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Rapid steroid hormone quantification for congenital adrenal hyperplasia (CAH) in dried blood spots using UPLC liquid chromatography–tandem mass spectrometry

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ABSTRACT

Newborn screening for congenital adrenal hyperplasia (CAH) is usually done by quantifying 17α-hydroxyprogesterone using immunoassay. However, this test produces high rates of false positive results caused by cross reacting steroids. Therefore we have developed a selective and specific method with a short run time (1.25 min) for quantification of 17α-hydroxyprogesterone, 21-deoxycortisol, 11-deoxycortisol, 11-deoxycorticosterone and cortisol from dried blood spots. The extraction procedure is very simple and steroid separation is ensured on a BEH C18 column and an ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS). Analysis was done in positive ionization mode (ESI+) and recorded in multiple reaction monitoring mode (MRM). The method gave linear results for all steroids over a range of 5–200 (cortisol: 12.5–500) nmol/L with coefficients of regression >0.992. Absolute recovery was >64.1%. Across the analytical range the inter-assay coefficient of variation (CV) was <3%. Newborn blood samples of patients with confirmed 21-CAH and 11-CAH could clearly be distinguished from samples of unaffected newborns falsely positive on immunoassay. The method is not influenced by cross reactions as found on immunoassay. Analysis of dried blood spots shows that this method is sensitive and fast enough to allow rapid analysis and can therefore improve the newborn screening program.

1. Introduction

Congenital adrenal hyperplasia (CAH) is an inherited defect of steroid biosynthesis. The two most frequent forms are 21α-hydroxylase and 11β-hydroxylase deficiency. Newborns with CAH are unable to produce sufficient amounts of cortisol and in two-thirds of cases of aldosterone. Clinical symptoms include salt wasting, dehydration and death. In females CAH causes virilization and girls may initially be wrongly assigned male [1].

In most newborn screening programs worldwide the detection of CAH is currently implemented. The standard test parameter is 17α-hydroxyprogesterone (17-OHP). Most laboratories use immunoassay techniques to quantify this steroid. Although immunoassays are easy to handle several publications state low specificity due to cross-reactivity of antibodies with steroids other than 17α-hydroxyprogesterone such as steroid sulfates [2], 17α-hydroxypregnenolone [3] and 17α-hydroxypregnenolone sulfate [4].

Mass spectrometry has the potential to replace conventional immunoassay for quantification of steroids [5]. False positive results are found especially in sick newborns [6] and preterm babies [7] due to cross reactions on the one hand and stress-related stimulation of the adrenal cortex on the other hand. Furthermore, EDTA which is used as an anticoagulant for blood samples may interfere with newborn screening tests based on lanthanide fluorescence and thus lead to false-positive results [8]. To avoid these problems other techniques have been developed to identify CAH. Gas chromatography-mass spectrometry combined with isotope dilution is being widely used in clinical and environmental laboratories measuring steroids [9]. The advantage of this method is generation of a steroid profile with high accuracy. However, the method requires a rather high sample volume. Furthermore, blood sample preparation, derivatization and measurement are very time consuming.

Other groups have tried to purify samples by liquid–liquid extraction [10] prior to immunoassay analysis in order to remove steroid sulfates from the sample. They measured lower values for 17-OHP from healthy newborns and infants with CAH and found a reduced rate of false positives.

In recent years, numerous groups have applied tandem mass spectrometry using analytical reversed phase separation techniques. Several ionization techniques like atmospheric pressure chemical ionization (APCI) [11], electrospray ionization (ESI) [12,13] and atmospheric pressure photo ionization (APPI) [14] were used to ionize steroids. The efficiency of each ionization method

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varied with the design of the utilized instrument. Matrix effects (ion suppression) were more marked in ESI mode compared to APCI or APPI [15]. The ESI mode, however, can be handled easily, and most clinical laboratories use this ionization technique. This ionization mode is powerful enough to enable sufficient transfer of charge to generate $[M+H]^+$ ions of the steroid molecules to quantify them in very small sample volumes.

In order to detect all steroids of interest, sample volume has to be increased or else the steroids have to be chemically converted into derivatized molecules [3,16]. Derivatization with 2-hydrazinopyridine facilitates ionization of the molecule. This enhances the sensitivity and specificity. Again, derivatization methods are more time consuming [17] and are less reliable.

Our work was based on the publication of Lacey et al. [18] who showed that steroid profiling from newborn dried blood spots with LC–MS/MS was able to detect 21-CAH and to minimize the false positive rate obtained by immunoassay. Soldin and Soldin [17] have summarized that LC–MS/MS technology affords specificity, imprecision and limits of quantification necessary for the reliable measurements of steroids in human fluids.

Our goal was to develop a very fast and robust method to eventually replace use of immunoassays. The method was designed to be fast enough to analyze the large quantity of samples of the routine screening program. Therefore, the extraction of steroids had to be simple enough.

2. Experimental

2.1. Material and methods

2.1.1. Materials

All steroids (17α-hydroxyprogesterone, 21-deoxycortisol, 11-deoxycortisol, cortisol and deoxycorticosterone) were from SIGMA (Deisenhofen, Germany). Deuterated steroids (d8 17α-hydroxyprogesterone, d8 21-deoxycortisol, d2 cortisol, d2 11-deoxycortisol and d7 androstenedione) were from Cambridge Isotopes Laboratories Inc. (LCG Euriso-Top, Saarbruecken, Germany). High purity solvents recommended for UPLC–MS/MS methods (acetonitrile, water, methanol, formic acid and acetone) were from Biosolve BV (Valkenswaard, The Netherlands).

2.1.2. Samples

Dried blood spots from the neonatal screening program and from clinically and genetically confirmed cases of 21-CAH and 11-CAH were anonymized prior to the analysis. The filter paper spots were stored at 4 °C. The study was approved by the Medical Ethics Review Board of Hannover Medical School.

2.1.3. Internal standard preparation

For internal standards stock solutions of deuterated steroids at a concentration of 300 μmol/L (cortisol: 100 μmol/L) were dissolved in methanol and stored at −18 °C. Immediately before use stock solutions were diluted with methanol to give final concentrations of 100 nmol/L for cortisol and 15 nmol/L for the other steroids.

2.1.4. Calibrators and controls

Calibrators and control material were produced following our method previously described [19]. In brief blood was centrifuged at 1.900 U/min, red cells were washed three times with saline, serum and supernatant was discharged. Then erythrocytes were mixed with steroid-free serum (MP Biomedicals, Eschwege, Germany) to produce an approximately steroid-free blood with a hematocrit of 55%. Steroids were dissolved in methanol/water 50:50 (v:v) to obtain stock solutions of 4 μmol/L and 10 μmol/L for cortisol. Stock solutions were added to the steroid-free blood to obtain calibrators at final concentrations of 0, 5 (12.5), 25 (62.5), 75 (187.5), 125 (312.5), 200 (500) nmol/L for steroids (cortisol). Samples were frozen at −20 °C. Subsequently, calibrators were spotted on filter paper cards (Munktel & Filtrak, Baerenstein, Germany), dried at room temperature for 24 h and stored at +4 °C in a refrigerator. For the determination of absolute recovery [20,21] the stock solution was diluted with methanol/water 50:50 (v:v) to obtain the same final concentrations as described for whole blood (see above).

2.1.5. UPLC and MS/MS spectrometer

The LC–MS/MS composed of an ACQUITY UPLC system combined with a XEVO tandem quadrupole mass spectrometer both from Waters (Eschborn, Germany) was controlled by the MassLynx v4.1 software.

For LC analysis an AQUITY UPLC BEH C18 1.7 μm, 2.1 × 50 mm (Waters) column was used. A binary gradient was applied. Solvent A and B consisted of UPLC purity water and highest purity acetonitrile, respectively. Both solvents contained 0.1% formic acid. The column temperature was held at 40 °C. Sample (7.5 μl) were filled in 'partial loop with needle overfill' mode. The UPLC requires two wash solutions for cleaning needle and port system. The strong wash solution consisted of methanol and water 90:10 (v:v), the weak wash solution had the same composition as the gradient at the starting point.

The flow was 0.6 mL/minute and started with 57% solvent B. After 0.38 min solvent B was stepped up to 66% within 0.01 min and held for 0.26 min. Then solvent B was increased to 100% within 0.01 min and held for 0.1 min. At 0.77 min solvent B was reduced to the initial concentration which was reached at 1.25 min.

All steroids were measured in positive electrospray (ESI+) ion mode $[M+H]^+$ since the abundances are significantly higher compared to negative ion mode $[M-H]^-$. To gain highest sensitivity each steroid dissolved in 1:1 methanol/water solution was injected separately and the ionization settings were optimized for best fragmentation through infusion in mobile phase.

The following settings were used: capillary voltage 0.55 kV, cone voltage 26–29 V, collision energy 17–26 eV, source temperature 150 °C, desolvation temperature 600 °C, dwell time 0.017 s, collision cell pressure filled with argon 4.5 × 10–3 mbar. The detailed parameters for each steroid as well as the functions are given in Table 1.

2.1.6. Assay procedure

Two 4.7 mm spots each of calibrators and patient samples were punched out from filter cards (DBS-puncher, Perkin–Elmer, Rottgau, Germany) and placed into microtiter plates (ABGENE, Hamburg, Germany). 20 μL internal standard and 150 μL acetonitrile/acetone 50:50 (v:v) were added to the blood spots. The microtiter plate was sealed with aluminum foil and shaken for 40 min at ambient temperature. The supernatant was transferred to a second plate and dried at 60 °C by a gentle nitrogen stream on a plate heater VLM BIO2 (VLM GmbH, Bielefeld, Germany). The residues were reconstituted in 80 μL 50% methanol in water with 50 mmol/L formic acid and shaken for 20 min at room temperature. The plate was centrifuged at 1000*g* for 10 min to separate small particles and wear from card material. The clear supernatant was transferred to a 384 microtiter plate (ABGENE) using a Matrix Impact2 multi pipette (Thermo Fisher Scientific, Dreieich, Germany) with variable interspaces.

For routine screening of 21-CAH, we used the Delfia® Neonatal 17α-hydroxyprogesterone competitive immunoassay from Perkin–Elmer (Turku, Finland) labelled with europium.

2.1.7. Statistics

Validation and correlation data was calculated using Microsoft Excel® 2003 and MedCalc® v11 (MedCalc software bvba, Mariakerke, Belgium).