Bedtime Salivary Cortisol and Cortisone by LC-MS/MS in Healthy Adult Subjects: Evaluation of Sampling Time

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The measurement of late-night salivary cortisol is a mainstay in the diagnosis of Cushing syndrome. Furthermore, the measurement of salivary cortisol is useful in assessing the cortisol awakening response. Because the salivary glands express 11-β-hydroxysteroid dehydrogenase, the measurement of salivary cortisone may improve the performance of salivary corticosteroid measurements. We measured salivary cortisol by enzyme immunoassay (EIA) and salivary cortisol and cortisone by liquid chromatography-tandem mass spectrometry (LC-MS/MS) in only 50 μL of saliva sampled from 54 healthy subjects (aged 20 to 64 years). We allowed patients to sample at their normal bedtime (2025 to 2400 hours) to answer a common question as to whether sampling at the normal bedtime is equivalent to the standard required sampling at 2300 to 2400 hours. We found that the salivary cortisol and cortisone results by LC-MS/MS correlated well with salivary cortisol measured with the US Food and Drug Administration-cleared EIA. Furthermore, the upper limit of normal of salivary cortisol by EIA for bedtime samples was lower than the previously published upper limit of normal with sampling required at 2300 to 2400 hours. There were no significant effects of age or sex on any of the salivary steroid measurements. We conclude that (i) salivary cortisol and cortisone can be reliably measured by LC-MS/MS in small volumes of saliva and (ii) that patients can be evaluated using saliva sampled at their normal bedtime, rather than being required to stay awake until 2300 to 2400 hours.

Freeform/Key Words: late-night salivary cortisol, awakening, immunoassay, mass spectrometry, sex

Measurement of late-night salivary cortisol is now one of the mainstays of the diagnosis of Cushing syndrome [1–10]. The majority of prior studies used immunoassays for the measurement of salivary cortisol with a sensitivity and specificity for Cushing syndrome of greater than 90% [11, 12]. Because the salivary gland expresses 11-β-hydroxysteroid dehydrogenase [13], salivary cortisone is significantly greater than salivary cortisol, and the assessment of salivary cortisone may be useful in the diagnosis of Cushing syndrome, as an index of cortisol exposure, and in the evaluation of the stress response in humans [13–23]. Furthermore, the measurement of salivary cortisol and cortisone has been used in the evaluation of the dynamics of the hypothalamic-pituitary-adrenal (HPA) axis in humans including the awakening response [13, 14, 20, 24–29]. This has led to the possibility that, under certain circumstances, the measurement of salivary cortisone by liquid chromatography-tandem mass spectrometry

Abbreviations: CV, coefficient of variation; EIA, enzyme immunoassay; HPA, hypothalamic-pituitary-adrenal; LC-MS/MS, liquid chromatography-tandem mass spectrometry.

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(LC-MS/MS) may be useful in addition to salivary cortisol, or perhaps even superior in the evaluation of the HPA axis.

The purpose of this study is to explore salivary cortisol and cortisone measurements in healthy subjects with a wide age range and compare those results to the standard, US Food and Drug Administration-cleared salivary cortisol enzyme immunoassay (EIA) currently in use [30]. In particular, our focus is whether there is a requirement to stay awake until 2300 to 2400 hours as is now routinely done, or whether subjects and patients can sample at their normal bedtime. We also sought to validate a modified LC-MS/MS method using only 50 μL of saliva as we often receive clinical samples with <100 μL of saliva, and compare its performance to our standard EIA.

1. Methods

A. Healthy Subjects

Adult subjects (N = 54) were recruited from employees at the Aurora Research Institute and Aurora St. Luke's Medical Center under a protocol approved by the Aurora Institutional Review Board. Because samples were de-identified, the institutional review board determined that written informed consent was not required. The only information obtained from the subjects was age [median 39 years (range 20 to 64 years)] and sex (35 females/19 males). All of the subjects were daytime workers (no nightshift workers), and all reported normal sleep-wake patterns. The only other exclusion was the use of any form of exogenous corticosteroids including topical, inhaled, nasal, and/or oral. Subjects were instructed to refrain from smoking for at least 2 hours before sampling. Subjects obtained saliva samples at their normal bedtime [median 2257 hours (range 2025 to 2400 hours)] and upon their normal awakening time [median 0611 hours (range 0500 to 0810 hours)]. Saliva samples were obtained using the Sarstedt Plain Cotton Salivette [9].

B. Assays

B-1. Enzyme immunoassay

Salivary cortisol was measured in duplicate by EIA (Salimetrics 1-3102, State College, PA) [31] as described and validated previously [30]. Briefly, the assay requires 50 μL of saliva for duplicate measurements. The lower detection limit is 0.3 nmol/L. The intraassay imprecision [coefficient of variation (CV)] is 5.2% at 3.1 nmol/L (n = 10) and 2.6% at 10.4 nmol/L (n = 10). Interassay (total) imprecision (CV) is 11% at 2.8 nmol/L (n = 10), 11% at 10.1 nmol/L (n = 10), and 6.9% at 25.0 nmol/L (n = 10). Relevant endogenous steroid cross-reactivities are cortisone (0.13%), 11-deoxycortisol (0.16%), and corticosterone (0.21%). Quality control samples are run in each assay, and no significant assay drift has been detected in the EIA for over 10 years. This was verified by reassaying College of American Pathologists Proficiency Testing Samples from 2013 to 2016, which gave similar results to the original Proficiency Testing submission.

B-2. LC-MS/MS

Salivary cortisol and cortisone were measured by LC-MS/MS using methods modified from [32–34]. Saliva and quality controls (50 μL) were combined with D.I. water (150 μL) and 10 μL of 138 nmol/L deuterium-labeled cortisol-d4 and cortisone-d7 (IsoSciences LLC, Ambler, PA) then extracted with 2 mL of methyl *tert*-butyl ether. We chose to sacrifice some sensitivity to use only 50 μL of saliva as compared with 100 to 500 μL typically used [16, 17, 21, 22, 35] because it is common for us to receive clinical samples with <100 μL of saliva. The ether phase was then transferred and evaporated to dryness under nitrogen in a 35°C water bath and reconstituted in 100 μL of 50:50 (volume-to-volume ratio) methanol-to-water ratio. Cortisol and cortisone were measured using a 1290 Infinity HPLC (Agilent Technologies, Palo Alto, CA) and a triple-quadrupole LC-MS

(Agilent Technologies) with an electrospray ionization ion source in positive mode. Cortisol and cortisone standards (0.05 to 89.4 nmol/L) were created in 50:50 (volume-to-volume ratio) methanol-to-water ratio using stock standards from Cerilliant Corporation (Round Rock, TX). Cortisol and cortisone were resolved on a Poroshell 120, EC-C18 (2.1 × 50 mm, 2.7 μm) analytical column (Agilent Technologies) at 50°C, combined with a 0.3-μm inline filter. The injection volume and mobile phase flow rate were 10 μL and 0.4 mL/min, respectively. Gradient elution of mobile phase consisting of 5 mM ammonium formate in water (solvent A) and 5 mM ammonium formate in methanol (solvent B) began at an initial concentration of A-B 45:55 (0 to 0.5 minutes). A linear gradient increased solvent B to 65% (0.5 to 4 minutes). Additionally, gradients were used for elution of waste components and equilibration of the column to initial conditions. MassHunter software (Agilent Technologies) was used to control the instruments and analyze the data. The MS scan type was multiple-reaction monitoring with cortisol quantified and qualified by the ion transitions m/z 363.2/121.1 and m/z 363.2/91.1, respectively, whereas cortisone was quantified and qualified by ion transitions m/z 361.2/163.1 and m/z 361.2/121.1, respectively. The Cortisol-d4 and Cortisone-d7 internal standards were analyzed at ion transitions m/z 367.2/121.1 and m/z 369.2/169.1, respectively. The following source conditions were used: gas temperature, 250°C; gas flow, 11 l/min; nebulizer pressure, 35 psi; sheath gas temperature, 350°C; sheath gas flow, 11 l/min; capillary voltage, 3000 V; nozzle voltage, 0 V; and electron multiplier voltage, 300 V. All cortisol-related compounds had a fragmentor voltage of 105 V, and all cortisone-related compounds had a fragmentor voltage of 120 V. Collision energy for cortisol quantification, qualification, and deuterated internal standard ions was 24, 60, and 24 V, respectively. Collision energy for cortisone quantification, qualification, and deuterated internal standard ions was 20, 32, and 20 V, respectively.

The functional sensitivity, set at a threshold CV of 10%, was 0.053 (SD 0.004) nmol/L for cortisol and 0.053 (SD 0.002) nmol/L for cortisone. Therefore, the analytic range of the LC-MS/MS method for cortisol and cortisone was conservatively set at 0.1 to 89.4 nmol/L without additional dilution, and to 27,600 nmol/L with additional sample dilutions. The intrassay variability (N = 10) for cortisol was 7.1% at 1.4 nmol/L, 3.1% at 5.8 nmol/L, and 2.7% at 10.3 nmol/L and for cortisone was 4.8% at 4.2 nmol/L, 3.2% at 23.3 nmol/L, and 2.9% at 31.9 nmol/L. The interassay variability (N = 20) for cortisol was 11.1% at 1.3 nmol/L, 6.5% at 4.6 nmol/L, and for cortisone was 8.5% at 3.6 nmol/L and 5.8% at 20.2 nmol/L. Deming (unbiased) regression of the cortisol results compared with College of American Pathologists Proficiency Testing LC-MS/MS median cortisol results (N = 24) was slope 0.91 [0.89 to 0.94 (95% CI)], Y-intercept −0.30 nmol/L [−1.15 to 0.55 (95% CI)], and $r^2 = 0.998$ ($P < 0.0001$). These results agree with our extraction recoveries for cortisol of 88% to 94% and for cortisone of 91% to 110%, and are similar to recoveries achieved by a similar method [14].

C. Statistical Analysis

Data are reported as mean/SD when normally distributed and median/25% to 75% CI when not normally distributed. A conservative approach to the upper limit of normal was calculated as mean plus <2, <3, and <4 SDs above the mean. Data were evaluated by *t* test when data were normally distributed and Mann-Whitney nonparametric test when data were not normally distribution (SigmaPlot 12.5, Systat Software, Inc., San Jose, CA). Salivary results vs clock time and cortisol-to-cortisone ratio vs salivary cortisol by LC-MS/MS were evaluated by linear regression. Regression/correlation sample size analysis for salivary results vs clock time were evaluated using a power of 0.80 and an α of 0.05. Unbiased comparisons of methods were performed by Deming regression [36].

2. Results

Fifty-four subjects returned late night (bedtime) and morning (awakening) saliva samples. One late-night (female) sample was excluded due to saliva contamination with topical hydrocortisone [15] and one morning (male) sample was excluded because the saliva volume was

insufficient (<75 μL) for measurement using both the EIA and LC-MS/MS methods. Therefore, 53 late-night and 53 morning results are presented.

Table 1 shows the results for all subjects and by sex. The upper limit of normal was conservatively calculated as less than the mean plus 3 and 4 SDs above the mean; for the late-night sample, it was <2.9 and <3.5 nmol/L, respectively, for the cortisol EIA. When using a less conservative calculation (less than the mean plus 2 SDs), it was <2.3 nmol/L which is 2.0 nmol/L less than the upper limit of normal for required 2300-hour sampling for the same EIA performed in our laboratory in samples from 73 healthy subjects (<4.3 nmol/L) [9, 30]. The upper limit of normal was significantly less for cortisol by LC-MS/MS and higher for cortisone by LC-MS/MS, and the median cortisol-to-cortisone ratio for LC-MS/MS measurement was 0.2. The morning samples were all greater than late-night samples as expected, and the median cortisol-to-cortisone ratio (0.3) was greater than the late-night samples. The morning salivary cortisone results were quite variable with a large reference range (*i.e.*, high upper limit of normal). There were no differences between men and women for any of the findings in Table 1.

Deming regression showed excellent correlation between salivary cortisol by EIA vs LC-MS/MS with a slope of 0.65 (Fig. 1) indicating that the LC-MS/MS yields lower cortisol results and upper limits of normal compared with EIA (see Table 1). Salivary cortisol by EIA and by LC-MS/MS correlated well with salivary cortisone, the latter being significantly higher than cortisol. Notice that the relationships between LC-MS/MS or EIA cortisol and LC-MS/MS cortisone are slightly concave again suggesting that the cortisol-to-cortisone ratio increases as cortisol secretion increases. This analysis was confirmed by a significant, positive linear relationship between awakening salivary cortisol by LC-MS/MS and the salivary cortisol-to-cortisone ratio by LC-MS/MS, which ranged from 0.16 to 0.46 (Fig. 2).

The time of normal bedtime sampling had no effect on salivary cortisol or cortisone (Fig. 3). To confirm that this conclusion would not change if more subjects were evaluated, we performed a sample size analysis (power 0.80; α 0.05) using the data with the highest r^2 value (0.03) in the salivary cortisol panel vs bedtime. This analysis revealed that over 8700 bedtime samples would be required to show a significant effect of bedtime, so it is unlikely that the lack of the effect of bedtime was due to the number of subjects studied. As described above, all of the late-night (bedtime) cortisol EIA results in the current study were at least 1 nmol/L less than the established upper limit of normal for the EIA with sampling performed at 2300 to 2400 hours (<4.3 nmol/L) and regardless of the subjects' normal bedtime [30]. There was also

Table 1. Salivary Cortisol by EIA and Salivary Cortisol and Cortisone by LC-MS/MS (nmol/L)

	All	All ULN			Male	Female
Late-night (bedtime)		<+2 SD	<+3 SD	<+4 SD		
Cortisol EIA	1.1 (0.6)	<2.3	<2.9	<3.5	0.9 (0.6–1.2)	1.1 (0.7–1.6)
Cortisol LC-MS/MS	0.7 (0.4)	<1.5[a]	<1.9	<2.3	0.5 (0.4–0.6)[a]	0.6 (0.5–1.0)[a]
Cortisone LC-MS/MS	4.0 (2.0)	<8.0	<10.0	<12.0	3.3 (2.4–5.0)	3.8 (2.6–5.0)
Cortisol:Cortisone	0.2 (0.1–0.2)				0.2 (0.1–0.2)	0.2 (0.1–0.2)
Morning (awakening)						
Cortisol EIA	11.5 (6.0)	<23.5	<29.5	<35.5	8.6 (6.5–20.6)	11.3 (7.8–14.0)
Cortisol LC-MS/MS	7.4 (4.1)	<15.3[a]	<19.7	<23.8	5.0 (4.5–13.2)[a]	6.7 (4.3–8.6)[a]
Cortisone LC-MS/MS	25.1 (9.5)	<44.1	<53.6	<63.1	23.5 (18.5–37.5)	27.1 (17.7–30.1)
Cortisol:Cortisone	0.3 (0.2–0.3)[b]				0.3 (0.2–0.4)[b]	0.3 (0.2–0.3)[b]

N values are 53 for "All." N values for late-night male and female are 19 and 34, respectively, and for morning are 18 and 35, respectively. [One late-night female sample had to be eliminated from inclusion because it appeared to be contaminated with topical hydrocortisone [15], and one morning male sample had to be eliminated from inclusion because the saliva sample volume was insufficient (<75 μL) for complete analysis using both assay methods.] "All" cortisol and cortisone data were normally distributed and are mean (SD). Other data were not normally distributed and are median (25%–75% CIs).

Abbreviation: ULN, upper limit of normal for All calculated as < mean +3 or +4 SDs from the mean.

[a]Cortisol by EIA is significantly greater than cortisol by LC-MS/MS within a column.

[b]Morning Cortisol:Cortisone ratios were significantly greater than late-night ($P < 0.001$).

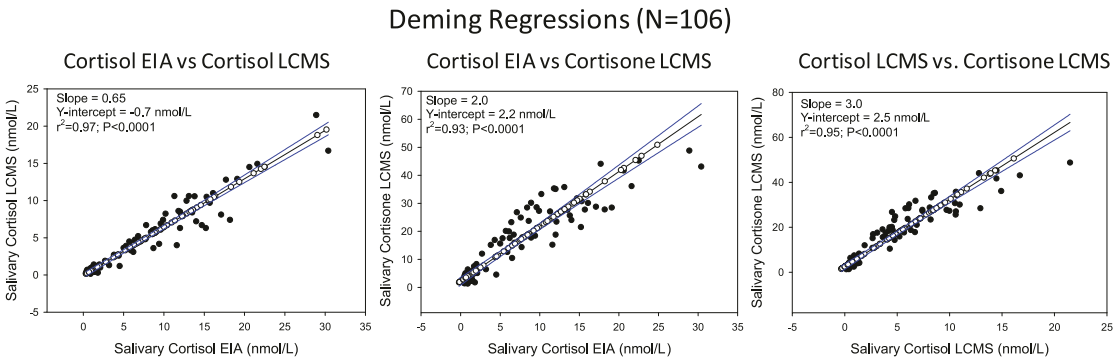

Figure 1. Deming regressions of salivary cortisol by EIA vs salivary cortisol or cortisone by LC-MS/MS (LCMS), and of salivary cortisol vs cortisone by LC-MS/MS. Deming statistics are shown in the figure labels. Open circles are imputed results that generated the slope; the outer lines are 99% confidence limits.

no significant effect of awakening time on any of the salivary measurements performed. However, a formal evaluation of the "cortisol awakening response" per strict criteria [37] would be required to more carefully examine the effect of awakening clock time. There was no significant effect of age on any of the results (data not shown).

3. Discussion

We have demonstrated that (i) healthy adult subjects can sample at their normal bedtime and achieve lower salivary cortisol results than if forced to remain awake until 2300 hours or later [21, 30], (ii) that a modified LC-MS/MS method using only 50 μL of saliva performs well in the measurement of salivary cortisol and cortisone, (iii) that the ratio of cortisol to cortisone is higher in the morning when both cortisol and cortisone concentrations are greater (compared with the evening) and that this ratio is positively correlated with salivary cortisol, (iv) morning cortisol and cortisone at awakening are quite variable and unlikely to be useful by themselves for the diagnosis of adrenal insufficiency, and (v) there was no effect on late-night cortisol or cortisone based on sex or age in adults <65 years of age.

One of the most common inquires to the clinical laboratory from patients being screened for Cushing syndrome using late-night salivary cortisol is whether the patient is really required to stay awake until 2300 hours to obtain a "late-night" salivary sample. Most previous studies

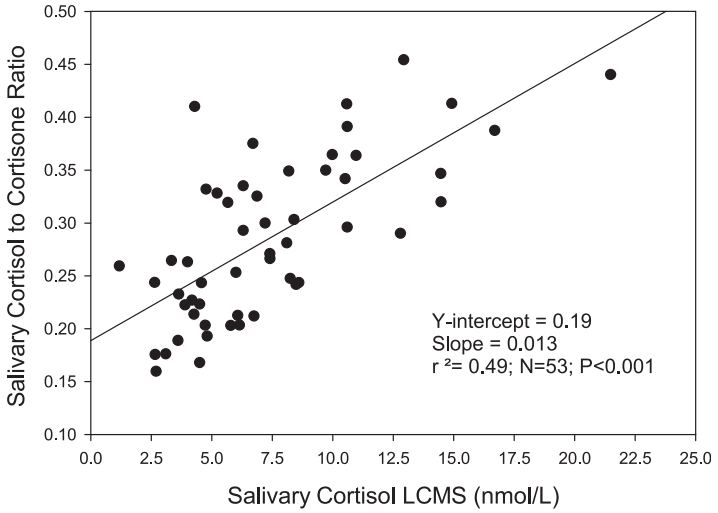

Figure 2. Correlation of awakening salivary cortisol by LC-MS/MS (LCMS) and the ratio of salivary cortisol to cortisone by LC-MS/MS. Regression statistics are shown in the figure.

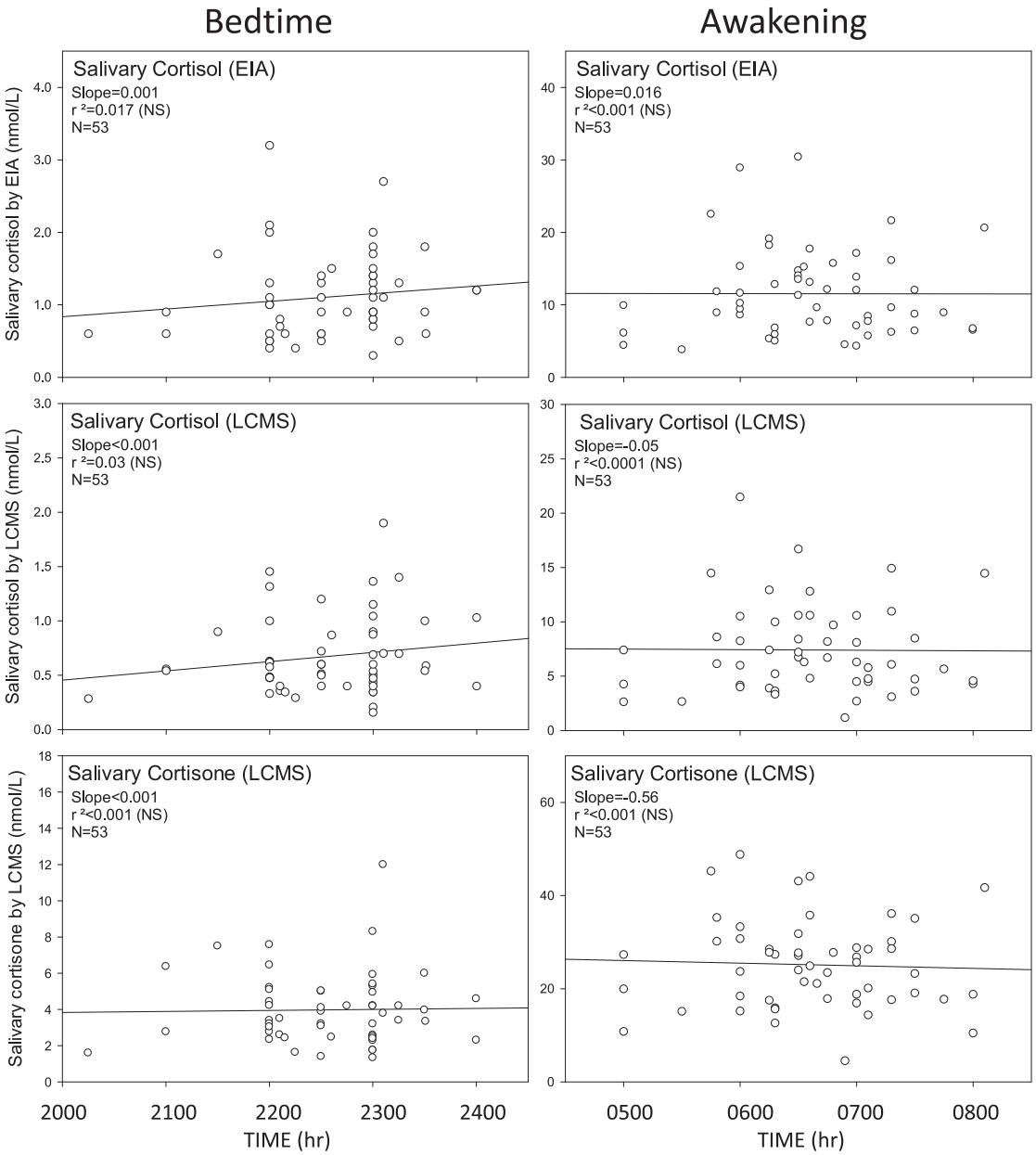

Figure 3. Lack of significant correlation between times of sampling (bedtime or awakening) vs salivary cortisol by EIA or LC-MS/MS (LCMS) or salivary cortisone. Regression statistics are shown in the figure label.

enforced a 2300-hour or later sampling time (reviewed in [7, 8]). One study demonstrating an excellent sensitivity (96%) and specificity (98%) for Cushing syndrome did state that patients were sampled at bedtime [22]. Although specific bedtimes were not given, this study was from Italy where, typically, bedtime is considerably later than the Midwestern United States [38], which makes this issue less of a confounder than in our patients with a bedtime significantly earlier than 2300 hours. A study from Budapest, Hungary, found consistently higher "late-night" (without defining the sampling time) immunoassay cortisol and LC-MS/MS cortisol and cortisone results compared with our results [21]. Investigators should be encouraged to record and report actual sampling times when doing these types of studies. In a study in healthy subjects in a clinical research center, we showed that serum and salivary cortisol reached its nadir by 2000 hours [39]. It seems reasonable to be assured from our results that

saliva sampling at the normal bedtime is as good if not better to achieve unstressed levels than requiring patients to stay awake past their normal bedtime to 2300 hours or later.

Our LC-MS/MS method reliably measured salivary cortisol and cortisone in only 50 μL of saliva. Salivary cortisol concentrations by LC-MS/MS were consistently lower than by the Salimetrics EIA. The magnitude of the difference agrees with a previous study [40] and is likely due to antibody cross-reactivity with other corticosteroid metabolites in saliva in addition to cortisone in the EIA.

Although the Salivette method typically yields at least 250 μL of saliva in healthy, normal subjects, one of our subjects had less than 75 μL in one of their samples. In our over 20 years of experience using late-night salivary cortisol to screen patients for Cushing syndrome [9, 29], it is not uncommon to receive clinical samples with <75 μL of saliva. We estimate that ~1 in 150 to 200 clinical samples have less than 50 μL of saliva. It is inconvenient and cumbersome to reorder the test and to obtain additional samples from a patient, so it is an advantage to minimize the volume of saliva necessary.

A previous study found a mean late-night salivary cortisol by LC-MS/MS of 19 ng/dL (0.5 nmol/L) and an optimal cutoff of 70 ng/dL (<1.9 nmol/L) [35]. This agrees with our mean bedtime LC-MS/MS cortisol result (0.7 nmol/L) and upper limit of normal set conservatively at <+3 SD above the mean (<1.9 nmol/L) (Table 1). Although the healthy elderly (~70 years of age) have a slightly increased upper limit of normal late-night salivary cortisol [41], our data demonstrate that this is not the case in subjects up to 64 years of age. This agrees with the CIRCORT database study [42]. We also confirm no male-female differences in salivary cortisol [41] except perhaps due to sex differences in the timing of the onset of puberty [42], which is not relevant in the current study in postpubertal adults.

The measurement of bedtime salivary cortisone may improve sensitivity and specificity for Cushing syndrome [21, 22]. However, the improvement would be, at most, 5% to 8% above the already excellent sensitivity and specificity (≥90%) [12]. It remains to be seen whether all reference laboratories switch to LC-MS/MS to measure and report *both* salivary cortisol and cortisone to improve the overall diagnostic performance for Cushing syndrome compared with immunoassay [43]. Using LC-MS/MS does not mitigate the lack of usefulness of late-night salivary cortisol measurements for the evaluation of patients with adrenal incidentalomas, although salivary cortisone clearly has potential in this regard [23, 44].

There are other clinical circumstances where the measurement of salivary cortisone may be useful in addition to cortisol. In particular, measurement of salivary cortisone may be useful in assessing endogenous glucocorticoid exposure over a day [19], the evaluation of hydrocortisone (*i.e.*, cortisol) replacement therapy [45, 46], and with cosyntropin stimulation testing for adrenal insufficiency [16, 27, 45]. In that regard, the ratio of cortisol to cortisone increases with stimulated cortisol secretion after cosyntropin administration [16, 27, 45]. This is consistent with the higher cortisol-to-cortisone ratio we observed in the morning vs evening sampling and the positive correlation of cortisol-to-cortisone ratio vs salivary cortisol in the current study. Finally, a major advantage of measuring salivary cortisone is to detect saliva sample contamination with topical or oral hydrocortisone (authentic cortisol) as these preparations do not contain cortisone [15]. In fact, one group has suggested "reflexing" any saliva samples with increased or equivocal late-night salivary cortisol immunoassay results to LC-MS/MS for measurement of cortisol and cortisone, which decreased the false-positive results significantly [47]. Because most screening late-night salivary cortisol measurements are typically normal and assay-mediated false-negative results are rare, reflexing all samples with elevated results to LC-MS/MS would require little additional work and expense on the part of the clinical laboratory and makes a lot of sense.

Our morning (awakening) salivary cortisol and cortisone results confirm that it is unlikely that one awakening salivary cortisol result will be a useful measurement for the diagnosis of adrenal insufficiency without actually formally measuring the cortisol awakening response [48] or without cosyntropin stimulation [16, 27, 45].

We conclude that normal bedtime salivary cortisol assessment yields equivalent or even better unstressed salivary cortisol and cortisone measurements compared with an enforced 2300-hour or later sampling time and that age (up to 64 years of age) and sex are not significant confounders when assessing HPA dynamics in adults.

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Additional Information

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References and Notes

1. Nieman LK, Biller BM, Findling JW, Newell-Price J, Savage MO, Stewart PM, Montori VM. The diagnosis of Cushing's syndrome: an Endocrine Society Clinical Practice Guideline. *J Clin Endocrinol Metab*. 2008;**93**(5):1526–1540.
2. Findling JW, Raff H. Cushing's Syndrome: important issues in diagnosis and management. *J Clin Endocrinol Metab*. 2006;**91**(10):3746–3753.
3. Findling JW, Raff H. Diagnosis and differential diagnosis of Cushing's syndrome. *Endocrinol Metab Clin North Am*. 2001;**30**(3):729–747.
4. Findling JW, Raff H. Newer diagnostic techniques and problems in Cushing's disease. *Endocrinol Metab Clin North Am*. 1999;**28**(1):191–210.
5. Findling JW, Raff H. Screening and diagnosis of Cushing's syndrome. *Endocrinol Metab Clin North Am*. 2005;**34**(2):385–402, ix–x.
6. Raff H. Cushing's syndrome: diagnosis and surveillance using salivary cortisol. *Pituitary*. 2012;**15**(1): 64–70.
7. Raff H. Cushing syndrome: update on testing. *Endocrinol Metab Clin North Am*. 2015;**44**(1):43–50.
8. Raff H. Update on late-night salivary cortisol for the diagnosis of Cushing's syndrome: methodological considerations. *Endocrine*. 2013;**44**(2):346–349.
9. Raff H, Raff JL, Findling JW. Late-night salivary cortisol as a screening test for Cushing's syndrome. *J Clin Endocrinol Metab*. 1998;**83**(8):2681–2686.
10. Raff H. The role of salivary cortisol determinations in the diagnosis of Cushing's syndrome. *Curr Opin Endocrinol Diabetes*. 2004;**11**(5):271–275.
11. Carroll T, Raff H, Findling JW. Late-night salivary cortisol measurement in the diagnosis of Cushing's syndrome. *Nat Clin Pract Endocrinol Metab*. 2008;**4**(6):344–350.
12. Carroll T, Raff H, Findling JW. Late-night salivary cortisol for the diagnosis of Cushing syndrome: a meta-analysis. *Endocr Pract*. 2009;**15**(4):335–342.
13. Blair J, Adaway J, Keevil B, Ross R. Salivary cortisol and cortisone in the clinical setting. *Curr Opin Endocrinol Diabetes Obes*. 2017;**24**(3):161–168.
14. Lee S, Kwon S, Shin HJ, Lim HS, Singh RJ, Lee KR, Kim YJ. Simultaneous quantitative analysis of salivary cortisol and cortisone in Korean adults using LC-MS/MS. *BMB Rep*. 2010;**43**(7):506–511.
15. Raff H, Singh RJ. Measurement of late-night salivary cortisol and cortisone by LC-MS/MS to assess preanalytical sample contamination with topical hydrocortisone. *Clin Chem*. 2012;**58**(5):947–948.
16. Perogamvros I, Owen LJ, Newell-Price J, Ray DW, Trainer PJ, Keevil BG. Simultaneous measurement of cortisol and cortisone in human saliva using liquid chromatography-tandem mass spectrometry: application in basal and stimulated conditions. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2009; **877**(29):3771–3775.

17. Magda B, Dobi Z, Mészáros K, Szabó É, Márta Z, Imre T, Szabó PT. Charged derivatization and on-line solid phase extraction to measure extremely low cortisol and cortisone levels in human saliva with liquid chromatography-tandem mass spectrometry. *J Pharm Biomed Anal*. 2017;**140**:223–231.
18. Mezzullo M, Fanelli F, Fazzini A, Gambineri A, Vicennati V, Di Dalmazi G, Pelusi C, Mazza R, Pagotto U, Pasquali R. Validation of an LC-MS/MS salivary assay for glucocorticoid status assessment: Evaluation of the diurnal fluctuation of cortisol and cortisone and of their association within and between serum and saliva. *J Steroid Biochem Mol Biol*. 2016;**163**:103–112.
19. Harrison RF, Debono M, Whitaker MJ, Keevil BG, Newell-Price J, Ross RJ. Salivary cortisone to estimate cortisol exposure and sampling frequency required based on serum cortisol measurements. *J Clin Endocrinol Metab*. 2019;**104**(3):765–772.
20. Bae YJ, Reinelt J, Netto J, Uhlig M, Willenberg A, Ceglarek U, Villringer A, Thiery J, Gaebler M, Kratzsch J. Salivary cortisone, as a biomarker for psychosocial stress, is associated with state anxiety and heart rate. *Psychoneuroendocrinology*. 2019;**101**:35–41.
21. Mészáros K, Karvaly G, Márta Z, Magda B, Tőke J, Szücs N, Tóth M, Rácz K, Patócs A. Diagnostic performance of a newly developed salivary cortisol and cortisone measurement using an LC-MS/MS method with simple and rapid sample preparation. *J Endocrinol Invest*. 2018;**41**(3):315–323.
22. Antonelli G, Ceccato F, Artusi C, Marinova M, Plebani M. Salivary cortisol and cortisone by LC-MS/MS: validation, reference intervals and diagnostic accuracy in Cushing's syndrome. *Clin Chim Acta*. 2015; **451**(Pt B):247–251.
23. Ceccato F, Barbot M, Albiger N, Antonelli G, Zilio M, Todeschini M, Regazzo D, Plebani M, Lacognata C, Iacobone M, Mantero F, Boscaro M, Scaroni C. Daily salivary cortisol and cortisone rhythm in patients with adrenal incidentaloma. *Endocrine*. 2018;**59**(3):510–519.
24. Raff H. Utility of salivary cortisol measurements in Cushing's syndrome and adrenal insufficiency. *J Clin Endocrinol Metab*. 2009;**94**(10):3647–3655.
25. Raff H, Biru N, Reisinger N, Kramer DJ. Dissociation of ACTH and cortisol in septic and non-septic ICU patients. *Endocrine*. 2017;**55**(1):307–310.
26. Raff H, Ettema SL, Eastwood DC, Woodson BT. Salivary cortisol in obstructive sleep apnea: the effect of CPAP. *Endocrine*. 2011;**40**(1):137–139.
27. Elder CJ, Harrison RF, Cross AS, Vilela R, Keevil BG, Wright NP, Ross RJ. Use of salivary cortisol and cortisone in the high- and low-dose synacthen test. *Clin Endocrinol (Oxf)*. 2018;**88**(6):772–778.
28. Elder GJ, Wetherell MA, Barclay NL, Ellis JG. The cortisol awakening response--applications and implications for sleep medicine. *Sleep Med Rev*. 2014;**18**(3):215–224.
29. Raff H. Salivary cortisol: a useful measurement in the diagnosis of Cushing's syndrome and the evaluation of the hypothalamic-pituitary adrenal axis. *Endocrinologist*. 2000;**10**(1):9–17.
30. Raff H, Homar PJ, Skoner DP. New enzyme immunoassay for salivary cortisol. *Clin Chem*. 2003;**49**(1): 203–204.
31. RRID:AB_2801306. https://scicrunch.org/resolver/AB_2801306.
32. Raff H, Hoeynck B, Jablonski M, Leonovicz C, Phillips JM, Gehrand AL. Insulin sensitivity, leptin, adiponectin, resistin, and testosterone in adult male and female rats after maternal-neonatal separation and environmental stress. *Am J Physiol Regul Integr Comp Physiol*. 2018;**314**(1):R12–R21.
33. George MP, Szczesniewski A. Analysis of testosterone in serum using LC/triple quadrupole MS/MS. 2010; Available at: www.agilent.com/cs/library/applications/5990-5228en_lo%20CMS.pdf. Accessed 15 May 2019.
34. Szczesniewski A. Endogenous steroids analysis in plasma by LC/MS. 2014; Available at: http://docplayer.net/45368930-Endogenous-steroids-analysis-in-plasma-by-lc-ms.html. Accessed 15 May 2019.
35. Sturmer LR, Dodd D, Chao CS, Shi RZ. Clinical utility of an ultrasensitive late night salivary cortisol assay by tandem mass spectrometry. *Steroids*. 2018;**129**:35–40.
36. Martin RF. General deming regression for estimating systematic bias and its confidence interval in method-comparison studies. *Clin Chem*. 2000;**46**(1):100–104.
37. Clow A, Thorn L, Evans P, Hucklebridge F. The awakening cortisol response: methodological issues and significance. *Stress*. 2004;**7**(1):29–37.
38. Kluger J. How you sleep depends on where you live. 2016; Available at: http://time.com/4318156/sleep-countries-style/. Accessed 15 May 2019.
39. Raff H, Trivedi H. Circadian rhythm of salivary cortisol, plasma cortisol, and plasma ACTH in end-stage renal disease. *Endocr Connect*. 2012;**2**(1):23–31.
40. Miller R, Plessow F, Rauh M, Gröschl M, Kirschbaum C. Comparison of salivary cortisol as measured by different immunoassays and tandem mass spectrometry. *Psychoneuroendocrinology*. 2013;**38**(1): 50–57.

41. Raff H, Raff JL, Duthie EH, Wilson CR, Sasse EA, Rudman I, Mattson D. Elevated salivary cortisol in the evening in healthy elderly men and women: correlation with bone mineral density. *J Gerontol A Biol Sci Med Sci*. 1999;**54**(9):M479–M483.
42. Miller R, Stalder T, Jarczok M, Almeida DM, Badrick E, Bartels M, Boomsma DI, Coe CL, Dekker MC, Donzella B, Fischer JE, Gunnar MR, Kumari M, Lederbogen F, Power C, Ryff CD, Subramanian SV, Tiemeier H, Watamura SE, Kirschbaum C. The CIRCORT database: Reference ranges and seasonal changes in diurnal salivary cortisol derived from a meta-dataset comprised of 15 field studies. *Psychoneuroendocrinology*. 2016;**73**:16–23.
43. Zerikly RK, Amiri L, Faiman C, Gupta M, Singh RJ, Nutter B, Kennedy L, Hatipoglu B, Weil RJ, Hamrahian AH. Diagnostic characteristics of late-night salivary cortisol using liquid chromatography-tandem mass spectrometry. *J Clin Endocrinol Metab*. 2010;**95**(10):4555–4559.
44. Debono M, Harrison RF, Chadarevian R, Gueroult C, Abitbol JL, Newell-Price J. Resetting the abnormal circadian cortisol rhythm in adrenal incidentaloma patients with mild autonomous cortisol secretion. *J Clin Endocrinol Metab*. 2017;**102**(9):3461–3469.
45. Perogamvros I, Keevil BG, Ray DW, Trainer PJ. Salivary cortisone is a potential biomarker for serum free cortisol. *J Clin Endocrinol Metab*. 2010;**95**(11):4951–4958.
46. Raff H. Measurement of salivary cortisone to assess the adequacy of hydrocortisone replacement. *J Clin Endocrinol Metab*. 2016;**101**(4):1350–1352.
47. Ceccato F, Marcelli G, Martino M, Concettoni C, Brugia M, Trementino L, Michetti G, Arnaldi G. The diagnostic accuracy of increased late night salivary cortisol for Cushing's syndrome: a real-life prospective study. *J Endocrinol Invest*. 2019;**42**(3):327–335.
48. Roa SL, Elias PC, Castro M, Moreira AC. The cortisol awakening response is blunted in patients with active Cushing's disease. *Eur J Endocrinol*. 2013;**168**(5):657–664.