CLINICAL STUDY

The clinical utility of alternative, less invasive sampling techniques in the assessment of oral hydrocortisone therapy in children and adolescents with hypopituitarism

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Abstract

Objective: The aim of glucocorticoid replacement therapy in ACTH-deficient patients is to mimic the normal diurnal variation of cortisol. However, current hydrocortisone (HC) replacement results in prolonged episodes of hypocortisolaemia and supraphysiological peaks. Plasma cortisol profiles are an accurate yet labour-intensive method of assessing HC replacement. Salivary and bloodspot cortisol sampling methods are less invasive and may be useful tools for assessing glucocorticoid replacement, particularly in children. Therefore, we aimed to define normal salivary and bloodspot cortisol levels in children and their correlations with the gold standard (plasma cortisol).

Design: Cross-sectional study in a paediatric teaching hospital.

Methods: Plasma, saliva and bloodspot cortisol profiles were performed on 30 ACTH-deficient children and 22 healthy siblings.

Results: In ACTH-deficient patients taking oral HC, the bloodspot–plasma correlation ($r = 0.90$) was stronger than the salivary–plasma correlation ($r = 0.49$). Using target ranges for salivary and bloodspot cortisol levels based on normal data from control subjects, the less invasive sampling methods had low rates of agreement with plasma cortisol target ranges (saliva 65% and bloodspot 75%). Using the plasma–bloodspot correlation regression equation to convert bloodspot to calculated plasma cortisol, there was a high concordance between calculated and actual measured plasma cortisol (88%).

Conclusion: Bloodspot cortisol sampling is a feasible and accurate method for monitoring oral HC replacement in paediatric patients without necessitating hospital admission, but salivary sampling is not useful.

Background

Although it is indisputable that extreme over- or under-replacement of glucocorticoids has clinically important consequences, it is more difficult to quantify the sequelae of lesser degrees of mismatch in replacement therapy. Whilst subclinical over-replacement has been associated with impaired glucose tolerance (1) and increased markers of bone turnover (2), it remains unclear whether unphysiological cortisol profiles and daily cortisol fluctuations result in acute adverse effects. Until there is definite evidence that no harm is caused by prolonged episodes of hypocortisolaemia alternating with supraphysiological peaks, the commonly accepted practice of attempting to mimic the normal diurnal variation of plasma cortisol when replacing glucocorticoids in adrenocorticotrophin (ACTH)-deficient patients should continue. Promising research to develop a delayed and sustained release hydrocortisone (HC) preparation is ongoing (3). Despite this, it is unlikely that any such future commercial preparation will be amenable to the small dose adjustments required for growing infants and children.

While cortisol profiles are an accurate method of assessing HC replacement and may prevent excessive doses by allowing dose reductions in response to post-dose cortisol peaks (4, 5), they are labour-intensive and other groups have been less convinced of their usefulness (6). Salivary cortisol collection, being non-invasive and pain-free (therefore relatively stress-free), may be a useful tool for assessment of the hypothalamo-pituitary-adrenal axis and glucocorticoid replacement particularly in children. It has also been successfully used to screen for Cushing’s syndrome in children (7, 8). Capillary bloodspot collection onto filter paper, rarely used in paediatric practice, has been used in research studies in the assessment of children with adrenal insufficiency (9), neonates suspected of having...
congenital adrenal hyperplasia (10) and ill preterm neonates (11). Bloodspot sampling is useful in monitoring HC replacement in ambulant adult patients (4) and has a high degree of concordance with plasma cortisol levels (12). The technique of bloodspot collection has been successfully taught to parents and used in the home to monitor paediatric cortisol levels (9).

As both salivary and bloodspot tests are minimally invasive, they may be an alternative to hospital admission for assessment of cortisol levels in paediatric practice. Therefore, we aimed to define normal diurnal salivary and bloodspot cortisol values for children and adolescents and assess the correlations between the gold standard (plasma total cortisol) and these less invasive sampling techniques.

Subjects and methods

Subjects

The study population consisted of 30 children and adolescents (18 males) 5.1–18.5 years of age with hypopituitarism attending the Endocrine Clinic at The Children’s Hospital at Westmead. Any patient previously diagnosed with ACTH deficiency was eligible for the study. Patients who had required stress dose steroids during the previous 3 months were excluded. Twenty-two patients were taking daily oral HC and eight patients who were asymptomatic took HC only during times of physiological stress. Other pituitary hormone deficiencies were satisfactorily replaced with thyroxine (n = 25), biosynthetic human growth hormone (GH) (n = 19), intranasal desmopressin acetate (n = 6), oral desmopressin acetate (n = 4), oral oestradiol valerate (two females), oral testosterone undecanoate (four males) and s.c. testosterone implants (one male). No patient had hyperprolactinaemia. Seven GH-deficient patients who were growing satisfactorily without GH therapy (n = 4) or had achieved near final height (n = 3) were not receiving GH therapy.

To assess the HC regimens in the patients with hypopituitarism plasma, salivary and bloodspot cortisol profiles were assessed from 0800 to 2100 h. The characteristics of the patients and control subjects are detailed in Table 1.

The study was approved by the Ethics Committee of The Children’s Hospital at Westmead. All participants and their parents gave informed consent/assent after receiving a detailed verbal explanation and written information about the study.

Experimental design

Patients were admitted at 0700 h to the Endocrine Testing Unit at The Children’s Hospital at Westmead.
by pricking a cleaned fingertip using a lancet. The capillary blood was blotted onto filter paper (Schleicher & Schuell, Bogen 100, Dassell, Germany) to fully saturate two 6 mm diameter circles. The bloodspots were air-dried at room temperature and stored in individual zip-locked bags at −20 °C until analysis.

**Laboratory assays**

Total cortisol concentration in venous plasma was evaluated using an Immulite 1000 Cortisol chemiluminescence immunoassay on an Immulite 1000 Analyser (Diagnostics Products Corporation, Los Angeles, CA, USA) as this is the method used in clinical practice at our institution.

Due to the low cortisol levels in saliva and bloodspot diluent, and specimen matrixes being incompatible with the Immulite Analyser, analysis using immunoassay was not possible. Therefore, cortisol levels in saliva and bloodspots were evaluated using an antibody coated-tube radioimmunoassay technique (Spectria Cortisol RIA, Orion Diagnostica, Espoo, Finland). For saliva, 150 µl standards, controls and unknown saliva samples were pipetted into antibody-coated tubes. Subsequently, 500 µl cortisol tracer were added. The tubes were incubated for 30 min at 37 °C in a water bath and after decanting, each tube was counted with a gamma counter (LKB Wallac 1261 Multigamma II, Turku, Finland).

Bloodspot standards were prepared by combining kit plasma standards with PBS-washed blood cells from heparinised blood (giving ≈ 45% haematocrit). Blood was then pipetted onto filter paper and treated like bloodspot controls and samples. For bloodspots, one disc (6 mm diameter) was obtained using a standard hole-puncher and inserted into the antibody-coated tube along with 500 µl cortisol tracer. The tubes were shaken for 30 min, incubated for 2 h at 37 °C in a water-bath, decanted and counted using a gamma counter.

The assay lower detection limits for plasma, salivary and bloodspot cortisol were 10, 0.6 and 50 nmol/l respectively. The mean inter-assay coefficients of variation were 6.0, 8.6 and 4.2% for plasma, saliva and bloodspot cortisol respectively. The cortisol conversion factor is 27.625 nmol/l = 1 µg/dl.

**Statistical analysis**

Data were analysed using Statistical Package for Social Sciences, version 13.0 (SPSS, Chicago, IL, USA) and graphs prepared in SigmaPlot, version 8.0.2. Normal ranges for plasma, salivary and bloodspot cortisol levels were derived using the 10th and 90th percentiles for control subjects at each time point (see Fig. 1).

Plasma and salivary cortisol values were not normally distributed (assessed by Kolmogorov–Smirnov test) therefore non-parametric correlation coefficients (Spearman’s $\rho$) were used. Bland–Altman plots were used to compare the performance of bloodspot and plasma cortisol (13), but not to compare saliva and plasma due to the difference in magnitude of results between the tests.

Height, weight and body mass index SDS were calculated using the Centres for Disease Control 2000 reference standards (14).

**Results**

Reference ranges for bloodspot, salivary and plasma cortisol levels from 0800 to 2000 h, derived from 22 control subjects, demonstrated diurnal variation with peak values at 0800 h, a gradual decline throughout the day and trough values at 2000 h (Fig. 1). A high percentage of salivary samples (patients on HC 52% and controls 44%) and bloodspot samples (patients on HC 29% and controls 17%) were below the lower detection limit of the assays (salivary cut-off 0.6 nmol/l and bloodspot cut-off 50 nmol/l) and were excluded from the correlation analyses. Seventy-four per cent of the salivary and bloodspot values falling below the assay detection limits were taken 0–2 h prior to the next HC dose being due. In addition, three extreme salivary cortisol outliers, all taken 1 h after oral HC, were excluded (salivary cortisol levels were 1078, 956 and 354 nmol/l with corresponding plasma cortisol values of 46, 267 and 259 nmol/l respectively).
In control subjects, bloodspot cortisol and salivary cortisol levels correlated significantly with the plasma cortisol (see Fig. 2a and b). In the 22 ACTH-deficient patients taking oral HC, the bloodspot–plasma correlation was similar to that seen in controls. The salivary–plasma correlation was lower but remained significant (see Fig. 2c and d).

Overall, bloodspot cortisol levels were higher than plasma cortisol (mean difference, plasma minus bloodspot: controls $K_{33.4}$G$109.2$ nmol/l, patients $K_{63.7}$G$100.1$ nmol/l). For mean cortisol values <200 nmol/l, plasma values were higher than bloodspot for controls but not for ACTH-deficient patients on oral HC. Above 200 nmol/l, control bloodspot values tended to be higher than control plasma values (see Fig. 3a and b).

Using target ranges for salivary and bloodspot cortisol levels based on normal data derived from control subjects (see Fig. 1), the less invasive sampling methods had low but similar rates of agreement with plasma cortisol target ranges (see Table 2). The concordance rate for saliva and plasma cortisol was 65% with a weighted $\kappa$ score of 0.68. For bloodspot and plasma, the concordance was 75% and the weighted $\kappa$ score 0.77.

Since there was a strong correlation between bloodspot and plasma cortisol values in both controls and patients, the linear regression equation obtained from Fig. 2c (bloodspot cortisol = 1.1649 (calculated plasma cortisol) + 30.6199) was used to convert bloodspot cortisol levels into calculated plasma cortisol levels. Using this method, the concordance of calculated plasma cortisol with actual measured plasma cortisol was 88% (see Table 2) and the weighted $\kappa$ score was 0.87.

In eight ACTH-deficient patients not taking regular HC, the bloodspot–plasma and salivary–plasma...
Table 2  Concordance rates between (a) saliva and plasma cortisol values (b) bloodspot and plasma cortisol values and (c) calculated and actual plasma cortisol values for ACTH-deficient patients on oral HC in relation to specific plasma, bloodspot and salivary normal ranges.

<table>
<thead>
<tr>
<th>Plasma values</th>
<th>Below normal range</th>
<th>Within normal range</th>
<th>Above normal range</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Saliva values (n=108)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Below normal range</td>
<td>39</td>
<td>18</td>
<td>1</td>
</tr>
<tr>
<td>Within normal range</td>
<td>4</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>Above normal range</td>
<td>3</td>
<td>6</td>
<td>23</td>
</tr>
<tr>
<td>(b) Bloodspot values (n=109)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Below normal range</td>
<td>34</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Within normal range</td>
<td>11</td>
<td>29</td>
<td>11</td>
</tr>
<tr>
<td>Above normal range</td>
<td>0</td>
<td>2</td>
<td>19</td>
</tr>
<tr>
<td>(c) Calculated plasma cortisol (n=109)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Below normal range</td>
<td>43</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Within normal range</td>
<td>1</td>
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<td>6</td>
</tr>
<tr>
<td>Above normal range</td>
<td>1</td>
<td>2</td>
<td>24</td>
</tr>
</tbody>
</table>

(a) The concordance for salivary plasma cortisol is (39 + 8 + 23)/108 = 65%, weighted $\kappa = 0.68$. (b) The concordance for bloodspot plasma cortisol is (34 + 29 + 19)/109 = 75%, weighted $\kappa = 0.77$. (c) The concordance for calculated and measured plasma cortisol is (43 + 29 + 24)/109 = 88%, weighted $\kappa = 0.87$.

The weak saliva–plasma correlation in HC-replaced patients can probably be explained by the known limitations of salivary cortisol sampling. The very high levels of salivary cortisol exhibited in some of our patients in Fig. 2d (and in the three major outliers excluded from the analysis) may be explained by residual oral HC in the mouth. In one study, hypoadrenal patients who had ingested oral HC produced contaminated saliva specimens for 1.8 ($\pm$ 1.5) h afterwards despite mouth washing and meal consumption (16). The adults in Lovas’s study (15) may have been more adept at swallowing HC tablets, perhaps explaining the different results from our paediatric patients. In addition, fluctuations in albumin and cortisol-binding globulin (CBG) could affect the free fraction of cortisol and therefore the salivary cortisol levels. All our patients had normal albumin levels and although we did not measure CBG, we have no reason to suspect low CBG in our hypopituitary patients. The extremely low salivary values relative to the corresponding plasma cortisol may reflect the time taken for ingested HC to pass into the saliva. This estimated time lag of between 5 (17, 18) and 15 (19) min may not be accurate during the rapid, dramatic fluxes in plasma cortisol occurring after a dose of oral HC.

The high percentage of samples falling below the lower limit of assay detection is a problem particularly for salivary testing. Fifty-two per cent of patient samples and 44% of control samples were below the salivary assay cut-off of 0.6 nmol/l. The fact that salivary cortisol cannot distinguish between plasma cortisol levels below $\approx$ 200 nmol/l, makes salivary cortisol of limited use. From our data, we conclude that salivary sampling is not useful in detecting cortisol nadirs in ACTH-deficient patients on oral HC replacement. However, salivary cortisol may be useful in other clinical settings, for example, when high cortisol levels are expected, in physiological stress or when excessive endogenous cortisol secretion is suspected. Indeed, midnight salivary cortisol measurement has been shown to be a sensitive, specific and reproducible screening test for the detection of Cushing’s syndrome (7, 8, 20, 21).

Sample collection difficulties may have caused some minor variation in bloodspot results shown in Fig. 2a and c. If filter paper blood distribution was uneven or if excessive squeezing was required to fill a bloodspot, the proportion of red cells to plasma could be disrupted resulting in an inaccurate result. Bloodspot collection was tolerated in 50 out of our 52 patients. A similar level of acceptability has been reported in other studies, most importantly in one study in which parents collected the samples (9). The easy repeatability of the sampling technique and thus the ability to consider a set of values, rather than one point may help to minimise the problem of discordant result between serum and bloodspot cortisol.

Using cortisol levels calculated from the bloodspot linear regression line equation, there was a high rate of concordance between the calculated and the actual

Correlations were $0.88 (n=26, P<0.005)$ and $0.93 (n=12, P<0.005)$.

Discussion

In this study, we have described normal reference ranges for salivary and bloodspot cortisol levels throughout the day and strong correlations between bloodspot cortisol and the gold standard, plasma cortisol. Salivary cortisol, although highly acceptable as a non-invasive sampling technique, had weaker correlations with plasma cortisol, which limits its clinical use in ACTH-deficient patients.

The stronger bloodspot–plasma correlation when compared with the salivary–plasma correlation has been described previously in adult patients with hypopituitarism (12). Despite the weaker correlation of saliva with plasma cortisol, we still deemed that salivary cortisol was worthy of further study, due to the anticipated benefits of a completely pain-free sampling method. In our study, although the salivary–plasma correlation was reasonable in control subjects not taking HC ($\rho = 0.79$), the correlation in HC replaced patients was poor ($\rho = 0.49$) with more scatter around the regression line (see Fig. 2b and d). Our results differ from the salivary–plasma correlation in HC-replaced patients ($\rho = 0.83$) reported by Lovas (15). However, by sampling only after doses, they avoided low pre-dose cortisol levels around which salivary assays are less discriminatory.
plasma cortisol levels when classified as falling below, within or above the normal range. More importantly, using this system, there was a low incidence of serious misclassification. Only one low actual plasma cortisol result was classified as being a high calculated cortisol level. One patient, who had a high actual cortisol level, was erroneously classified as low based on calculated cortisol. It is not surprising that this result was at 2100 h when the range for normal plasma cortisol is narrow making misclassification more likely. Overall, this method resulted in greater concordance between bloodspot and plasma than when using the normal ranges of bloodspot cortisol from our 2.2 control subjects.

The acceptability of the bloodspot collection technique along with the high rate of agreement between calculated cortisol and actual plasma cortisol makes bloodspot sampling a feasible and accurate method for monitoring oral HC in paediatric patients without necessitating hospital admission.

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References


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