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Simultaneous analysis of cortisol and cortisone in saliva using XLC–MS/MS for fully automated online solid phase extraction

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ABSTRACT

Salivary cortisol measurements are increasingly being used in the investigation of disorders of the hypothalamic–pituitary–adrenal axis. In the salivary gland, cortisol is metabolised to cortisone by the action of 11β-hydroxysteroid dehydrogenase type 2, and cortisone is partly responsible for the variable interference observed in current salivary cortisol immunoassays. The aim of this study was to validate an assay for the simultaneous analysis of salivary cortisol and cortisone using the Spark Holland Symbiosis™ in eXtraction liquid chromatography–tandem mass spectrometry (XLC–MS/MS) mode for fully automated online solid phase extraction (SPE). Saliva samples were diluted in water with the addition of internal standard (d4-cortisol and d7-cortisone). Online SPE was performed using the Spark Holland Symbiosis™ with HySphere™ C18 SPE cartridges and compounds were eluted onto a Phenomenex® C18 guard column attached to a Phenomenex® Onyx monolithic C18 column for chromatography. Mass spectrometry used the Waters® Xevo™ TQ MS in electrospray positive mode. Cortisol and cortisone eluted with their internal standards at 1.95 and 2.17 min, respectively, with a total run time of four minutes. No evidence of ion-suppression was observed. The assay was linear up to 3393 nmol/L for cortisol and 3676 nmol/L for cortisone, with lower limits of quantitation of 0.75 nmol/L and 0.50 nmol/L, respectively. Intra- and inter-assay imprecision was <8.9% for cortisol and <6.5% for cortisone across three levels of internal quality control, with accuracy and recovery within accepted limits. High specificity was demonstrated following interference studies which assessed 29 structurally-related steroids at supra-physiological concentrations. We have successfully validated an assay for the simultaneous analysis of salivary cortisol and cortisone using XLC–MS/MS and fully automated online SPE. The assay benefits from increased specificity compared to immunoassay and minimal sample preparation which allows high sample throughput and is thus suitable for use in a routine clinical laboratory.

1. Introduction

Cortisol is the major glucocorticoid produced by the body. In the circulation, over 90% of cortisol is protein bound [1], primarily to cortisol binding globulin (CBG) but also to albumin, with the remaining non-protein bound 'free' fraction generally regarded as being physiologically active. The concentration of total plasma cortisol varies according to the levels of binding proteins and may be decreased in people with congenital CBG deficiency and increased in high oestrogen states resulting in elevated CBG levels. In contrast, the concentration of free cortisol in plasma is relatively constant under basal conditions and independent of the levels of binding proteins [1]. Free cortisol measurements reflect the active cortisol fraction and results are more physiologically relevant, however, assays to measure plasma free cortisol are complex, expensive and time-consuming and so are offered by relatively few laboratories. Free cortisol readily diffuses across cell membranes, including those of the ductar and ascini cells of salivary glands, allowing its detection in saliva. Salivary cortisol has previously been shown to strongly correlate with both serum total cortisol [2,3] and serum free cortisol [2,4].

Cortisol is metabolised *in vivo* via the action of the enzyme 11β-hydroxydehydrogenase type 2 (11β-HSD2), which oxidizes cortisol to cortisone. 11β-HSD2 has been shown to be expressed in various tissues, including the salivary gland [5], which results in an increased ratio of cortisone to cortisol in saliva relative to the free fraction of these steroids in plasma [4]. Salivary cortisone has also been shown to be a potential biomarker for serum free cortisol [4]. Studies have also looked at the potential use of salivary cortisol to cortisone ratios to detect contamination for oral hydrocortisone in

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patients with unexpectedly high concentrations of cortisol; a discrepant ratio can suggest contamination from oral hydrocortisone [4].

There is a growing interest amongst endocrinologists to use salivary cortisol and cortisone in the investigation of disorders of the hypothalamic–pituitary–adrenal (HPA) axis. Midnight salivary cortisol is recommended as a frontline test in the Endocrine Society guidelines for the diagnosis of Cushing's syndrome [6]. The non-invasive sampling technique minimizes any artefactual elevations in cortisol that may occur as a result of venepuncture, potentially reducing the rate of false-positive test results. Salivary cortisol and cortisone measurements may also be used as non-invasive alternatives in other HPA axis investigations, including the diagnosis of adrenal insufficiency and for assessing the adequacy of hydrocortisone replacement, particularly in patients with altered binding protein concentrations [7,8].

Cortisol in saliva is present at concentrations less than ten times lower than in serum [3] and current salivary cortisol immunoassays have been shown to be subject to variable positive interference from cortisone and other structurally related steroids also present in saliva [9]. Therefore, successful measurement of these steroids relies upon using a very sensitive and specific method of analysis. Analysis of steroids by liquid chromatography–tandem mass spectrometry (LC–MS/MS) has previously been shown to be superior to immunoassay, in terms of both increased specificity and sensitivity and because of its ability to measure multiple steroids simultaneously [10]. We have developed and validated an assay for the simultaneous analysis of salivary cortisol and cortisone using the Spark Holland Symbiosis™ in eXtraction liquid chromatography (XLC–MS/MS) mode for fully automated online solid phase extraction, and the Waters® Xevo™ TQ MS.

2. Materials and methods

2.1. Materials

HPLC grade methanol and acetonitrile, 2-propanol, hydrocortisone, cortisone and phosphate buffered saline (PBS) tablets were purchased from Sigma (Dorset, UK). Formic acid (99%) was purchased from VWR (Lutterworth, UK). d4-cortisol and d7-cortisone were purchased from CDN Isotopes (Quebec, Canada).

Powdered cortisol and cortisone were dissolved in methanol to produce calibrator superstocks with concentrations 14.0 mg/mL and 11.7 mg/mL, respectively. Working calibrators containing both cortisol and cortisone were produced by diluting the superstocks in PBS (0.01 mol/L, pH 7.4) to give the following concentrations: 150, 75, 37.5, 18.8, 9.4, 4.7, 2.3, 1.2, 0.6, 0.3 and 0.2 nmol/L (cortisol), and 200, 100, 50, 25, 12.5, 6.3, 3.1, 1.6, 0.8, 0.4, 0.2 and 0.1 nmol/L (cortisone). The calibrators were aligned to those used in our serum cortisol assay, which are traceable to European Reference Material (ERM)-DA 192 and 193. Separate superstocks for cortisol (12.3 mg/mL) and cortisone (10.6 mg/mL) were diluted together in PBS to produce internal quality control (IQC) material with concentrations: 75, 15 and 3 nmol/L (cortisol) and 100, 20 and 4 nmol/L (cortisone). A combined internal standard of d4-cortisol and d7-cortisone was prepared by diluting superstocks in methanol to give concentrations of 60 μg/L and 80 μg/L, respectively.

2.2. Sample collection and preparation

Saliva samples were collected using Salivette® Cortisol devices (Sarstedt Ltd., Leicester, UK). Samples were vortex mixed, and centrifuged at 1000 × *g* for 2 min, according to manufacturer's instructions. Samples underwent one freeze–thaw cycle prior to analysis. For analysis, sample, internal quality control or calibrator (50 μL), 10 μL internal standard solution and 150 μL water were added to wells of a polypropylene 1.2 mL 96-deep well plate (Abgene, Epsom, UK). The plate was thermo-sealed (Abgene) before being vortex mixed for 2 min and centrifuged at 880 × *g* for 5 min at room temperature.

2.3. Solid phase extraction

Online solid phase extraction (SPE) was performed using the fully automated Spark Holland Symbiosis™ (Emmen, The Netherlands) in eXtraction Liquid Chromatography (XLC) mode. The Symbiosis™ has previously been described in detail [11]. HySphere™ C18 HD 7 μm SPE cartridges were used (Spark Holland). Each cartridge was initially conditioned in the left clamp position with 1000 μL methanol and then equilibrated with 1000 μL water, both at flow-rates of 5000 μL/min. Sample (150 μL) was aspirated and loaded onto the cartridge with 1000 μL water at a flow-rate of 2000 μL/min. The cartridge was then washed with 1000 μL 35% methanol at a flow rate of 5000 μL/min. After washing, the cartridge was transferred to the right clamp and cortisol and cortisone were eluted by mobile phase gradient for 1 min. Meanwhile, the left clamp was flushed with 500 μL 10% acetonitrile at flow rate of 5000 μL/min. The autosampler was washed with 500 μL 20% methanol + 0.1% formic acid, followed by 700 μL 33% methanol, 33% acetonitrile, 33% 2-propanol and then 700 μL 20% methanol + 0.1% formic acid again. A new cartridge was placed in the left clamp allowing the next sample to undergo SPE whilst chromatography was simultaneously being performed on the previous sample.

2.4. Chromatography

Mobile phase A consisted of water and 0.1% (v/v) formic acid and mobile phase B consisted of methanol and 0.1% (v/v) formic acid. Cortisol and cortisone were eluted from the SPE cartridges onto a Phenomenex® C18 guard column (Phenomenex, Macclesfield, UK) attached to a Phenomenex® Onyx monolithic C18 25 mm × 4.6 mm column. Initial conditions were 50:50 (v/v) mobile phase A:mobile phase B, at a flow rate of 0.6 mL/min. The proportion of mobile phase B was gradually increased to 68% over 2.5 min and then increased step-wise to 95% for 0.75 min at a flow-rate of 1.0 mL/min. The mobile phase composition was returned to starting conditions for a further 0.75 min but at a flow-rate of 1.0 mL/min. The total run time was 4.0 min.

2.5. Mass spectrometry

Mass spectrometry was performed using the Waters® Xevo™ TQ MS in electrospray positive ionization mode (Waters, Manchester, UK). The mass spectrometer was optimized following tuning in multiple reaction monitoring (MRM) mode and was maintained with a capillary voltage of 1.0 kV, desolvation temperature of 550 °C, desolvation gas flow of 900 L/h, cone gas flow of 0 L/h and collision gas (argon) pressure of 0.3×10^{-3} mbar. Both primary (quantitative) and secondary (qualitative) mass to charge (m/z) transitions were used to monitor cortisol and cortisone, with single transitions for the internal standards (Table 1). Cone voltages and collision energies were optimized for each. For quantitation of the analytes, peak area response ratios of cortisol:d4-cortisol and cortisone:d7-cortisone were calculated using the TargetLynx™ software.

2.6. Breakthrough

To ensure that none of the compounds of interest were being washed to waste during SPE and that neither was being retained on

Table 1
Optimized mass to charge (m/z) transition, cone voltage and collision energy for each analyte and its respective internal standard.

Compound	m/z	Cone voltage (V)	Collision energy (eV)
Cortisol			
Quantitative	363.3 > 121.1	28	25
Qualitative	363.3 > 97.05	28	28
Cortisone			
Quantitative	361.5 > 163.1	28	26
Qualitative	361.5 > 145.1	28	25
d4-Cortisol	367.6 > 121.05	28	26
d7-Cortisone	369.6 > 169.2	30	27

the tubing, breakthrough experiments were conducted. A cartridge in the left clamp position was conditioned and equilibrated and sample injected onto it. During the wash step with 35% methanol, the eluent was directed onto a second cartridge in the right clamp position rather than being diverted to waste. Three consecutive and complete mobile phase gradient cycles followed. The first cycle was used to elute the contents of the cartridge in the right clamp position which would determine if any of the analytes had been washed off the initial cartridge during the washing step. The cartridge in the left clamp position was then transferred into the right clamp position and a second mobile phase gradient was performed to elute the analytes off the cartridge, as usual. Finally, the mobile phase gradient was run for a third time with no SPE cartridge to see if any cortisol or cortisone had been retained on the tubing. Breakthrough experiments were performed using saliva samples ($n = 5$), saliva samples spiked with additional cortisol/cortisone ($n = 5$) and calibrators ($n = 5$).

2.7. Ion suppression

Ion suppression was assessed by post-column infusion of internal standard into the mass spectrometer at a flow-rate of 15 μL/min, with the simultaneous injection of saliva samples ($n = 10$) and deionized water ($n = 5$) which had undergone sample preparation as described previously in Section 2.2. Ion suppression was observed by monitoring the ion counts for each m/z transition throughout the 4 min run time and noting the position and extent of any reductions in ion count.

2.8. Imprecision and accuracy

To assess intra-assay imprecision, the three levels of IQC material were assayed 12 times within a single batch and for inter-assay imprecision, the IQC material was assayed on 10 separate occasions. For each level of IQC the mean concentration, standard deviation and percentage coefficient of variation (CV) were calculated. To assess accuracy, the percentage deviation of the measured concentration from the target concentration was calculated. Imprecision and accuracy were deemed acceptable if the CV and percentage deviation of the mean from target were <15%, respectively [12].

2.9. Linearity

Calibrators were prepared from separate superstocks spanning the concentration range 0–3393 nmol/L (cortisol) and 0–3676 nmol/L (cortisone). The peak area response ratios were plotted against the cortisol/cortisone concentration and a calibration curve fitted using the TargetLynx™ software. The assay was deemed to be linear if the correlation coefficient (R^2) was >0.99. For routine use of the assay, calibration curves spanned the concentration ranges 0–150 nmol/L (cortisol) and 0–200 nmol/L (cortisone), and the run was accepted if $R^2 > 0.99$.

2.10. Lower limit of quantitation

To assess the lower limit of quantitation, low concentrations of cortisol (0.15–0.75 nmol/L) and cortisone (0.2–2.0 nmol/L) were assayed 10 times each within a single run. The mean concentration, standard deviation and CV were calculated. The lower limit of quantitation of each analyte corresponded to the minimum concentration with acceptable imprecision and accuracy (<20%) [12].

2.11. Recovery

Recovery was assessed by spiking 6 different patient saliva samples and a PBS blank with known amounts of a combined cortisol/cortisone stock solution, prepared in 50% methanol, to give additional concentrations of cortisol: 5, 50, 100 nmol/L, and cortisone: 7.5, 75, 150 nmol/L. The patient saliva samples were analysed both before and after spiking and recovery was calculated as the difference between the two results divided by the known concentration of spike added. Recovery of each analyte was deemed acceptable if it was between 80% and 120%.

2.12. Interferences

An extensive interference study was conducted using 29 structurally-related steroids: 11-deoxycortisol, 17-hydroxyprogesterone, 19-nortestosterone, 21-deoxycortisol, aldosterone, androstenedione, beclomethasone dipropionate, budesonide, corticosterone, cyproterone acetate, dehyroepiandrosterone, dehydroepiandrosterone sulphate, dexamethasone, dihydrotestosterone, epitestosterone, estrone, ethinylestradiol, flucinolone acetonide, fludrocortisone acetate, levonorgestrel, methylprednisolone, norethisterone, oestradiol, prednisolone, prednisone, pregnenolone, progesterone, testosterone and triamcinolone acetonide. Solutions of each steroid were prepared in PBS at the supra-physiological concentration of 1 μmol/L. In addition, for prednisolone and prednisone, serial doubling dilutions of the 1 μmol/L were performed down to a concentration of 15.6 nmol/L. Each solution was prepared and analysed as described above and the ion counts for the cortisol and cortisone m/z transitions were monitored throughout the 4 min run. Compounds were deemed to interfere if any signal was detected at the expected time of elution of the analytes.

2.13. Cartridge stability

In order to assess whether the SPE cartridges were stable over multiple injections, 4 different saliva samples were spiked using a combined cortisol/cortisone stock solution, prepared in 50% methanol, to increase both the cortisol and cortisone concentration by approximately 25 nmol/L. Each spiked saliva sample was injected onto a single SPE cartridge 15 times. The SPE cartridges were deemed stable if no systematic decrease in peak area response ratio was observed across the repeat injections.

3. Results

3.1. Chromatography and breakthrough

Effective SPE and chromatography resulted in separation of cortisol and cortisone to produce clean, discrete peaks with cortisone eluting first at 1.9 min followed by cortisol at 2.2 min and co-elution of their respective internal standards (Fig. 1). Breakthrough experiments confirmed that all cortisol and cortisone in the samples was being retained on the SPE cartridge during the wash step with 35% methanol and not lost to waste. In addition there was no evidence

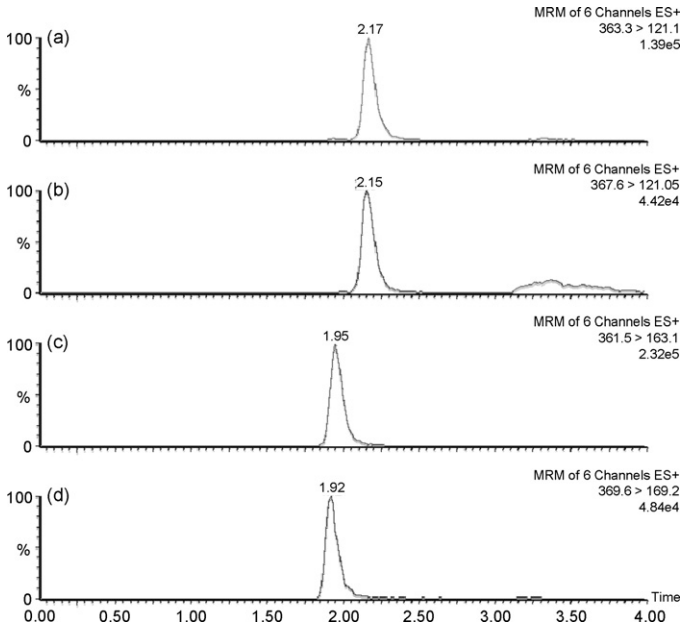

Fig. 1. MRM chromatograms of cortisol and cortisone and their respective internal standards. Concentrations of cortisol and cortisone were 150 nmol/L and 200 nmol/L, respectively and the internal standards were d4-cortisol (60 μg/L) and d7-cortisone (80 μg/L). (a) Cortisol elution at 2.17 min (*m*/*z* 363.3 > 121.1); (b) d4-cortisol elution at 2.15 min (*m*/*z* 367.6 > 121.05); (c) cortisone elution at 1.95 min (*m*/*z* 361.5 > 163.1); (d) d7-cortisone elution at 1.92 min (*m*/*z* 369.6 > 169.2).

that either cortisol or cortisone were being retained on the tubing. Breakthrough experiment results were replicated for all saliva samples and calibrators assessed.

3.2. Ion suppression

No ion suppression was observed at the time of elution of either cortisol or cortisone (Fig. 2). Between 1.5 and 2.5 min, ion counts for each *m*/*z* transition were stable with minimal decrease and the location and extent of ion suppression was found to be reproducible following the injection of different patient samples and water. No differences in ion suppression were noted between matrix and water.

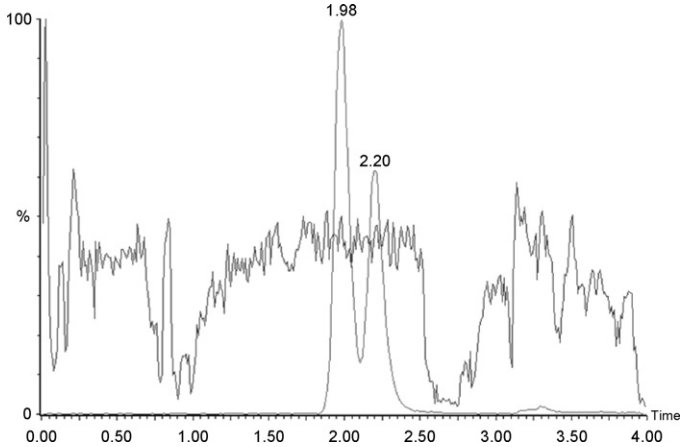

Fig. 2. Ion suppression. The total ion counts following post-column infusion of internal standard have been superimposed with a total ion count chromatogram showing the elution of cortisone at 1.98 min and cortisol at 2.20 min in a region of minimal and stable ion suppression.

3.3. Imprecision and accuracy

Excellent imprecision and accuracy was demonstrated for the salivary cortisol and cortisone assay, both within batch and between batches (Table 2). For all three concentrations of IQC both intra- and inter-assay CVs and accuracy were well within acceptable limits [12].

3.4. Linearity and lower limit of quantitation

The calibration curve used in the routine salivary cortisol assay was shown to be linear up to a concentration of 150 nmol/L with an R^2 value consistently >0.997. However, analysis of high concentration calibrators demonstrated that the cortisol assay was linear up to a concentration of at least 3393 nmol/L (R^2 > 0.9998). Similarly, for the salivary cortisone assay, the calibration curve used routinely was linear up to 200 nmol/L (R^2 > 0.997), however, it was shown that the assay was linear up to a concentration of at least 3676 nmol/L with an R^2 value >0.9988. The lower limit of quantitation was found to be 0.75 nmol/L for cortisol and 0.5 nmol/L for cortisone.

3.5. Recovery

Recovery of both cortisol and cortisone was found to be within acceptable limits. Six saliva samples were spiked to contain additional cortisol and cortisone concentrations of 5, 50 and 100 nmol/L and 7.5, 75 and 150 nmol/L, respectively. For cortisol, the mean recoveries for each increasing spike concentration were 112.2%, 114.1% and 117.5%, with a range of 100.3–124.0%. Mean recoveries for cortisone were 104.1%, 96.9% and 95.6% with increasing spike concentration, with a range of 86.3–126.7%.

3.6. Interferences

The XLC–MS/MS assay was shown to be very specific to cortisol and cortisone following the analysis of 29 structurally-related steroids at supra-physiological concentrations. No signal above baseline noise was detected in either the cortisol or cortisone channel for 26 of the steroids assessed. Peaks were detected in both the cortisol and cortisone channels when a 1 μmol/L solution of aldosterone was analysed, however, the retention times of the peaks observed differed from those expected, at 1.8 min for both *m*/*z* transitions, and therefore would not interfere. No signal above baseline noise was detected in the cortisol channel around the expected retention time when solutions containing prednisone over the concentration range 15.6–1000 nmol/L were analysed. However, an increase in signal above the limit of quantitation was observed in the cortisone channel when the prednisone concentration exceeded 31.3 nmol/L. At a prednisone concentration of 1000 nmol/L the measured cortisone was 10.9 nmol/L, equivalent to 1% interference. Solutions of prednisolone over the same concentration range were also analysed. For the cortisone *m*/*z* transition, signal was detected but the retention time was incorrect (2.18 min) and interference was excluded. However, when present at a concentration greater than 125 nmol/L, prednisolone caused an increase in signal above the limit of quantitation in the cortisol channel. The measured cortisol concentration was 3.9 nmol/L at a prednisolone concentration of 1000 nmol/L, equivalent to 0.3% interference.

3.7. Cartridge stability

The SPE cartridges were found to be stable for up to 15 repeated injections. Comparison of the peak area response ratios

Table 2
Intra- and inter-assay imprecision and accuracy for the salivary cortisol and cortisone assay.

	Intra-assay (n = 12)			Inter-assay (n = 10)		
Cortisol						
Mean concentration (nmol/L)	2.7	14.5	72.0	3.0	14.6	71.7
Standard deviation	0.2	0.9	3.9	0.2	0.7	3.8
Coefficient of variation (%)	8.9	6.3	5.4	7.9	4.5	5.4
Deviation of mean from target (%)	−10.4	−3.2	−4.0	−1.6	−2.7	−4.4
Cortisone						
Mean concentration (nmol/L)	4.0	21.1	108.7	4.2	20.6	102.3
Standard deviation	0.3	0.5	2.1	0.3	0.5	3.8
Coefficient of variation (%)	6.5	2.3	1.9	6.1	2.6	3.7
Deviation of mean from target (%)	0.6	5.6	8.7	5.4	2.8	2.3

for four patient samples demonstrates that there was no systematic decrease in the response over 15 injections for either cortisol (Fig. 3a) or cortisone (Fig. 3b). The mean salivary cortisol concentrations in the patient samples ranged from 27.7 to 47.4 nmol/L and mean salivary cortisone concentrations were 47.8–69.4 nmol/L. For all four patient samples over the 15 repeat injections the CVs of the analyte concentrations were <4.2% for cortisol and <4.6% for cortisone. It was noted that there was some deterioration in the quality of the chromatography over the 15 injections, with slightly poorer baseline resolution of the two compounds observed in the total ion count chromatogram (Fig. 4). However, multiple reaction monitoring of the specific m/z transitions for each compound meant that peak integration and compound analysis using TargetLynx™ was not affected. In the example shown in Fig. 4, a patient sample injected 15 times on the same SPE cartridge had a peak area response ratio for cortisol:d4-cortisol of 0.6443 in the first injection and 0.6449 in the last injection, with the salivary cortisol concentration calculated to be 36.1 nmol/L for both. Mean salivary cortisol concentration for this patient across the 15 injections was 37.4 nmol/L with a CV of 3.9%. For cortisone, the peak area response ratio (cortisone:d7-cortisone) was 1.6807 in the first injection and 1.7578 in the last, corresponding to salivary cortisone concentrations of 64.7 nmol/L and 67.7 nmol/L, respectively. The mean salivary cortisone concentration was 66.4 nmol/L with a CV of 2.9% over the 15 injections.

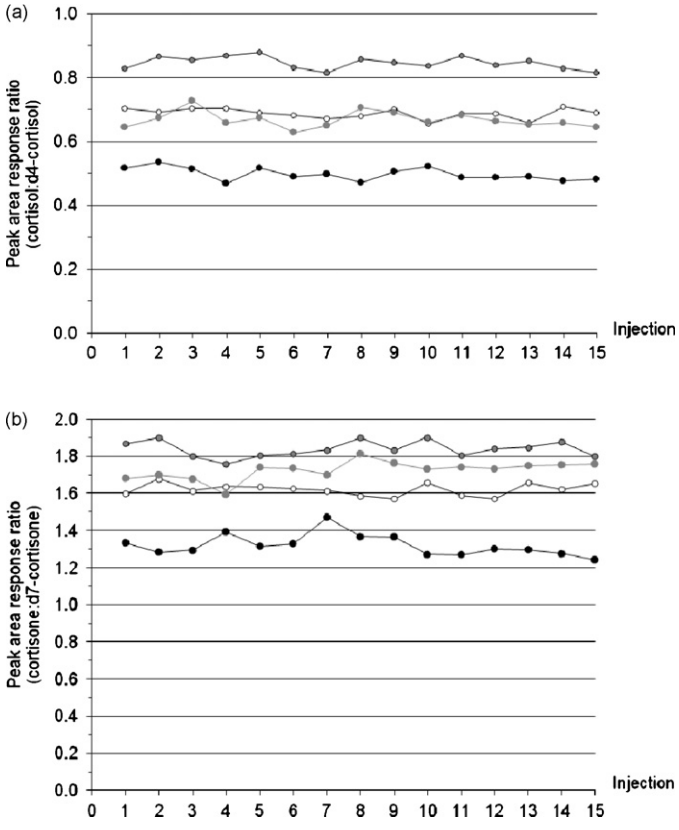

Fig. 3. Peak area response ratios for four patient saliva samples which were repeatedly injected onto the same solid phase extraction cartridge 15 times. (a) Mean salivary cortisol concentrations ranged from 27.7 to 47.4 nmol/L for the four patient samples. The CV of the cortisol concentration was <4.2% over the 15 replicates for all four patients; (b) mean salivary cortisone concentrations ranged from 47.8 to 69.4 nmol/L. The CV of the cortisone concentration was <4.6% over the 15 replicates for all four patients.

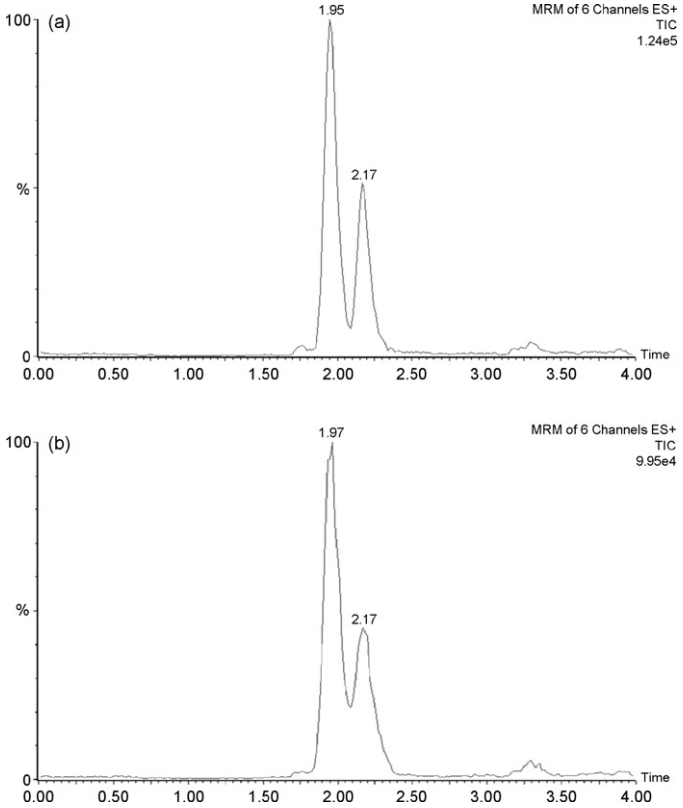

Fig. 4. Total ion count chromatograms of the first and last injection of a patient sample which had been repeatedly injected on the same solid phase extraction cartridge a total of 15 times. Multiple reaction monitoring of the specific m/z transitions for each compound meant that peak integration and compound analysis using TargetLynx™ was not affected. (a) First injection. Peak area response ratios were 0.6443 for cortisol and 1.6807 for cortisone, corresponding to analyte concentrations of 36.1 nmol/L and 64.7 nmol/L respectively; (b) last injection (15). Peak area response ratios were 0.6449 for cortisol and 1.7578 for cortisone, corresponding to analyte concentrations of 36.1 nmol/L and 67.7 nmol/L respectively.

4. Discussion

Salivary cortisol and cortisone measurements have been shown to have an increasingly important role in the investigation of disorders of the hypothalamic–pituitary–adrenal axis [7]. In response to increased demand, partly driven by recommendations for salivary cortisol to be used as a frontline test for Cushing's syndrome [6], we have successfully developed and validated an XLC–MS/MS assay for the simultaneous analysis of cortisol and cortisone in saliva. The assay has been shown to be linear over a wide concentration range with good imprecision and accuracy. Excellent sensitivity and specificity were also demonstrated.

Sarstedt Salivette® Cortisol devices were used for saliva sample collection as the synthetic swab has been specifically designed for cortisol determination in saliva. For the assay, sample preparation involves a simple dilution and addition of internal standard, which therefore minimizes the amount of manual bench-time required for the assay and allows the analysis of large numbers of samples efficiently within a single batch. The fully automated online SPE provided by the Spark Holland Symbiosis™ ensures maximal clean-up of samples prior to elution of the compounds for chromatography, thus considerably increasing the lifespan of the analytical column. Unlike online SPE using a guard column, as described previously [3,13], the Symbiosis™ is able to utilize washes containing multiple solvents and so washes are not restricted to combinations of mobile phase. This is clearly advantageous for assays of compounds present in more complex matrices which require extensive sample clean-up and saliva samples are not exempt from this. Online SPE with a guard column is sufficient for the relatively clean saliva obtained using collection devices such as the Sarstedt Salivette® Cortisol, however swab-based collection devices are not suitable for certain groups of patients, such as neonates and those under sedation on intensive care units, due to the risk of choking. Saliva collected by other techniques, including passive drool, is more viscous and analysis of such samples using the previous method of online SPE would result in blockage of the guard column, which would increase the back-pressure through the system and be detrimental to instrument performance. However, using individual cartridges which allow SPE to be performed off the main path of flow it is possible to analyse saliva collected by all sampling techniques without the risk of blocking the entire analytical system. As such, the Symbiosis™ ensures that the assay is suitable for the analysis of all saliva samples, regardless of the collection technique used, and it is therefore suitable for use in a referral laboratory. In addition, the assay still utilized a guard column as this further increased the number of injections that could be performed on the analytical column before it would need replacing. The HySphere™ C18 HD 7 μm SPE cartridges used in this assay were shown to be stable for up to 15 injections with no systematic decrease in response ratio observed across multiple injections. This maximum efficiency of the SPE cartridges results in considerably reduced consumable costs per injection. The analytical column used for this assay was the Phenomenex® Onyx monolithic C18 column whose solid phase is composed of porous silica rod. The column has been specifically designed to allow fast flow rates under relatively low pressures with no detrimental effect on chromatography. This column allows the assay to have flow-rates of up to 1 mL/min and a run time of only 4 min whilst also achieving good separation of cortisol and cortisone. Furthermore, the Symbiosis™ is able to perform the simultaneous SPE of one sample and analytical chromatography of another [11], which reduces the time from injection to injection and increases the throughput of the assay.

The assay was shown to be very specific for both cortisol and cortisone, with only 0.3% interference from prednisolone observed in the salivary cortisol assay and 1% interference from prednisone observed in the salivary cortisone assay, out of the 29 structurally-related steroids tested at a supra-physiological concentration of 1 μmol/L. In the salivary cortisol assay, the concentration of prednisolone had to exceed 125 nmol/L (45 μg/L) before interference was observed at a level greater than the limit of quantitation of the assay and prednisone had to be present at a concentration greater than 31.3 nmol/L (11 μg/L) to interfere in the salivary cortisone assay. The level of interference observed would not be clinically relevant, especially as these steroids would not be expected to reach concentrations in saliva which could give rise to significant interference [14]. Furthermore, it is also unlikely that patients being investigated for disorders of the HPA axis will be prescribed these steroids. However, should prednisolone or prednisone be present in saliva at interfering concentrations then a discrepant cortisol to cortisone ratio would be observed. As with contamination from residual oral hydrocortisone [4], this finding would alert to the possibility of interference and a full drug history would need to be taken to ensure the patient is not taking these steroids. The most likely cause of the interference observed is from the so-called M+2 effect, whereby the natural isotopes of prednisone and prednisolone containing two ^{13}C atoms are detected as these have the same molecular mass as cortisone and cortisol, respectively. Prednisolone interference in a salivary cortisol LC–MS/MS assay due to the M+2 effect has previously been described [13] although this was not subsequently observed in another validation of a salivary cortisol method [3]. The interference from prednisone and prednisolone observed here and not in previously published methods may be due to the increased sensitivity of the Waters® Xevo™ TQ MS and also possibly due to the different fragmentation patterns which occur with this instrument.

This assay is currently in routine use in our laboratory and has replaced the methods which we had previously been developed and validated [3,13]. Throughput of the assay is increased compared with our previous method [3], with a shorter run-time of four minutes per sample and reduced time from injection to injection. Current clinical applications in our laboratory include: midnight salivary cortisol measurements for the diagnosis of Cushing's syndrome [6]; assessment of the adequacy of hydrocortisone replacement using salivary cortisol day curves and for other investigations of the HPA axis in patients with binding protein abnormalities, for whom total plasma cortisol measurements may produce equivocal results [7], *e.g.* women prescribed oestrogen preparations.

In conclusion, we have successfully developed and validated an assay for the measurement of cortisol and cortisone in saliva using eXtraction liquid chromatography–tandem mass spectrometry with fully automated online solid phase extraction.

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