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1 **Salivary cortisone reflects cortisol exposure under physiological conditions**
2 **and after hydrocortisone**

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Abstract

Context: Measuring serum cortisol to evaluate stress, adrenal disease, and monitor hydrocortisone replacement requires venepuncture. Conversely, salivary measurements are non-invasive.

Objective: To investigate measurement of salivary cortisol and cortisone as alternatives to serum cortisol.

Design & Setting: A prospective cross-over study in a Clinical Research Facility.

Participants & method: Over 3 periods (period 1: 24h physiological cortisol rhythm; periods 2 and 3: after 20mg oral and intravenous hydrocortisone) 14 male volunteers had serum and saliva cortisol and cortisone, serum albumin, CBG, and free cortisol measured. Data were analysed for rhythm parameters and correlations. Linear mixed effects modelling was performed to determine the relationship between serum cortisol and salivary cortisone.

Results: Serum cortisol and cortisone showed similar circadian rhythms with large peak:trough ratios (cortisol median ratio 11). Albumin and CBG showed minor peak:trough ratios <1.2. When serum cortisol was <74 (SD 29) nmol/L, salivary cortisol was not detectable but salivary cortisone was always detected. Salivary cortisol post-oral hydrocortisone produced spurious results due to contamination. Under physiological conditions, salivary cortisone correlated strongly with serum cortisol [ρ (95%CI): 0.91 (0.89-0.93);P<0.001]. Similarly, following intravenous or oral hydrocortisone, salivary cortisone correlated strongly with serum cortisol [ρ (95% CI) = 0.91 (0.89-0.92); P<0.001]. A mixed effects model showed that in this population 94% of the variation in salivary cortisone could be predicted from serum cortisol.

Conclusion: Salivary cortisol is frequently undetectable and contaminated by oral hydrocortisone. In contrast, salivary cortisone reflects serum cortisol and provides a non-invasive alternative to measuring serum cortisol levels.

Introduction

Cortisol measurement is important in the assessment of adrenal function and also used for assessing the adequacy of hydrocortisone replacement and as a marker of stress in studies of human behaviour. Cortisol levels have a distinct circadian rhythm being low in the evening and at sleep onset, rising from around 0300h-0500h, peaking shortly after waking and then declining over the day with small peaks at meal times (1,2). Therefore the timing of cortisol samples is very important when assessing cortisol exposure; for example a midnight cortisol is used to diagnose Cushing's syndrome (3), an early morning cortisol measurement to examine for adrenal insufficiency and some clinicians use multiple cortisol samples when assessing hydrocortisone replacement therapy (4). The measurement of serum cortisol is inconvenient and expensive involving venepuncture and either a visit to the clinic or hospital admission. In contrast, the measurement of salivary cortisol is relatively non-invasive and convenient for the patient as it can be done at home and posted to the laboratory (5). The measurement of salivary cortisol however, has limitations as it is undetectable at low levels of serum cortisol (6), and oral hydrocortisone contaminates the mouth resulting in spuriously high salivary cortisol levels (7).

It is important to consider the transport of cortisol in the circulation when measuring serum cortisol; 80% is bound to cortisol binding globulin (CBG), 10% to albumin and approximately 10% is free cortisol (unbound fraction), the latter providing biological activity (8). As cortisol concentration exceeds ~550nmol/L CBG saturates so that the biologically active free cortisol increases. At these levels the clearance of total cortisol increases (9), and the disappearance rate is negatively correlated with CBG (10). CBG has a diurnal rhythm in rats (11), and the metabolic clearance rate of cortisol in humans is significantly higher at

81 0500h to 1100h compared to that at 2000h to 0200h (12). Salivary cortisol has been shown to
82 reflect serum free cortisol (7).
83
84 The enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) is expressed in the
85 salivary glands and rapidly inactivates cortisol by conversion to cortisone (13). In serum,
86 levels of cortisone are approximately 4-fold less than those of cortisol whereas in saliva
87 levels of cortisone are approximately 6-fold higher than those of cortisol and presumed to be
88 generated during the production of saliva from free serum cortisol (6). Thus, salivary
89 cortisone predominantly reflects serum free cortisol.
90
91 Cortisone is present at higher concentration than cortisol in saliva and has previously been
92 shown to have a linear relationship with serum cortisol and therefore has the potential to
93 provide a good marker of serum cortisol (14). We propose that the measurement of salivary
94 cortisone can be used to estimate serum cortisol and serum free cortisol and to test this we
95 examined the relationship between serum cortisol, cortisone, free cortisol and salivary
96 cortisol and cortisone under physiological conditions and after the administration of
97 hydrocortisone orally and intravenously.
98

99 **Methods and subjects**

100 A single centre, prospective, cross over study was carried out at Simbec Research Limited
101 Clinical Centre. Fourteen healthy male volunteers with a median (IQR) age of 28 (25 – 36)
102 years, weight 83 (75 – 90) kg and BMI 25.3 (23.1 – 26.3) kg/m^2 participated in the study.
103 Inclusion criteria included ages 18 to 60 years of age, a BMI of 21 to 28 kg/m^2, and no
104 clinically significant liver, renal or cardiac disease. Exclusion criteria were the presence of
105 gastrointestinal disorders that could influence drug absorption; renal, hepatic, central nervous

106 system, respiratory, cardiovascular or metabolic dysfunction, drug or alcoholic abuse,
107 presence of infections, regular medications 14 days prior to study start, use of
108 glucocorticoids, smokers and rotating work shifts. None of the subjects had diabetes or other
109 significant comorbidities. The study was approved by the South East Wales Research Ethics
110 Committee and all participants gave written informed consent. The study was divided into
111 three study periods:

112

113 **Study period 1, Physiological Circadian Cortisol rhythm:** Volunteers were admitted to the
114 Clinical Centre and serum and saliva sampling was commenced at 1500h on Day 1 under
115 stable conditions. Standard mixed meals were given on Day 1 at 1300h and 1900h and on
116 Day 2 at 0800h and 1300h. Sleep disruption was minimised and subjects slept with lights out
117 from 2300h to 0600h. Serum samples for circulating cortisol and cortisone, cortisol binding
118 globulin and albumin were collected every hour until Day 2 at 1500h whereas salivary
119 samples for cortisol and cortisone were collected every hour between 1500h and 2200h and
120 between 0700h and 1500h. Subjects were asked not to eat, drink or wash their teeth 30
121 minutes prior to each sample collected. Serum free cortisol was measured at 2200h, 0700h
122 and 0900h.

123

124 **Study periods 2 & 3, oral and intravenous (IV) hydrocortisone administration:** During
125 period 2 each volunteer was administered 20mg oral hydrocortisone and during period 3
126 20mg IV at 07:00h. Serum samples for cortisol, cortisone, cortisol binding globulin and
127 albumin and salivary samples for cortisol and cortisone were taken at -10min, 15min, 30min,
128 45min, 60min, 1 hr 15min, 1 hr 30min, 2hr, 2hr 30min, 3hr, 4hr, 5hr, 6hr, 8hr, 10hr and 12hr.
129 Free cortisol levels were measured at -10min and at 2 hours post drug administration.
130 Volunteers received dexamethasone 1mg orally at ~2200h on Day 1 and at ~0600h and

131 ~1200h on Day 2 during each study period to suppress the hypothalamo-pituitary-adrenal
132 axis. All volunteers were given a standard mixed meal on Day 1 at 1900h and on Day 2 at
133 0800h and 1300h.
134
135 **Assays:** LC-MS/MS analysis for serum, free and salivary cortisol/cortisone was performed
136 using a Waters Xevo TQ-MS^{TM} mass spectrometer and a Waters Acquity^{TM} LC system with
137 an electrospray source operated in positive ionisation mode (15). The lower limit of
138 quantitation (LLOQ) for serum cortisol was was 12.5nmol/L. The inter-assay imprecision
139 was 8, 7 and 6% at concentrations of 80, 480 and 842nmol/L respectively. The cumulative
140 intra-assay and inter-assay coefficients of variation (CVs) for the LC-MS/MS method for free
141 cortisol were <8% and <9.5% respectively. Salivary cortisol and cortisone were measured
142 with a modified LC-MS/MS assay with lower limits of detection 0.80nmol/L (salivary
143 cortisol) and 0.50nmol/L (salivary cortisone). Intrassay CVs were less than 9.3% and less
144 than 7.9%; and interassay CVs were less than 9.7% and less than 10.3% at 1.8–52.2 nmol/L
145 of salivary cortisol and 3.6–96 nmol/L of salivary cortisone, respectively (16). Free cortisol
146 was measured using the same method as saliva but serum samples were filtered with a
147 molecular weight centrifugal filter (cut off 30,000Da) prior to analysis to remove binding
148 proteins. Inter assay CVs were less than 8% at 40-85 nmol/L of free cortisol.
149
150 **Statistical Analysis:** All statistical analyses were performed using MatLab and Microsoft
151 Excel 2010. Means or medians and 95% confidence intervals (CI), inter-quartile ranges or
152 ranges were calculated for continuous variables. The free cortisol index (FCI) was calculated
153 as serum total cortisol/CBG (nmol/mg). The peak-to-trough ratio was estimated by the
154 maximal concentration/minimal concentration of the substrate over 24 hours. The serum free
155 cortisol (FF) was expressed as a percentage of total serum cortisol (SF): (FF/SF*100%). The

156 difference in FF/SF*100% between separate time points was assessed using non-parametric
157 Wilcoxon test for related samples. Linear mixed effects models were used for both cosinor
158 and regression analysis to account for intra- and inter-subject variability. Model selection was
159 by likelihood-ratio test between models and statistically significant but more complex models
160 with only marginal improvement in Akaike Information Criterion (AIC) or Bayesian
161 Information Criterion (BIC) were rejected in favour of simplicity. The selected mixed effects
162 models were all found to be superior to their fixed effects equivalents ($P<=0.001$) although
163 only marginally so for the cosinor models of albumin and CBG.

164

165 **Results**

166 **During physiological conditions (study period 1)**, serum cortisol, cortisone and the
167 calculated free cortisol index [FCI; serum total cortisol/CBG (nmol/mg)] all showed a similar
168 circadian rhythm as defined by a cosinor model, with similar times for acrophase and nadir
169 and similar peak to trough ratios (**Table 1; Fig.1**). The peak to trough ratios were large in
170 amplitude for both serum cortisol and cortisone; median (range) 11.05 (6.57-66.77) and 6.16
171 (3.37-15.24), respectively. Serum CBG and albumin showed a statistically significant but
172 very low amplitude circadian rhythm, the acrophase at around 1600h to 1700h was out of
173 phase with serum cortisol, and the peak to trough ratios for CBG and albumin were small at
174 <1.2. Serum cortisol, FCI and free cortisol showed similar changes over the day being high
175 first thing in the morning and low at night. Serum free cortisol when expressed as a
176 percentage of total serum cortisol (FF/SF*100) was lower in the evening when compared to
177 morning (Supp **Table 1**).

178

179 There was considerable individual variability in the serum cortisol:cortisone ratio, however
180 there was a circadian rhythm ($p<001$): the median (range) mesor was 4.3 (3.0 — 5.8)

181 peak:trough ratio 1.4 (1.3 – 1.5), peak 5.0 (3.7 — 6.6), trough 3.6 (2.4 — 5.0), acrophase
182 08:08h (07:17–08:51h), and nadir 20:04h (19:13 – 20:47h) (Supp Fig. 1a). Individual cross-
183 correlation analysis of serum cortisol with serum cortisone for relative shifts from -24 to 24
184 hours showed a clear maximum at zero (P<0.001) for all subjects (Supp **Fig. 1**). This
185 suggests that whilst the changes in cortisone concentrations might lag cortisol, any such lag is
186 undetectable at the one hour resolution of the data.

187

188 95% CI for correlations (ρ) of salivary cortisone and salivary cortisol with serum cortisol
189 rhythm varied between 0.89 - 0.93 and from 0.86 - 0.92; P<0.001, respectively. When serum
190 cortisol was <74 (SD 29) nmol/L, salivary cortisol was not detectable (19% of total values).
191 After excluding these undetectable values salivary cortisol and cortisone both showed very
192 strong correlations with serum cortisol, cortisone, FCI, and free cortisol (**Table 2**). The
193 strongest correlation was between salivary cortisone and serum cortisol. The median (range)
194 ratio between salivary cortisone and salivary cortisol was 6.4 (Range 2.4 - 14.6).

195

196 **Study periods 2 & 3, following oral and IV administration of hydrocortisone:** After oral
197 hydrocortisone administration salivary cortisol levels showed no correlation with serum
198 cortisol (**Table 3a**). This was related to some salivary cortisol values showing spuriously high
199 levels presumed to be contamination from the oral hydrocortisone which was not seen after
200 intravenous. By contrast, salivary cortisone maintained a strong correlation with serum
201 cortisol, and FCI and the correlation coefficient for salivary cortisone with serum cortisol was
202 the same as that seen under physiological conditions (ρ = 0.91; P<0.001) (**Table 3b**).

203

204 **Pooling data from periods 1, 2 & 3:** there was a strong linear relationship between salivary
205 cortisone and serum cortisol (**Fig. 2a**). Logarithmic transformation reduced heteroscedasticity

206 in the variance and was used in a linear mixed effects model grouped by subject (**Fig. 2b**).
207 The best fit model required only random intercepts for each subject (likelihood ratio test,
208 P<=0.001) and explained 94% of the variability in the data. The explanation for the inter-
209 subject variability was further explored by examining age, body mass index (BMI), body
210 surface area (BSA), height, and weight however none of these variables could contribute
211 significantly to the model. The final, best-fit model is given by $\log_{10}$ serum F = 1.24 + 0.89 ×
212 $\log_{10}$ salivary E + b_j, where b_j represents the modification of the intercept for subject j, j =
213 1,2, …,14 and values ranged from -0.13 to 0.14.
214
215 **Normative salivary cortisol and cortisone levels:** To calculate the 24 hour rhythm of
216 salivary cortisol and salivary cortisone we used the fixed-effects components of the fitted
217 regression models to "back transform" the 2-cosinor model for serum cortisol. The full,
218 mixed effects regressions were also used to provide estimates of the missing night-time
219 salivary cortisone levels (2300h to 0600h) **(Fig. 3)**.
220
221 **Discussion**
222 Salivary cortisol and salivary cortisone show a very strong correlation to serum cortisol and
223 salivary cortisone provides a better marker of serum cortisol as it is detectable at low levels of
224 serum cortisol and not contaminated by oral hydrocortisone. We found that CBG and albumin
225 showed a circadian rhythm that was out of phase with the circadian rhythm of cortisol.
226 However, the peak to trough ratio for both CBG and albumen was <1.2 and therefore the
227 circadian changes in serum cortisol and serum free cortisol within an individual over 24h is
228 predominantly due to either secretion of cortisol or absorption of hydrocortisone with
229 changes in CBG and albumen having a minimal effect. Salivary cortisone has previously
230 been shown to have a linear relationship to serum cortisol (6,14). We have now extended this

231 observation by modelling the relationship in fine detail over 24 hours, confirming it exists
232 under both physiological cortisol secretion and after the administration of hydrocortisone, and
233 by defining the key parameters of salivary cortisone levels over 24 hours.
234
235 Cortisol is inactivated through oxidation to cortisone by 11β-HSD Type 2 in organs such as
236 kidney, salivary glands and the colon and cortisone is either excreted in urine or re-shuttled
237 back into the circulation, to be reduced back to cortisol by 11β-HSD Type 1 in the tissues
238 (17,18). By activating cortisone to cortisol, the Type 1 enzyme amplifies the effect of cortisol
239 in tissues, including liver and adipose tissue (19). Our results showed that serum cortisone
240 correlated strongly with serum cortisol. Cross correlation analysis showed correlation
241 between serum cortisol and simultaneously measured cortisone suggesting that both under
242 physiological conditions and after administration of hydrocortisone there is rapid conversion
243 of serum cortisol to serum cortisone, at least within the sampling period of the study. This
244 conversion of cortisol to cortisone predominantly reflects the function of 11β-HSD Type 2 in
245 the kidneys as it protects the mineralocorticoid receptor from excess active cortisol by
246 converting it to inactive cortisone (20). From our results serum cortisol levels exceed those of
247 cortisone with a ratio of 4.3 cortisol:cortisone. In contrast in saliva cortisone levels exceed
248 those of cortisol with a ratio of 6.4 cortisone:cortisol. There was considerable individual
249 variability in the serum cortisol:cortisone ratio, however there was a low amplitude circadian
250 rhythm with a mesor of 4.3. Previous publications have demonstrated that salivary cortisone
251 closely reflects serum free cortisol (6,14). We have now shown that both salivary cortisol and
252 cortisone have a much stronger correlation with serum cortisol than with serum cortisone
253 consistent with salivary cortisone being derived from serum free cortisol through conversion
254 by 11β-HSD Type 2 in the salivary gland. We have established that repetitive sampling of

255 salivary cortisone is a suitable alternative to measuring serum cortisol both under
256 physiological conditions and after the administration of hydrocortisone.
257
258 We further examined the relationship between salivary cortisone and serum cortisol using a
259 mixed effects model. Log transforming the data provided a minor improvement in the model
260 and reduced the heteroscedasticity in the residuals. In this population around 94% of the
261 variability in salivary cortisone could be explained by the change in serum cortisol. We
262 examined demographic characteristics (age, weight, height, BMI, BSA) to see if they
263 explained the minor inter-subject variability but they were found not to do so. We would
264 therefore propose a fixed effects model could be used to back calculate serum cortisol from
265 salivary cortisone but this needs to be tested in prospective studies in different populations.
266 By inverting the mixed effects model to estimate night-time salivary cortisone levels, we
267 developed a full 24-hour salivary cortisone rhythm model (Fig. 3), and this could be used in
268 clinical practice.
269
270 The use of salivary methods to measure cortisol is becoming increasingly common both in
271 research and in the clinical management of patients as an alternative, or an adjunct, to the
272 more frequently used serum or urinary cortisol measurements. The introduction of LC-
273 MS/MS has further increased the sensitivity and specificity of the assays and allows the
274 simultaneous measurement of both salivary cortisol and cortisone (6). Immunoassays are
275 complicated by cross-reactivity with other steroids including cortisone, which impacts on
276 specificity and test accuracy (21). The use of LC-MS/MS as in this study is important for the
277 individual measurement of steroids, including cortisol and cortisone. The methods used are
278 becoming simpler, faster and have a quick turn-around achieving speeds similar to
279 immunoassays. They are therefore used extensively in hospital laboratories (22), are an ideal

280 tool for researchers and the measurement of salivary cortisol has been recommended in major
281 guidelines (3). However, despite the improved sensitivity of LC-MS/MS we were still unable
282 to measure salivary cortisol when the serum cortisol was low.
283
284 Disturbances in cortisol rhythm are associated with ill-health. A flattened diurnal cortisol
285 slope is associated with cardiovascular mortality (23), Type 2 diabetes (24) and obesity (25).
286 Therefore, measuring the cortisol rhythm by stress free, non-invasive salivary cortisone
287 sampling could potentially be an additional means of assessing patient health risk on an
288 everyday basis; though this still needs to be established in future studies. Furthermore, no
289 consensus exists on how to titrate hydrocortisone doses in patients on glucocorticoid
290 replacement (26). The use of two salivary cortisol samples, measured 1 hour and 30 minutes
291 after cortisone acetate, as an indication of 24-hour cortisol exposure has been suggested but
292 this does not give an indication of fluctuating changes (27). The use of multiple salivary
293 cortisone samples allows an objective assessment of cortisol exposure after hydrocortisone
294 and has been used during hydrocortisone infusion (28). Moreover, measuring salivary
295 cortisone after hydrocortisone does not have the same risk for drug contamination as
296 observed when measuring salivary cortisol in this setting (29). As a wide variability in
297 hydrocortisone pharmacokinetics and glucocorticoid sensitivity exists between individuals
298 multiple salivary cortisone sampling can be used in assessing hydrocortisone replacement.
299 Interestingly early morning salivary cortisone has been proposed to be a superior test to
300 salivary cortisol in detecting severe adrenal insufficiency in patients on glucocorticoids (30).
301
302 Our study is limited by the relatively small number of subjects and its cross-sectional nature
303 but the analysis was carried out in detail, under carefully controlled environmental conditions
304 with strict supervised sampling of multiple samples and we have undertaken a detailed

305 analysis. Furthermore the study is only carried out in men. It has been shown that in women

306 on oestrogens besides a rise in the bound cortisol pool, free cortisol pharmacokinetics may be

307 affected resulting in excess cortisol exposure especially in patients on hydrocortisone in

308 whom the HPA axis might not adjust (7). Salivary cortisone reflects free cortisol

309 concentrations irrespective of elevations in CBG (14) but detailed modelling of the

310 mathematical relationship with total circulating cortisol levels in this setting has not been

311 performed. Salivary cortisone has been shown to be a more useful tool to assess the cortisol

312 circadian rhythm under both physiological conditions and after oral hydrocortisone

313 replacement. We have shown how normative salivary cortisone levels can be calculated and

314 displayed. Future studies should assess salivary cortisone measurement prospectively in

315 clinical and research settings to evaluate salivary cortisone use as a diagnostic tool.

316

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Tables

Table 1 Time and concentration taken from best fit individual cosinor models (likelihood ratio test P<=0.001) in 14 healthy male volunteers.

	Clock time Median (range)		**Concentration Median (range)**		**Peak:trough ratio Median (range)**
Variable	**Acrophase**	**Nadir**	**Peak**	**Trough**	
Cortisol[1] (nmol/L)	07:24 (06:48-08:08)	23:23 (22:43-00:03)	323.9 (289.0-443.9)	30.7 (5.0-67.6)	11.05 (6.57-66.77)
Cortisone[1] (nmol/L)	07:31 (06:48-08:51)	23:05 (22:36-00:24)	64.8 (44.4-86.1)	10.2 (3.7-22.7)	6.16 (3.37-15.24)
FCI[1] (nmol/mg)	07:24 (06:41-08:08)	23:08 (22:29-23:55)	15.5 (14.9-18.4)	1.16 (0.38-3.36)	12.86 (5.46-40.67)
CBG[2] (mg/L)	16:52 (16:12-17:32)	04:49 (04:16-05:28)	23.2 (19.4-27.2)	20.8 (17.1-24.7)	1.12 (1.10-1.14)
Albumin[2] (g/L)	14:27 (13:40-14:52)	02:38 (01:44-03:18)	46.1 (42.7-48.5)	42.3 (38.9-44.7)	1.09 (1.09-1.10)

[1] Two-harmonic model is best fit (P≤0.001)

[2] One-harmonic model is best fit (P≤0.001)

FCI: Free Cortisol Index, CBG: Cortisol Binding Globulin

Table 2 Correlation matrix Period 1 with significant correlation coefficient ρ (P<=0.001) (N=14)

P (95% CI)	Serum F	Serum E	FCI	Free F[1]	Salivary F[2]	Salivary E
Serum F	1	0.83 (0.79-0.86)	0.97 (0.96-0.98)	0.91 (0.83-0.96)	0.90 (0.87-0.92)	0.91 (0.89-0.93)
Serum E		1	0.81 (0.78-0.85)	0.72 (0.50-0.86)	0.52 (0.40-0.61)	0.77 (0.71-0.81)
FCI			1	0.90 (0.80-0.95)	0.90 (0.87-0.92)	0.96 (0.95-0.97)
Free F[1]				1	0.89 (0.76-0.95)	0.85 (0.72-0.93)
Salivary F[2]					1	0.87 (0.83-0.90)
Salivary E						1

[1] Undetectable values excluded (6% values)

[2] Undetectable values excluded (19% values)

F: cortisol, E: cortisone, FCI: Free Cortisol Index

Table 3a Correlation matrix Period 2 after oral HC with ρ (P<=0.001) (N=14)

ρ (95% CI)	Serum F	Serum E	FCI	Salivary F[1]	Salivary E
Serum F	1.0	0.51 (0.40-0.60)	0.97 (0.96-0.97)	0.30 (0.16-0.43)	0.84 (0.79-0.87)
Serum E		1.0	0.53 (0.43-0.62)	NS	0.61 (0.52-0.68)
FCI			1.0	0.30 (0.16-0.43)	0.88 (0.84-0.90)
Salivary F[1]				1.0	NS
Salivary E					1.0

NS "not significant p>0.001

[1] Undetectable values excluded (19%)

Table 3b Correlation matrix Period 3 after intravenous HC with ρ (P<=0.001)

ρ (95% CI)	Serum F	Serum E	FCI	Salivary F[1]	Salivary E
Serum F	1.0	0.68 (0.6-0.75)	0.97 (0.97-0.98)	0.89 (0.85-0.91)	0.94 (0.92-0.95)
Serum E		1.0	0.68 (0.60-0.74)	0.33 (0.20-0.46)	0.66 (0.58-0.73)
FCI			1.0	0.87 (0.84-0.91)	0.96 (0.95-0.97)
Salivary F[1]				1.0	0.87 (0.83-0.90)
Salivary E					1.0

[1] Undetectable values excluded (19%)

F:cortisol, E:cortisone, FCI: Free Cortisol Index

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Figure 1: Cosinor models for serum cortisol, cortisone, Free Cortisol Index (FCI), Cortisol Binding Globulin (CBG) and albumin along with their corresponding 95% prediction intervals overlaid on observed data (+) (N=14). The number of harmonics for the models was 2 for cortisol, cortisone and FCI and 1 for albumin and CBG.

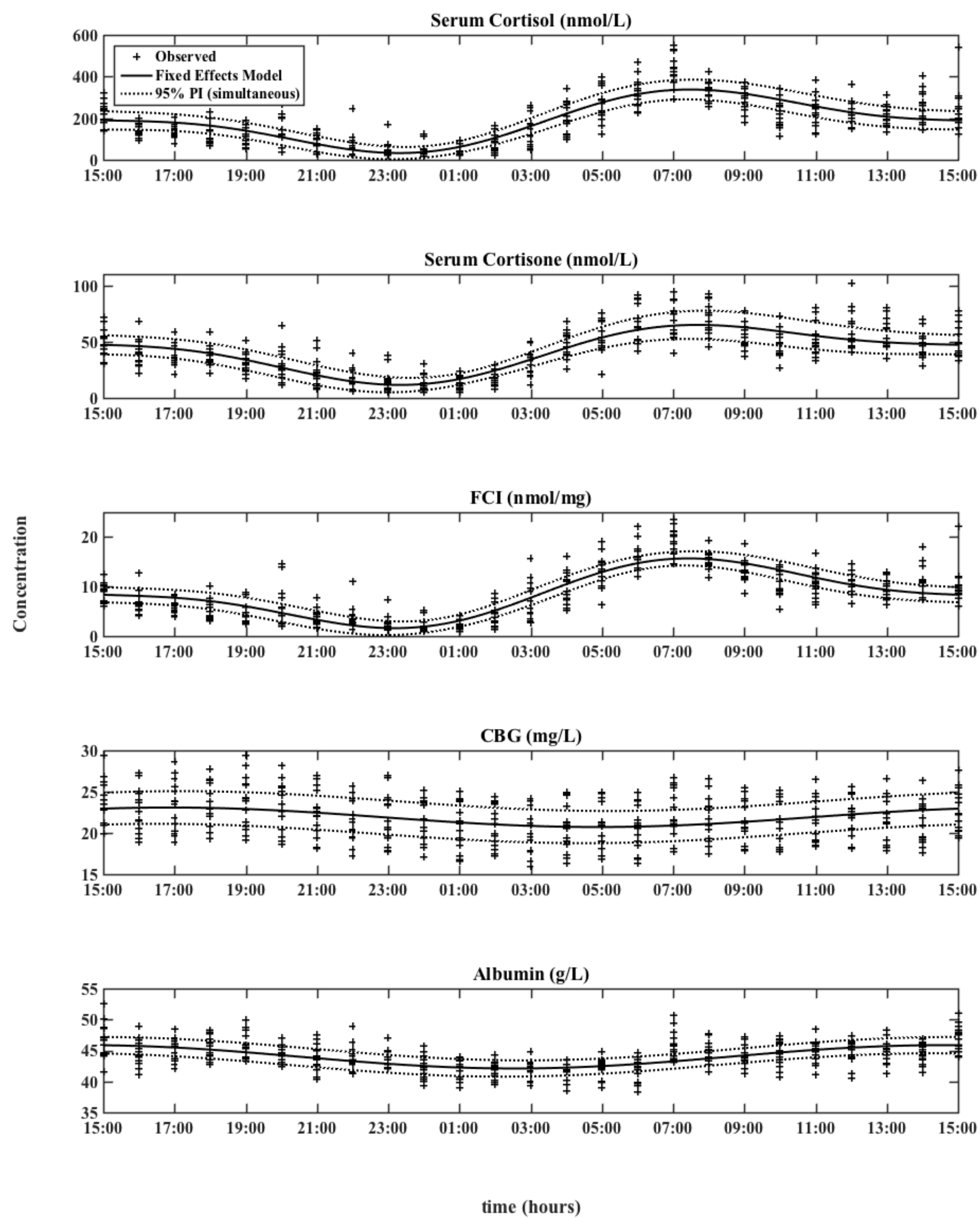


Figure 2: **(a)** Scatter graph showing relation of serum cortisol to salivary cortisone by Period. **(b)** Mixed-effects model showing per subject variation. The lines are the individual subject regression lines and all have the same slope with minor variation in intercept. The fixed effects line is shown in cyan.

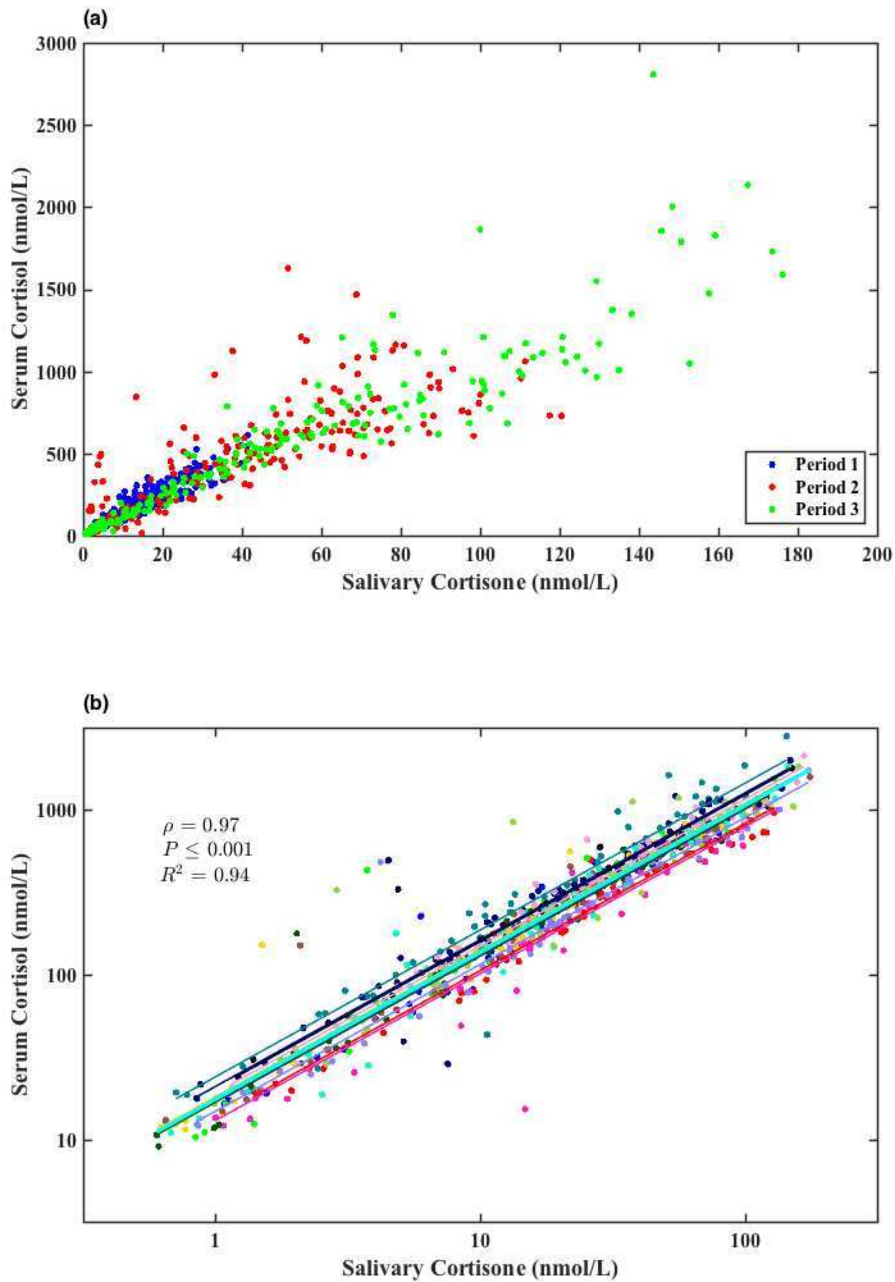
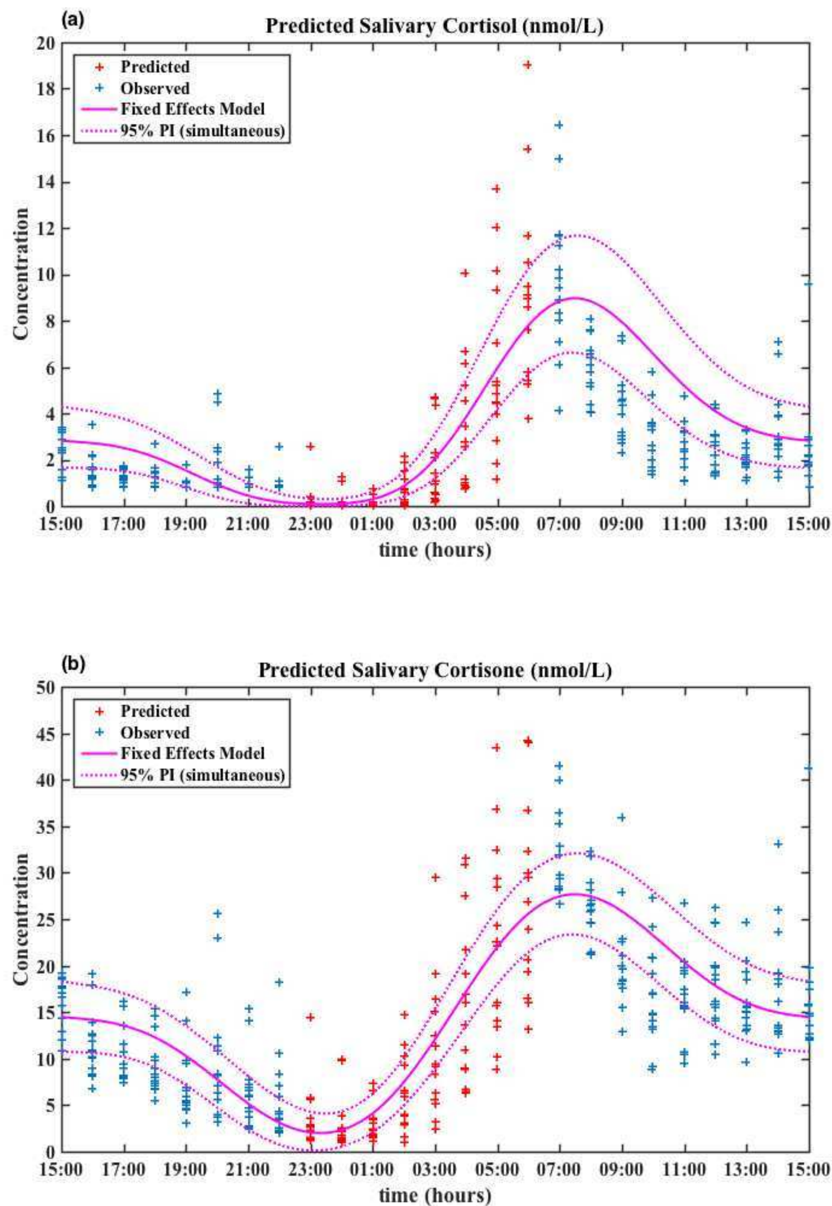
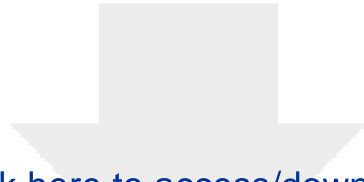


Figure 3: Concentration-time profiles for salivary cortisol (a) and salivary cortisone (b) including 2-cosinor fixed-effects models and their corresponding 95% prediction intervals. These were obtained via "back transformation" using the fixed effects components of their respective regression models. Salivary cortisone values between 2300h and 0600h were predicted by inverting the mixed effects model and applying it to the night-time serum cortisol observations. By cosinor analysis the median (range) was estimated as (a) salivary cortisol acrophase 07:24 (06:48 – 08:08), nadir 23:23 (22:43 – 00:03), peak 8.3nmol/L (6.6 - 15.5nmol/L), trough 0.1nmol/L (0.0 - 0.4nmol/L) and peak:trough ratio 122.8 (43.3 – 4505.0), and (b) salivary cortisone acrophase 07:24 (06:48 – 08:08, nadir 23:23 (22:43 – 00:03), peak 27.1nmol/L (18.0 – 45.1nmol/L), trough 1.8nmol/L (0.3 – 5.0nmol/L) and peak:trough ratio 14.8 (8.3 – 111.6).





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