Combined measurement of cortisol and cortisone in human saliva using UPLC tandem-mass spectrometry

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Introduction

Measurement of salivary cortisol has become increasingly popular in studies of the hypothalamic-pituitary-adrenal (HPA) axis. It is used as a biomarker of psychological stress (1), in the diagnosis of adrenal insufficiency (2), and it has become a first-line marker for diagnosis of Cushing's syndrome (3). Salivary cortisol correlates strongly with the serum free cortisol concentration (4) and might be a more reliable indicator of cortisol status in patients with altered binding protein concentrations. Salivary cortisol, rather than serum cortisol, can be collected non-invasively and on an outpatient basis. The stability at room temperature allows postal mailing using commercially available devices (e.g. Salivettes, Sarstedt) (5). Salivary cortisone is a consequence of the salivary glands expressing 11ß hydroxysteroiddehydrogenase type 2 (11ß-HSD2) which converts cortisol to cortisone. Consequently, in patients with altered 11ß-HSD2 activity, e.g. in apparent mineralocorticoid excess syndrome or liquorice-induced hypertension, a combined test for cortisol and cortisone has diagnostic significance.
We developed a method for combined measurement of cortisol and cortisone in saliva using isotope-dilution Ultra Performance Liquid Chromatography (UPLC)-tandem mass-spectrometry (MS/MS).

Materials and Methods

Sample preparation

Saliva was routinely collected by use of Salivettes with polyester wad (Sarstedt Ltd). To 500 µl of patient saliva, 50 µl of internal standard (IS, 7.7 µmol/L cortisol-1,2-d2, Ritmeester BV, Utrecht, The Netherlands) and 6 ml of dichloromethane (DCM) were added. The samples were incubated for 15 min on a rotary shaker and subsequently centrifuged for 10 min at 16,000 g at 4 °C. After removal of the aqueous layer, the organic layer was transferred to a new glass tube and the content was evaporated under a continuous nitrogen stream. The residue was dissolved in 300 µl MeOH (53%)/formic acid(3%) (v/v) and transferred to vials which were sealed. Six calibration standards (range 1-1100 nmol/l) were made by diluting 1 mmol/l stock solutions (prepared in MeOH) of cortisol and cortisone (Sigma Aldrich, Zwijndrecht, The Netherlands) in water. Control samples with low, intermediate and high analyte concentrations were prepared from pooled saliva at 3 pm, 11 and 8 am, respectively.

UPLC-MS/MS system

20 µL of the reconstituted samples were injected onto an ACQUITY Ultra Performance LC (UPLC) BEH C18, 1.7 µm, 2.1 mm x 50 mm column (Waters Milford, MA, USA) and chromatographed at 45 °C at a flow rate of 0.25 mL/min on a ACQUITY UPLC system (Waters). Mobile phases A and B consisted of 5% acetonitrile (AcN) with 0.1% (v/v) acetic acid (AcOH), and 95% AcN with 0.06% (v/v) AcOH, respectively with the following gradient program: initial: 15% B; 0-2.0 min: increase to 45% B; between 2.0-2.5 min; increase to 100% B, maintained between 2.5-3.5 min, with reversion of the mobile phase to 15% B between 3.5-5.0 min. We quantified the analytes by using selected reaction monitoring (SRM) on a Waters ACQUITY TQ tandem quadrupole mass spectrometer, interfaced with an Atmospheric Pressure Electrospray Ionisation (AP-ESI) source operating in the positive ion mode. We monitored mass-to-charge (m/z) transitions 363.30→121.05 (cortisol), 361.34→163.10 (cortisone), and 365.30→122.22 (IS). Secondary transitions for confirmation were monitored at m/z 363.30→97.10 (cortisol) and 361.34→105.00 (cortisone). All aspects of system operation and data acquisition were controlled using Masslynx v4.1 software with automated data processing using the Quanlynx Application Manager (Waters).

Method validation

Intra-assay variation was obtained from 20 replicate measurements in a single series of three human saliva pools containing low, medium and high concentrations of cortisol (1.81, 3.49 and 7.49 nmol/l, respectively) and cortisone (10.94, 23.73 and 27.00 nmol/l,

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Abbreviations. UPLC: ultra performance liquid chromatography; MS/MS: tandem-mass spectrometry; AcN: acetonitrile; MeOH: methanol; AcOH: acetic acid; DCM: dichloromethane; GA: glycyrrhetinic acid; 11ß-HSD2: 11ß-hydroxysteroiddehydrogenase type 2; AP-EI: Atmospheric Pressure Electrospray Ionisation; IS: internal standard; SRM: selected reaction monitoring; SPE: solid phase extraction; LLE: liquid-liquid extraction.

respectively). Inter-assay variation was obtained from 20 assays over a 17 days period of three saliva pools containing low, medium and high concentrations of cortisol (0.50, 4.49 and 8.77 nmol/l, respectively) and cortisone (3.29, 12.14 and 20.66 nmol/l, respectively). The limit of detection (LOD) and of quantification (LOQ) was based on analyte signal/noise ratio of 3 and 10, respectively in saliva samples after serial dilution in water. Linearity was evaluated by measuring five replicates of six calibrator solutions of cortisol and cortisone (range 1-1100 nmol/l). Analyte recovery was tested by adding standard solutions of cortisol (28.8 nmol/l) and cortisone (33.9 nmol/l) to plain saliva with basal concentrations of 5.1 nmol/l and 30.6 nmol/l for cortisol and cortisone, respectively. Basal and spiked

saliva were added to polyester swabs, and the swabs were incubated for 30 min at RT before centrifugation and subsequent measurement in a single analytical run. The UPLC-MS/MS method was compared to an in-house RIA after paper chromatography for cortisol using 47 patient saliva samples with cortisol concentrations up to 30 nmol/l (6). To establish reference values, we measured morning and evening saliva samples from 66 apparently healthy individuals (34 males/ 32 females).

Results and Discussion

Using solid phase extraction (SPE) for analyte purification from urine samples, we experienced variable cortisol responses which prompted us to use liquid-liquid extraction (LLE) of both urine and salivary by DCM despite its practical inefficiency and difficulty to automate.

Cortisol and cortisone were partially separated chromatographically eluting at 2.1 min and 2.2 min, respectively, with a 5 min total runtime. No attempt was made to resolve the analytes chromatographically, because the specificity of the mass selection and fragmentation (m/z transitions) gave the necessary compound specificity. Assays for cortisol and cortisone were linear to at least 1100 nmol/l. Regression curves were y = 1.012x + 0.04 for cortisol (r^2 = 0.9998) and y= 1.007x – 0.04 for cortisone (r^2 =0.9985). For cortisol, intra- and interassay CV's were 2.8-7.9% and 5.1-20.1%, respectively. The inter-assay CV was 20% at a concentration of 0.5 nmol/l, reaching the assay's functional sensitivity. For cortisone, intra- and interassay CV's were 1.5-3.6% and 9.2-10.2%, respectively. LOD and LOQ were 0.2 and 0.5 nmol/l for cortisol and 0.5 and 1 nmol/l for cortisone. The mean (±SD) percentage recoveries were 104.3 (±5.3) % for cortisol and 116.4 (±14.5) % for cortisone. Passing & Bablok regression analysis of the RIA to UPLC-MS/MS for cortisol gave the equation: UPLC-MS/MS = 0.83 (95% Confidence Interval (CI): 0.76-0.90) x RIA -0.15 (95%CI: -0.51 to 0.21) (figure 1a). Correlation coefficient (r^2) was 0.997, with bias (± SD) of -1.24 (± 1.39) nmol/l (figure 1b), most likely to be caused by differences in calibration between both assays. Preliminary reference values were established in 64 (33 M / 31 F) individuals. Median (range) late-night (23: 00 h) and morning (07: 00 h) cortisol and cortisone concentrations are shown in table 1. Salivary cortisone concentrations were approximately 4 times higher than cortisol concentrations, reflecting the 11ß-HSD2 enzyme activity of the salivary glands. The presence of relatively high concentrations of cortisone in saliva constitutes a risk for cross-reactivity using direct immunoassays, consequently, we applied one with
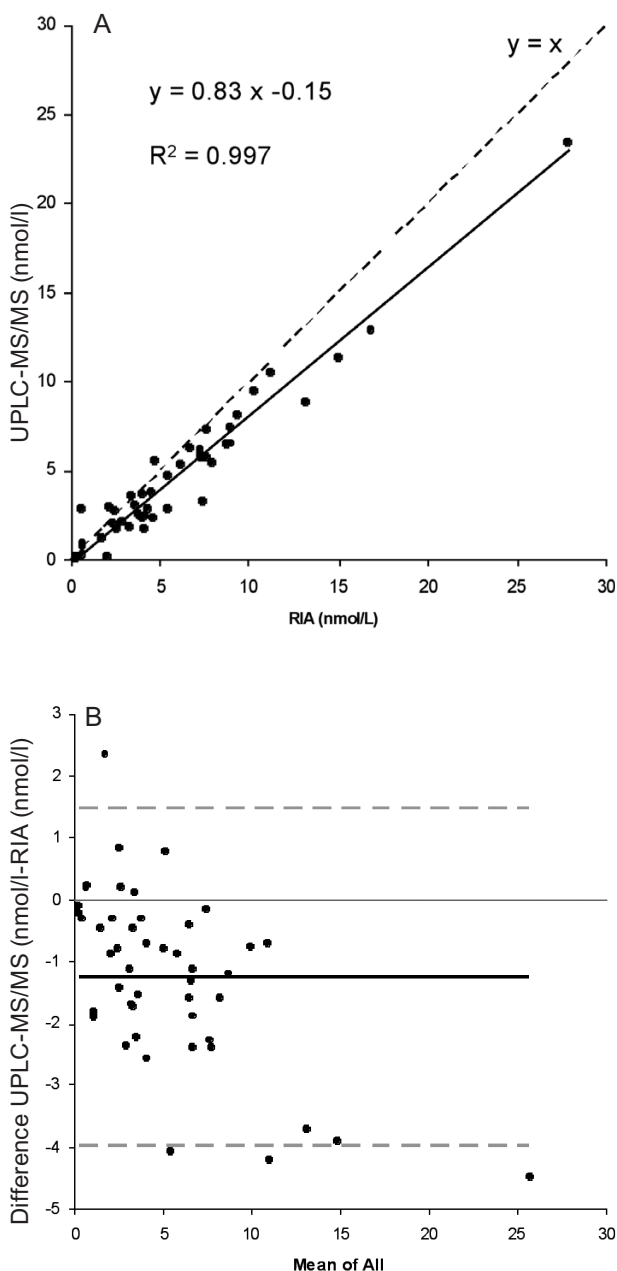
Figure 1. Inter-method comparison of salivary cortisol in 47 clinical saliva samples with RIA and UPLC-MS/MS. A: scatter plot; B: difference plot.

Table 1. Diurnal salivary cortisol and cortisone concentrations

Time of day	Cortisol (nmol/l)	Cortisone (nmol/l)
07:00h	10.8 (2.2-24.0)	36.0 (7.5-75.0)
23:00h	0.8 (0.2-2.8)	4.2 (2.0-12.7)

Values are median (range) of 66 measurements (34M/32F) by UPLC-MS/MS

pre-purification by paper chromatography. The activity of the 11ß-HSD2 can be inhibited by glycyrrhetinic acid (GA), a constituent of liquorice. Administration of GA results in increased ratios of cortisol to cortisone in saliva, plasma and urine (7). In patients where liquorice-induced hypertension is suspected, screening for increased ratios of cortisol to cortisone in saliva might become a non-invasive and fast alternative for measurement of urinary GA (8).

Conclusion

We have developed a rapid, robust UPLC-MS/MS assay for the combined measurement of salivary cortisol and cortisone. This method can be used as a 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 11ß-HSD2 activity, e.g. in studies on liquorice-induced hypertension.

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Determination of serum 25-OH vitamin D$_3$ and 25-OH vitamin D$_2$ using LC-MS/MS with comparison to radioimmunoassay and automated immunoassay*

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Introduction

In addition to the well known effect of vitamin D deficiency on bone metabolism, there is now growing evidence that vitamin D deficiency is involved in other diseases such as certain cancers (1). The most reliable assessment of vitamin D status is measuring the concentration of serum 25-OH vitamin D (25(OH)D). The volume of 25(OH)D testing has markedly increased over the last few years. Serum 25(OH)D concentration can be measured by protein binding assay, radioimmunoassay, HPLC and more recently liquid chromatography (LC)-tandem mass spectrometry (MS/MS) as well as automated immunoassay. Due to its hydrophobic character and strong protein binding, measurement of 25(OH)D is technically demanding. We employed isotope-dilution LC-MS/MS for the measurement of both serum 25(OH)D$_3$ and 25(OH)D$_2$ and compared the assay to popular comparison methods, being radioimmunoassay (RIA) from DiaSorin and a recently re-standardised version of the automated chemiluminescence-based immunoassay (ECLIA) from Roche.

Materials and Methods

Sample preparation

After addition of 50 µl of internal standard (IS, 6.3 µmol/L hexadeuterated 25(OH)D$_3$, Synthetica AS,

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Abbreviations. UPLC: ultra performance liquid chromatography; MS/MS: tandem-mass spectrometry; AcN: acetonitrile; MeOH: methanol; AP-EI: Atmospheric Pressure Electrospray Ionisation; IS: internal standard; SRM: selected reaction monitoring; DEQAS: vitamin D external quality assessment scheme.