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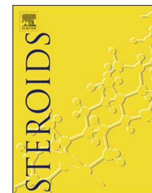

Clinical utility of an ultrasensitive late night salivary cortisol assay by tandem mass spectrometry

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ABSTRACT

Background: Late night salivary cortisol measurement is a clinically important and convenient screening test for Cushing's syndrome. Tandem mass spectrometry (LC-MS/MS) assays have superior sensitivity and specificity compared to immunoassays. Our goal was to improve a LC-MS/MS method to measure salivary cortisol in both adult and pediatric patients and to characterize its analytical performance by method validation and clinical performance by chart review.
Methods: We improved a LC-MS/MS method originally developed for urine cortisol to measure low level salivary cortisol. The sample preparation was by liquid-liquid extraction using dichloromethane followed by stepwise washing with acidic, basic and neutral solutions. The assay's analytical performance was characterized and retrospective patient chart review was conducted to evaluate the assay's clinical diagnostic performance.
Results: The LC-MS/MS assay showed enhanced functional sensitivity of 10 ng/dL for salivary cortisol and was linear within an analytical measurement range of 10–10,000 ng/dL. Assay accuracy was within 84–120% as determined by recovery studies and correlation with a reference method. Data from healthy adult volunteers was compiled to establish the reference interval for late night salivary cortisol. Patient chart review determined subjects with diagnosis of Cushing's syndrome or disease, and assay's clinical diagnostic sensitivity of 100% and specificity of 92% when the cutoff value was 70 ng/dL.
Conclusions: The improved LC-MS/MS method is sensitive and specific with enhanced analytical performance and clinical diagnostic utility for screening Cushing's syndrome. The assay may have broad clinical application due to its high sensitivity and wide dynamic range.

1. Introduction

Cushing's syndrome (CS) is caused by excessive and prolonged exposure to the hormone cortisol. CS can result from long-term use of glucocorticoid medications (iatrogenic CS) or overproduction of cortisol due to pituitary tumor (Cushing's disease), adrenal tumor (CS), tumor in other parts of the body (ectopic CS), or other stress-inducing medical conditions (pseudo-CS). The loss of diurnal cortisol release, where normally it is lowest at midnight and highest in early morning, usually precedes the typical signs and symptoms. The classic clinical presentations of CS – central obesity, moon facies, hirsutism, and plethora – are well established [1], and the most sensitive features in distinguishing CS from pseudo-CS are the presence of supraclavicular fat pads, proximal muscle weakness, thin skin, and easy bruising [2]. However, CS cannot be diagnosed with clinical findings alone. Evaluation of CS relies on laboratory testing and usually starts with measuring excess endogenous production of cortisol. Differential diagnosis, confirmation, and localization of the disease require further laboratory and radiological testing [3].

Measuring late night salivary cortisol (LNSC) and 24h urine cortisol are among the first line screening tests for CS [4]. LNSC is gaining increasing clinical importance due to reported high diagnostic sensitivity and specificity, as well as the convenient and noninvasive nature of sample collection which is usually by patients at home [5–8]. The appearance of late night cortisol secretion is one of the first indications of CS [4]. Salivary cortisol reflects biologically active free hormone [9–11] albeit the level is much lower than serum total cortisol levels which includes protein-bound and free cortisol levels. Sensitive immunoassays such as RIA were traditionally used to measure LNSC, and most clinical performance evaluation of LNSC were by RIA method [12–18]. In recent years, liquid chromatography-tandem mass spectrometry (LC-MS/MS) has become the method of choice due to enhanced specificity and sensitivity over conventional immunoassays including RIA. Despite the apparent advantages, LNSC determination by

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LC-MS/MS has not been widely adopted into clinical service and there was limited number of reports of its clinical diagnostic values [19–21]. Clinical application of LNSC by LC-MS/MS is further limited by poorly standardized protocol and lack of clinically proven single reference range or cutoff value for diagnosis. As a result, there has been conflicting information regarding the clinical diagnostic sensitivity and specificity of LNSC by LC-MS/MS [22].

We aim to set up a LC-MS/MS method with enhanced sensitivity by using advanced instrumentation, and with accuracy better characterized by using certified pure standards spiked in appropriate sample matrix. We adopted a LC-MS/MS method originally developed for urine cortisol measurement [23] and optimized it for both salivary and urine samples with enhanced sensitivity and accuracy. The assay was validated for clinical diagnostic use with performance characteristics such as sensitivity, specificity, precision, and accuracy characterized. Healthy volunteers were recruited to establish a reference interval and a diagnostic cutoff value for LNSC. We conducted retrospective evaluation of clinical diagnostic sensitivity and specificity of the test for CS in our local patient population. The diagnostic cutoff values of LNSC were compared and updated as the result of retrospective evaluation.

2. Materials and Methods

2.1. Reagents, standards, and quality control materials

Cortisol (MW 362.46 g/mol; 99% purity) was purchased from Steraloids Inc. (Newport, RI). Stable isotope labeled internal standard (SIL-IS) was deuterated cortisol (cortisol-9,11,12,12-d4; MW 366.49 g/mol; 98% isotope enrichment) from C/D/N Isotopes (Pointe-Claire, Quebec, Canada). Dichloromethane and methanol (HPLC grade) were from VWR (Radnor, PA). Formic acid (95%), Congo red, and bovine serum albumin (BSA) were from Sigma-Aldrich (St. Louis, MO). Potassium hydroxide (KOH; 1 M) and hydrochloric acid (HCl; 12.1 M) were from Fisher Scientific (Pittsburgh, PA). All other chemicals used were of highest purity commercially available. Pure cortisol was weighed and dissolved in 100% methanol to obtain a 1 mg/mL stock solution, and was further diluted in methanol to obtain working stock solutions. Calibration standards were made by spiking cortisol stock solutions in 0.2% w/v BSA with organic solvent content being kept to < 5% v/v. A separate stock solution of 1 mg/mL (certified; Cerilliant, Round Rock, TX) was diluted in methanol and was used for accuracy study. The same pure certified solution was spiked into 0.2% w/v BSA to produce two levels of quality control (QC) samples for routine use. Cortisol SIL-IS solution was checked for unlabeled cortisol impurity on the LC-MS/MS system. The 0.2% w/v BSA solution was checked for any endogenous cortisol and was used as salivary sample blank when needed.

2.2. Sample collection

Two separate and mostly consecutive late night salivary samples were collected using Salivette swabs and container tubes (Sarstedt, Nümbrecht, Germany). The collection kit included written instructions in both English and Spanish for patients to collect their own samples, either in their home or as in-patient in the hospital. Healthy volunteers, used as normal controls, were provided the same collection kit and instructions to collect samples from their home. A separate swab (Salimetrics; State College, PA) was used in children < 6 years of age and collection was under direct parental or caregiver supervision. Instructions were to collect salivary samples in a relaxed state just before going to sleep, without any physical or emotional perturbations, and without brushing teeth, eating, or drinking for at least 15 min prior to the collection. Samples were collected by rolling the absorbent swab in the mouth for approximately 2–3 min before placing it back into the collection tube and tightly capping the lid. Collected samples were to be stored in the refrigerator before using the provided package to ship them to the central laboratory via US Postal Service. For in-patients, samples were sent by the caring unit directly to the laboratory. Upon arrival in the lab, samples were centrifuged (10 min; 800*g*), and liquid saliva recovered at the bottom of container was refrigerated until analysis.

2.3. Sample pretreatment

A 500 μL aliquot of each patient's salivary sample was transferred to a pre-labeled, 2 mL microcentrifuge tube. Samples of < 500 μL (but more than 100 μL) were measured and brought to 500 μL by adding 0.2% w/v BSA, with the dilution factor noted. A mixture consisting of 100 μL SIL-IS (200 ng/dL) and 0.1% w/v Congo red was added to all patient samples, pre-prepared calibration standards (levels: 5, 10, 50, 200, 1000, 5000 ng/dL), and QC materials and briefly vortexed. Subsequently protein-bound cortisol was released by the addition of 20 μL 10% v/v formic acid to each sample. Samples were then incubated at room temperature (RT) for 20 min, with vortex mixing twice during the time. Cortisol was then extracted by the addition of 1.2 mL dichloromethane (0.6 mL twice) followed by mixing and incubation at RT for another 20 min. Samples were placed in a −20 °C freezer for a minimum of 30 min followed by centrifugation at 8 °C (15 min; 800*g*) to generate two solution phases. The aqueous phase on top was discarded with the remaining dichloromethane phase being washed sequentially with 0.8 mL HCl (0.1 M), 0.8 mL KOH (0.1 M), and 0.8 mL distilled water. For each wash the samples were vortexed and centrifuged prior to aspirating the top aqueous phase into waste. The remaining organic solvent phase was dried under nitrogen gas on a heating block set to 37 °C. The completely dried sample was reconstituted using 120 μL of 25% aqueous methanol and incubated at 37 °C for 10 min. Tubes were centrifuged briefly to collect the sample at the bottom and 120 μL of the sample was transferred to glass inserts for LC-MS/MS injection.

2.4. Urinary cortisol assay

Twenty-four-hour urinary free cortisol was determined using the sample preparation step indicated above alongside salivary samples, followed by LC-MS/MS method described below. The upper limit of reference interval was set at 50 μg/24 h.

2.5. LC-MS/MS analysis

A high-performance liquid chromatography system (Shimadzu model LC-20AD), coupled to an API5000 triple quadruple mass spectrometer (Sciex, Redwood Shores, CA) equipped with an electrospray ionization (ESI) source, was used for the quantitative analysis. Cortisol and its SIL-IS were chromatographically resolved from potential interfering compounds by a reverse-phase analytical column (Kinetex 2.6 μm C18, 100 × 3 mm; Phenomenex, Torrance, CA) housed in a column oven set at 45 °C. Samples (30 μL) were injected into 50% methanol containing 0.1% v/v formic acid at a flow rate of 0.5 mL/min and methanol concentration maintained for 1 min before ramping up to 95% over the next min and being maintained for another 3 min to elute cortisol. Methanol concentration was brought down to 50% over 1 min and maintained for another min to equilibrate column and system to be ready for next sample injection. Total run time was 6 min from injection to injection. Eluting compounds were subjected to ESI operating in positive ionization mode with a source temperature of 500 °C, ion spray voltage of 5000 V, curtain gas of 30 psi, and nebulizer gas (GS1) of 50 psi. Cortisol was detected and quantitated by selected reaction monitoring (SRM) with the quantifier transition 363.2 $m/z \rightarrow$ 121.1 m/z (collision energy (CE) of 30 V), and qualifier transition 363.2 $m/z \rightarrow$ 97.0 m/z (CE of 35 V), while SIL-IS was detected by monitoring the 367.3 $m/z \rightarrow$ 121.0 m/z transition (CE of 30 V). Data acquisition and quantitation were accomplished by using Analyst software (Ver. 1.6.1; Sciex, Redwood Shores, CA). A calibration curve was constructed by

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