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Short communication

Development of a urinary free cortisol assay using solid-phase extraction–capillary electrophoresis

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Abstract

In clinical practice, the measurement of urinary free cortisol (UFC) provides the most sensitive and specific diagnostic information for excess adrenal production of cortisol. The existing methodologies (RIA and HPLC) are time consuming, costly, involve tedious extractions, derivatizations and problems with non-specific interactions with cortisol metabolites in urine. In the present study, we describe the development of an SPE–CE method for the rapid analysis of UFC. UFC was concentrated using SPE C_{18} cartridges (3M Empore) under a vacuum and eluted with acetonitrile–SDS. The use of 10% acetone to wash cartridges before final elution with acetonitrile–SDS showed significant improvements in the free cortisol recovery. The complete extraction was accomplished in 10–15 min with a recovery of 89–94%. CE analysis was done on a Beckman P/ACE 5010 with detection at 254 nm using a neutral capillary. Detection limits of free cortisol in urine was improved to 10 μg/l with SPE compared to 500 μg/l without SPE. No interferences either from BSA or other urinary cortisol metabolites affected the free cortisol determinations. The results showed the feasibility of a rapid UFC detection with improved sample handling capacity.

Keywords: Cortisol; Steroids

1. Introduction

Cortisol is the major glucocorticoid produced by the adrenal glands in humans and regulates a myriad of biological functions and processes. Excess production of cortisol over a prolonged period of time can cause a disease known as Cushing's syndrome. Free cortisol is filtered by the glomerulus of the kidney and most is reabsorbed by the renal tubules. A discrete amount, however, is excreted in the urine. Thus, the cortisol in a 24-h urine represents the integrated or mean concentration of free cortisol in plasma over a 24-h period and provides an excellent diagnostic sensitivity and specificity [1]. In contrast, serum cortisol levels are not always an accurate measure of over active adrenocorticoid function and can be elevated in pregnancy, obesity, diabetes, or hyperthyroidism. Therefore, urinary free cortisol (UFC) measurement is the most reliable single approach to screening patients for Cushing's syndrome [1,2]. The problem with the measurement of urine cortisol is the presence of cortisol metabolites that can falsely elevate the apparent UFC.

There are a variety of methods to measure UFC, ranging from some relatively easy immunoassays

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[3–5] to laborious equilibrium dialysis and high-performance liquid chromatography (HPLC) [6]. Immunoassay methods, including the traditional radioimmunoassay (RIA) and non-isotopic chemiluminescence assay are routinely used to measure UFC's. Although relatively inexpensive, immunoassays often overestimate the concentration when compared to chromatographic assays. This is due to the use of antibodies that cross-react with various metabolites and/or synthetic corticosteroids [6,18]. Even development of automated immunoassays has only improved the precision leaving the problems of urinary interferences unresolved [17]. The extent of interference depends on the specificity of the antibody that varies between commercially available kits. HPLC, on the other hand, separates cortisol from other interfering substances, but has the disadvantage of being complicated in addition to long turn around time, limiting its use only to a few specialized laboratories.

Capillary Electrophoresis (CE) on the other hand can overcome many of these problems using an 'open tubular format'. It has exceptional resolving power, requires no derivatization, low sample volume, and fast turn around times. However, not all forms of CE are useful in separations of steroids. Steroids being neutral molecules can only be resolved by using some form of micellar electrokinectic chromatography (MEKC). In addition to our laboratory, separation of neutral steroids by MEKC has been shown by various groups [7–12]. Although separation of steroids is possible a major concern for clinical applications is the lack of adequate sensitivity and reproducibility. To overcome this problem several strategies have been suggested including sample stacking, use of solid-phase or membrane concentrators or performing an isotachophoretic step before free zone electrophoresis [13–15]. In a manner similar to our previous publications [7] Quirino et al. [16] demonstrated the application of field-enhanced sample injection with reverse migrating micelles (FESI–RMM) for determination of testosterone and progesterone. As noted by these authors and from our own experience sensitivity observed for pure standards does not translate to complex biological matrix such as serum or urine. Thus for steroids a prior extraction from these complex matrix is a requirement for clinical testing. In the present study, we developed a solid-phase extraction (SPE) coupled MEKC as a potential analytical method for the rapid separation and detection of UFC. To measure overall performance and feasibility, we evaluated linearity, recovery and lower limit of detection of free cortisol in human urine and compared the results with a commercially available immunoassay.

2. Materials and methods

2.1. Chemicals and reagents

Cortisol (hydrocortisone) and corticosterone was purchased from Sigma (St. Louis, MO, USA), 4-pregnen-11β,17,21-triol-3,20 dione 21-acetate (cortisol acetate), 21-sulfate (cortisol sulfate), 21-glucosiduronate (cortisol glucuronides) were purchased from Steraloids (Wilton, NH, USA). Steroid stock solutions (1 mg/ml) were made were made by dissolving the respective steroids in ethanol.

2.2. Preconcentration

Normal saline or urine (15 ml) was spiked with 100 μg/dl Corticosterone (Internal Standard; I.S.) and different concentrations of cortisol and its conjugated metabolites (10–160 μg/dl). Preconcentration of cortisol from urine samples was achieved by using 3M Empore extraction disc cartridges (Fisher Scientific, USA). The discs are C_{18} modified 12 μm fused-silica particles immobilized on an inert matrix of polytetrafluoroethylene fibrils (7 mm diameter, 0.5 mm thick) secured in 3 ml polypropylene columns. The urine was rapidly passed through the SPE Cartridges, preconditioned with 250 μl of methanol and washed with 1 ml of deionized water (DIW), using vacuum. After passing the respective spiked urine/saline samples, the discs were washed twice with 1 ml of 10% Acetone (acetone:water v/v) followed by 1 ml of DIW. The final elution of steroids was achieved using 80 μl of acetonitrile followed by 320 μl 10 m*M* SDS and immediately used for separation by MEKC.

2.3. Capillary electrophoresis

Analysis was done using a Beckman P/ACE 5010 (Beckman Instruments, Fullerton, CA, USA)

equipped with system Gold software for data analysis. MEKC as previously described [7] was used to separate UFC. The final run buffer for electrophoresis comprised 75 mmol/l SDS, 20% acetonitrile (v/v), and 20 mmol/l MES buffer, pH 6.0. Separation was done on a 50 μm (I.D.)×375 μm (O.D.) neutral eCAP capillary tube (Beckman Instruments), 37 cm injection to detection. Samples were pressure injected (5 p.s.i.) for 20 s and the capillary temperature was maintained at 16±0.1°C. Detection was at 254 nm and 10 kV voltage with reversed polarity was employed for all separations done in this study.

2.4. Immunoassay

Cortisol immunoassay was performed on a Technicon Immuno 1 system (Bayer Diagnostics, Tarrytown, NY, USA). This analyzer uses a heterogeneous competitive magnetic separation format for in vitro quantitative measurement of cortisol in serum and plasma. urine samples requires a prior extraction and preconcentration step. This typically involves a 30 min extraction by methylene chloride, after extraction the organic solvent is evaporated and cortisol is reconstituted with a reconstitution diluent supplied by Bayer Diagnostic. This method is used routinely in our laboratory for testing patient serum and urine samples.

3. Results and discussion

In this study we report the development of a CE method for the determination of free cortisol in human urine. Fig. 1 shows the separation of cortisol acetates, sulfates, glucuronides and free cortisol. It was achieved by a system similar to one described by us involving a coated neutral eCAP and SDS buffer at pH 6 [7]. The electroosmotic flow in this system is less than the electrophoretic mobility of SDS micelles, hence in order to allow the detection of steroids it is important to reverse the polarity. Janini et al. in 1996 termed it as reverse flow MEKC [22]. The method gave a detection limit of 500 μg/l which is far above the cortisol levels found in normal human urine of 20–90 μg/day and 5–55 μg/day for adults and children, respectively. Therefore in order

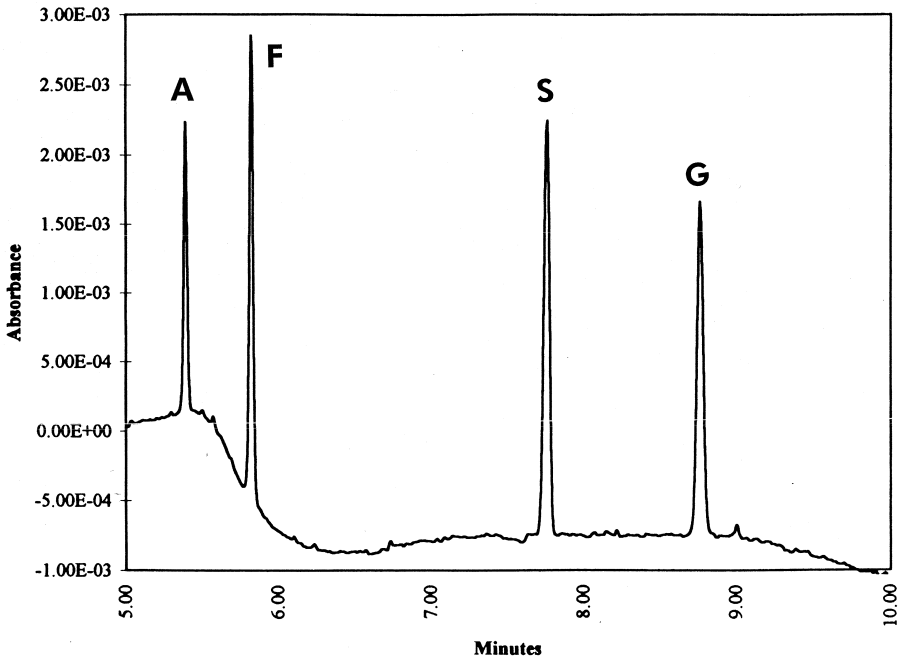

Fig. 1. Separation of cortisol acetate (A), free cortisol (F), cortisol sulfates (S) and cortisol glucuronides (G) by micellar electrokinetic capillary chromatography. The run buffer used was 100 m*M* SDS, 20% acetonitrile and 20 m*M* MES, pH 6.0, 15 kV).

to detect normal levels of UFC, preconcentration of urine samples is necessary.

Solid phase extraction disc cartridges were used for sample clean-up and preconcentration. Sample clean-up and extraction procedure was optimized by using different concentrations of acetone and acetonitrile in elution buffer. Acetonitrile is preferred over acetone because of its transparency at 254 nm. Recoveries ranging from 89–94% for free cortisol and its metabolites in urine are achieved by using a two step elution protocol involving acetonitrile and 10 mmol/l SDS respectively. Percent recoveries were estimated by both capillary electrophoresis and immunoassay (Immuno-1; Bayer Diagnostics). Thus by using this protocol it is possible to achieve a 37.5 fold preconcentration. The lower limit of normal for normal adults is 5 μg/day, which is approximately 3–4 μg/l. Cortisol at these physiological levels can be easily detected by subjecting a 15 ml of urine sample to the sample clean-up and preconcentration protocol discussed above.

Normal urine contains variable quantities of proteins ranging from 30–100 mg/dl. Cortisol and steroidal hormones are known to bound to albumin at varying concentrations. Therefore the effect of variable quantities of proteins in urine on extraction of cortisol by extraction disc was evaluated by spiking saline standards with different amounts of bovine serum albumin. No interference was observed for albumin concentrations ranging from 5 to 100 mg/dl.

Another problem that is encountered with human urine is the numerous compounds such as creatinine, porphyrins, urobillinogen, lactate etc present in it that are also separated and detected during the electrophoresis step. The sample clean-up although reduces these components significantly but still there are some interference from hydrophobic components that are eluted from disc with cortisol. We tried to reduce these peaks by using different SPE disks with 8–18% carbon loads, pretreating urine sample through ion-exchange resins, and treatment with a variety of acids and basic wash solutions with no significant improvements. Using 2×2.0 ml of a 10% acetone wash of the SPE disk prior to elution of the cortisol with acetonitrile gave the best results. Fig. 2 shows the free cortisol peak at different concentrations, very well separated from the endogenous substance in normal urine. This separation of UFC peak is significantly enhanced by reducing capillary temperature to 16°C.

The run to run and capillary to capillary irreproducibility (both in terms of retention time and peak area) is resolved by adding an internal standard. After evaluating several steroids to use as an internal standard, we chose corticosterone because of its non-interference by other peaks found in urine (Fig. 2). The calibration curved prepared by plotting peak area of cortisol/peak area of I.S versus cortisol concentration was linear between 10–160 μg/dl with a correlation coefficient of 0.998. Addition of internal standard also allowed us to use relative migration time and relative peak area for qualitative identification of cortisol peak and its quantification in urine controls. Both relative migration time and relative peak area gave better precision when compared to absolute migration time and absolute peak area. This is very obvious from the coefficient of variation for these parameters, which has improved from ~15% to less than 5% (n=21) for both between and within run estimation. The urinary free cortisol measured on 20 patients samples by this method compared very well with automated immunoassay giving a correlation coefficient of 0.95 with a slope and intercept of 0.934 and 0.012 respectively.

4. Concluding remarks

The present observations showed the feasibility of a method for the determination of urinary free cortisol using SPE–CE with a neutral capillary. In this method separation was achieved by partitioning steroids between a pseudo stationary organic phase (acetonitrile) and a SDS micellar phase. The complete extraction and electrophoresis is accomplished in 30 min. Although the use of neutral capillary increases the cost per assay and they do have a significant lot to lot variability in terms of neutral coating but even with these shortcomings it can be used for determination of UFC. The one advantage with neutral capillary is low currents observed when compared to fused-silica capillary this allows use of high ionic strength buffer which helps in performing FESI–RMM [13,16,19–22]. The method discussed here is accurate, precise and can be used for de-

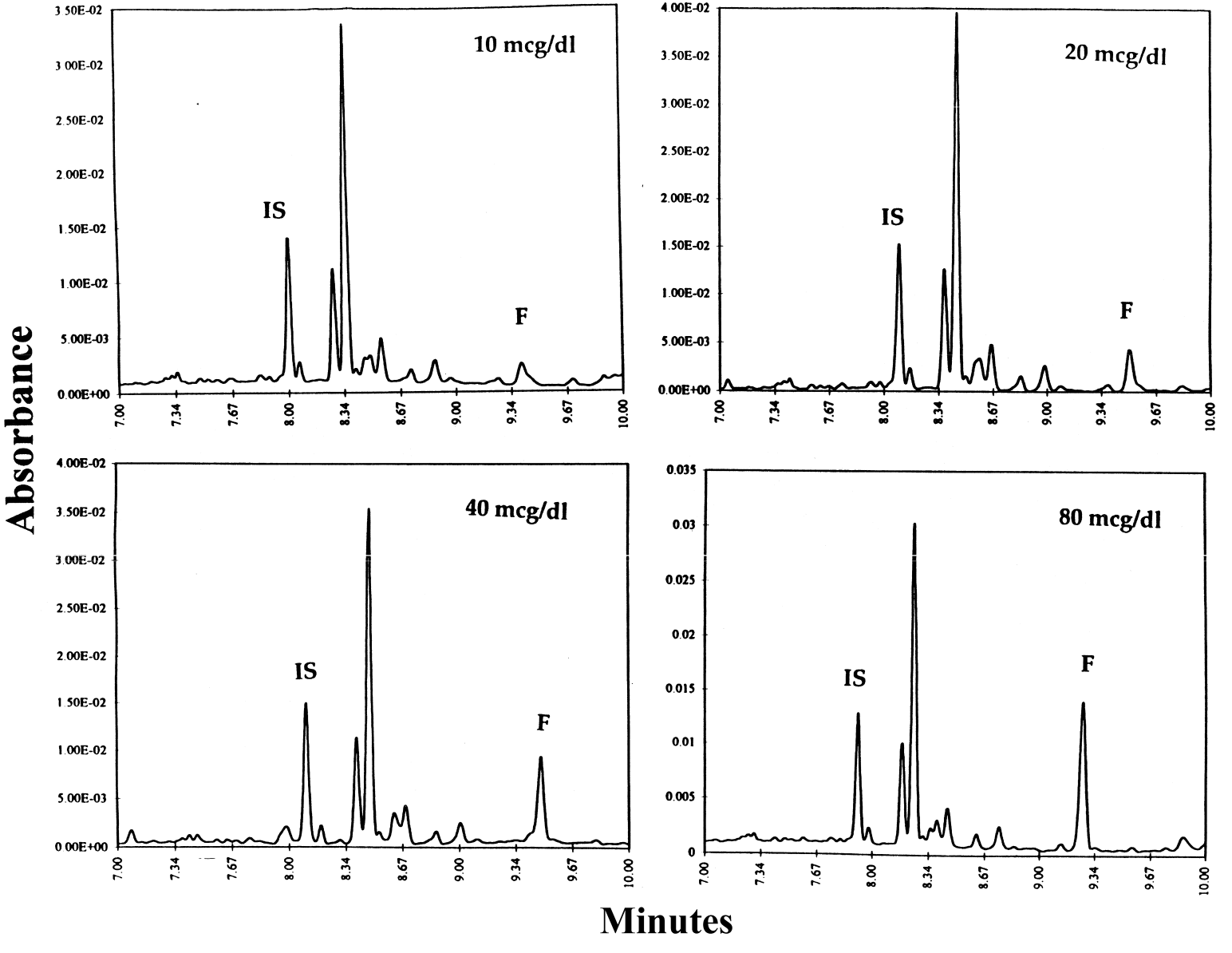

Fig. 2. Urinary free cortisol at different concentration in human urine. CE conditions 100 m*M* SDS, 20% acetonitrile and 20 m*M* MES pH 6.0, 10 kV.

termination of urinary free cortisol in clinical samples.

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