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A simplified method for the measurement of urinary free cortisol using LC–MS/MS

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Abstract

The measurement of 24 h urinary free cortisol is used in the investigation of patients with symptoms of hypercortisolism. Many different methods have been published for the measurement of cortisol, but most of these methods involve cumbersome pre-extraction of the cortisol prior to analysis. We have developed a method using in-well protein precipitation which serves to clean up the sample without requiring lengthy sample preparation. A Shimadzu SIL-HT autosampler was used to inject 50 μL of extract onto a Phenomemex® Gemini C18 guard column attached to a Waters™ Xbridge C18 column. The eluant was introduced directly into a Waters™ Quattro Micro tandem mass spectrometer. The method was found to be linear up to 3448 nmol/L with a lower limit of detection of 5.3 nmol/L. Precision and accuracy were acceptable, and no interference was noted from compounds such as prednisolone or fenofibrate. This assay was compared to a previously published method, which uses solid phase extraction prior to LC–MS/MS analysis. We have developed a simplified, robust assay for the quantitation of urinary free cortisol that will increase the throughput of the assay and avoid the use of neurotoxic solvents such as dichloromethane.

Keywords: Urinary free cortisol; Liquid chromatography tandem mass spectrometry

1. Introduction

Cushing's syndrome is the production of excess cortisol. The symptoms of Cushing's syndrome including truncal obesity, hypertension and mood disorders are non-specific; therefore biochemical tests are necessary to enable diagnosis. One of the first-line tests for Cushing's syndrome is the measurement of 24-h urinary free cortisol, although due to the variability of cortisol excretion from day to day, it is advisable to collect three 24-h urine collections for measurement.

Many different methods for the measurement of urinary free cortisol have been described including immunoassay and chromatography techniques. Many of these involve extraction prior to analysis using solid phase extraction [1–4] or a solvent such as dichloromethane [5], which adds to the cost and complexity of the assay and decreases its throughput. Some online purification methods have been described using liquid-chromatography tandem mass spectrometry [6,7] but these methods use atmospheric pressure chemical ionisation instead of electrospray ionisation as this is said to increase the ionisation efficiency of cortisol [6]. We feel our simplified method offers significant advantages over the currently used solvent extraction methods which are used for the measurement of urinary free cortisol using LC–MS/MS instruments with an electrospray ionisation interface.

2. Experimental

2.1. Materials

HPLC grade methanol was obtained from Fisher Scientific UK (Loughborough, UK). Ammonium acetate, hydrocortisone and trichloroacetic acid were purchased from Sigma

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(Dorset, UK). Formic acid, sodium chloride, potassium chloride, disodium hydrogen phosphate and potassium dihydrogen phosphate were purchased from VWR International (Leicestershire, UK) and cortisol-1,2-d2 was purchased from QMx laboratories (Essex, UK). Polypropylene 1.2 mL, 96-deep well plates were purchased from VWR International (Leicestershire, UK).

Aqueous trichloroacetic acid 5% (w/v) was prepared. A calibrator superstock was prepared by dissolving hydrocortisone powder in methanol to a concentration of 2759 nmol/L (1 mg/L). Working calibrators were then prepared by diluting the superstock in phosphate buffered saline (PBS), pH 7.2, to concentrations of 690, 172, 86, 43 and 10.7 nmol/L. Biorad lyphocheck quantitative urine control level 1 was used as the low control, (Biorad Laboratories, Hertfordshire, UK), along with Randox assayed urine control levels 2 and 3 (Randox Laboratories Ltd., Co Antrim, UK), as the medium and high controls. All QC material was used according to manufacturer instructions. A 1 mg/L (2759 nmol/L) superstock solution of D2-cortisol was prepared in methanol as the internal standard. This was diluted in methanol to a working concentration of 350 nmol/L by taking 4 mL of superstock and adding 27.5 mL of methanol, to a final volume of 31.5 mL.

2.2. Sample preparation

D2-cortisol (25 μL of a 350 nmol/L solution) was added as internal standard to 100 μL of calibrator, QC or sample in a 96-deep well plate. 100 μL of trichloroacetic acid 5% (w/v) was then added to each well and the plate was thermosealed and vortex mixed. This was then centrifuged at 8000 × *g* for 5 min.

2.3. Liquid chromatography

The plate was placed on a Shimadzu SIL-HT autosampler (Shimadzu, Buckinghamshire, UK) and 50 μL injected onto a Phenomenex Gemini C18 4 mm × 3 mm guard column (Phenomenex, Cheshire, UK) attached to a Waters XBridge C18 3.5 μm, 2.1 mm × 20 mm column (Waters, Hertfordshire, UK). The column was maintained at a temperature of 60 °C to reduce the back pressure on the column. Cortisol was eluted from the column using a stepwise gradient of 68% mobile phase A, 32% mobile phase B at 0 min with a flow rate of 0.6 mL/min, then 100% mobile phase B at a flow rate of 0.6 mL/min at 1.2 min. The gradient returned to starting conditions at 1.8 min for a further 3.2 min, but at a flow rate of 0.5 mL/min. The total run time was 5 min. Mobile phase A consisted of 2 mmol/L ammonium acetate and 0.1% (v/v) formic acid in distilled water (pH 2.6), mobile phase B contained 2 mmol/L ammonium acetate and 0.1% (v/v) formic acid in methanol (pH 4.2). The first 1.5 min and final 2 min of flow were diverted to waste to prevent instrument contamination. The retention time for both cortisol and its internal standard was 2 min.

2.4. Tandem mass spectrometry

The column eluant was injected into a Waters Quattro Micro tandem mass spectrometer with Z spray source (Waters, Hertfordshire, UK). The mass spectrometer was maintained in positive ion mode, with a desolvation gas flow of 630 L/h. The capillary voltage was 1 kV, the source temperature was 140 °C, and the pressure of the collision gas (argon) was 3.58×10^{-3} mbar. Cortisol was monitored with a transition of *m/z* 363.4 > 120.9, with a dwell time of 0.2 s, cone voltage of 26 volts (V) and collision energy of 22 electrovolts (eV). Deuterated cortisol was monitored with a transition of *m/z* 365.5 > 122.0, with a dwell of 0.2 s, cone energy of 22 V and collision energy of 22 eV. The extractor voltage was 3 V and RF lens voltage 0.4 V. Resolution was 14 for MS1 and MS2 and the photomultiplier energy was 650 V.

2.5. Ion suppression

Ion suppression experiments were carried out by postcolumn infusion of 290 nmol/L of cortisol (in 50:50 (v/v) mobile phase A:mobile phase B) directly into the mass spectrometer. Urine samples ($n = 6$) prepared as described above were simultaneously introduced into the mass spectrometer via the autosampler, and the degree and position of ion suppression noted by observing any drop in the ion counts. In addition, two different concentrations of cortisol (200 and 400 nmol/L) were spiked into five urine samples. The response ratio of cortisol:D2-cortisol for these samples was compared to the response obtained for the same concentrations of cortisol spiked into 50% (v/v) methanol in water.

2.6. Calibration

In addition, a set of calibrators with concentrations of 0, 11, 23, 46, 92, 183, 365 and 730 nmol/L were prepared by diluting hydrocortisone superstock (as described in materials section) in cortisol free urine obtained from a patient receiving high dose dexamethasone to suppress endogenous cortisol production. The detector response from this set of calibrators was compared to that from a set of calibrators of the same concentrations prepared in PBS pH 7.2.

2.7. Lower limit of quantitation

We determined the lower limit of quantitation (LOQ) by measuring low concentrations of cortisol (43, 21.5, 10.7 and 5.3 nmol/L) 10 times each and quantifying the CV, SD and percentage deviation of the mean from the target at each of these concentrations. The LOQ was taken to be the lowest concentration with a CV < 20% and a mean value within 20% of the target [8].

2.8. Linearity

We determined the linearity of the method by analysing a set of cortisol calibrators with concentrations ranging from 0 to 3448 nmol/L, prepared from a separate superstock to the calibrators in routine use. These values were then plotted against the LC–MS/MS response (cortisol:D2-cortisol peak area ratio) using QuanLynx™ software (Waters, Hertfordshire, UK). Cali-

bration lines were judged to be linear if the correlation coefficient was better than 0.99 as calculated by weighted linear regression [8]. A urine sample with a cortisol concentration of 528 nmol/L was serially diluted up to 128 times in PBS. The measured value was then plotted against the expected value and the curve judged to be linear if the correlation coefficient was greater than 0.99.

2.9. Precision and accuracy

Three cortisol samples with concentrations of 31, 85 and 312 nmol/L were prepared. These were assayed 15 times within a single run (within batch) and also on 15 separate occasions (between batch), and the CV and percentage deviation of the mean from the target were calculated. Precision and accuracy were judged to be acceptable if these were <15% [8].

2.10. Recovery

The recovery of each analyte was assessed by comparing the cortisol concentration of urine samples before and after the addition of known amounts of cortisol (100, 200 and 300 nmol/L) ($n = 5$). The recovery was calculated using the formula: ((detector response of spiked urine-detector response of unspiked urine)/cortisol concentration spiked into urine) × 100.

2.11. Detector stability

Stability of the prepared samples was determined by repeat analysis of an 85 nmol/L standard every 5 min over a 17-h period. The assay was considered stable if no systematic decrease in response was observed over this period.

2.12. Interference studies

Solutions of the steroids fludrocortisone, prednisolone, methyl prednisolone, spironolactone, dexamethasone and 17 hydroxyprogesterone (1 μmol/L) were prepared for analysis as described in sample preparation section. A 1 μmol/L solution of fenofibrate was also prepared as this has been shown to cause interference in other cortisol assays [6]. These substances were deemed not to interfere if they did not give a signal in the chromatogram at the time at which cortisol elutes. In addition, the effect of the above substances on the ionisation efficiency of cortisol was investigated. Fludrocortisone, dexamethasone, fenofibrate, methylprednisolone, prednisolone and spironolactone were spiked into aliquots of urine to a final concentration of 1 μmol/L. The cortisol concentration of the urine sample was 73 nmol/L. The cortisol ion counts of the spiked samples were compared to those obtained when unspiked urine was analysed. There was deemed to be no effect on ionisation efficiency if the difference in ion counts between the spiked and unspiked samples was less than 10%.

2.13. Method comparison

Urine samples ($n = 65$) were analysed using the simplified method and the solid phase extraction method published by McCann et al. [4]. Results were compared using Microsoft® Excel (Microsoft UK, Berkshire, UK) and Analyse-It™ software (Analyse-It Software Ltd., Leeds, UK).

3. Results and discussion

3.1. Sample preparation and liquid chromatography

The chromatograms showed that cortisol and the internal standard (D2-cortisol) coeluted with a retention time of 2 min, and that there were no interfering peaks in this region of the chromatogram (Fig. 1).

Ion suppression experiments showed some signal interference in the region of the chromatogram where cortisol elutes from the column, indicating that there is ion suppression at this point under the conditions used. Comparison of these chromatograms with that of a water blank containing TCA showed that TCA was not responsible for any ion suppression seen. The response ratios of 5 urine samples spiked with 200 or 400 nmol/L cortisol were compared to the response ratios of 50% methanol spiked with the same concentrations of cortisol. The response ratio of the urine samples was at least 94% of that of the methanol (94.8–111.9%), indicating that ion suppression is compensated by the internal standard and does not affect cortisol analysis under these conditions. It was therefore decided to validate the assay using these conditions, according to published acceptance criteria [8].

3.2. Linearity and lower limit of quantitation

The assay was found to be linear up to 3448 nmol/L. The plotted line showed good correlation with the assigned standard values, and had an R^2 value of 0.996. A urine sample with a

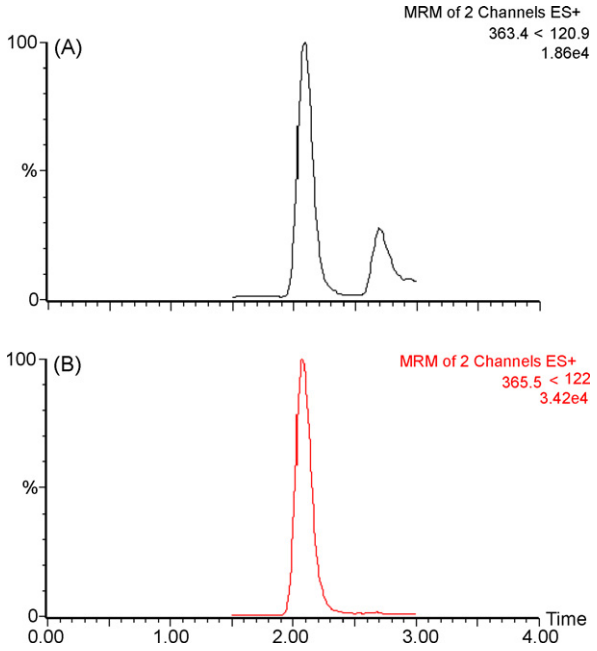

Fig. 1. Chromatogram of a urine sample with a cortisol concentration of 12.4 nmol/L: (A) cortisol; (B) D2-cortisol (350 nmol/L).

cortisol concentration of 528 nmol/L was diluted and measured values compared with the expected values. The resulting line had an R^2 value of 0.998 and therefore was shown to dilute linearly.

All the calibrators for this assay are made up in PBS pH 7.2 as it is very difficult to obtain cortisol free urine. It was decided to confirm the validity of the calibrators by comparing the mass spectrometer detector response from a set of calibrators made using PBS to the response obtained from a set of calibrators prepared using cortisol free urine from a patient receiving high dose dexamethasone. The mass spectrometer detector responses obtained were very similar for the two sets of calibrators (Fig. 2). The plotted line had an R^2 value of 0.9971 and the equation of the line was: detector response of calibrators prepared in PBS = 1.0026 × detector response of calibrators prepared in cortisol free urine −0.5152. This indicates that the results obtained for the two sets of calibrators were comparable and that it is valid to routinely use calibrators prepared using PBS in this assay.

The lower limit of quantitation, which is the lowest concentration which can be measured with a CV <20% and a mean <20% from the target, was found to be 2.5 nmol/L [8]. At this concentration, the CV was 11% and the mean was 13.6% below the target. The lower limit of detection of the assay, however, which is the lowest concentration at which the cortisol peak was detected with a signal to noise ratio >3 times that of the blank, was found to be 5.3 nmol/L. This means that the assay can be used to measure cortisol concentrations between 5.3 and 3448 nmol/L, which more than spans the expected range of concentrations of urinary free cortisol in patient samples.

3.3. Imprecision and bias

The within batch imprecision and accuracy of the assay was calculated at three different concentrations of cortisol: 31, 85 and 312 nmol/L. At all concentrations the CV was <5%, and the mean was <15% away from the target (Table 1). The between batch imprecision and accuracy were calculated at the same three concentrations of cortisol. In this case, the CV was <12% at all concentrations, and the mean concentration was <10% away from the target (Table 2).

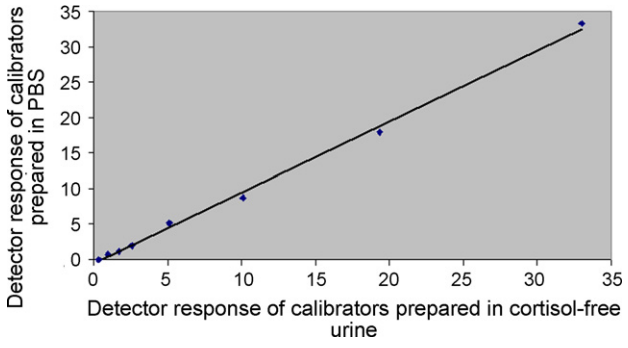

Fig. 2. Mass spectrometer detector responses obtained from calibrators prepared using PBS compared to response obtained using calibrators prepared in cortisol-free urine.

Table 1
Within batch precision and accuracy

Mean cortisol concentration (nmol/L)	S.D.	CV (%)	Deviation of mean from target (%)
33.5	1.5	4.3	8.2
72.6	2.1	2.8	−14.6
300.3	7.4	2.5	−3.8

Three different concentrations of cortisol (31, 85 and 312 nmol/L) were analysed 15 times each within a single run and the precision and accuracy calculated.

Table 2
Between batch precision and accuracy

Mean cortisol concentration (nmol/L)	S.D.	CV (%)	Deviation of mean from target (%)
33.9	3.5	11.3	9.4
79.6	8.5	10.0	−6.3
297.1	14.8	4.7	−4.8

Three different concentrations of cortisol (31, 85 and 312 nmol/L) were each analysed on 15 separate occasions and the precision and accuracy calculated.

3.4. Recovery and stability

The mean recovery of the assay was 104.8% with a range of 89–128%. No systematic loss in sensitivity was noted after repeated injection of an extracted sample every 5 min for 17 h (Fig. 3). This indicates that the extracted sample is stable over this time period, and it would be possible to analyse a large number of samples in a single analytical run without detriment to the samples at the end of a batch.

3.5. Interference studies

No peaks were observed at the point of cortisol elution in response to fludrocortisone, dexamethasone, fenofibrate, methylprednisolone, prednisolone or spironolactone. This indicates that these substances do not interfere in this assay. The difference in cortisol ion counts between urine samples spiked with the above substances and unspiked samples was 6% or less, indicating that none of these compounds have an effect on the ionisation efficiency of cortisol.

3.6. Method comparison

The cortisol concentrations of 65 urine samples were measured using both the simplified method and the solid phase

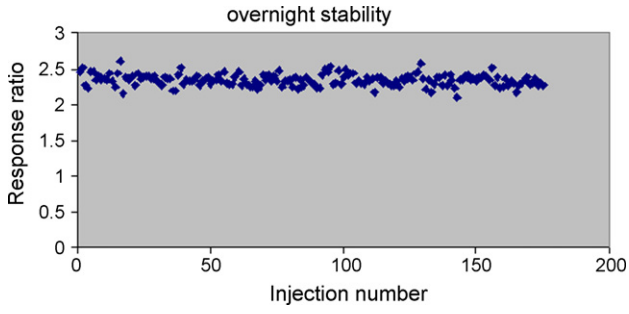

Fig. 3. Overnight stability of the assay. The response ratio of cortisol:D2-cortisol is shown for each injection.

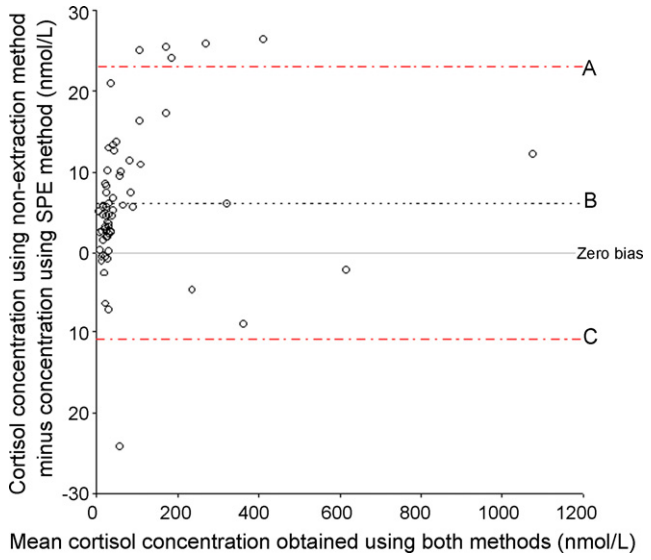

Fig. 4. Bland–Altman plot of non-extraction method compared to the solid phase extraction method. Line A shows the mean difference between the two methods + 2S.D., line B indicates the mean difference, line C indicates the mean difference − 2S.D.

extraction method published by McCann et al. [4]. Results produced using both methods were very similar (Fig. 4). Using the published reference range of <165 nmol/24 h [4], 10/65 samples were found to have abnormal cortisol concentrations measured by the simplified method compared to 8/65 measured using the solid phase extraction method. Both discrepant samples had concentrations around the cut-off limit.

4. Conclusions

We have developed a simplified sample preparation method for the measurement of urinary free cortisol using LC–MS/MS with electrospray ionisation. Previously published non-extraction methods have used atmospheric pressure chemical ionisation as this is said to improve the ionisation efficiency of cortisol [6,7]. However, many of the mass spectrometers available in NHS clinical biochemistry laboratories have electrospray ionisation sources and this method therefore allows high throughput measurement of urinary free cortisol in laboratories that only have access to an electrospray instrument. In addition, the run time of our method is significantly shorter than published methods, with an injection-to-injection time of 5 min compared to 8 min [6]. This method has been shown to compare well to a previously published method [4] and is therefore suitable for routine use in the laboratory.

The use of trichloroacetic acid precipitates the proteins in the sample to prevent column blockage [9,10]. The low pH of the prepared sample effectively keeps salts such as calcium phosphate in solution and prevents precipitation and therefore possible contamination of the instrument. Instrument contamination is further reduced by diverting the LC eluant away from the mass spectrometer to waste just before and then just after the peak elutes. The throughput of the assay is higher than those methods which use liquid–liquid extraction prior to analysis, and this method also has the advantage that the use of neurotoxic and teratogenic solvents such as dichloromethane is also avoided. This method also avoids the drawbacks of online sample clean up including the longer analytical run times due to lengthy column re-equilibration and the increased potential for carry over.

The method is highly specific due to the combination of HPLC and tandem mass spectrometry. Hydrophobic cortisol binds to the C18 column in the presence of 68% aqueous mobile phase, and more hydrophilic compounds in the matrix are washed away. The gradient then changes to 100% methanol to elute cortisol from the column with a retention time of 2 min. Using the transition of m/z 363.4 > 120.9, cortisol is the only analyte which elutes from the column with this retention time that is detected by the tandem mass spectrometer. Under the conditions used in this method, compounds such as prednisolone and fenofibrate which have been shown to interfere in previous assays for urinary free cortisol [6] were shown not to interfere in this assay.

The assay has been in routine use since January 2006. Despite the injection of solution containing TCA, the column has been resistant to chemical attack and we have injected in excess of 700 samples on a single analytical column without loss of performance. We routinely measure up to 30 samples in a batch once per week and the guard column is changed monthly. We are enrolled in the UK NEQAS urinary free cortisol EQA scheme and our results compare well to the other laboratories using LC–MS/MS. The introduction of a simplified method for urinary free cortisol has been useful in our laboratory as it reduces the time spent by highly trained staff on performing complex sample preparation tasks and therefore makes staff deployment easier. This is particularly of benefit in times of staff shortage.

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