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Dried urine and salivary profiling for complete assessment of cortisol and cortisol metabolites

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ABSTRACT

Aims/objectives: The primary aim of this study was to determine the utility of dried urine sampling in obtaining measures of cortisol and cortisol metabolites. Additional aims were to evaluate if a 4-spot dried urine collection is representative of a 24-hour urine collection and if expected diurnal cortisol patterns can be observed in samples from both urine and saliva.

Methods: Data from individuals with cortisol measures available from both a 4-spot dried urine collection and a 24-hour urine collection (n = 28) were evaluated. Of these 28, 20 also had concurrent liquid and dried 24-hour urine measures. Consistency between these methods was evaluated using paired t-tests and intraclass correlation coefficients (ICCs). In addition, data from individuals with concurrent measures of both urinary and salivary cortisol (n = 68) were assessed for consistency in the diurnal pattern of change in cortisol.

Results: Near ideal consistency was observed between liquid and dried urine for measures of total urine free cortisol, total urine cortisone, and total cortisol metabolites (n = 20; ICCs = 0.99, 0.97 and 0.96, respectively). Good to excellent consistency was observed between the 4-spot method and the 24-hour collection (n = 28; ICCs = 0.89, 0.95 and 0.92, respectively). In mixed model analysis, no difference was seen in the diurnal pattern of cortisol between salivary and urinary free cortisol (n = 68; P = 0.83).

Conclusion: Dried urine is a viable alternative to liquid urine for the measurement of cortisol and cortisol metabolites. Additionally, if the 4 measures are added together, 4-spot urine collections can be representative of diurnal cortisol patterns commonly assessed using saliva and 24-hour urine collections.

Introduction

Accurate assessment of the adrenal axis is critical when evaluating a patient exhibiting hallmark signs of cortisol excess or deficiency. A similar approach may also be warranted for patients reporting nonspecific symptoms such as depressed mood, poor sleep, or weight changes, as these complaints could be indicative of hypothalamic–pituitary-adrenal (HPA) axis dysfunction. Assessing HPA axis activity is most often accomplished by measuring cortisol levels. Cortisol concentrations can be measured in serum, plasma, saliva, urine and hair [1–3]. Although cortisol levels are necessary for evaluating HPA axis function, they may not be sufficient in all clinical scenarios.

While free cortisol is essential for assessment of the HPA axis, it represents less than 5% of total cortisol secretion [4,5]. The other 95% of cortisol's metabolic clearance is represented by the excretion of the major cortisol metabolites including cortisone, α-tetrahydrocortisol (aTHF), β-tetrahydrocortisol (bTHF), and tetrahydrocortisone (THE) [4]. As a result, adding assessment of cortisol metabolites offers an improved approximation of total glandular output compared to evaluating free cortisol measurements alone. This strategy can better illuminate alterations in cortisol metabolism that may be seen in idiopathic obesity, thyroid disorders, and possibly myalgic encephalomyelitis. Because alterations in cortisol production and metabolism have been associated with an increasing number of pathophysiological processes such as Alzheimer disease [6], metabolic syndrome [7], polycystic ovary syndrome [8], and primary male osteoporosis [9], it is becoming more important to consider assessment of the full range of cortisol and cortisol metabolites when evaluating patients.

For laboratory tests ordered in the outpatient setting, saliva and urine are the most commonly used sampling methods; however, cortisol metabolites can only be measured in urine. The 24-h urine collection is a widely used sampling method for urinary cortisol measures including

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urine free cortisol (UFC), one of the 4 first-line recommended tests for the diagnosis of Cushing syndrome [10]. However, the collection process is notoriously cumbersome and inconvenient for patients [11]. Studies have found over 30% of 24-h urine collections to be inaccurate due to incomplete collection [12,13]. The challenging collection process also limits the utility of 24-h collections in field settings [11]. As a result of this limitation, population-based field studies often use salivary cortisol samples as they are easier to collect and store compared to a 24-h urine [11]. Clinicians evaluating patients in the outpatient setting have the option of either or both methods of sampling. Choosing the best test, or combination of tests, for specific clinical scenarios is paramount to being able to elucidate meaningful inferences from measures of cortisol. The increased role of cortisol metabolites as important biomarkers makes this choice more difficult as comprehensiveness comes at the cost of convenience and accuracy in 24-h urine collections.

A potential method for mitigating the issues inherent in 24-h urine collections includes both the use of multiple spot urine collections throughout a single day as well as the use of dried urine collected on filter paper. Dried urine samples analyzed by mass spectrometry can provide measurements of both cortisol and cortisol metabolites. Additionally, compared to 24-h collections, the collection process is significantly less cumbersome. Dried urine samples do not require refrigeration or the large collection containers needed for 24-h collections. Dried urine spot samples have been validated and used for a variety of applications including monitoring dietary-related metabolite excretion [14], measuring levels of glycosaminoglycans in the investigation of mucopolysaccharidoses [15], measuring hippuric acid and creatinine to determine occupational exposure to toluene [16], and in the detection and quantification of cytomegalovirus in newborns [17].

Currently, however, no studies have been published evaluating the viability of using dried urine in place of liquid urine for the measurement of cortisol and cortisol metabolites. Therefore, the primary aim of this study was to determine if measures of cortisol and cortisol metabolites are consistent between dried urine and liquid urine. Secondary aims were to determine if 1) a 4-spot collection method is representative of a 24-h urine collection and 2) if the expected diurnal cortisol pattern seen in salivary samples could be observed in multiple dried urine samples collected over the day. If the 4-spot dried urine collection accurately reflects measures obtained from a 24-h liquid collection as well as the diurnal pattern seen in salivary cortisol, it could offer a novel sampling method that can be used for diagnostic purposes, research, and field collection.

Materials and methods

Study design

This was a cross-sectional study conducted using a databank from Precision Analytical Inc. that included data from 144,561 laboratory visits by 129,883 individuals collected between January 1, 2016 and December 9, 2019. All data reports were deidentified during extraction so the National University of Natural Medicine Institutional Review Board determined that written informed consent could be waived. Nevertheless, informed consent was obtained from the 28 individuals who provided both a 4-spot and a 24-h urine collection. The study was registered on Clinicaltrials.gov (Clinical Trials ID: NCT04305093).

Subjects

Of the 129,883 individuals included in the databank, 28 individuals had cortisol measures available for both a 4-spot dried urine collection and a 24-h urine collection on the same day, and of these, 20 had data available for concurrent liquid and dried 24-h urine measures. Having the study participants complete both the 4-spot dried urine collection and the 24-h urine collection on the same day allowed for direct comparison of the two sampling methods. Ordering providers were able to select one of two possible laboratory panels, either the dried urine test for comprehensive hormones (DUTCH) Plus, which included urinary cortisol metabolites and salivary measures of free cortisol, or DUTCH Complete, which included urinary measures of both free cortisol and cortisol metabolites. There were 68 individuals with measures for both urine and salivary cortisol collected at multiple time points on the same day for assessment of the diurnal pattern of free cortisol. Data were extracted from the databanks for salivary and urinary free cortisol and cortisone measurements at multiple time points throughout the day along with urinary measures of cortisol metabolites, aTHF, bTHF, and THE. Exclusion criteria included pregnancy, use of hormonal medications, known diagnoses of Cushing Disease, adrenal insufficiency, thyroid disease, self-reported kidney disease, or evidence of overly dilute urine (urine creatinine less than 0.1 mg/dl).

Dried urine sample collection

Subjects were instructed to collect dried urine samples at 4 different time points during the day (4-spot method): immediately upon awakening, 2 h after awakening, between 4 pm and 5 pm (before evening meal), and at bedtime. Urine samples were collected on EBF 903 sample collection filter paper (Eastern Business Forms, Inc., Mauldin, SC, USA) and subjects were instructed to saturate the filter paper by urinating directly on it or collecting urine in a clean collection cup and dipping the filter paper in it for 5 s. Samples were left open to dry for at least 24 h. Subjects were also instructed to limit fluid intake following the second collection to no more than 32 oz for the remainder of the day. Food and fluids were to be avoided during the hour preceding the final 2 collections of the day. Additionally, subjects were instructed to avoid caffeine and alcohol on the day of collection and the night before.

Liquid urine sample collection

Liquid urine samples were obtained from 24-h urine collections. Four dried urine samples were collected simultaneously which resulted in a negligible amount of urine (<8mL) not being collected in each of the 24-h collections. Subjects were instructed to discard the first urine void of the day and note the time as the start of the 24-h collection. All subsequent voids were to be collected ending with the first void on the following day. Samples were collected in cooled, 2-liter collapsible low-density polyethylene plastic containers (ES Robbins, USA) that contained 1 g of boric acid as a preservative. Subjects were instructed to keep the container refrigerated or on ice both during and after the collection. Once the collections were received in the laboratory, the total volumes were measured, and liquid and dried aliquots were frozen and stored at −80 °C until analyzed.

Saliva sample collection

Subjects were instructed to collect saliva samples at 5 different time points during the day: immediately upon awakening, 30 min after awakening, 60 min after awakening, between 4 pm and 5 pm (before the evening meal), and between 10 pm and 12 am. Saliva samples were collected using Cortisol-Salivette swabs (Sarstedt, Numbrecht, Germany). For the first morning sample and the two subsequent samples, subjects were instructed to avoid consuming any food or drink, refrain from brushing or flossing, and to limit themselves to light activities such as showering and getting dressed. Subjects were also instructed not to floss on the day of collection, and to avoid caffeine and alcohol on the day of collection and the night before. Samples were required to be frozen within 12 h of collection and to remain frozen until they were ready to ship. Subjects were advised that it is best to collect saliva samples on a relatively normal day and that if sleep was significantly disturbed, it may be best to select a different day.

Laboratory methods

Urine analysis

The urinary free cortisol, free cortisone, and other cortisol metabolites were analyzed using proprietary in-house assays referred to as Dried Urine Testing for Comprehensive Hormones (DUTCH) on a Waters Acquity/TQD LC–MS/MS (Waters, Milford, MA, USA). The equivalent of approximately 600 uL of urine was extracted from the filter paper using 2 mL of 100 mM ammonium acetate adjusted to a pH of 5.9. Each of the four dried filter paper collections was analyzed separately for cortisol and cortisone; however, the extractions were combined for aTHF, bTHF, and THE. Aliquots of the free and conjugated hormones were transferred to a C18 solid phase extraction (SPE) column (UCT LLC, Briston, PA, USA). The free hormones were then eluted using methyl tert-butyl ether (MTBE) and dried under nitrogen at 40 °C. The conjugated hormones were eluted using methanol and the four methanol extracts were combined. The free hormones were then reconstituted in a water/MeOH mixture. The conjugated hormones were subsequently hydrolyzed from their glucuronide and sulfate forms to free forms using enzymes from *Helix pomatia* (Sigma-Aldrich, St. Louis, MO, USA) in acetate buffer (55 °C, 90 min). The enzymatic reaction was quenched with sodium carbonate and the hormones extracted with ethyl acetate. The ethyl acetate extracts were dried under nitrogen at 40 °C. The analytes were reconstituted using a mixture of water/MeOH. Internal standards for the free hormones (Cortisol-d4) were spiked prior to MTBE elution from the SPE column. Internal standards for the conjugated hormones (B-THF d4, THE d5, Steraloids, Newport, RI, USA) were added prior to ethyl acetate extraction, and the percentage recovery after all assays was greater than 90%. Reconstituted extract (20 uL for cortisol and cortisone, 40 ul for THE and THFs) was injected into the LC-MS/MS. Samples were analyzed along with a standard curve spanning the expected range of concentrations and a series of controls. Multiple reaction monitoring transitions were 363 > 121 for cortisol, 361 > 163 for cortisone, 331 > 295 for aTHF, 349 > 301 for bTHF and 365 > 304 for THE. Creatinine was measured using a conventional colorimetric (Jaffe) method, after initial extraction from the filter paper. The 24-h creatinine excretion was estimated using a formula by Kawasaki et al. that accounts for age, sex, weight and height [18]. Based on this formula, a correction factor for creatinine was applied to measures of cortisol, cortisone, and the cortisol metabolites. An additional creatinine correction was applied to measures of cortisol using a proprietary equation developed by Newman and colleagues to adjust for the correlation between creatinine and cortisol in subjects with low creatinine values. The average interassay coefficients of variation (CV) for the urine measures were 11.7% for cortisol, 7.4% for cortisone, bTHF, and THE, and 15% for aTHF. Urinary cortisol, cortisone, and cortisol metabolites from liquid urine were analyzed using a method similar to what is described above.

Saliva analysis

Salivary cortisol and cortisone were measured using the above method but starting with 200ul of saliva. No additional adjustments or corrections were applied to these measurements. The average interassay CVs for salivary measures were 11% for cortisol and 8.3% for cortisone. The lower level of detection for the assays were 0.1 ng/ml for cortisol and cortisone, 25 ng/ml for bTHF, 2.5 ng/mL for aTHF, and 50 ng/ml for THE.

Statistical analysis

A sample size of 20 individuals provides a power of 90% to detect an intraclass correlation coefficient (ICC) of at least 0.6 with an alpha of 0.05 [19]. The statistical analyses were performed using SAS/STAT® software, Version 9.3 (SAS Institute Inc., Cary, NC, USA). Alpha was set to 0.05.

Variables are described as means ± standard deviation or median (interquartile range (IQR)) depending on whether they were or were not normally distributed. Spearman correlation coefficients were used to determine the initial associations between variables. Student t-tests were used to assess differences between males and females. The 30-minute cortisol awakening response in saliva (CAR_{30}) was calculated as the difference between the 30-minute salivary measure and the first morning collection salivary measure. Total urine free cortisol and total urinary cortisone values were obtained by adding the results of the four measurements from the 4-spot sampling together (e.g., $Cortisol_{AM}$ + $Cortisol_{9AM}$ + $Cortisol_{afternoon}$ + $Cortisol_{HS}$ = Total Urine Cortisol). Total salivary cortisol and cortisone values were obtained similarly, by adding together the values from all 5 salivary collections (e.g., $Cortisol_{7AM}$ + $Cortisol_{7:30AM}$ + $Cortisol_{8AM}$ + $Cortisol_{afternoon}$ + $Cortisol_{HS}$ = Total Salivary Cortisol).

Consistency between cortisol measures for an individual using 4-spot dried urine (DUTCH) versus 24-hour urine collections (n = 28) and dried versus liquid collections (n = 20) were compared using paired t-tests and ICCs, a measure of agreement between tests [20]. ICCs differ from interclass correlation coefficients (e.g., Pearson and Spearman) in that they assess the agreement of a measure between groups [21]. ICC values range from 0 to 1, with higher values indicating better agreement between the two measures. As 4-spot measures are reported in ng/mg-Cr and 24-hour urine collections are reported in ug/d, z-scores ((individual measurement – mean)/S.D.) for the cortisol metabolites were created to standardize the measures for direct comparison. Similarly, z-scores were created for both urine and salivary cortisol concentrations for assessment of consistency (with ICCs) in the 68 individuals with measures in both body fluids. A mixed model to account for repeated measures over time was used to determine whether the pattern of diurnal variation in cortisol differed between saliva and urine; time was modeled as a quadratic relationship.

Results

Subject characteristics

Of the 28 subjects with both a 24-h urine collection and the 4-spot urine collection, the majority were female (61%; n = 17; Table 1). Of these (12 female, 8 male), 20 had measures from both the liquid 24-h collection and dried urine from the 24-h collection. Characteristics of the 68 individuals (46 female, 22 male) with concurrent measures in saliva and urine are shown in Table 2.

Table 1
Urine measures of the 28 individuals with both a 24-h urine collection and the 4-spot urine collection. Data are presented as mean ± standard deviation. The p-value for differences between males and females as assessed by a Student *t*-test are provided. Cr = creatinine, aTHF = urinary α-tetrahydrocortisol, bTHF = urinary β-tetrahydrocortisol, THE = urinary β-tetrahydrocortisone, TCM = total urinary cortisol metabolites.

Variable	All	Females (n = 18)	Males (n = 11)	p-value
Age (years)	36.3 ± 14.9	36.2 ± 13.4	36.5 ± 17.8	0.96
BMI (kg/m^2)	26.6 ± 6.8	27.1 ± 7.1	25.9 ± 6.6	0.64
Urine Creatinine (mg/dl)	0.56 ± 0.24	0.52 ± 0.24	0.63 ± 0.24	0.18
Total Urine Free Cortisol (ug/24 h)	129.3 ± 99.6	121.4 ± 92.6	141.6 ± 113.0	0.61
Total Urine Cortisone (ug/24 h)	312.9 ± 170.1	326.5 ± 193.6	292.0 ± 131.9	0.61
aTHF (ug/24 h)	202.7 ± 183.2	145.8 ± 105.3	290.8 ± 242.6	0.08
bTHF (ug/24 h)	1953.5 ± 930.9	1564.8 ± 689.5	2554.2 ± 962.0	0.004
THE (ug/24 h)	2870.0 ± 1616.4	2491.8 ± 1362.5	3454.4 ± 1861.5	0.13
TCM (ug/24 h)	5026.2 ± 2461.7	4202.4 ± 1802.5	6299.4 ± 2868.3	0.02

Table 2
Characteristics and hormonal measures of the 68 individuals with concurrent measures of urine and saliva. Data presented as mean ± standard deviation. The urine measures are from the DUTCH 4-spot dried urine assay. The p-values for differences between males and females as assessed by a Student *t*-test are provided.

Variable	All	Females (n = 46)	Males (n = 22)	p-value
Age (years)	40.6 ± 12.3	41.0 ± 13.1	39.7 ± 10.9	0.68
BMI (kg/m^2)	26.3 ± 5.9	27.1 ± 6.6	24.5 ± 3.3	0.04
Urine Creatinine (mg/dl)	0.85 ± 0.47	0.75 ± 0.39	1.05 ± 0.53	0.19
7 AM Salivary Cortisol (ng/ml)	2.58 ± 1.30	2.62 ± 1.35	2.50 ± 1.23	0.72
30 min Salivary Cortisol (ng/ml)	5.23 ± 2.22	5.46 ± 2.05	4.76 ± 2.53	0.26
CAR_{30} (ng/ml)	2.65 ± 1.96	2.84 ± 1.88	2.26 ± 2.11	0.25
60 min Salivary Cortisol (ng/ml)	3.28 ± 1.63	3.32 ± 1.67	3.19 ± 1.56	0.76
4 PM Salivary Cortisol (ng/ml)	0.67 ± 0.44	0.63 ± 0.45	0.75 ± 0.40	0.30
10 PM Salivary Cortisol (ng/ml)	0.34 ± 0.49	0.27 ± 0.20	0.51 ± 0.79	0.17
Total Salivary Cortisol (ng/ml)	12.11 ± 4.20	12.30 ± 4.29	11.71 ± 4.06	0.59
Total Salivary Cortisone (ng/ml)	42.46 ± 8.88	42.37 ± 9.52	42.66 ± 7.55	0.90
7 AM Urine Cortisol (ng/mg-Cr)	36.0 ± 24.8	34.9 ± 23.0	38.5 ± 28.4	0.58
9 AM Urine Cortisol (ng/mg-Cr)	92.2 ± 55.9	83.0 ± 48.3	111.3 ± 66.3	0.08
4 PM Urine Cortisol (ng/mg-Cr)	25.8 ± 16.3	23.0 ± 14.1	31.7 ± 19.1	0.06
10 PM Urine Cortisol (ng/mg-Cr)	10.3 ± 8.4	8.6 ± 6.2	13.9 ± 11.0	0.04
Total Urine Cortisol (ng/mg-Cr)	164.3 ± 77.4	149.4 ± 63.5	195.4 ± 94.8	0.05
Total Urine Cortisone (ng/mg-Cr)	225.0 ± 89.2	216.8 ± 86.0	242.2 ± 95.3	0.28
aTHF (ng/mg-Cr)	335.4 ± 245.5	257.2 ± 164.8	498.8 ± 304.4	0.002
bTHF (ng/mg-Cr)	1905.2 ± 877.0	1693.0 ± 772.3	2349.1 ± 933.1	0.003
THE (ng/mg-Cr)	3150.9 ± 1762.4	2861.1 ± 1729.2	3756.9 ± 1712.7	0.05
TCM (ng/mg-Cr)	5391.6 ± 2730.1	4811.3 ± 2574.4	6604.8 ± 2701.6	0.01

BMI = body mass index, CAR_{30} = 30-minute cortisol awakening response, Cortisol = free cortisol, Cr = creatinine, aTHF = urinary α-tetrahydrocortisol, bTHF = urinary β-tetrahydrocortisol, THE = urinary β-tetrahydrocortisone, TCM = total urinary cortisol metabolites

Consistency of cortisol measures in liquid versus dried urine samples

There were no differences in the measurements of cortisol or cortisol metabolites from a 24-h urine collection (as assessed by paired t-tests) whether analyzing liquid urine or dried urine samples extracted from filter paper (n = 20). The ICCs all demonstrated almost ideal consistency between the two samples (Table 3). Correlations between the two methods are shown in Fig. 1.

Consistency of cortisol measures in a DUTCH 4-spot urine collection versus a 24-hour urine collection

Z-scores for the 4-spot (DUTCH) collection and 24-h measures were created to allow for direct comparison. ICCs showed good consistency between the two methods of collection (n = 28; Table 4) with no directionality to the differences between the Z-scores. Correlations of the raw measurements done using these two methods of collection are shown in Fig. 2.

To verify the use of the creatinine correction for the DUTCH measures, sensitivity analyses with the uncorrected, raw measures (ng/ml) with no creatinine correction were conducted. Accounting for creatinine when determining cortisol measures from the filter paper urine resulted in better agreement between the 4-spot urine collection and the 24-h urine measures. For example, the ICC between the 4-spot collection and the 24-h collection urinary free cortisol measures was reduced from 0.89 [95%CI: 0.79, 0.95] to 0.69 [0.45, 0.84] without the creatinine correction, the ICC for urine cortisone was reduced from 0.95 [0.91, 0.98] to 0.45 [0.11, 0.69], and the ICC for the total cortisol metabolites was reduced from 0.92 [0.84, 0.96] to 0.47 [0.14, 0.71].

Table 3
Comparison of dried versus liquid urine analysis (n = 20). Data presented as mean ± SD for dried and liquid measurements and the difference is presented as mean [95% CI].

Variable	Dried	Liquid	Difference [95% CI][a]	ICC [95% CI]
Total Urine Free Cortisol (ug/24 h)	141.33 ± 113.19	141.82 ± 116.14	−0.50 [−8.02, 7.03]	0.99 [0.98, 0.99]
Total Urine Cortisone (ug/24 h)	320.03 ± 195.19	313.20 ± 206.21	6.84 [−14.85, 28.52]	0.97 [0.94, 0.99]
aTHF (ug/24 h)	149.47 ± 135.65	162.76 ± 141.90	−13.29 [−33.23, 6.66]	0.95 [0.88, 0.98]
bTHF (ug/24 h)	1857.13 ± 761.37	1748.09 ± 777.67	109.04 [−49.17, 267.25]	0.90 [0.77, 0.96]
THE (ug/24 h)	2750.55 ± 1462.20	2854.27 ± 1481.34	−103.71 [−262.18, 54.75]	0.97 [0.93, 0.99]
TCM (ug/24 h)	4757.15 ± 2016.95	4765.11 ± 2133.79	−7.96 [−275.12, 259.19]	0.96 [0.91, 0.99]

Cr = creatinine, ICC = intraclass correlation coefficient, aTHF = urinary α-tetrahydrocortisol, bTHF = urinary β-tetrahydrocortisol, THE = urinary β-tetrahydrocortisone, TCM = total urinary cortisol metabolites
[a] All p-values for differences by paired *t*-test > 0.16

Consistency of cortisol measures in dried urine versus saliva

The mean diurnal changes in salivary and urinary free cortisol measurements in the 68 individuals with concurrent measures are shown in Fig. 3. A mixed model revealed no difference in the pattern of change over the day between the two measures (p = 0.83).

There was no directionality to the mean difference in the Z-scores between total urine free cortisol and total salivary cortisol over the day (Δ = -0.01 [95% CI: −0.22, 0.22], p = 0.92). There was good consistency between the two measures of total free cortisol (ICC = 0.77 [95% CI: 0.65, 0.85]). As expected, based on predicted appearance of cortisol in the urine versus saliva, there was no agreement of the first awakening cortisol measurements (ICC = 0.01 [-0.23, 0.24]); however, the peak 10 AM urine free cortisol measure showed moderate agreement with the peak 30-minute post awakening salivary cortisol measure (ICC = 0.58 [0.39, 0.70]). In a sensitivity analysis using cortisol without the creatinine correction, the ICC for agreement with salivary total free cortisol was 0.35 [0.11, 0.54] and the ICC for agreement between the peak cortisol was 0.45 [0.24, 0.62], further verifying the need for creatinine correction in the spot urine measures.

Discussion

Measuring cortisol and cortisol metabolites using dried urine samples collected on filter paper produced results that demonstrate excellent agreement with those collected from liquid urine. Additionally, a 4-spot dried urine collection using the DUTCH methodology produced results reflective of those seen in 24-h urine collections and displayed the diurnal pattern of cortisol commonly seen with saliva sampling. Finally,

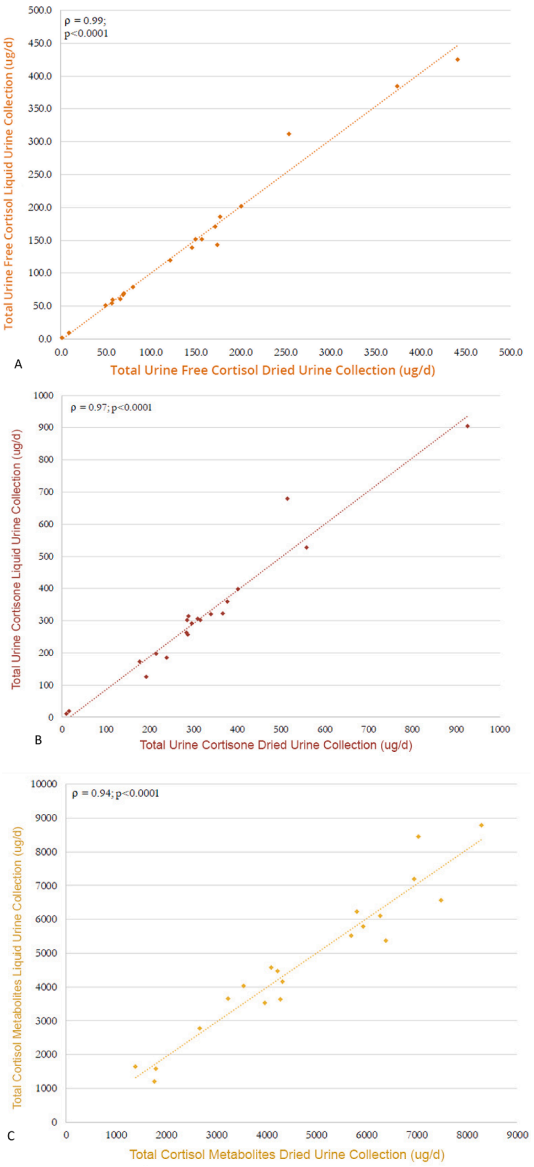

Fig. 1. Correlations between the liquid versus dried 24-h urine collection measurements for (A) total urine free cortisol, (B) total urine cortisone, and (C) total cortisol metabolites (aTHF + bTHF + THE). Reported correlation coefficients are Spearman correlations. Cr = Creatinine.

dried urine measures of total free cortisol and peak cortisol were consistent with values measured in saliva.

The feasibility of using dried urine spot samples in place of traditional urine collections in the measurement of biomarkers other than cortisol and cortisol metabolites has been previously studied [14,16,17,22]. Published studies have also compared dried urine samples to samples obtained from other bodily fluids, including a study published by this group that demonstrated the utility of dried urine as a substitute for serum sampling for the measurement of estrogen, progesterone and their respective metabolites [23]. That study also showed that the DUTCH 4-spot sampling method is an acceptable surrogate for a 24-h urine collection. However, to the best of our knowledge, this is the first study to compare these sampling methods in the measurement of cortisol and cortisol metabolites.

All sampling methods employed in laboratory testing have distinct advantages and disadvantages. An oft-cited advantage of 24-h urine collections is their ability to show an integrated index of corticosteroid production over a 24-h period, whereas salivary and serum measures

Table 4
Comparison of 4-spot (DUTCH) versus 24-h urine collection (n = 28). The ICCs were calculated between measurements standardized as Z-scores. Data presented as mean ± SD for 24-h urine and 4-spot collections and the difference in Z-scores is presented as mean [95% CI].

Variable	24-h urine collection (ug/d) (z-score)	4-spot (ng/mg-Cr) (z-score)	Z-score Difference [95% CI][a]	ICC [95% CI]
Total Urine Free Cortisol	129.3 ± 99.6 (0.00 ± 0.98)	158.1 ± 119.8 (0.00 ± 0.98)	0.00 [−0.18, 18]	0.89 [0.79, 0.95]
Total Urine Cortisone	312.9 ± 170.1 (0.00 ± 0.98)	360.2 ± 173.2 (0.00 ± 0.98)	0.00 [−0.12, 0.12]	0.95 [0.91, 0.98]
aTHF	202.7 ± 183.2 (0.00 ± 0.98)	209.7 ± 201.6 (0.00 ± 0.98)	0.00 [−0.23, 0.23]	0.87 [0.73, 0.93]
bTHF	1953.5 ± 930.9 (0.00 ± 0.98)	2213.3 ± 1148.5 (0.00 ± 0.98)	0.00 [−0.21, 0.21]	0.89 [0.79, 0.95]
THE	2870.0 ± 1616.4 (0.00 ± 0.98)	3062.1 ± 1474.8 (0.00 ± 0.98	0.00 [−0.17, 0.17]	0.91 [0.81, 0.96]
TCM	5026.2 ± 2461.7 (0.00 ± 0.98)	5485.1 ± 2500.6 (0.00 ± 0.98	0.00 [−0.15, 0.15	0.92 [0.84, 0.96]

Cr = creatinine, ICC = intraclass correlation coefficient, aTHF = urinary α-tetrahydrocortisol, bTHF = urinary β-tetrahydrocortisol, THE = urinary β-tetrahydrocortisone, TCM = total urinary cortisol metabolites.
[a] All p-values for Z-score differences by paired *t*-test > 0.90.

only provide information pertaining to a single point in time. Dried urine offers this same advantage while also avoiding many of the disadvantages associated with 24-h liquid urine collections. Moreover, similar to saliva and serum, dried urine sampling allows for assessment of adrenal axis activity at multiple time points while requiring only a single day of collection. While the individual values at different timepoints are not exactly analogous to those seen in saliva, the 4-spot dried urine collection does allow for construction of a representation of the diurnal pattern of cortisol secretion and we found moderate agreement between the peak cortisol Z-scores. The diurnal pattern observable using 4-spot dried urine sampling is shifted compared to what is seen in saliva and serum as a result of the time lapse between cortisol production and its excretion in the urine.

Another advantage of dried urine sampling is the ability to measure concentrations of cortisol metabolites. Measures of cortisol metabolites obtained from dried urine are not only similar to those obtained from liquid urine, they also have the added benefit of being more convenient to collect. Although saliva sampling has an arguably equally convenient collection process and provides measures of saliva's major cortisol metabolite, cortisone, measurement of other cortisol metabolites in saliva do not accurately represent the rate of production [24,25]. Even though cortisol metabolites can be measured in serum, these measurements only represent a single moment in time and thus do not provide a representation of total secretion. Further, the invasiveness required for serum sampling makes it prohibitive for outpatient laboratory and field research studies. When examining these sampling methods concurrently, dried urine provides the optimum balance of convenience and comprehensiveness.

Comprehensive assessment of cortisol metabolism has become increasingly important as a result of recent research illuminating cortisol's metabolic pathway and its role in an array of disease processes. Investigations into the diagnosis and treatment of these disease processes has resulted in both the targeting of enzymes involved in cortisol metabolism as well as the use of cortisol metabolites as biomarkers [26–30]. Cortisol metabolites were found to be a better indicator than urinary cortisol in the setting of cyclical Cushing syndrome and in patients with adrenal incidentalomas [31]. It has also been suggested that measures of the major metabolites of cortisol may be better surrogates

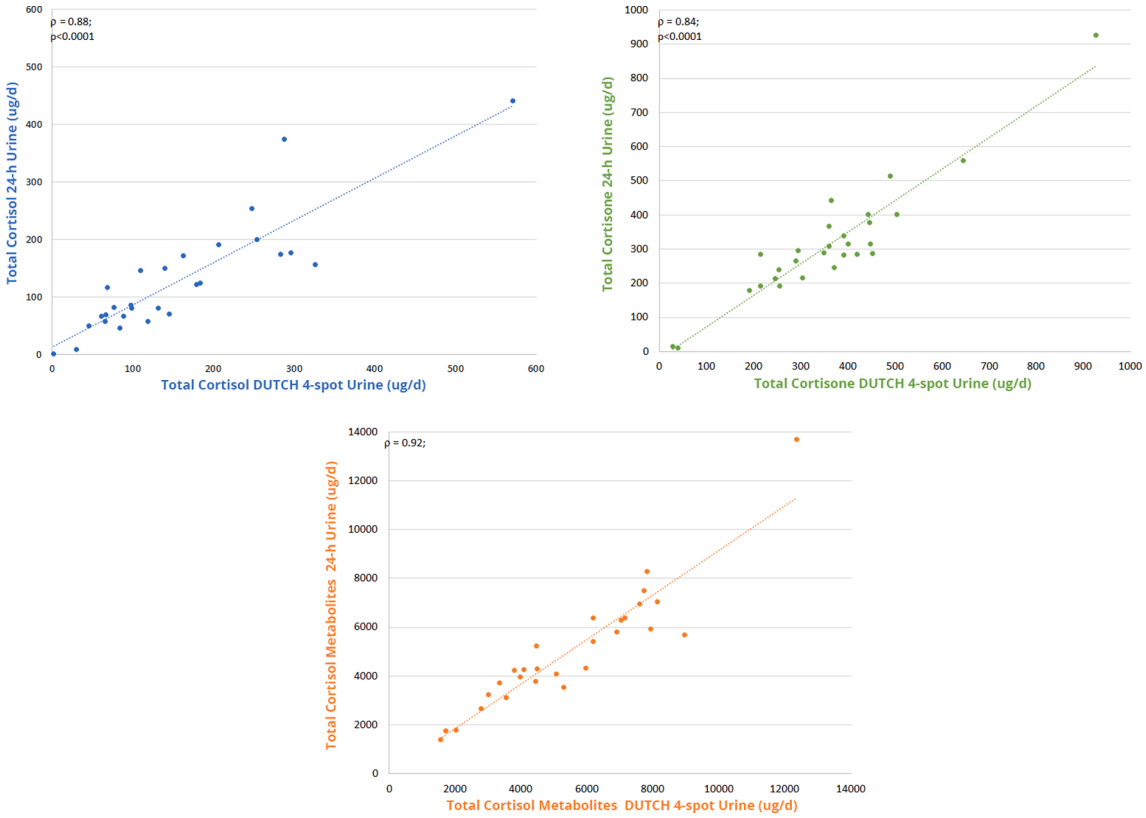

Fig. 2. Correlations between 24-h urine collection and 4-spot urine collection raw measurements for (A) total free cortisol, (B) total cortisone, and (C) total cortisol metabolites (aTHF + bTHF + THE). Reported correlation coefficients are Spearman correlations. Cr = Creatinine, DUTCH = Dried Urine Testing for Comprehensive Hormones.

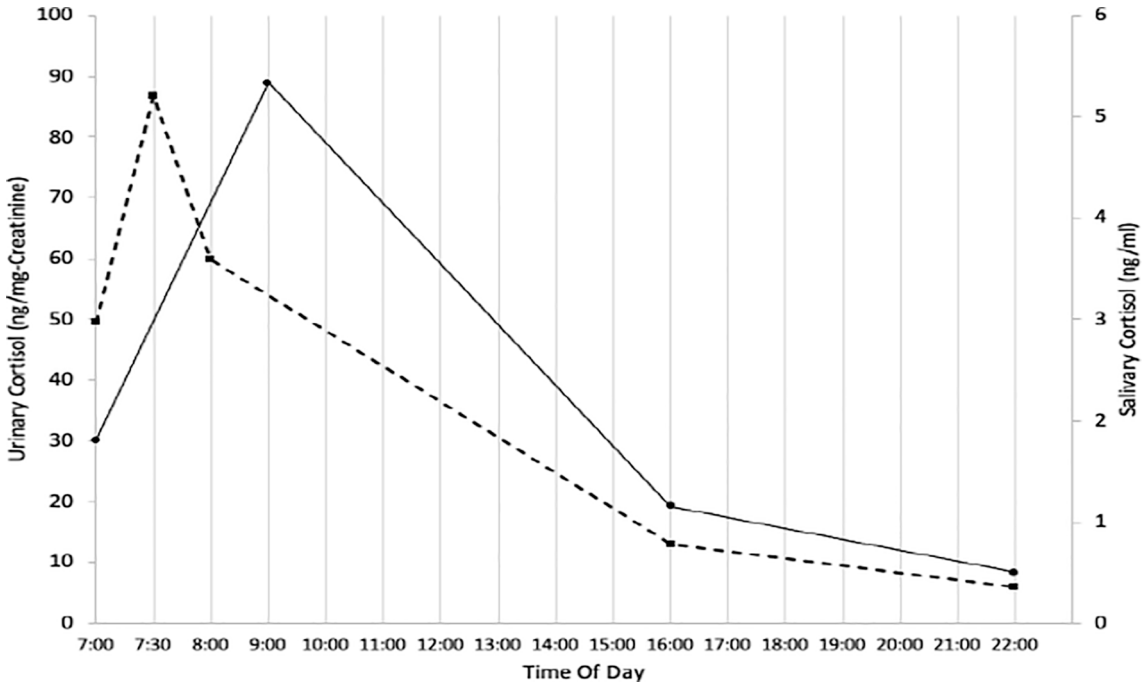

Fig. 3. Diurnal changes in mean salivary cortisol (squares with dashed line) and mean urinary cortisol (circles with solid line) in the 68 individuals with concurrent measures in urine and saliva.

for total cortisol secretion than measures of cortisol alone [32]. While urinary free cortisol is indicative of cortisol's metabolic activity, with a significant amount of cortisol being bound to either cortisol-binding globulin or albumin, the cortisol metabolites are more indicative of total cortisol production. Importantly, the metabolites do not independently represent the activity of the pituitary-adrenal axis, but they do

still provide useful and actionable information through their estimation of cortisol metabolism and clearance. Given the importance of measuring cortisol metabolites, value exists in being able to measure them via a convenient and reliable method such as the one provided by dried urine sampling.

A caveat to using dried urine on filter paper is the necessity to adjust for creatinine to control for fluctuations in analyte concentration that may occur as a result of variable dilutions of each sample [33]. Other methods exist for adjusting the urinary concentrations of analytes; however, creatinine adjustment is the method that is most often employed [33]. Despite the fact that adjustments using creatinine help to bring the unadjusted concentrations closer to those that would be seen in serum, an undefinable degree of accuracy is still sacrificed. In our analysis, correcting the cortisol and cortisol metabolites for creatinine after 4-spot dried urine collection improved agreement with both 24-h urine and salivary measures.

Although dried urine provides a great deal of information about HPA axis activity, especially activity related to measures of cortisol and cortisol metabolites, it does not eliminate the need for other methods of measuring cortisol. An example of this is seen in patients with moderate to severe renal impairment. In these patients, 24-h urine free cortisol excretion may be reduced and therefore cannot be used to identify elevated levels of cortisol [34]. Urinary excretion of cortisone and other cortisol metabolites are also thought to be affected by declining renal function. Additionally, increased dietary sodium consumption has been shown to increase urinary free cortisol excretion [35]. In clinical scenarios such as these, measuring cortisol using another sampling method would be a better option. Saliva sampling shares important characteristics with dried urine sampling such as being non-invasive, stable at room temperature, and amenable to collections at multiple time points and thus is a potential alternative to urine sampling [11].

Salivary cortisol has utility in an expanding number of clinical scenarios, such as in the evaluation of adrenal insufficiency and congenital adrenal hyperplasia [36], distinguishing subclinical hypercortisolemia from Cushing disease [37], and, arguably, in the monitoring of glucocorticoid replacement therapy [38]. It has been suggested that the primary reason salivary cortisol is a desirable biomarker is because serum free cortisol diffuses into saliva independently of the salivary flow rate [39,40]. Perhaps one of the most validated applications is the late-night salivary cortisol test used in the diagnosis of Cushing disease [10,40].

Another common application of salivary sampling is in the assessment of the cortisol awakening response (CAR_{30}) [41–43]. Publications involving assessment of the CAR_{30} have increased significantly over the past 2 decades [41]. These publications suggest associations with a number of disorders, including cardiovascular disease and psychiatric conditions [42,43]. The CAR_{30} has also proved to be a useful biomarker in neuroendocrinology research [41]. The ability to measure the CAR_{30} is an advantage that salivary sampling holds over both dried and liquid urine sampling.

Although the CAR_{30} cannot be measured directly from a 4-spot dried urine collection, the 10 am value may provide a possible surrogate. However, if comprehensive analysis of HPA axis activity is the goal, including both saliva and dried urine analysis would accomplish this better than either method alone. Because of the ease of sampling, storage and transport provided by both saliva and dried urine sampling, using them together is an option that maintains the convenience offered to patients or study participants by each method individually.

One particular strength of this study was that saliva and urine were collected on the same day, allowing for an accurate comparison of the two collection methods. Another key feature of this study was the use of Salivette swabs as opposed to cotton swabs or passive drool collections for salivary sampling. Cotton swabs may interact with analytes which can affect assay performance and produce inconsistent results. In addition to providing better, more consistent results, this collection device is also easier to use for study participants and patients.

This analysis did have limitations. First, two adjustments related to creatinine were applied to cortisol measures . The first adjustment, adapted from an equation developed to predict 24-h creatinine excretion based on an individual's age, body weight and height, was used to adjust all urine values of cortisol, cortisone, and cortisol metabolites. The second adjustment, based on a proprietary equation developed by Newman et al., was applied only to values of cortisol to account for dilutional effects on urine free cortisol. Sensitivity analyses were conducted to confirm the necessity of accounting for creatinine. These analyses verified that agreement of the 4-spot urine measures with both 24-h urine collection and salivary measures was markedly improved when the spot urine collections were corrected for creatinine. Further, because we did not have measures of specific gravity, creatinine had to be used as a surrogate to identify and exclude overly dilute samples.

Because this study was conducted in healthy subjects, the results cannot necessarily be extrapolated to patients with HPA axis dysfunction. Although the patient population in this study was considered healthy, excluding all possible confounding variables was not feasible given the design of the study. Some of these potential confounders include binge drinking, atypical sleep schedules, and complete adherence to sample collection protocol. However, this could be viewed as a strength since this is representative of real-world data.

A final point is that data on race/ethnicity were not collected so no adjustments for race/ethnicity could be made in the multivariate model. Despite these limitations, the findings of this study represent a major addition to clinical practice and offer the prospect of improving the HPA axis laboratory testing experience for both patients and clinicians.

A greater understanding of the clinical utility of dried urine sampling in measuring cortisol and cortisol metabolites could be gained in the future by conducting a larger, prospective cohort study in which both healthy patients and patients with HPA axis dysfunction are included. Nonetheless, the data presented here provide a basis to consider dried urine as a viable alternative to traditional liquid urine collection for assessing cortisol and cortisol metabolite levels in patients. Furthermore, the findings of this study, in combination with the decreased participant burden compared to other sampling methods, support the use of dried urine sampling in large scale clinical trials.

Another potential application for dried urine sampling is in the area of urine metabolomics. Interest in urine metabolomics has increased rapidly as researchers attempt to identify potential biomarkers for the disease processes determined to be associated with HPA axis dysfunction. Identifying adrenal tumors is one of the more prevalent potential roles for steroid profiling currently, but many additional applications are being investigated [31,45]. Although this study only evaluated measures of cortisol and cortisol metabolites, the LC-MS/MS analysis along with the proprietary DUTCH method is capable of measuring many of the metabolites measured in studies conducting urinary steroid metabolite profiling [44–46] including 8-OHdG, melatonin, reproductive hormone metabolites, organic acids, and more.

Conclusion

The results obtained in this study clearly demonstrated that dried urine collected on filter paper provides a novel sampling method for assessment of cortisol and cortisol metabolites. This approach holds multiple advantages over more traditional sampling methods including convenience and simultaneous evaluation of both total urine free cortisol and the diurnal pattern of cortisol production. Furthermore, current advances in the understanding of the role of cortisol production and metabolism in numerous disease states has created the need for a convenient, minimally invasive, comprehensive method of measuring cortisol and cortisol metabolites. Dried urine sampling alone fulfills these criteria; however, the addition of salivary measures of cortisol and cortisone could provide perhaps the most comprehensive assessment of cortisol production, secretion, and metabolism possible in the outpatient setting.

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CRediT authorship contribution statement

Mark Newman: . **:** Conceptualization, Methodology, Validation, Investigation, Writing - original draft, Writing - review & editing, Visualization, Supervision. **Desmond A. Curran:** Methodology, Data curation, Investigation, Writing - original draft, Writing - review & editing. **Bryan P. Mayfield:** Conceptualization, Writing - original draft, Writing - review & editing, Visualization.

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