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Short communication

Simultaneous measurement of cortisol and cortisone in human saliva using liquid chromatography–tandem mass spectrometry: Application in basal and stimulated conditions

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ABSTRACT

Immunoassays used for the measurement of salivary cortisol are limited by variable interference from cortisone. Salivary cortisone is a consequence of the salivary glands expressing 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) which converts cortisol to cortisone. We report a combined salivary cortisol and cortisone (SalF and SalE respectively) liquid chromatography–tandem mass spectrometry (LC–MS/MS) assay to address the cortisone cross-reactivity in cortisol immunoassays and as a tool to study 11β-HSD2 activity. The method was linear up to 400 nmol/L for SalF and 200 nmol/L for SalE and the lower limits of quantitation were 0.39 nmol/L (SalF) and 0.78 nmol/L (SalE). No evidence of ion suppression was found and precision, accuracy and recovery were within internationally accepted limits. No interference was identified from 13 structurally related steroids. SalF, SalE and SalF/SalE were significantly greater in the morning than at bed-time and following stimulation of the adrenal glands. As serum cortisol increased, an exponential rise was observed in SalF and a linear increase in SalE which reached a plateau at higher SalF concentrations. We have developed a novel, robust LC–MS/MS assay for the combined measurement of SalF and SalE. Our results confirm the 11β-HSD2 activity of the salivary glands resulting in high SalE concentrations and the enzyme saturation at high substrate concentrations. This method can be used as a simple, non-invasive and highly specific tool to assess the value of salivary cortisol as a surrogate for free serum cortisol and as a potential novel way to assess 11β-HSD2 activity.

1. Introduction

In recent years measurement of cortisol in saliva has gained an important role in the study of the hypothalamic-pituitary-adrenal (HPA) axis, in both psychological and endocrine research. Moreover, its value in the screening for Cushing's syndrome has been widely acknowledged and it is now recommended as a first-line diagnostic test [1].

The advantages of measuring salivary cortisol include the non-invasive sampling but most importantly the evidence of good correlation between cortisol in saliva and the unbound, biologically active serum cortisol fraction [2]. However, a major limitation of the determination of cortisol concentrations by routinely used immunoassays, especially at the very low levels found in saliva, is the cross-reactivity by exogenous glucocorticoids and endogenous cortisol precursors and metabolites. Although a good correlation exists between results generated by different assays, absolute values show significant discrepancies and assay-specific reference ranges are needed [3].

An interesting characteristic of the parotid glands is the expression of the enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), that inactivates cortisol by converting it to cortisone and is also present in tissues such as the kidney, colon and placenta [4]. 11β-HSD2 and the cortisol–cortisone shuttle have been implicated in the pathophysiology of essential hypertension and an inherited genetic defect of the enzyme causes the syndrome of apparent mineralocorticoid excess (SAME) [5]. A similar clinical picture can arise following inhibition of 11β-HSD2 by glycyrrhetinic acid, a compound contained in many widely consumed products such as liquorice [6].

A highly sensitive and specific method is needed in order to study two very similar compounds such as cortisol and cortisone in

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the very low levels found in saliva. Liquid chromatography–tandem mass spectrometry (LC–MS/MS) can provide the specificity required to eliminate interference by steroids related to cortisol and has been used previously for the combined quantification of cortisol and cortisone in serum, plasma and urine but not saliva [7–10]. We aimed to develop an LC–MS/MS assay for the simultaneous measurement of cortisol and cortisone in saliva, with the intention of understanding the factors regulating salivary cortisol concentration and as a tool to study the cortisol–cortisone shuttle.

2. Material and methods

2.1. Materials

Stock solutions of cortisol and cortisone (both >98% pure) were prepared by dissolving hydrocortisone and cortisone powder (Sigma, Poole, UK) in methanol to concentrations of 11.8 and 10.8 mg/mL respectively. Working calibrators were prepared by dilution of stock solutions in phosphate buffered saline (PBS, pH 7.4) to concentrations of 0, 0.32, 0.64, 1.27, 2.55, 5.1, 10.2, 20.4, 40.7, 81.5 and 163 nmol/L for cortisol and 0, 0.29, 0.58, 1.17, 2.34, 4.7, 9.4, 18.75, 37.5, 75 and 150 nmol/L for cortisone. QCs were prepared in PBS from separate hydrocortisone and cortisone stocks to concentrations of 1.8, 10.8, 52.2 nmol/L and 3.6, 21.6, 96 nmol/L respectively.

2.2. Sample preparation

d4-cortisol and d2-cortisone (CDN Isotopes, Quebec, Canada), 10 μL of 10 μg/L each, were added as internal standards to 100 μL of calibrator, QC or sample and 100 μL of H_2O in a 96-well plate. The plate was then thermo-sealed, vortex-mixed for 2 min and centrifuged at 1500 × *g* for 5 min.

2.3. Liquid chromatography

The plate was placed in a Acquity UPLC system (Waters, Manchester, UK) and 40 μL of extract was injected onto a 4 mm × 2 mm C8 Gemini guard cartridge which was attached to a 30 mm × 3 mm 4 μm Synergy Hydro-RP C18 analytical column (both Phenomenex, Macclesfield, UK), for on-line solid phase extraction as described previously [11,12]. The mobile phases were: A = water with 2 mmol/L of ammonium acetate and 0.1% (v/v) formic acid and B = methanol with 2 mmol/L of ammonium acetate and 0.1% (v/v) formic acid. The guard cartridge was washed with 10% B for 0.6 min at 1 mL/min. Cortisol and cortisone were eluted isocratically at 60% B for 2.5 min followed by a column wash with 100% B for 1 min before returning to starting conditions (60% B). The run time was 5 min.

2.4. Tandem mass spectrometry

The eluant was injected into a Quattro Premier tandem mass spectrometer (Waters, Manchester, UK) operated in electrospray positive mode (ES+). Following tuning with 1 mg/L cortisol, d4-cortisol, cortisone and d2-cortisone the optimised conditions were: capillary at 1 kV, source temperature at 140 °C, desolvation gas flow at 798 L/h, cone energy 28 V, collision energy 26 eV. The transitions used were *m/z* 363.2 > 120.9 (cortisol), 367.2 > 120.8 (d4-cortisol), 361.2 > 162.9 (cortisone), 362.9 > 165 (d2-cortisone). Secondary transitions for cortisol and cortisone were monitored for confirmation at *m/z* 363.2 > 96.8 and *m/z* 361.2 > 147.0 respectively.

2.5. Method validation

All validation steps were performed according to published international guidelines [13]. Ion suppression was evaluated by post-column infusion and pre-extraction addition (recovery). Cortisol (55 nmol/L) and cortisone (139 nmol/L), both in 50:50 (v/v) mobile phase A:B, were infused post-column and the reduction in ion counts was used to detect any ion suppression following injection of *n* = 6 saliva samples. Recovery experiments included spiking six saliva samples and water with three different concentrations for cortisol (4, 8 and 16 nmol/L) and cortisone (3.7, 7.5 and 15 nmol/L) and the comparison of responses from samples to the ones from water.

The assay linearity was studied using the squared correlation coefficient (R^2) on a separate stock of calibrators with concentrations ranging 0–400 nmol/L for cortisol and 0–200 nmol/L for cortisone.

The lower limit of quantitation was the lowest measured concentration with a CV and deviation from target <20%. For the study of intra-assay and inter-assay precision, three QCs were assayed 10 times within the same run and in 10 consecutive runs respectively and the CV was calculated. Accuracy was estimated for three different cortisol/cortisone concentrations based on the percentage deviation of the mean value from the target following 10 measurements of each of the three QCs.

Interference was studied by the injection of 1 μmol/L of corticosterone, prednisolone, methylprednisolone, dexamethasone, 11-deoxycortisol, 21-deoxycortisol, spironolactone, progesterone, pregnenolone, 17-hydroxyprogesterone, aldosterone, dehydroepiandrosterone and dehydroepiandrosterone-sulphate and the identification of any peaks at the cortisol and cortisone channels.

2.6. Method and sampling device comparison

Saliva samples (*n* = 41) collected using the Salivette synthetic swab (Sarstedt, Nümbrecht, Germany) were also analysed by an electrochemiluminescent cortisol immunoassay (ECLIA, Roche Diagnostics GmbH, Mannheim, Germany). Results were also compared to those obtained from samples collected simultaneously using the Salivette cotton swab with citric acid preparation (Sarstedt, Nümbrecht, Germany).

2.7. Clinical application

The samples used in the circadian study were collected from healthy volunteers (*n* = 14) at 09.00 and bed-time. For the stimulation studies, patients (*n* = 54, details published previously [12]) had a Short Synacthen Test (SST, 250 μg IM). Matched serum and saliva samples (*n* = 331) were also used from a circadian study of oral modified-release hydrocortisone in healthy volunteers (*n* = 6, details published in [14]).

Serum cortisol was measured by an automated immunoassay (Siemens Centaur, lower limit of quantitation 50 nmol/L, within-run CV 3.1%, 3.8%, 3% and between-run CV 3.8%, 1.9%, 4% at 155, 760 and 1025 nmol/L respectively).

Samples were analysed following communication with the UK National Research Service that defined the procedure as service evaluation.

2.8. Statistical analysis

Statistical analysis was performed using the SPSS software (SPSS 15.0; SPSS, Inc., Chicago, IL). Bland–Altman analysis was applied for the method and sampling device comparison. Values in the clinical application study were not normally distributed and are reported as

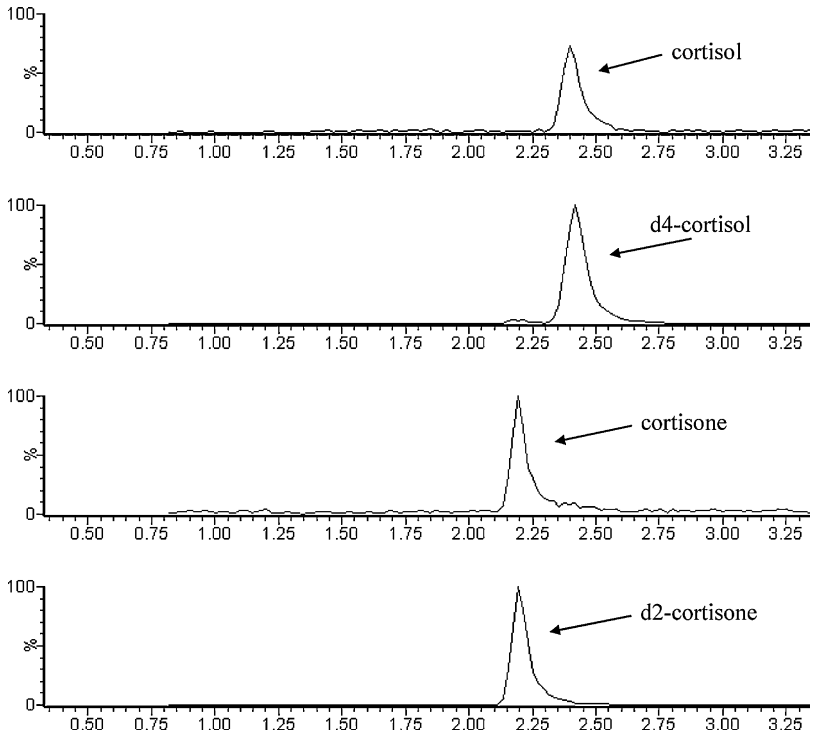

Fig. 1. Multiple reaction (MRM) chromatogram of cortisol and cortisone.

median (range) and the Wilcoxon matched pairs test was used for the comparisons. $P \leq 0.05$ was considered statistically significant.

3. Results and discussion

3.1. LC–MS/MS characteristics

Cortisol and cortisone were fully separated chromatographically (Fig. 1). The retention time for cortisol was 2.45 min and for cortisone 2.22 min, while the full run time was 5 min. There was no evidence of ion suppression in the experiments conducted and mean recoveries were 104%, 114% and 107% for cortisol and 96%, 100% and 110% for cortisone for the three concentrations studied. No interfering peaks were observed for any of the steroids examined.

3.2. Linearity, lower limit of quantitation, precision, accuracy and interferences

A set of calibrators covering a wider range of concentrations than the one used routinely was used in order to assess linearity. The assay was found to be linear up to 400 nmol/L for cortisol and 200 nmol/L for cortisone ($R^2 = 0.999$ and $R^2 = 0.996$ respectively). The lower limit of quantitation was 0.39 nmol/L for cortisol and 0.78 nmol/L for cortisone.

Precision and accuracy are described in detail in Table 1. Intra-assay CV were all <9.3% for cortisol concentrations of 1.8, 10.8 and 52.2 nmol/L and <7.9% for cortisone concentrations of 3.6, 21.6 and 96. The mean concentration was <10% from the target for cortisol and <10.5% for cortisone. The inter-assay CV at the above cortisol/cortisone concentrations was <9.7% and <10.3% respectively and the mean concentration was <8% from the target for cortisol and <12% for cortisone.

3.3. Method and sampling device comparison

Samples analysed for cortisol by both LC–MS/MS and the ECLIA revealed the latter having a positive bias of 2.9 nmol/L with SD = 7.9 nmol/L (Fig. 2A). The comparison of two commercially available Salivette devices showed good agreement with a bias of 0.2 nmol/L and SD = 2.8 nmol/L (Fig. 2B), although the samples collected with the synthetic swabs produced cleaner chromatograms.

3.4. Clinical application

Salivary cortisol, cortisone and their ratio were significantly greater in the morning (Table 2, 09.00 vs bed-time, $P < 0.001$). Salivary cortisone was approximately 4 times higher than cortisol in the morning and 10 times higher at bed-time. This demonstrates an inverse cortisol–cortisone relationship in saliva than the one

Table 1
Within and between-batch imprecision and accuracy for salivary cortisol (SalF) and cortisone (SalE).

	Concentration (nmol/L)	CV (%)		Deviation of mean from target (%)	
		Intra-assay	Inter-assay	Intra-assay	Inter-assay
SalF	1.8	8.7	8.1	3.5	1.0
	10.8	9.3	9.7	11.7	3.1
	52.2	8.1	5.8	10.1	7.2
SalE	3.6	7.9	9.4	8.6	5.0
	21.6	4.2	10.3	6.6	11.9
	96	4.6	4.4	5.8	4.1

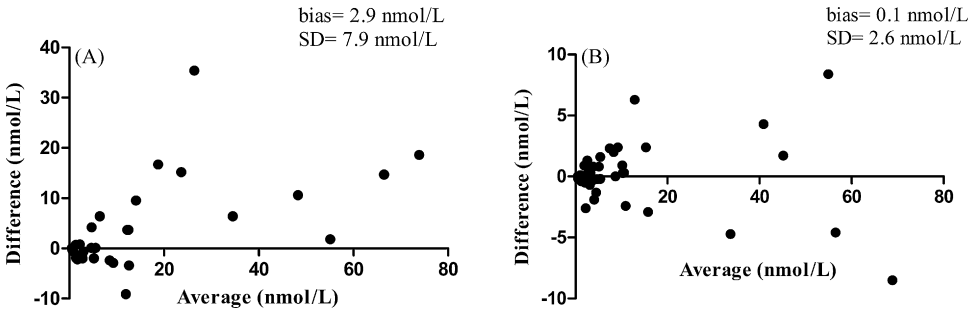

Fig. 2. Method and sampling device comparison of salivary cortisol measure Bland–Altman plots. (A) Bland–Altman plot of the method comparison. The *x*-axis represents average values of salivary cortisol measured by LC–MS/MS and ECLIA. The *y*-axis corresponds to the difference in salivary cortisol concentrations measured by LC–MS/MS and the ECLIA. Positive bias indicates higher values in the immunoassay. (B) Bland–Altman plot of the sampling device comparison. The *x*-axis represents the average values of salivary cortisol measured by LC–MS/MS in saliva collected using the synthetic and the cotton swab with citric acid Salivettes. The *y*-axis corresponds to the difference in salivary cortisol concentrations in the samples collected with the different sampling devices. Positive bias indicates higher values from the synthetic Salivettes.

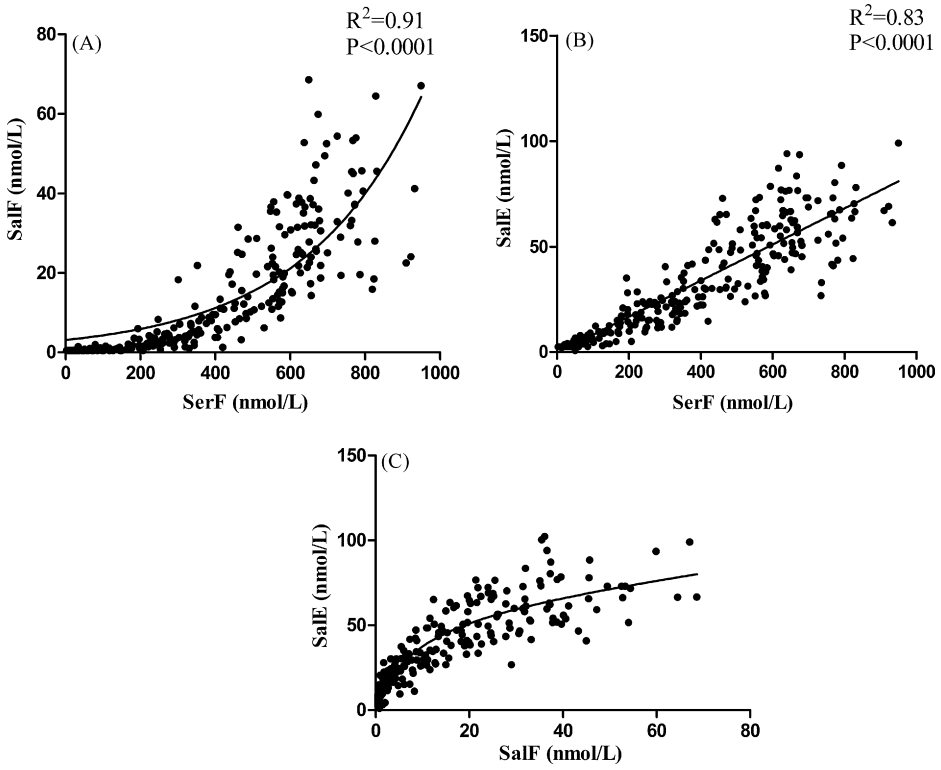

Fig. 3. Correlation plots and curve fitting in a wide range of cortisol and cortisone concentrations. (A) Curve fitting of salivary cortisol vs serum cortisol data; (B) curve fitting of salivary cortisone vs serum cortisol data; (C) salivary cortisol vs salivary cortisone concentrations plot.

observed in serum [15,16] and provides further evidence for the 11β-HSD2 activity of the salivary glands that results in the conversion of cortisol to cortisone. These findings also confirm the importance of our specific LC–MS/MS assay as some routinely used cortisol immunoassays with up to 30% cross-reactivity for cortisone [17] may be actually reflecting salivary cortisone rather than cortisol.

Table 2
Salivary cortisol (SalF) and cortisone (SalE) concentrations (nmol/L) under basal and stimulated conditions.

Times	SalF	SalE	SalF/SalE ratio
am	8.3 (3.2–14.8)	34.6 (18.6–47)	0.2 (0.1–0.4)
pm	0.6 (0.4–1.9)	5.9 (3.4–18.4)	0.1 (0.1–0.3)
$t = 0$[a]	4.2 (1.1–21.6)	23.8 (3.7–54.5)	0.2 (0.1–0.7)
$t = 60$[a]	33.7 (12.8–68.6)	65.1 (26.8–102.4)	0.6 (0.3–1.1)

[a] SST.

The concentrations of both compounds and their ratio were significantly greater following stimulation with ACTH (Table 2, $t = 0$ vs $t = 60$, $P < 0.0001$). Data from the Synacthen studies and the modified-hydrocortisone administration study were combined to produce cumulative plots of serum cortisol against salivary cortisol and salivary cortisone. Although salivary cortisol increased exponentially (Fig. 3A, exponential fit $R^2 = 0.91$, $P < 0.0001$), the increase in salivary cortisone was linear (Fig. 3B, linear fit $R^2 = 0.83$, $P < 0.0001$). Fig. 3C confirms that the increase in salivary cortisone tended towards a plateau at higher cortisol levels, possibly as a result of the 11β-HSD2 saturation at high substrate concentrations.

4. Conclusions

Our LC–MS/MS method for the combined measurement of salivary cortisol and cortisone involves minimal sample preparation, a total run time of 5 min and shows good sensitivity, precision and

accuracy. In contrast to routinely used immunoassays, this assay is free of interference by all steroids tested. Although LC–MS/MS has been used previously for the measurement of plasma, serum and urinary cortisol and cortisone [7–10] and for salivary cortisol [12,18], to the best of our knowledge this is the first report of a combined LC–MS/MS assay for salivary cortisol and cortisone.

The high cortisone concentrations found in saliva are rarely accounted for when reporting salivary cortisol measurements and imply that variable specificities for cortisone in cortisol immunoassays may cause discrepancies in reference ranges and cut-offs between different methods, with clear clinical implications, especially since bed-time salivary cortisol is currently included in the first-line screening tests for Cushing's syndrome [1].

In conclusion, we have developed a robust LC–MS/MS assay for the simultaneous measurement of cortisol and cortisone in saliva that addresses the problem of variable cortisone interferences in salivary cortisol immunoassays. This could lead to a useful and non-invasive tool in the study of the cortisol–cortisone shuttle as saliva is a post-enzyme tissue, similarly to urine that has been traditionally used for the measurement of free cortisol, cortisone and their metabolites in the study of 11β-HSD2 activity.

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