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Commentary

Development and Validation of a Quantification Method of Urinary Free Cortisol in 24 Hours Urine by Mass Detection

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Abstract

Introduction: Measurement of urinary excretion of corticosteroids and their metabolites is used to evaluate adrenal function in Cushing's syndrome or adrenal cancer. Quantification of Urinary Free Cortisol (UFC) is recommended for Cushing's syndrome diagnosis.

Objective: To develop an easy, reproductible and specific assay technique to quantify UFC by High Performance Liquid Chromatography (UHPLC) and mass detection.

Methods: We developed a method based on an UHPLC assay coupled to a single quadrupole mass (QDa) detector. Urines were pre-treated with 1% formic acid and the internal standard before the Supported Liquid Extraction (SLE). Elution was performed with dichloromethane that was then evaporated. Samples were reconstituted with water. The compounds were separated on a HSS T3 column with a gradient elution (0.1% formic acid in water and acetonitrile). Injection volume was 1 µl and the flow rate 0.6 ml/min. We investigated the method regarding linearity, precision, recovery, accuracy, limits of detection and quantification, robustness, and carry-over.

Results: The linear range of the method was from 5 µg/l to 1000 µg/l with a determination coefficient of 0.999. The precision was evaluated by the Relative Standard Deviation (RSD) for intra- and inter- assay and was below 9% for both of them. The method showed adequate cortisol recovery (> 90%). The bias between the mean of between-day results and the target value was < 4.7% while the biases between measured and expected values from an external quality control program were <6%. The limit of detection was 0.284 µg/L and the limit of quantification was 0.755 µg/L. The variation representing the method robustness was 4.1% that is lower than the between-day variation. The carry-over was <0.02%.

Conclusion: The UHPLC-QDa method with a SLE step for UFC quantification shows adequate performances. It is easy, reproductible and suitable for routine laboratory use.

Keywords: Cushing's syndrome; Urinary free cortisol; Mass detection; QDa; Supported liquid extraction; Ultra-high performance liquid chromatography.

Abbreviations: UFC: urinary free cortisol; UHPLC: Ultra-High Performance Liquid Chromatography; LOD: Limit of Detection; LOQ: Limit of Quantification; RSD: Relative Standard Deviation; SD: Standard Deviation; SLE: Supported Liquid Extraction; LLE: Liquid-Liquid Extraction

INTRODUCTION

Cortisol (hydrocortisone) is the main and most abundant glucocorticoid hormone synthesized from cholesterol by a multienzyme cascade in the adrenal glands [1,2]. Cortisol secretion is subjected to a nycthemeral rhythm, its blood concentration being maximal in the morning between 7 and 10 AM and minimal at midnight. This secretion is regulated by the

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Hypothalamo-Hypophyso-Adrenal (HHA) axis. Cortisol plays a critical role in controlling protein, lipid and carbohydrate metabolism, in regulating blood pressure, fighting infections and in the body's response to stress.

In the blood, circulating cortisol is mainly bounded to transcortin or Cortisol Binding Globulin (CBG). Only the free cortisol is biologically active [1,3]. Urinary Free Cortisol (UFC) correlates well with the plasma free cortisol concentration. The cortisol quantification is recommended to confirm hypercorticism, such as Cushing's syndrome, and hypocorticism, such as Addison disease which is an adrenal insufficiency [4,5].

Cushing's syndrome results from chronic and excessive circulating levels of glucocorticoids. Biochemical tests are needed to confirm the clinical suspicion [6-25]. The three first-line screening tests recommended are a measurement of 24-hours UFC, a low-dose dexamethasone suppression test, and a midnight serum or salivary cortisol measurement [9,10]. UFC is non-invasive and unlike plasma cortisol secretion, it smoothes out the nycthemeral variation. The test has a reported sensitivity of 95%-100% and a specificity of 94-98% for the diagnosis of Cushing's syndrome [6,24,25]. A very high level of UFC negates the need for other test procedures in patients with obvious symptoms and signs of Cushing's syndrome.

Wood and al [7] showed that immunoassays have significant positive analytical bias compared to HPLC due to antibody cross-reactivity in urines with cortisol metabolites and synthetic glucocorticoids. These cross reactions lead to an overestimation of the diagnosis of hypercorticism [17]. High-performance liquid chromatography (HPLC) can separate interfering substances and gives a more specific measurement of urinary cortisol [8,20].

The aim of this study was to develop an easy, rapid and sensitive UHPLC method connected with a mass detector and using Supported Liquid Extraction (SLE) as a clean-up procedure. SLE is a cartridge-based variant of Liquid-Liquid Extraction (LLE) in which extraction is achieved by passing a water immiscible organic mobile phase through an aqueous stationary phase formed on an inert diatomaceous earth or a synthetic support. Compared to LLE, SLE support allows an intimate contact between the aqueous and the organic phases, possibly leading to analyte recoveries higher than the ones obtained with LLE [15,16,19]. SLE avoids the delicate steps as the phases separation, but also the emulsion present in the classic LLE method and is efficient with less volumes of solvents [13].

Although the standard for urinary free cortisol is LC-MS/MS, the cost equipment and the easy use of the mass detector (QDa, Waters) make it an interesting option for clinical laboratory.

MATERIALS AND METHODS

Samples pretreatment

Patients' 24-hours urines were frozen immediately after collection. After the thawing at room temperature the day of analysis, 200 µl of urine, standard or control were mixed with 20 µl of 10% formic acid, 40 µl of 10 µg/ml internal standard, and 140 µl of water.

Cortisol solution

A 50 µg/ml stock solution of cortisol was obtained by diluting 5 mg of hydrocortisone (Lot 20E14-B11-201954, Infinity Pharma, Oldenzaal, Netherlands) in 100 ml of water. This stock solution was aliquoted and frozen for thawing on the day of analysis.

Internal Standard (IS)

A 50 µg/ml methylprednisolone solution was obtained by diluting 5 mg of methylprednisolone (Lot LRAC4830, Sigma-Aldrich, Saint-Louis, USA) in 100 ml of water. The solution was then diluted 1:5 in water to obtain the 10 µg/ml working solution. This stock solutions was aliquoted and frozen for thawing on the day of analysis.

Standards and controls preparation

Six standards (5, 10, 50, 100, 250, 500 µg/l) were prepared each day of analysis from the cortisol solution by dilutions in water. Two levels of quality control, corresponding to physiologically low and high cortisol concentrations (20 and 150 µg/l), were prepared from the cortisol solution. Standards and controls were prepared from two different lots of cortisol solution.

Supported Liquid Extraction

The pretreated samples were extracted by supported liquid extraction using a 400 µl Novum 96-Well plate (Phenomenex, Torrance, California). A drying time of 5 min was waited after loading. The elution was performed with 2 x 900 µl of dichloromethane (Biosolve, Dieuze, France). The collected eluate was then evaporated to dryness at 40°C for 75 min (SPD121P-230, ThermoScientific, Waltham, Massachusetts USA) and reconstituted in 100 µl of water for 5 min on an orbital shaker.

Chromatographic conditions

The chromatographic conditions were defined using an Ultra-High Performance Liquid Chromatography (UHPLC) system (Acquity H-Class, Waters, Milford, USA) coupled with a QDa detector (Acquity, Waters). A HSS T3 pre-column (Acquity UPLC® HSS T3 1.8 µm VanGuard™ Pre-Column 2.1 X 5 mm, lot 0216390091, Waters) and a HSS T3 reverse phase column (Acquity UPLC HSS T3 1.8 µm 2.1X50 mm, lot 0213382772, Waters) were used as stationary phase. Separation was achieved with an acid mobile phase consisting of water with 0.1% formic acid (lot 1198871, Biosolve, Dieuze, France) and acetonitrile (lot 1387001, Biosolve) that was used in gradient at a flow rate of 0.6 mL/minute. The run time was set to 6 minutes (t = 0 minute: 20% acetonitrile; t = 2 minutes: 35%; t = 3 minutes: 95%; t=5: 20%). The column temperature was maintained at 40°C. The injection volume was 1 µl. Ionisation was in positive mode. The capillary voltage was set to 1 kV and the probe temperature was held at 600°C. Cortisol (m/z: 363.2 Da) and methylprednisolone (IS) (m/z: 375.2 Da) were detected in Single Ion Recording (SIR) mode with a cone voltage of 10 V. Data was acquired with the software Empower 3 (Waters).

The sampling rate was 2 points per second. Cortisol peak and methylprednisolone peak were detected at a retention time of 1.9 min and 2.4 min.

Method Validation

Linearity: The linearity was evaluated by diluting a 1000 µg/l cortisol solution in water (1, 5, 10, 50, 100, 250, 500, 1000 µg/l). 200 µl of each dilution underwent the extraction step and were analysed just as the samples.

Precision: The precision of the method was checked at two levels - within-day (repeatability) and between-day (intermediate precision) - by analysing 20 measurements of 2 cortisol concentration levels (20 and 150 µg/l). These solutions corresponded to control solutions. The precision was expressed by the Relative Standard Deviation (RSD).

Accuracy: The accuracy was evaluated by calculating the bias between the mean of results (C_{mean}) obtained in the inter-assay experiment and the target value (C_{target}). It was calculated for two levels of concentration (20 and 150 µg/l).

$$Bias(\%) = \frac{C_{mean} - C_{target}}{C_{target}} * 100$$

Trueness: The trueness was evaluated by calculating the difference between the measured concentration ($C_{measured}$) and the expected concentration ($C_{expected}$) of the 2022 ProBioQual (Lyon, France) external quality control testing.

$$Bias(\%) = \frac{C_{measured} - C_{expected}}{C_{expected}} * 100$$

Limits of detection and quantification: The limits of detection (LOD) and quantification (LOQ) were obtained by injecting 10 blanks (purified water) and quantifying the background noise at the retention time of cortisol. LOD and LOQ were calculated as follows:

LOD = mean+3* Standard Deviation (SD)

LOQ = mean +10* SD.

Recovery: The recovery percentage was assessed by spiking a patient's urine with different amounts of cortisol (0, 10, 50 and 100 µg/l). Samples were extracted and injected in triplicate. The mean of replicates (C_{mean}) were compared to the expected values ($C_{expected}$).

$$Recovery\ (\%) = 100 - \left(\frac{C_{expected} - C_{mean}}{C_{expected}}\right) * 100$$

Matrix effect: The matrix effect was estimated from the comparison between the slopes of calibrations curves obtained in standard solutions (linearity experiment) and in urine (recovery experiment).

Carry-over: Carry-over was evaluated by injecting 3 high concentrations of cortisol (H) (500 µg/l) followed by 3 low concentrations of cortisol (L) (5 µg/l).

$$Carry - over\ (\%) = \frac{(L1 - L3)}{(mean\ H - L3)} * 100$$

Robustness: To test the robustness of the method, small variations in method parameters were deliberately performed. The extraction and the injection of the 6 standards and the 2 controls were carried out with:

- 3 evaporation durations: 70, 75 and 80 minutes.
- 3 reconstitution durations on the orbital shaker: 3, 5 and 10 minutes.
- 3 mobile phases (variation of the aqueous phase): 0.98, 1 and 1.2% formic acid in water.

RESULTS

Chromatographic conditions determination

Examination of cortisol and methylprednisolone structures suggested a reverse phase analysis given that molecules were both hydrophobic (log P: cortisol: 1.275, methylprednisolone: 1.525), thereby a HSS T3 column (Waters) was chosen. Separation was achieved using an acid mobile phase composed of water with 0.1% formic acid and acetonitrile. Acidic pH (2.3) allowed reproducible separation given that only one neutral species was in solution for both molecules, therefore avoiding secondary interactions with residual silanol groups of the column. Using acetonitrile as organic phase gave lower retention times than those obtained with methanol given that acetonitrile is a stronger solvent than methanol [22].

Different flow rates (0.3, 0.6 ml/min) and injection volume (1, 5 µl) were tested before choosing the parameters allowing good peak shapes without any tailing: 0.6 ml/min and 1 µl [Figure 1]. The chromatographic method optimisation was continued with the variation of the cone voltage (5, 10, 15, and 20 V) and the capillary voltage (0.8, 0.9 and 1 kV). QDa parameters producing the greatest peak area for both cortisol and IS were: cone voltage = 10 V and capillary voltage = 1 kV.

Extraction method selection

Two extraction methods were tested: Liquid-Liquid Extraction (LLE) and Supported Liquid Extraction (SLE). LLE presents some disadvantages, including an emulsion formation and the necessity of phases separation and more solvent than SLE. Therefore, LLE was tested but excluded. SLE consists in a liquid-liquid extraction in the presence of a sorbent, it enables an efficient extraction with less organic solvent and without any emulsion formation. It was tested on 96 well plates. It produced easily and quickly clear products ready for analysis without conditioning or equilibrating steps [15,16,19]. It allowed a good recovery rate and produced clean chromatograms.

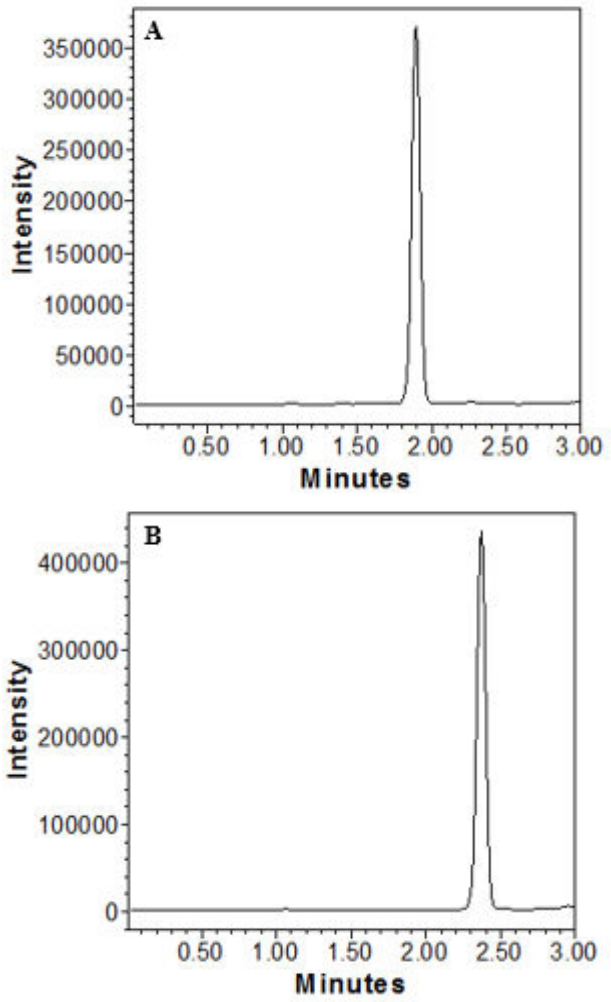

Figure 1: Cortisol (m/z: 363.2 Da) (A) and methylprednisolone (375.2 Da) (B) chromatograms obtained from the extracted standard solution 500 µg/l cortisol.

SLE optimisation

Before being loaded on the SLE plate, samples have to be diluted 1:1 in aqueous solvent. For this purpose, water and 1% formic acid were tested. The acidic dilution provided higher intensity peaks than the ones obtained with water dilution (Figure 2). Elution was performed in two steps with dichloromethane and optimised by varying the evaporation time (70, 75, 80 min). Total organic phase evaporation was achieved after 75 minutes. The bias between the mean of results obtained in the inter-assay experiment and the target value was <4.7%. The LOD was 0.284 µg/l and LOQ was 0.755 µg/l based on the background noise.

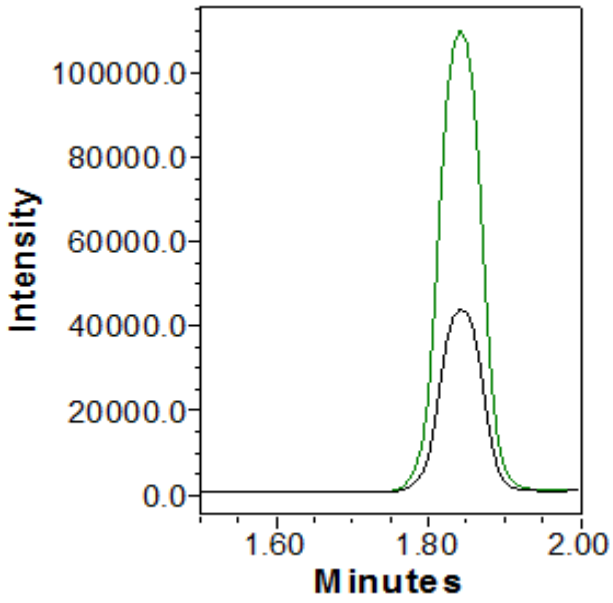

Figure 2: Cortisol chromatograms obtained from urine sample diluted 1:1 with water (black) or with 1% formic acid (green) prior to SLE.

Validation of the method

Cortisol concentrations exhibited consistent linearity over a range from 5 µg/l to 1000 µg/l (equation: 0.9997x + 0.0411) with a correlation coefficient of 0.999. The lower limit of the linearity range was 5 µg/l, which was the lowest concentration giving a deviation percentage lower than 20% between the observed and the expected value. Calibration curves carried out each day of analysis were defined based on the clinical interest in the linearity range (5-500 µg/l). Repeatability coefficient of variation was <1.24%. Random effects like different operators or analysis days were evaluated by the inter-assay precision. The coefficient of variation was <8.6% (Table 1). The trueness was assessed through external quality control testing. The difference between assays and expected value was 3.28% for low concentration and 5.97% for high concentration (Table 2). Cortisol extraction recovery was greater than 90% with a variation <2.53%. The slope of the spiking curve obtained in urine was 0.9873, indicating a slight ion suppression when compared to the slope obtained for standard solutions in the linearity experiment. However, this matrix effect is low and well compensated given that the slope is close to 1. The injection of 3 low concentration samples after 3 high concentration samples showed a carry-over of 0.02%. The quantification of UFC was not affected by small variations in method parameters given that results variation was <5.8% which is lower than the between-day variation.

Table 1: Within-day and between day precision of the method, obtained from the analyse of 20 measurements of 2 cortisol concentration levels.

	Within-day precision (n=20)			Between-day precision (n=20)		
	Mean (µg/l)	SD (µg/l)	RSD (%)	Mean (µg/l)	SD (µg/l)	RSD (%)
Low concentration (20 µg/l)	19.207	0.238	1.238	19.071	1.638	8.587
High concentration (150 µg/l)	148.069	0.935	0.632	146.361	9.786	6.686

Table 2: Trueness of the method evaluated by calculating biases between measured concentrations and expected values of external quality controls.

Measured concentrations (µg/l)	Expected value (µg/l)	Bias (%)
45.93	44.473	3.28
631.968	596.376	5.97

DISCUSSION

Cortisol is recognised as a reliable biomarker for diagnosing Cushing's syndrom and is measurable in multiple biological matrices (urine, blood, saliva) [1,21]. UFC is not influenced by the nycthemeral rhythm, so it offers a consistent indicator of steroid production over 24 hours [26,27]. Various techniques allow the cortisol quantification including immunoassays and HPLC [8,11,20]. Chromatography coupled with mass detector is usually preferred because of its good specificity and sensitivity [30,32]. Interferences from matrices affect more immunoassay results than chromatography results [17,32]. Using a mass detector instead of tandem mass spectrometry (MS/MS) is less costly for laboratories with budget constraints, but is more limited in terms of sensitivity and specificity [33]. However, it was not an inconvenient for this application given that this method based on mass detection have shown suitable performances for UFC quantification in the clinical range of interest.

SLE was validated as an efficient and reproducible extraction method, using less organic solvent than LLE and avoiding emulsion issues. It was tested on 96-well plates, enabling fast and simple sample preparation without prior conditioning [13,15,16,19]. This led to clean chromatograms and robust recovery rates.

Typically, mass spectrometry analyses use deuterated IS, mirroring at best the target molecule's behaviour. Nevertheless, their disadvantage is their high cost [15,35]. An alternative strategy employing methylprednisolone as IS because of its structural similarity with cortisol presents a cost-effective option for clinical laboratory. Interestingly, this alternative demonstrated adequate intermediate precision in UFC quantification (Table 1), staying within the recommended limits for HPLC-based methods (<15%) [15, 34], indicating that methylprednisolone was suitable as IS and could be used to take UFC loss into account during the extraction and the mass analysis.

The method was validated over the range of 5 µg/l to 1000 µg/l, allowing the quantification of both elevated concentrations associated with Cushing's syndrome and lower concentrations seen in healthy individuals or those with Addison's disease [4,5, 18, 28]. The method showed high precision (RSD <15%), acceptable bias (<15%), high (>70%) and reproducible (RSD <15%) recovery rate, and negligible carry-over (<0.05), fulfilling the stringent criteria for analytical performance [29, 34].

This easy UHPCL method allows clinical laboratories to provide specific and reproducible quantitative results without investing in expensive equipment such as tandem mass spectrometry (MS/MS).

CONCLUSION

This report describes the development of a 24-hours urinary free cortisol quantification method by Ultra-High Performance Liquid Chromatography (UHPLC) coupled with a mass detector with a Supported Liquid Extraction as a clean-up procedure. The method is simple, cost-effective, quick, and reproducible. This method allows the quantification of high concentrations of cortisol typical of Cushing's syndrome as well as low concentrations found in patients with Addison disease.

Author contributions

M.C., Data curation: D.Q., Formal analysis: M.A, M-L C., Investigation: M.A., D.Q., G.N., M-L C., Methodology: M-L C., Project administration: M C., Resources: M.A., Supervision: M-L C., MC., Validation: M-L C., MC., Visualization: M.A., Writing–original draft: M.A.,Writing–review & editing: M-L C., M.C.

Author approval

All authors have read and agreed to the published version of the manuscript.

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