Not collected in accordance with guidelines”) and their data were thus discarded from the data analysis. Complete urine collections were obtained from 72 males and 70 females, mean age (SD) 8.6 (1.9), median 8.8 (range 5.0–11.8) years. Demographic data stratified by gender is given in Table 1.

In the overall population of 142 children the geometric mean OUFC was 5.7 (range 1.1–24.8 nmol/mmol). The 95% prediction interval was 1.7–19.8 nmol/mmol (coefficient of variation 69.1%). For boys the geometric mean OUFC was 5.9 (range 1.4–23.5 nmol/mmol), 95% prediction interval 1.7–20.1 nmol/mmol (coefficient of variation 67.5%); and for girls the geometric mean OUFC was 5.6 (range 1.1–24.8 nmol/mmol), 95% prediction interval 1.6–20.3 nmol/mmol (coefficient of variation 71.2%). Individual data on OUFC by age, weight and height are presented in Figure 1. The distribution of OUFC for boys and girls is given in Figure 2. There was no statistical difference in OUFC between boys and girls: Exp (LS mean) 5.9 (95% CI: 5.1, 6.8 nmol/mmol) and 5.6 (95% CI: 4.8, 6.5 nmol/mmol), respectively, ratio 0.96 (95% CI: 0.78, 1.18), p=0.67; or when adjusted for weight: Exp (LS mean) 5.9 (95% CI: 5.1, 6.8 nmol/mmol) and 5.6 (95% CI: 4.8, 6.5 nmol/mmol), respectively, ratio 0.95 (95% CI: 0.77, 1.16), p=0.59.

In the overall population of 142 children the geometric mean overnight urinary free cortisol without correction (OUFC) was 56.7 (range 5.2–245.4) nmol, 95% prediction interval 16.2, 198.6 (coefficient of variation 70.1%). In boys the geometric mean OUFC was 62.1 (range 17.4–245.4) nmol, and 95% prediction interval 20.4, 189.3 nmol (coefficient of variation 60.1%). In girls the geometric mean OUFC was 51.5 (range 5.2–175.2) nmol/L, and 95% prediction interval 12.8, 207.8 nmol (coefficient of variation 78.7%).

Discussion

Despite the considerable body of data supporting the use of OUFC, and the obvious potential benefits of an overnight (vs. 24-h) non-invasive urinary index of the systemic bioactivity of topical corticosteroids in children, and despite the use of OUFC assessments in paediatric clinical studies for more than a decade, the present study is the first to establish a reference range for OUFC in healthy children [3, 16, 17].

Since the total amount of cortisol secreted during the night is only 40–45% of the daily total, OUFC is heavily influenced by start and end times. Overnight urine collection for assessment of cortisol needs to encapsulate the morning spike in cortisol excretion, thus, urine must be collected from bedtime until around 8 am. Urine collection over a brief period of time which encapsulates the morning spike in cortisol excretion will lead to a marked overestimation of OUFC. Conversely a prolonged nocturnal collection which omits collection of the morning cortisol peak will lead to an underestimation of OUFC. The requirement to collect morning urine may be problematic in outpatient populations of Danish children who at this time of the day are preparing for and travelling to school. The present protocol used a pragmatic solution to improve the completeness of urine collections, especially, to secure collection of the 8 am morning void. Urine collections were performed overnight on Friday or Saturday to avoid conflicts with early morning rising on school days. The urine bottles were stored for a maximum of 2 days in a refrigerator which would not have influenced cortisol levels since urine samples may be stored for more than a week [18]. The present observations showed that outpatient overnight urine collections were feasible as only six cases of incomplete collections were recorded.

We used an LC-MS/MS assay method for assessment of urine cortisol excretion. The assay is quite labour-intensive and time-consuming. However, whereas more frequently used immunoassays rely upon antibody-binding and may therefore result in varying degrees of cross-reactivity with cortisol metabolites and other endogenous steroid...
hormones and, hence, quite different estimates of cortisol within a given sample depending on the immunoassay used, the LC-MS/MS exclusively measures cortisol [19–21]. This is advantageous as even low levels of assay cross-reactivity may elevate estimated cortisol levels [19, 22]. Indeed, one study in adults estimated urine cortisol levels to be 1.6 and 1.9-fold greater with two different immunoassays compared to a mass spectrometric (MS) method [20]. Similar results have been noted for several other cortisol immunoassays in comparison to gas chromatography mass spectrometry (GC-MS) and LC-MS/MS assays [19, 22].

Consistent with such observations the geometric mean OUFFC reported in children with mild asthma off-treatment using the Siemens Coat-A-Count radioimmunoassay was approximately 60% higher [12]. These findings indicate that assay-specific reference ranges must be used for correct data interpretation [12, 23, 24]. Where the available cortisol assay is not supported by an assay-specific reference range and when OUFCC measurement is desired to screen for HPA-axis suppression in children on high doses of topical corticosteroids in clinical practice or to assess systemic activity of new formulations and devices of topical corticosteroids in clinical trials MS assays should be preferred. The lack of cross-reactivity of such assays indicates that similar results may be anticipated for a given sample with different MS methods [22].

Finally, pubertal assessment by children and parents is not a reliable measure of exact pubertal staging and should be augmented by a physical examination. In the present study, however, we were not aiming at a detailed classification of pubertal stages as such. We simply aimed at discriminating between prepuberty and puberty. Good evidence has been provided that for a simple distinction between prepuberty and puberty self-assessment can be sufficiently accurate [11].

In conclusion, the present study has provided the first normative LC-MS/MS OUFCC reference data in children. They facilitate the use of LC-MS/MS OUFCC in the clinical management of children on high dose exogenous corticosteroid regimens and in studies to assess the systemic activity of topical corticosteroids in children.
Acknowledgments: Thanks to research nurse Anne Karina Kjaer for helping with recruiting children and collecting urine samples. Thanks to children, parents and teachers at Randers Realskole for creating helpful working conditions in all involved classes and for taking part in the study.

Research funding: The study was supported by a grant from “The Foundation for Research and Development in Secondary Referral Centres”. The funding organization played no role in the study design; in the collection, analysis and interpretation of the data, in the writing of the report; or in the decision to submit the report for publication.

Author contributions: Ole D. Wolthers wrote the protocol, required funding, executed the protocol, took part in data processing and interpreted the data. He also wrote the paper. Mark Lomax commented on the protocol, took part in data processing and performed the statistical analysis. He also commented on the paper. Anne Vibeke Schmedes commented on the protocol, was responsible for the urine measurements and commented on the paper. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Competing interests: None declared.

Informed consent: Oral and written information about the study rationale were given to children and parents separately. Firstly, parents were informed of the study at parent-teacher conferences arranged by the school (Randers Realskole) and were asked for their written informed consent. Thereafter, children whose parents had consented were informed about the study procedures in their classrooms and were asked for their assent to participate in the study.

Ethical approval: The study did not require authorization from Ethics Committee.

References


