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Measuring steroids from dried blood spots using tandem mass spectrometry to diagnose congenital adrenal hyperplasia

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Measuring steroids from dried blood spots using tandem mass spectrometry to diagnose
congenital adrenal hyperplasia

by

Deema Omar Qasrawi

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
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Abstract

Congenital adrenal hyperplasia (CAH) is a group of autosomal recessive disorders that occur due to defects in the steroidogenesis pathway within the adrenal glands. CAH is characterized by numerous clinical manifestations resulting from dysregulation of various enzymes in the steroidogenesis pathway. Approximately 90% of CAH cases can be diagnosed by the measurement of serum 17-hydroxyprogesterone alone. However, the quantification of six additional steroids, cortisol, 21-deoxycortisol, 11-deoxycortisol, androstenedione, pregnenolone and dehydroepiandrosterone could significantly improve CAH laboratory diagnosis. Although clinical laboratories predominantly use immunoassays to measure some of the above steroids, these assays are hampered by low specificity due to cross reactivity of antibodies to molecules with similar structures and high cost of antibodies used in the system. As CAH is diagnosed in neonates, using dried blood spot (DBS) as specimen of choice can further improve patient care due to the very small sample volume. This study aimed to develop a more specific and rapid assay to measure seven steroids using liquid chromatography mass spectrometry (LC-MS/MS) in DBS. An optimized DBS sample preparation method was employed without the need of derivatization. A LC-MS/MS assay was developed and optimized using reverse phase-ultra performance liquid chromatography (RP-UPLC) system combined with a triple quadrupole mass spectrometry using positive electrospray ionization (+ESI) mode. Each steroid was quantified using its deuterated or isotopically labelled internal standard. Calibration curve and quality control specimens were prepared in saline-washed erythrocytes mixed with steroid free serum to achieve a $55\pm1\%$ hematocrit level. Prepared specimens were enriched with predetermined concentrations of the seven steroids and spotted onto Whatman 903[®] filter papers. The assay was validated according to CLSI analytical guidelines, including limit of detection (LOD) and lower limit of quantification

(LLOQ), linearity, precision, and method comparison. The analytical measuring range of the method for all steroids was 2.5 were 0.11-1.8 ng/ml, 0.001-6.4 ng/ml, 1.8–11.5%, and 5.3-13.8%, respectively. This robust and inexpensive assay can be readily implemented in clinical laboratories equipped with LC-MS/MS and can provides superior analytical performance over traditional immunoassays for the diagnosis of CAH.

Preface

This thesis is an original work by the author. No part of this thesis has been previously published.

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Dedication

*This thesis is dedicated to the memory of my father, **Dr. Omar Qasrawi** and to my beloved mother, **Mrs. Samírah Al-Bakrí**. I also dedicate this thesis to my intelligent husband, **Abdulhameed** and to my lovely son, **Majd**.*

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List of Abbreviations

11 β -OH	11 β -hydroxylase
11-OHD	11-hydroxylase deficiency
17-OHP	17 α -Hydroxyprogesterone
21-OH	21 α -hydroxylase
21-OHD	21-hydroxylase deficiency
3 β -HSD	3 β -hydroxysteroid dehydrogenase
ACTH	Adrenocorticotrophic hormone
AUC	Area under the curve
APCI	Atmospheric pressure chemical ionization
APPI	Atmospheric pressure photoionization
BEH	Ethylene Bridge Hybrid
CAH	Congenital Adrenal Hyperplasia
CDC	Centers for Disease Control and Prevention
CE	Collision energy
CI	Chemical ionization
CID	Collision induced dissociation
CLS	Calgary Laboratory Services
CLSI	Clinical and Laboratory Standards Institute
CPS	Count per second
CRH	Corticotropin-releasing hormone
CV	Cone voltage
DBS	Dried blood spot

DC	Direct current
DHEA	Dehydroepiandrosterone
ESI	Electrospray ionization
FDA	Food and Drug Administration
GC	Gas chromatography
GC-MS	Gas chromatography mass spectrometry
Hct	Hematocrit
HPA	Hypothalamic-pituitary-adrenal
HPLC	High performance liquid chromatography
HQC	High quality control
IEM	Inborn errors of metabolism
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LLOQ	Lower limit of quantification
LOD	Limit of detection
LQC	Low quality control
m/z	Mass over charge ratio
min	Minute
mL	Milliliter
mm	Millimeter
mM	Millimolar
MRM	Multiple reaction monitoring
MS	Mass spectrometry
ms	Millisecond

MTBE	Methyl tert-butyl ether
MQC	Middle quality control
N ₂	Nitrogen gas
ng	Nanogram
NSQAP	Newborn Screening Quality Assurance Program
PKU	Phenylketonuria
PT	Proficiency testing
QC	Quality control
QqQ	Triple quadrupole
RF	Radio frequency
RP	Reverse phase
S/N	Signal to noise ratio
TDM	Therapeutic drug monitoring
TQS	Triple quadrupole
μl	Microliter
UHPLC	Ultra high pressure liquid chromatography
UPLC	Ultra pressure liquid chromatography

Epigraph

“Be less curious about people and more curious about ideas”

Marie Curie

1. INTRODUCTION

1.1 Congenital Adrenal Hyperplasia (CAH):

Congenital adrenal hyperplasia (CAH) is a group of autosomal recessive disorders, that are caused by enzymatic defects of the steroid biosynthesis pathway in the adrenal glands (**Figure 1.1**) [1]. This predominantly results in an accumulation of 17α -hydroxyprogesterone (17-OHP) due to the adrenal cortex's inability to produce cortisol [2]. Decreased production of cortisol prevents negative feedback inhibition leading to increased production of corticotropin-releasing hormone (CRH) and Adrenocorticotrophic hormone (ACTH) by the hypothalamus and the pituitary gland, respectively (**Figure 1.2**) [3].

Adrenal steroids are derived from cholesterol by action of several important enzymes that are shown in **Figure 1.1** [4]. Cholesterol's conversion to pregnenolone is catalyzed by the side-chain cleavage enzyme (P450_{scc}), which is considered as the first step in the adrenal steroidogenesis [4, 5]. Subsequently, pregnenolone is converted into progesterone via the activity of 3β -hydroxysteroid dehydrogenase (3β -HSD) enzyme. Then, progesterone is hydroxylated by 21-hydroxylase (21-OH) enzyme to 11-deoxycorticosterone which is later converted into aldosterone by 11β -hydroxylase (11β -OH) enzyme. Conversion of pregnenolone into cortisol is mainly mediated by two major steps: first, the production of 17-hydroxypregserton (17-OHP) via 3β -HSD enzyme, second, the production of cortisol through the action of 11β -OH enzyme on the intermediate substrate 11-deoxycortisol [4, 5].

CAH is caused by 21-hydroxylase deficiency (21-OHD) (~90%), due to mutations in the CYP21A2 gene [6]. In CAH, serum cortisol is reduced and 17-OHP and androstenedione are elevated possibly due to hyperplasia of adrenal cortex [6]. The clinical manifestation of CAH is variable due to the spectrum of enzymatic activities resulting from different types of mutations [7].

A cardinal feature of classic or severe virilization CAH in newborn females is genital ambiguity [7]. If the disorder is not recognized and treated, both girls and boys undergo rapid postnatal growth and sexual precocity or, in the case of severe enzyme deficiency, neonatal salt loss can result in death [8]. About 75% of classic CAH cases suffer from aldosterone deficiency with salt wasting, failure to thrive, and potentially fatal hypovolemia and shock [8]. Overall, CAH reported incidence ranges from 1 in 15,000 to 1 in 16,000 births in North America [9].

Diagnosis of CAH relies on both a physical exam as well as laboratory testing including: blood analysis, urine testing, and genetic profiling [10]. National awareness of CAH diseases started in the 1980s when the first newborn screening program was established in Sweden [9]. In this screening program, 17-OHP levels detected by immunoassays were used as exclusive markers for CAH diagnosis [11]. However, multiple studies suggested that screening of biomolecules by immunoassay suffers from several drawbacks such as low specificity due to cross reactivity of antibodies with other molecules with similar structure, and the high cost of the immunoassay [9, 12]. For example, cross reactivity in immunoassays for 17-OHP has produced false positive results in highly stressed, premature and sick infants [10, 13, 14]. Using a more specific system such as liquid chromatography tandem mass spectrometry (LC-MS/MS) has improved laboratory diagnosis of CAH significantly [2, 12, 15].

Traditionally, 17-OHP has been the test of choice for diagnosis of CAH. Indeed 90% of CAH cases can be diagnosed by measuring serum 17-OHP [14]. However, including more steroids in the battery of tests for CAH diagnosis can improve CAH diagnosis. Specifically, the measurement of six additional steroids (cortisol, 11-deoxycortisol, 21-deoxycortisol, dehydroepiandrosterone, androstenedione and pregnenolone) significantly improves CAH diagnosis by detecting impairment in three additional enzymes (11 β -hydroxylase, 17 α -

hydroxylase and 3 β -hydroxysteroid dehydrogenase) [14, 16, 17]. Failure of timely and accurate diagnosis can lead to multiple clinical complications (adrenal crisis) and mortality [8]. Appropriate screening and accurate diagnosis can improve patient care by early intervention, (e.g. hormone replacement therapy) [12, 18].

Fundamentally, glucocorticoids and mineralocorticoids are the core treatments of CAH diseases. Basically, glucocorticoids substitute the deficient cortisol and restore the negative feedback mechanism on the hypothalamus and pituitary. Thus, glucocorticoids suppress adrenal androgen overproduction which leads to an attenuation of CRH and ACTH production [4, 19]. CAH treatment can be monitored by measuring levels of 17-OHP and androstenedione [2, 20].

Immunoassays have been used to measure different steroids for laboratory diagnosis of CAH. However; as briefly mentioned above, immunoassays suffer from low specificity and high cost. Thus, there has been a major struggle to come up with an accurate and specific method that can provide better results with lower cost. LC-MS/MS is one of the better choices for laboratory diagnosis of CAH and is described in the following section.

Figure 1.1 Adrenal steroidogenesis pathway

A simplified schematic representation of the adrenal steroidogenesis pathway demonstrating the action of the major enzymes involved in this signaling and impact of their deficiency on steroids levels associated with CAH. 21-hydroxylase (21-OH, blue), 11 β -hydroxylase (11 β -OH, red), 17-hydroxyprogesteron (17 α -OHP, green) and 3 β -hydroxysteroid dehydrogenase (3 β -HSD, pink).

Figure 1.2 Hypothalamic-pituitary-adrenal (HPA) axis: Negative feedback

A schematic overview of the hypothalamic-pituitary-adrenal (HPA) axis. Stress activates the HPA-axis and thereby enhances the secretion of cortisol from the adrenal cortex. Cortisol negatively affect the release of further stimulating secretions of both the hypothalamus and the pituitary gland, reducing the production of cortisol once an adequate amount has been released.

1.2 Liquid chromatography-tandem mass spectrometry (LC-MS/MS):

1.2.1 High Performance Liquid Chromatography (HPLC)

High performance liquid chromatography (HPLC) is a widespread analytical tool used for separation, identification, and quantification of molecules of interest in a complex matrix [21, 22]. It relies on pumps to pass a pressurized liquid solvent (mobile phase) containing the sample mixture through a column packed with a solid adsorbent material (stationary phase) [22, 23]. Each molecule in the mixture interacts slightly differently with the stationary phase, leading to the separation of molecules based on their structures as they elute from the column [22, 24] (**Figure 1.3**).

HPLC can be classified based on the relative polarity of the mobile phase and the stationary phase as normal phase and reverse phase HPLC. Reverse phase HPLC (RP-HPLC) is the most commonly used form of HPLC [24]. It has a non-polar stationary phase or less polar than the mobile phase [22, 23]. One common stationary phase is silica which has been surface-modified with long straight chain alkyl group such as $C_{18}H_{37}$ (C18 column). With such a stationary phase, the retention time is longer for compounds which are less polar, while hydrophilic or polar compounds are eluted first [22, 23] (**Figure 1.3**).

Recently, ultra high performance liquid chromatography (UHPLC) has been introduced that can enhance the movement of the compounds through the column, significantly [25]. UHPLC uses smaller particle sizes (2 μm in diameter or less) and shorter columns than HPLC with a higher pressure limit (15,000 psi) compared to HPLC (5000 psi) [26]. The advantage of using UHPLC is the ultrafast, high throughput and high resolution analysis [25].

Figure 1.3 HPLC system components

A flow diagram representation of a HPLC system and its major compartments and compound's elution in reverse phase-HPLC (RP-HPLC). A) Mobile phase (polar in RP-HPLC), B) Pump, C) Injector port (autosampler), D) Column (non-polar) and column oven (to maintain column temperature), E) Detector and F) Data acquisition to display separated peaks (non-polar compound will be eluted first in the RP-HPLC).

1.2.2 Mass spectrometry (MS)

Mass spectrometry (MS) is a detection system that can identify and quantify different molecules after their conversion into ions in the gas phase according to their mass-to-charge ratio (m/z) values [27]. Typically there are three major components of a mass spectrometer: 1) an ionization source, 2) a mass analyzer, and 3) a detector to amplify ions and to measure their relative signal abundance (**Figure 1.4**) [27]. A computer displays the data output as a mass spectrum where the x axis corresponds to m/z and the y axis corresponds to the relative ion abundance [27, 28]. Generally MS systems operate under a high vacuum to avoid the loss of charged ions until they reach the detector [29].

Figure 1.4 Mass spectrometry components

A simplified schematic representation of the basic components of a mass spectrometer.

The first MS was built by Nobel laureate Sir J.J. Thomson, who used it in a cathode tube [30]. MS was most used by physicists and scientists in the oil and gas field. Eventually, it found its way to chemistry and biology laboratories and was introduced into clinical laboratories in 1987 when president Regan issued a zero tolerance for drugs in American armed forces [31]. Thus, clinical laboratories employed mass spectrometry to confirm the presence of drugs of abuse in patient specimens [31, 32].

However, MS was suitable for low molecular weight compounds only for few decades because of the ionization techniques available at that time [33]. In 1980s, MS was significantly developed when Nobel laureate John Fenn introduced a new soft ionization technique for the large biomolecules called Electrospray ionization (ESI) (**Figure 1.5**) [34].

Gas chromatography (GC) was the first chromatographic technique to be coupled to MS detection and has been used as far back as the 1960's for metabolomics applications [35]. GC-MS is only applicable to volatile and thermostable analytes and requires relatively complicated sample purification and derivatization for non-volatile compounds. GC-MS was used in toxicology laboratories for drug and toxin analysis until mid-1990s [29].

In liquid chromatography mass spectrometry (LC-MS), a lower temperature is needed in comparison to GC-MS and analytes don't need to be volatile or derivatized, thus less sample preparation is necessary [36]. Also, LC-MS benefits from high dynamic range and excellent sensitivity [37].

1.2.2.1 Ionization methods

The method of ionization in MS should be chosen according to the nature of the analyte(s) (molecular weight and/or structure) [37]. Electron ionization (EI) involves chemical modifications (rearrangement of the analyte structure) and ion production that are shown within a mass spectrum

of molecule of interest [37]. Molecular weight information is required in chemical ionization (CI) settings where a number of molecular fragments are generated [37].

The above approaches have been used gas chromatography coupled with mass spectrometry, in order to couple LC to MS a different source of ionization than CI and EI was needed. Thus, researchers introduced an atmospheric pressure-based ionization methods (API), such as electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) systems, and atmospheric pressure photoionization (APPI) [38].

These ionization methods have different applications. ESI is the most commonly used ionization technique [38], and has been emerged as a highly sensitive and specific ionization strategy for small and large molecular weight polar compounds [39]. However, APCI and APPI offers an alternative and highly efficient approach to ionize non-polar compounds [40] (**Figure 1.6**).

Figure 1.5 History of mass spectrometry

This timeline represents accomplishments in mass spectrometry (Noble laureate).

Figure 1.6 Ionization sources application range

Representation of range of ionization by ESI, APCI, and APPI sources as a function of compound polarity and molecular weight.

1.2.2.1.1 Electrospray ionization (ESI)

ESI (liquid phase ionization) is a so-called 'soft ionization' technique [41], used for the production of gas phase ions with few fragments [37]. The general ESI process involves four stages; formation of charged droplet from the LC mobile phase, evaporation of droplets, repulsion of highly charged droplets and formation of ions [28]. Electrospray are produced by passing the mobile phase stream through a metal capillary maintained at high voltage (typically 3–4 kV) which is sufficient to nebulize the liquid into highly charged droplets on exiting the needle [37]. Subsequently, these charged droplets are continuously condensed following evaporation of the solvent by the high source temperature and applying a stream of a drying gas (nitrogen). As the solvent evaporates and the droplet radius shrinks, the electrostatic repulsion of the same charges becomes stronger than the surface tension holding the droplet together. Thus, a series of coulomb explosions occur until gas phase ions are generated [42, 43] (**Figure 1.7**).

Figure 1.7 Schematic representation of the electrospray ionization (ESI) process

This figure represents a detailed description of the main stages and mechanism of action of ESI source.

1.2.2.1.2 Atmospheric pressure chemical ionization (APCI)

APCI (gas phase ionization) is similar in its ionization technique to chemical ionization (CI) method and is a more aggressive ionization than ESI, so more fragments are produced [27]. APCI is mainly used for polar and relatively less polar thermally stable compounds with molecular weight of less than 1000-2000 Da [44]. In APCI, LC mobile phase mixture with the sample is vaporized into the gas phase by applying a nebulizing gas (N_2) and a very high temperature (300–500 °C). Consequently, the ionization is initiated by a complex series of reactions by means of a corona discharge needle (up to 5 kV is applied to the needle to produce a current of 2–5 μA) [38] (Figure 1.8).

Figure 1.8 Schematic representation of the atmospheric pressure chemical ionization process (APCI)

This figure represents a detailed description of the main stages and mechanism of action of APCI source.

1.2.2.2 Triple quadrupole (QqQ, TQ) mass analyzer

The quadrupole (Q) consists of four electrodes arranged parallel to each other [45] (**Figure 1.9**). Two opposite quadrupole rods have a positive and the other two a negative polarity. The polarities of the two rod pairs are exchanged with alternating radio frequency (RF) and fixed direct current (DC) voltages [45]. Thus, making ions circulate in a spiral motion between these four rods. The separation of the masses is based on the attraction and repulsion forces as well as the ion size making ions of a particular m/z to pass through to the detector [46].

Triple quadrupole (TQ) consists of three sets of quadrupole rods in series [46]. The first (Q1) and third (Q3) quadrupoles are radio frequency (RF) and direct current (DC) mass filtering quadrupoles, whereas the second quadrupole (q2) is only a radio frequency (RF), so it is used as a collision cell not a mass scanning quadrupole. The ions of interest selected in the Q1 (called precursor ions) avoid exclusion and completely pass through Q1 to q2. The q2 is called a collision cell where the precursor ions will be fragmented to product ions by applying an inert gas (N₂ or Argon) via a process called collision induced dissociation (CID) [33]. Q3 acts as a mass analyzer to scan and to select the product ions (**Figure 1.10**). Triple quadrupole is the most common hardware arrangement in tandem mass spectrometry (MS/MS) [37]. Triple quadrupole can perform multiplex analyses (quantitative and qualitative) simultaneously. This system has a relatively low cost. Also, it has a small size, robust and is easy to operate and maintain [46].

1.2.2.3 Multiple reaction monitoring (MRM)

The triple quadrupole arrangement can be switched from the product ion configuration to a multiple reaction monitoring (MRM) [47] (**Figure 1.11**). In this setting, the identity of a certain compound can be assessed by quantifying multiple product ions from a single precursor ion [33]. Normally, quantification and identification of any analyte is achieved via detecting two mass

transitions generated from a single precursor ion; one is a quantifier ion and the other one is a qualifier ion [48]. The consistent production of fragment ions from ionized analytes facilitates correlation of molecular identity to quantitative analysis of analytes of interest, especially isobaric compounds that have different structures but identical m/z [46].

Figure 1.9 A schematic representation of the quadrupole mass analyzer structure and mechanism of action

The analyzer consists of four rods or electrodes arranged across from each other. These electrodes are connected in pairs. The application of radio frequency (RF) causes ion transmission according to their m/z value by alternately focusing them in different planes via switching the charges on the rods.

Figure 1.10 Schematic representation of the triple quadrupole mass analyzer (QqQ).

This figure represents a detailed description of the quadrupoles settings and function in the QqQ.

Figure 1.11 Schematic representation of multiple reaction monitoring (MRM)

A schematic of a triple quadrupole mass spectrometer commonly used in multiple reaction monitoring mass spectrometry (MRM-MS) analysis; Q represents a quadrupole in a triple quadrupole mass spectrometer. In this system, multiple analytes (colored in red, navy, blue, yellow and green) are eluted at different retention times which allows this model to detect multiple analytes in a single run, simultaneously [47].

1.3 Dried blood spot (DBS):

Dried blood spot (DBS) sampling simply involves collection of blood samples on a filter paper [49]. This filter paper is made of pure cotton linters (Cellulose) (**Figure 1.12**) and it is a very precise and reproducible collection device which is manufactured according to the specific criteria set forth by the Food and Drug Administration (FDA) [49] (**Figure 1.13**). Initially, this approach was utilized by Dr. Robert Guthrie to screen newborns affected by phenylketonuria (PKU) in early 1960s [50]. Since then, DBS has become very attractive as the specimen of choice in many newborn screening programs. DBS has been extensively and successfully used in many newborn screening programs, therapeutic drug monitoring programs, molecular biology and toxicology testing [51, 52]. Clinical and Laboratory Standards Institute (CLSI) has established detailed clear instructions on collections, transportation, and storage of DBS [53, 54]. Compared to liquid specimens (e.g. blood), collecting for DBS offers the following advantages: (i) does not require a phlebotomist; (ii) relatively less invasive; (iii) generally considered as a nonhazardous sample; (iv) convenient handling, shipping and storage; and (v) low sample volume (a few drops of blood) on pre-printed circles on a filter paper [50, 54]. Like collecting liquid specimens, collection of DBS can be impacted by variables such as; humidity, hematocrit and the degree of homogeneity in the collection papers [55, 56]. To address these variables, the Centers for Disease Control and Prevention (CDC) along with the Newborn Screening Quality Assurance Program (NSQAP) routinely evaluates newly synthesized papers from all manufactures and compares their performance to previous ones [57]. In addition, this program provides an external quality assurance to the laboratories using DBS in their screening programs by providing them with both known quality control (QC) materials and blind-coded proficiency-testing (PT) panels on a periodic basis. Furthermore, CDC and NSQAP offer technical counseling to the laboratories, if needed [28, 53,

58]. Beside laboratories, kit manufacturers can join this program to standardize and to harmonize their results [9].

Figure 1.12 An illustration of cotton linters

This figure shows the cotton linters where filter papers made from. Figure obtained from Dried blood spots: applications and techniques [49].

Figure 1.13 Whatman 903® filter card

Whatman GE Healthcare 903 Protein Saver Cards. Each card contains five half-inches of pre-printed circles and each circle holds around 75 μ l of blood to be fully covered.

1.4 Hypothesis:

We hypothesize that measuring seven steroids by a highly sensitive and specific methods such as LC-MS/MS can improve diagnosis of CAH. We further hypothesize that using DBS will improve overall patient care by collecting less blood, transporting and processing specimen faster for shorter turnaround time (TAT).

1.5 Objectives:

- (i) Develop and validate an extraction method for seven individual steroids: 17-hydroxyprogesterone, pregnenolone, cortisol, 11-deoxycortisol, 21-deoxycortisol, dehydroepiandrosterone, and androstenedione, from DBS.
- (ii) Develop a sensitive and specific LC-MS/MS method that simultaneously measures seven individual steroids extracted: 17-hydroxyprogesterone, pregnenolone, cortisol, 11-deoxycortisol, 21-deoxycortisol, dehydroepiandrosterone, and androstenedione, from DBS.
- (iii) Validate the completed method based on CLSI guidelines for use in clinical laboratories.

1.6 Clinical Significance:

Using a seven steroid panel can improve the overall laboratory diagnosis of CAH. Measuring the steroids by LC-MS/MS can improve specificity of the test and will reduce false positive results that occur when using immunoassays. In immunoassays, antibodies can cross-react with molecules with similar structures and generates erroneous results. Current assessment of 17-OHP by immunoassays for laboratory diagnosis of CAH can be significantly improved by measuring other steroids. Using a highly specific method such as LC-MS/MS can reduce false positive results seen in immunoassay system. Also, measuring seven (instead of one) steroids can

improve CAH diagnosis. Immunoassays are bioanalytical methods in which the quantitation of the analyte depends on the reaction of an antigen with an antibody. Biochemical phenotype of CAH is characterized by steroidal imbalance. In some CAH cases, elevation of 17-OHP is associated with increased levels of androstenedione, 21-deoxycortisol and decreased cortisol levels. Measuring more steroids such as 21-deoxycortisol and 11-deoxycortisol, will be important to identify the specific disease markers. Thus, measuring more steroids by a very specific and precise technique like LC-MS/MS will greatly improve screening for CAH. Also, highly sensitive LC-MS/MS system facilitates measuring steroids in very low specimens such as those in DBS. DBS will be an ideal specimen for neonates, a major patient population for CAH testing, considering their limited blood volume and difficulties in blood sample collection.

2. MATERIALS AND METHODS

2.1 Materials:

Steroids (17-hydroxyprogesterone, 21-deoxycortisol, 11-deoxycortisol, cortisol, dehydroepiandrosterone, pregnenolone and Androstenedione) and their deuterated internal standards (17-hydroxyprogesterone-d8, 21-deoxycortisol-d8, cortisol-d4, 11-deoxycortisol-d5, DHEA-d5 and androstenedione-¹³C₃) were procured as certified reference standards from Cerilliant (Round Rock, TX United States of America). High purity solvents recommended for HPLC–MS/MS methods (acetonitrile and methanol) were purchased from EMD (Gibbstown, NJ, USA). Steroid free-serum DC Mass Spec Gold purchased from Golden West Biologicals Inc. (VWR, Mississauga, ON Canada). Buffer composition required the use of MS-grade formic acid purchased from Fisher Scientific (Ottawa, ON, Canada) and MS-grade ammonium formate purchased from Sigma Aldrich (St Louis, MO United States of America). Whatman 903[®] protein saver snap-apart cards utilized for DBS sampling were purchased from GE Healthcare Life Sciences (Mississauga, ON Canada).

2.2 Instrumentation:

The ultra-performance liquid chromatography (UPLC) system was from Waters (ACQUITY UPLC I-Class system) coupled to Xevo[™] TQ-S micro tandem mass spectrometry with positive electrospray ionization (+ESI) source. The UPLC chromatographic separation utilized ACQUITY UPLC[™] BEH C18 1.7 µm, 2.1 × 50 mm column from Waters (Mississauga, ON Canada). ACQUITY UPLC[™] in-line filter unit with 0.2 µm stainless steel filter from Waters (Mississauga, ON Canada).

2.3 Samples:

A total 82 of de-identified unknown whole blood leftover samples in heparinized PICO syringes were provided by chemistry laboratory at Alberta children's hospital, Calgary, AB, CA.

2.4 External quality control (QC) and proficiency testing (PT) materials:

External quality control (QC) DBS and proficiency testing (PT) specimens were obtained from the Newborn Screening Quality Assurance Program (NSQAP) and Center for Disease Control and Prevention (CDC) (Atlanta, GA). Five blind-coded DBS PT panels were enriched with the major steroids involved in the diagnosis of CAH; 17-OHP, cortisol, androstenedione, 11-deoxycortisol and 21-deoxycortisol. Each enriched panel is characterized by different concentrations and variable steroid ratios. The proficiency testing panel produced and offered by CDC were classified as: “un-enriched, true-negative, challenge, false positive” and “true-positive” cases [58, 59]. Clinical ratio was calculated using the ratio of the sum of 17-OHP and 21-deoxycortisol (or androstenedione), divided by the cortisol level. Ratio equal or greater than 1 is an indication for abnormal result (CAH) [60, 61].

- (i) “Un-enriched” is the base washed and adjusted hematocrit level blood pool used as an endogenous-level pool for the steroids
- (ii) “True positive” is defined as a whole blood specimen that contains a high level of 17-OHP and a ratio value below one.
- (iii) “True negative” is defined as a whole blood specimen that contains a low level of 17-OHP and a ratio value above one.
- (iv) “Challenge” is defined as a whole blood specimen that contains medium-high level of 17-OHP and a ratio value below one.

- (v) “False positive” is defined as a whole blood specimen that contains a high level of 17-OHP and a ratio value below one, as often seen in cases with CAH.

2.5 Methods:

2.5.1 Standards solutions preparation

Steroid standards and their internal standards were dissolved in methanol to obtain stock solutions with concentration of 1 mg/ml. Steroid working solutions were prepared by diluting the stock solution with saline. Internal standards working solutions (500 ng/ml for all except for DHEA-*d*5 that was 5000 ng/ml) were prepared by diluting the stock solution with methanol. All stock solutions were stored at -20 °C and brought to room temperature (RT) before use.

2.5.2 Preparation of calibration standards and quality control (QC) samples

Calibrators and quality controls (QC) were prepared by using saline-washed red blood cells [59]. Briefly, leftover blood samples (from heparinized PICO syringes; arterial blood samplers) pooled, mixed and centrifuged at $1,500 \times g$ for 15 minutes at room temperature. Supernatant and buffy coat were discarded. The remained erythrocytes were washed ($1,500 \times g$ for 15 minutes) three times with normal saline (0.9 N NaCl) at room temperature. The washed erythrocytes were mixed with steroid-free serum to achieve a $55 \pm 1\%$ hematocrit. Steroids working solutions were added to the prepared steroid-free blood to prepare quality control samples (ranging from 2.5 to 250 ng/ml in whole blood) and the calibration curve. Four levels of quality control samples were prepared as follows: LLOQ (2.5 ng/ml), LQC (5 ng/ml), MQC (30 ng/ml), and HQC (100 ng/ml).

To prepare blood spots 70 μ l whole blood aliquots were spotted onto the center of the pre-printed circles on Whatman 903[®] filter papers. Spotted samples were dried for at least 3 hours at room temperature and stored in airtight bags with desiccant to minimize humidity [53, 60].

2.5.3 Sample extraction and preparation

Extraction of steroids was carried out using one 6.4 mm diameter DBS punched out from the center of the spot and placed in a 2 ml microcentrifuge tube. 200 μ l of internal standard mixture (0.5 ng/ml prepared in 95% methanol) was added to the sample. The sample was vortexed thoroughly for 5 min at room temperature. An aliquot was transferred into another clean 2 ml microcentrifuge tube, and the DBS punch was discarded. Extracted sample was dried under a gentle flow of nitrogen for approximately 20 minutes at room temperature. The residue (dried sample) was reconstituted into 50% methanol in water containing 5.0 mM ammonium formate with 0.01% formic acid (100 μ l) and centrifuged at $6440 \times g$ for 10 minutes at 8°C. 90 μ l aliquot of this mixture was transferred into conical glass insert assay vial and placed into the LC-MS/MS autosampler for analysis (**Figure 2.1**).

Figure 2.1 Schematic workflow of the experimental design

This multi-step workflow illustration of experimental design. Blood samples were collected and spotted on the pre-printed circles filter paper and dried. A 6-mm diameter circle was punched and placed in a micro centrifuge tube to extract and measure the steroids as described in materials and methods section.

2.5.4 LC-MS/MS analysis

Sample aliquots of 25 μ l were injected into the column and eluted using a gradient flow of 250 μ l/min with 7.3 min total run time. The mobile phase consisted of two solvents: Solvent A (HPLC-grade water containing 5.0 mM ammonium formate), and solvent B (methanol containing 0.01% formic acid) which was pumped by a binary pump. The LC gradient program initiated from 45% solvent A and 55% solvent B. The gradient increased up to 45% solvent B within 5.75 min with a rate of 250 μ l/min, then ramped to 95% solvent B within 0.25 min with a flow rate of 500 μ l/min and held for 0.5 min for washing. Returning via a steep gradient to the initial LC state after 0.5 min to re-equilibrate the system (**Table 2.1**). The autosampler temperature was adjusted to 8 °C to avoid any sample evaporation. The column was maintained at 50 °C during the run and subsequently washed with water followed by 90% acetonitrile after every use.

Multiple Reaction Monitoring (MRM) was used to detect the following seven steroids and their internal standards; cortisol, 11-deoxycortisol, 21-deoxycortisol, dehydroepiandrosterone, androstenedione and pregnenolone. MRM was used to monitor two transitions for each analyte of interest and its internal standard. The ratio of the quantifier ion to qualifier ion was monitored in order to confirm their identification. Tuning solution was prepared using mobile phase in a concentration of 1 μ g/ml. Specific MS/MS product-ion scans and parameters were acquired in continuous flow mode by combined infusion with LC mobile phase pump utilizing MassLynx[®] 4.1 software. Instrument tune page (MS Tune) was used for manual tuning and Intelstart was used for auto-tuning (**Table 2.2**). Waters Xevo[™] TQ S micro tandem mass spectrometry was used in this study under the following optimized conditions; the capillary voltage was 1.00 kV, desolvation temperature was 600 °C, desolvation gas flow was 900 L/hr, and source temperature

was 150 °C. Sample analysis was performed in the MRM mode using a dwell time of 19 ms per transition to detect the product ions.

Each steroid was quantified using its deuterated internal standard. For pregnenolone and androstenedione, isotopically labelled androstenedione- $^{13}\text{C}_3$ was used as an internal standard. Steroids concentrations were quantified by linear regression using TargetLynx[®] software.

Table 2.1 Mobile phase gradient program

Time (min)	Flow rate (ml/min)	%A	%B	Curve
initial	0.250	45.0	55.0	initial
0.50	0.250	45.0	55.0	6
3.00	0.250	20.0	80.0	6
5.00	0.500	10.0	90.0	6
5.75	0.250	5.0	95.0	11
6.25	0.250	45.0	55.0	11
6.6	0.250	45.0	55.0	6
7.3	0.250	45.0	55.0	6

Figure 2.2 Liquid chromatography (LC) condition for the analysis of steroids

LC separation utilized a gradient of (A) water containing 5 mM ammonium formate and (B) methanol containing 0.01% formic acid.

Table 2.2 MS/MS Operating conditions

Steroid	Precursor ion	Quantifier transition			Qualifier transition			Ion ratio
		Product ion	CV	CE	Product ion	CV	CE	
Cortisol	363.26	120.99	44	24	91.05	44	56	2.4
Cortisol- <i>d4</i>	37.26	121.05	46	22	96.97	46	34	3.2
21-deoxycortisol	347.26	120.99	44	26	311.17	44	14	1.2
21-deoxycortisol- <i>d8</i>	355.30	319.21	46	14	125.15	46	24	1.5
11-deoxycortisol	347.26	96.98	48	24	108.95	48	26	1.1
11-deoxycortisol- <i>d5</i>	352.26	99.97	52	26	112.99	52	30	1.4
Androstenedione	287.26	96.98	44	22	108.95	44	24	1.5
Androstenedione- ¹³ C ₃	290.26	99.97	46	22	111.95	46	26	1.6
17 OHP	331.23	96.98	48	24	108.96	48	28	1.2
17 OHP- <i>d8</i>	339.32	99.97	40	28	112.99	40	30	1.4
DHEA	271.20	213.12	52	12	91.02	52	42	1.1
DHEA- <i>d5</i>	276.30	218.09	52	10	96.66	52	22	2.2
Pregnenolone	299.26	91.02	52	48	104.95	52	38	1.2

2.6 Method validation:

Method validation was performed using the Clinical and Laboratory Standards Institute (CLSI) guidelines [62, 63]. Parameters that were assessed included: precision, sensitivity, linearity and sample carryover. Linearity was assessed by measuring seven calibrators (2.5-250 ng/ml) in addition to the blank sample (0 ng/ml) for seven consecutive days in duplicates. Linearity was determined by plotting the ratio of the sum of the peak areas that corresponded to each steroid and the peak area of their deuterated internal standards. A linear least square analysis was conducted with 1/x as weighting factor, and the slope, intercept and coefficient of determination (r^2) were determined to assess linearity of the method. Linearity is expressed mathematically as:

$$y = mx + b$$

Where y is the analyte response, x the concentration of the analyte, m the slope of the calibration curve (sensitivity), and b the intercept of the calibration curve.

The sensitivity of analytical measurement range was evaluated to define the limit of detection (LOD). This was defined as the highest possible value from a blank (blood free-steroids). Chromatographically, LOD is the detected peak with a height at least three times higher than the baseline (signal to noise ratio). It was verified that the Lower limit of quantification (LLOQ) was above the LOD by comparing the measurement of the LLOQ to blank samples with defined accuracy and precision.

Sample carryover was assessed by running a few negative blanks followed by a high steroid concentration specimen followed by more negative blanks and the measured responses were compared. Any carry over greater than 20% of the LLOQ response was considered significant.

Precision was assessed by running identical QC samples three times a day for seven days and was described as intra-day and inter-day. Specifically, twenty replicates of four different QC level samples were tested in seven consecutive days. Precision was defined by calculating coefficient of variation ($CV = SD/mean$) for each run. In addition, Intra-day precision was evaluated using five levels (0, 10, 50, 100, and 500 ng/ml serum) of quality control materials obtained from CDC.

Recovery was assessed by comparing the measurement of each QC sample after samples had undergone all the preparation steps. Internal standard was added at two different steps in the sample preparation; first, internal standard was added with the extraction solvent, second, internal standard mixture was added after the sample extraction was completed. Analyte loss was monitored by comparing the area under the curve (AUC) for both the samples that underwent sample preparation and those that were spiked with internal standards mixture after sample preparation.

Autosampler stability was evaluated by comparing the concentration of each QC sample after samples had remained within the autosampler for 24 hours. These samples were analyzed at 0 hours for inter-day precision and after 24 hours for within the autosampler.

Correlation assessment evaluates the performance of the new method versus a “gold standard” or a similar method. The proficiency testing (PT) specimens obtained from the Newborn Screening Quality Assurance Program (NSQAP) were used to assess correlation. We performed the correlation study via linear regression analysis of the mean of duplicates of the comparative (CDC/APCI) method. Detailed description of these samples was described in section 2.3.

2.7 Statistics

Data are presented as the mean $\pm$ S.E.M., with “n” indicating the number of independent experiments. Data were analyzed by Student’s t-test, and $p < 0.05$ was considered to indicate statistical significance. In addition, *Mann–Whitney* test, and *Bland–Altman* plots were used for comparison and bias assessment, respectively. All statistical analyses were performed using the GraphPad Prism 6.0 program and Microsoft Excel 2016.

3. RESULTS

3.1 Sample extraction

3.1.1 Selection of optimal extraction solvent

To determine the most efficient extraction solvent, quality control (QC) samples were prepared (100 ng/ml) for five steroids (Androstenedione, 17-OHP, 11-deoxycortisol, 21-deoxycortisol and Cortisol) and extracted using various prototypical extraction solvent mixtures (v/v %); methanol and acetonitrile (50:50), acetonitrile and water (80:20), methanol and water (80:20), 100% acetonitrile (ACN), and 100% methyl tert-butyl ether (MTBE) (**Figure 3.1**). Extraction solvent was further examined using other extraction solvent combinations (v/v %); acetone and acetonitrile (50:50), methanol and water (90:10), acetonitrile and water (90:10), and methanol and acetonitrile (50:50) (**Figure 3.2**). AUC data showed a notable signal (peak) corresponding to samples extracted with methanol and water (**Figure 3.1** and (**Figure 3.2**). These data suggest potential utility of methanol as an extraction solvent in our experimental conditions. To further analyze the most appropriate methanol to water ratio for sample extraction, several ratios (v/v %) of water and methanol (50:50, 80:20, 85:15, 90:10, 95:5) were examined. Results showed that 90% and 95% methanol to water ratios showed the maximum extraction efficiency (**Figure 3.3**). However, 95% methanol showed a slightly better signal to noise ratio (S/N).

Figure 3.1 Extraction solvent optimization (experiment# 1)

Spiked dried blood spot (DBS) samples were prepared in a concentration of (100 ng/ml) for each steroid, and extracted using four different extraction solvents; acetonitrile and water (80:20 by volume, red), methanol and water (80:20 by volume, green), acetonitrile (100% by volume, blue), and methyl tert-butyl ether (MTBE) (100% by volume, orange). Response of each extraction condition was expressed as count per second (CPS) on y-axis with respect to time (min) on x-axis.

Figure 3.2 Extraction solvent optimization (experiment# 2)

Spiked dried blood spot (DBS) samples were prepared in a concentration of (100 ng/ml) for each steroid, and extracted using another four different extraction solvents; acetone and acetonitrile (50:50 by volume, black), methanol and water (90:10 by volume, red), acetonitrile and water (90:10 by volume, green), and methanol and acetonitrile (50:50 by volume, blue). Response of each extraction condition was expressed as count per second (CPS) on the y-axis with respect to time (min) on x-axis.

Figure 3.3 Optimization of methanol ratio as the extraction solvent

Spiked dried blood spot (DBS) samples were prepared in a concentration of (100 ng/ml) for each steroid and extracted using five different ratios of methanol: water (v/v %); 50:50 (orange), 80:20 (blue), 85:15 (red), 90:10 (green), and 95:5 (black). Response of each extraction condition was expressed as count per second (CPS) on y-axis with respect to time (min) on x-axis.

3.1.2 Evaluation of carryover from puncher tip

Possible puncher tip carryover of steroids from one sample to another (i.e., from high sample to low sample), was evaluated by punching one spot from high quality control sample for the steroids (androstenedione, 17-OHP, 11-deoxycortisol, 21-deoxycortisol and cortisol), with a concentration of 200 ng/ml. The puncher tip was then washed with the extraction solvent and analyzed in LC-MS/MS as described in materials and methods section. These data demonstrated no observable peak (AUC) detected from the puncher tip, as compared with the peak (AUC) of lower limit of quantification (LLOQ) indicating no carryover (**Figure 3.4A** and **Figure 3.4C**). These data suggested that carryover of puncher tip was very minimal, and the peak (AUC) was very similar to double blank (zero concentration mobile phase) sample (**Figure 3.4B**).

Figure 3.4 Evaluation of cross-contamination (carryover) from puncher tip

Possible puncher tip carryover was analyzed by measuring steroids response (AUC) (light green) from the puncher tip after punching a spot from a high concentration DBS (200 ng/ml) of the steroids; cortisol, 21-deoxycortisol, 11-deoxycortisol, 17-OHP, and androstenedione (**A**), and mobile phase (double blank) (**B**), in comparison with the lower limit of quantification (LLOQ) (2.5 ng/ml) (**C**). Response of each extraction condition was expressed as count per second (CPS) on y-axis with respect to time (min) on x-axis.

3.1.3 Optimization of extraction time

Time required to achieve maximal DBS sample extraction was examined in a specific concentration (150 ng/ml) of different steroids (androstenedione, 17-OHP, 11-deoxycortisol, 21-deoxycortisol and cortisol). DBS sample was extracted using 95% methanol in water at different time points [0, 2.5, 5, 10, 15 min]. These data demonstrated that the highest response (AUC) of all steroids was achieved at 2.5 min. Samples showed no change of the signal response after this time point (**Figure 3.5**).

Figure 3.5 Effect of time of extraction on steroid's response

Different steroids; androstenedione (black), 17-OHP (red), 11-deoxycortisol (green), 21-deoxycortisol (blue) and cortisol (purple) were extracted at different time points [0-15 min]. A specific concentration (150 ng/ml) of each steroid was used to analyze response values which is given as area under the curve (AUC), as described under materials and methods. Values represent means $\pm$ S.E.M. for $n = 3$ independent experiments.

3.1.4 Evaluation of the necessity of a second extraction

DBS samples were subjected to two consecutive extractions to study the total sample recovery from the punched sample. Samples containing 2.5, 15, and 150 ng/ml of steroids were used for this study and were extracted twice, and percent recovery for each steroid was calculated (% recovery at 2.5, 15, and 150 ng/ml, respectively). Androstenedione (91%, 92%, and 93%), 17-OHP (94%, 92%, and 94%), 11-deoxycortisol (91.5%, 90%, and 92.5%). Response (AUC) analysis showed that greater than 90% of steroids were extracted during the first extraction. However, for 21-deoxycortisol recovery at 2.5 ng/ml was 89% and for 15 ng/ml and 150 ng/ml recovery was greater than 90% (**Figure 3.6**). Thus, all DBS were extracted using one extraction cycle.

Figure 3.6 Analysis of sample recovery from extraction

Response (AUC) of: androstenedione (black), 17-OHP (green), 11-deoxycortisol (blue) 21-deoxycortisol (pink), and cortisol (orange) was analyzed from spots with steroid concentration of 2.5 ng/ml (A), 15 ng/ml (B), and 150 ng/ml (C). Each sample was extracted twice, and recovery was assessed for each of both extractions: first extraction (E1) and second extraction (E2), as described under materials and methods section. Values represent means $\pm$ S.E.M. for n = 4 independent experiments.

3.1.5 Drying gas assessment

Following the extraction step, the solution (organic solvent and steroids) were dried under a stream of different gases to assess the effect of a drying gas on steroids. The concern was the possible oxidation effect of oxygen (in air) on steroids. Thus, air and nitrogen (an inert gas) were used to dry the solutions. Response (AUC) analysis revealed that no significant difference detected from samples dried using both gases (**Figure 3.7**).

Figure 3.7 Gas optimization for drying sample extracts

Sample extracts were dried using Nitrogen (N₂) (black) or Air (orange). Responses (AUC) were calculated at both conditions for androstenedione, 17-OHP, cortisol, 11-deoxycortisol, and 21-deoxycortisol, as described under materials and method section. Values represent means $\pm$ S.E.M. for n = 5 independent experiments. *ns*- not significantly different (Student's t test, p < 0.05).

3.2 DBS Quality controls preparation

Calibrators and quality controls (QC) steroids DBS samples were prepared by using saline-washed red blood cells as described under materials and methods section. 70 μ l blood of 55% hematocrit level was sufficient to fill the whole pre-printed circles on Whatman 903™ filter papers (**Figure 3.8**).

3.2.1 Hematocrit effect evaluation

The effect of hematocrit (hct) on steroids quantitation was assessed in six QC samples containing 5, 30, 80, 100, 300 and 450 ng/ml of androstenedione, 17-OHP, 11-deoxycortisol, 21-deoxycortisol, cortisol, DHEA, and pregnenolone. These QC samples were prepared at three different hematocrit levels; low (45%), medium (55%), and high (65%). The concentrations of the seven steroids were measured at the three hematocrits levels, according to the equation generated from a linear calibration curve derived from standards of 55% hematocrit. These data showed no clear trend between hematocrit levels and steroids concentration (**Figure 3.9**).

Figure 3.8 Dried blood spotting on Whatman 903® papers

A representative image for the prepared blood samples (55% hematocrit) spotted on DBS cards.

Figure 3.9 Hematocrit effect evaluation

Six QC samples (5, 30, 80, 100, 300, and 450 ng/ml) for steroids were prepared at three different hematocrit levels; low (45%, blue), medium (55%, orange), and high (65%, black). Hematocrit effect on steroids quantitation was assessed for cortisol, 21-deoxycortisol, 11-deoxycortisol, androstenedione, 17-OHP, DHEA and pregnenolone. Values represent means $\pm$ S.E.M. for $n = 3$ independent experiments.

3.3 Optimization of LC-MS/MS for separation and quantitation of steroids

3.3.1 MS/MS instrumental method

ESI probe position and angle were adjusted and tuned manually to achieve the optimal signal which corresponded to each steroid from the tuning solution (**Figure 3.10**). Precursor ions were selected for each steroid in full MS scan mode as protonated ions $[M+H]^+$ except for DHEA and pregnenolone which exhibited a water loss and are given as $[M+H-H_2O]^+$. The product ions were determined from the MS/MS spectra, the first and the second most abundant signals were selected as the quantifier and qualifier transitions, respectively (**Figure 3.11**). Precursor ion values were previously shown in **Table 2.2** and described in detail under materials and methods section.

Figure 3.10 ESI probe position for steroids analysis

This image shows ESI Probe position and alignment to maintain optimal sensitivity and stability for the analytes signal.

Figure 3.11 Product ion transitions spectrum for each steroid

m/z

Product ion scan spectra for each steroid. Target analytes were profiled using electrospray ionization source in positive mode, as described under materials and methods section. Product ions represented for cortisol, 21-deoxycortisol, 11-deoxycortisol, androstenedione, 17-OHP, DHEA and pregnenolone. Relative response was expressed as % abundance (y-axis) and m/z (x-axis).

3.3.2 Liquid chromatography method

Chromatographic baseline separation of the steroids was achieved in 7.3 min. Chromatographic separation was employed to separate 11-deoxycortisol and 21-deoxycortisol as they possess the same m/z 347.2 (isobaric compounds). 17-OHP and DHEA were eluted at the same time; 2.9 and 2.95 min, respectively (**Figure 3.12** and **Figure 3.13**). The peak resolution and the relative response of each steroid was determined at the lower limit of quantification (LLOQ) at 2.5 ng/ml (5 ng/ml for DHEA and pregnenolone) and at the upper limit of quantification (ULOQ) (250 ng/ml). This data demonstrated a valid peak resolution of each steroid (**Figure 3.14**).

Figure 3.12 Total ion chromatogram (TIC) for the seven steroids

Total ion chromatogram (TIC) obtained for simultaneous detection of cortisol, 21-deoxycortisol, 11-deoxycortisol, androstenedione, 17-OHP, DHEA, and pregnenolone, in a spiked dried blood spot QC sample. Response was expressed as % abundance (y-axis) and time (x-axis).

Figure 3.13 MRM analysis

Representative multiple reaction monitoring (MRM) functions for simultaneous detection of cortisol, 21-deoxycortisol, 11-deoxycortisol, 17-OHP, pregnenolone, androstenedione and DHEA in a spiked dried blood spot QC sample. Response was expressed as % abundance (y-axis) and time (x-axis).

Figure 3.14 Evaluation of peak resolution

Multiple reaction monitoring (MRM) for the lower limit of quantification (LLOQ) for seven steroids using concentration of 2.5 ng/ml. For DHEA and pregnenolone a 5 ng/ml was used (**A**). The MRM for the upper limit of quantification (ULOQ) is shown for each steroid using concentration of 250 ng/ml (**B**). Response was expressed as % abundance (y-axis) and time (x-axis).

3.4 Method validation based on CLSI guidelines

3.4.1 Linearity, sensitivity, recovery, and carryover

To assess linearity of the assay, seven different DBS calibrators (2.5, 5, 10, 25, 50, 150 and 250 ng/ml) were used. For DHEA and pregnenolone DBS calibrators of 5-250 ng/ml was used. Linearity was determined by plotting the ratio of the sum of the peak area that corresponded to each steroid and the peak area of their internal standards (**Figure 3.15**). A linear regression calibration curve range of 2.5 to 250 ng/ml was also conducted as described in materials and methods section and the results are shown in **Table 3.1**.

The sensitivity of analytical measurement range was evaluated by defining the limit of detection (LOD) which was done by using the highest possible value from a blank (blood free-steroids). The Lower limit of quantification (LLOQ) was defined as the concentration that an analyte can be detected with precision and accuracy. The latter was verified to be above the LOD. Results showed that DHEA and pregnenolone exhibited a higher LLOQ values of 4.6 and 6.4 ng/ml, respectively (**Table 3.1**).

Carryover was studied by running a series of blanks (specimen with no or very low concentration of steroids) and immediately followed by series of samples with high concentrations of steroids as described under materials and methods section. Results showed acceptable carryover values (< 20% the area of LLOQ) (**Table 3.1**).

Recovery experiments showed an acceptable range of recovery for the seven steroids that varied from 104.2-111.6% of the amount of the steroid added (**Table 3.1**).

Figure 3.15 Linearity study

Seven steroids at concentrations 2.5, 5, 10, 25, 50, 150, and 250 ng/ml were measured as described under materials and methods section. Values in the y-axis represent response (standard/internal standard ratio), and x-axis represent concentration (ng/ml). Values obtained from 3 independent experiments.

Table 3.1 Linearity, sensitivity, recovery and carryover parameters

Steroid	Linearity range (ng/ml)	Slope (a)	Intercept (b)	Coefficient of determination (r^2)	LOD (ng/ml)	LLOQ (ng/ml)	Carryover (%)	Recovery (mean%)
Cortisol	2.5-250	0.12	-0.11	0.997	0.38	0.49	10.3	105.0
21-deoxycortisol	2.5-250	0.15	-0.46	0.997	0.16	0.19	4.4	111.6
11-deoxycortisol	2.5-250	0.15	-0.49	0.996	0.16	0.001	7.5	104.5
Androstenedione	2.5-250	0.10	-0.29	0.996	0.11	0.18	7.4	104.8
17-OHP	2.5-250	0.10	-0.33	0.997	0.58	0.001	9.2	111.0
DHEA	5-250	0.02	0.00	0.999	1.80	4.6	16.8	108.2
Pregnenolone	5-250	0.002	0.02	0.995	1.70	6.4	0.1	104.2

3.4.2 Precision

Precision was defined by calculating coefficient variation (CV) for intra-day and inter-day as described under materials and methods section. CV is a percentage value calculated by the ratio of the standard deviation to the mean. Intra-day CV range was from 1.8 to 11.5% and the inter-day CV was from 5.3 to 13.8% (**Table 3.2**). In addition, intra-day precision was evaluated using external quality control materials obtained from CDC at four different concentrations (10, 50, 100, and 500 ng/ml serum) and the calculated CV ranges were from 1.8 to 8.0% (**Table 3.2**).

Table 3.2 Coefficient of variation (CV) for the intra-day and inter-day precision and the intra-day of the external QCs from CDC

Steroid	CV (%) intra-day (n= 10)				CV (%) Inter-day (n= 20)				CV (%) intra-day (CDC) (n= 10)			
	2.5 ng/ml	5 ng/ml	30 ng/ml	100 ng/ml	2.5 ng/ml	5 ng/ml	30 ng/ml	100 ng/ml	10 ng/ml	50 ng/ml	100 ng/ml	500 ng/ml
Cortisol	11.5	8.3	6.0	2.2	13.8	9.1	5.3	5.5	3.6	4.3	3.9	2.5
21-deoxycortisol	7.0	4.6	5.7	4.9	11.9	13	13	10.3	8.0	4.4	5.0	2.8
11-deoxycortisol	5.7	7.9	4.8	1.8	11.2	7.3	5.4	7.2	4.5	7.2	4.9	1.9
17 OHP	2.5	5.5	4.6	2.5	6.5	8.4	7.4	7.1	5.2	6.4	4.2	3.4
Androstenedione	3.1	5.0	5.2	2.5	9.0	9.0	13.0	8.6	1.8	4.2	4.2	1.9
DHEA	-	8.8	6.2	3.3	-	13.0	9.6	6.3	-	-	-	-
Pregnenolone	-	-	8.6	4.9	-	-	8.5	9.6	-	-	-	-

* (-): Values were not reported, either lower than the LLOQ or not included in the steroids QC panel.

3.4.3 Correlation study

Some of the steroids (cortisol, 21-deoxycortisol, 11-deoxycortisol, androstenedione and 17-OHP) have been consistently measured by LC-MS/MS using APCI as the gold-standard ionization method. Thus, we compared the performance of the new method which uses ESI versus a method with APCI. Due to the difficulty in finding positive patients specimens, we participated in a proficiency testing program (NSQAP) from CDC. These specimens represent different steroid levels and ratios and were used for this analysis.

3.4.3.1 A comparative analysis of ESI and APCI

This method using ESI compared quite well with the APCI method ($r^2 > 0.99$) except for 21-deoxycortisol (0.94), slope of 1 and intercept of zero as shown in **Figure 3.16** and **Table 3.3**.

A bland Altman analysis (bias) of this method represents the percentage of bias (difference between data obtained from ESI and APCI (y-axis) compared to average results between the two methods (x-axis)) (**Figure 3.17**). All steroids, except 21-deoxycortisol, showed very low bias (**Table 3.3**).

Figure 3.16 Mann-Whitney scatter plot for both LC-MS/MS methods

Scatter plot with regression fit represents method comparison between the two LC-MS/MS methods; ESI vs. APCI for cortisol, 21-deoxycortisol, 11-deoxycortisol, 17-OHP, and androstenedione. This graph also shows a line with a slope of 1.0 going through the origin, where the dotted red line represents the theoretical best fit between the two methods, and the blue line represents the real obtained correspondence. X-axis represents the APCI values and y-axis represents the test method (ESI values).

Figure 3.17 Bland Altman analysis (bias) for both LC-MS/MS methods

A bland Altman analysis (bias) represents the percentage difference between data obtained from ESI and APCI (y-axis) compared to average results between the two methods (x-axis) for cortisol, 21-deoxycortisol, 11-deoxycortisol, 17-OHP, and androstenedione. The horizontal centerline of this graph has the value of zero.

Table 3.3 Correlation study parameters between both LC-MS/MS methods

	Cortisol	17OHP	21Deoxycortisol	11Deoxycortisol	Androstenedione
N	20	20	20	20	20
Mean X	92.47	56.27	3.94	16.44	28.84
Mean Y	89.10	57.09	4.39	16.06	28.71
Slope	0.99	1.00	0.97	1.05	0.99
Intercept	-2.87	0.61	0.59	-1.12	0.16
R²	1.00	1.00	0.94	1.00	0.99
S_{yx}	3.52	1.03	1.13	1.12	1.02
Av. Bias	-3.37	0.82	0.46	-0.38	-0.14
S_{yx}/X Mean	3.8%	1.8%	28.7%	6.8%	3.5%

3.4.3.2 A comparative analysis of ESI method and CDC reported values

This method using ESI compared quite well with the values reported by CDC ($r^2 > 0.99$) except for 21-deoxycortisol (0.95), slope of 1 and intercept of zero as shown in **Figure 3.18** and **Table 3.4**.

A bland Altman analysis (bias) of this method represents the percentage of bias (difference between data obtained from ESI and CDC (y-axis) compared to average results between the two methods (x-axis)) (**Figure 3.19**). All steroids, except 21-decoxycortisol, showed very low bias (**Table 3.3**).

Figure 3.18 Mann-Whitney scatter plot for the LC-MS/MS method and CDC values

Scatter plot with regression fit represents method comparison between the two LC-MS/MS methods; ESI vs. CDC reported values for cortisol, 21-deoxycortisol, 11-deoxycortisol, 17-OHP, and androstenedione. This graph also shows a line with a slope of 1.0 going through the origin, where the dotted red line represents the theoretical best fit between the two methods, and the blue line represents the real obtained correspondence. X-axis represents the CDC values and y-axis represents the test method (ESI values).

Figure 3.19 Bland Altman analysis (bias) for CDC reported values and the LC-MS/MS method

A bland Altman analysis (bias) represents the percentage difference between data obtained from ESI and CDC (y-axis) compared to average results between the two methods (x-axis) for cortisol, 21-deoxycortisol, 11-deoxycortisol, 17-OHP, and androstenedione. The horizontal centerline of this graph has the value of zero.

Table 3.4 Correlation study parameters between CDC reported values and LC-ESI-MS/MS method

	Cortisol	17OHP	21Deoxycortisol	11Deoxycortisol	Androstenedione
N	19	19	20	19	20
Mean X	72.64	47.29	5.058	12.99	27.16
Mean Y	87.73	54.65	5.258	13.46	28.71
Slope	1.27	1.12	1.024	1.06	1.04
Intercept	-4.68	1.65	0.081	-0.31	0.55
R²	0.99	0.99	0.947	0.99	0.98
S_{yx}	4.82	3.58	1.251	1.28	1.66
Av. Bias	15.09	7.36	0.201	0.48	1.55
S_{yx}/X Mean	7.0%	7.6%	24.7%	9.9%	6.1%

3.4.3.3 Clinical ratio of CAH

The seven steroids were measured simultaneously in eighty-two de-identified left over samples. Clinical ratio was calculated using the ratio of the sum of 17-OHP and 21-deoxycortisol, divided by the cortisol level. Values equal or above one is diagnostic for CAH. Although several of the specimens had low cortisol levels, one of the criteria for CAH diagnosis; however, the clinical ratio was below one in those samples. These data showed that all 82 samples were normal (**Figure 3.20**).

Sample#	Cortisol	17OHP	21deoxycortisol	11deoxycortisol	Androstendione	DHEA	Pregnenolone	Clinical Assessment Ratio
1	480.4	3.3	0.7	12.9	0.6	0.3	1.8	0.01
2	19.2	2.4	0	0.7	0.6	1.7	0.8	0.13
3	112.1	2	0.3	2.6	1.1	2.6	0	0.02
4	255.8	2.1	0.3	6.9	0.5	1.7	0	0.01
5	143.5	1.5	0	4.4	0.5	0.8	0	0.01
6	43.2	1.5	0	0.7	0.5	1.1	0	0.03
7	35.5	2	0.4	0.9	0.6	2	0	0.07
8	183.9	1.4	0.4	1.7	0.3	0.6	0	0.01
9	17.1	1.3	0	0.8	0.4	1.1	0	0.08
10	30.6	1.3	0.5	0.7	0.3	0.1	0	0.06
11	51.1	1.3	0.3	1	0.4	0.4	0	0.03
12	14	1.5	0.4	0.7	0.5	1.5	0	0.14
13	133.5	1.6	0.4	2.7	0.4	0.9	0	0.01
14	3.7	1.3	0.3	0.7	0.5	0	0	0.43
15	171.5	1.2	0.4	1.8	0.4	0	0	0.01
16	245.8	2	0.4	6.6	0.4	0	0	0.01
17	98.8	1.5	0.3	1.3	0.4	0	0	0.02
18	70.9	1.3	0.3	0.9	0.9	2.3	0	0.02
19	1667.4	0	0.3	0.3	0.3	0	0	0.00
20	75.5	1.9	0.5	2.6	1	6.4	2.7	0.03
21	3.4	1.5	0.3	0.6	0.5	3.5	0	0.53
22	34.6	1.6	0.3	0.8	0.6	4.8	0	0.05
23	80.4	1.9	0.3	1.4	1.1	2.4	0	0.03
24	102.1	1.4	0	3	0.4	0.9	0	0.01
25	9.9	1.4	0	0.7	0.9	4.9	0	0.14
26	99	1.3	0	1.7	0.4	1.1	9.1	0.01
27	494.4	3.2	0.6	11.7	1.7	11.7	10.1	0.01
28	17.2	1.8	0.3	0.7	0.5	3.5	0	0.12
29	64.3	1.4	0.3	1.1	0.6	0.3	0	0.03
30	219.2	1.5	0.3	4.6	0.4	0.2	0	0.01
31	77.2	1.5	0.3	1.3	1.2	5.7	0	0.02
32	267.2	2.9	1.5	12	0.5	0.1	0	0.02
33	46.9	1.5	0.3	1	1.1	1.2	0	0.04
34	95.4	1.3	0.5	2	0.6	1.6	0	0.02
35	50.7	1.5	0	2.4	0.6	0.3	0	0.03
36	243.7	1.7	0.3	4.4	0.6	0.7	1.1	0.01
37	81	0	0	1	0.6	0.8	4.7	0.00
38	21.6	1.6	0	0.9	1.2	1.8	2.6	0.07
39	286.8	1.8	0.3	3.1	0.9	1.3	1	0.01
40	83.4	1.5	0	1.1	0.7	0.1	0	0.02
41	7	0	0.3	0.7	0	1.6	0	0.04
42	4.2	2.3	0.3	1.1	0.8	2.4	0	0.62
43	90.8	1.5	0.3	1	0.6	0.6	0	0.02
44	23.4	1.7	0	1.3	0.6	2.8	0	0.07
45	16.2	1.7	0	0.7	0.9	4.6	6.8	0.10
46	139.5	1.5	0.3	1.6	0.6	1.2	0	0.01
47	454.7	6.1	1.9	19.2	1	1.9	3.1	0.02
48	32.5	2	1.4	1.4	0.9	3.4	5.7	0.10
49	146.6	1.6	0.5	9.2	0.6	1	4	0.01
50	132.2	1.4	0	1	0.6	1	0	0.01
51	74.6	1.5	0	1.8	0.6	1	0	0.02
52	24.6	1.3	0	0.8	0.9	3.1	0	0.05
53	163.7	2.1	0	1.8	1.3	9.2	10	0.01
54	129.7	1.6	0	1.4	0.6	2.4	0	0.01
55	179.7	2.3	0	5.1	1.2	2.7	1.3	0.01
56	120.9	1.4	0.2	1.7	0.7	1.7	0	0.01
57	152.1	1.9	0	2.5	0.8	2.1	1	0.01
58	175.8	3.4	1.2	10.5	0.7	0.7	0	0.03
59	6.9	1.4	0	0.7	0.6	1.1	0	0.20
60	70.1	1.7	0.3	2.1	0.7	0.4	0	0.03
61	511.2	5.1	0.9	11.6	1.3	16.1	20	0.01
62	89.9	1.4	0	0.9	0.6	2.2	0	0.02
63	179.4	2.2	0	2	1.5	2.8	5.8	0.01
64	9.4	1.4	0.3	0.7	0.6	1.4	0	0.18
65	192.9	2.1	1	5.7	1.3	2.1	0	0.02
66	83.4	1.7	0	2.4	0.6	1.3	0	0.02
67	30.7	1.7	0.3	2	0.7	3.5	0	0.07
68	38.9	1.3	0.2	2.6	0.6	1.5	0	0.04
69	27.9	1.4	0.2	0.7	0.8	0.8	0	0.06
70	78.3	1.5	0	0.8	0.6	1	0	0.02
71	139.7	1.7	0	1.6	0.6	0.4	0	0.01
72	106	1.5	0.3	2.8	0.6	1	2.8	0.02
73	7.2	0	0	0.8	0	1.6	0.4	0.00
74	9.2	1.7	0.2	0.7	0.8	3.1	0	0.21
75	147.1	1.7	0.3	3.6	0.7	0.3	0	0.01
76	34.3	1.7	0.3	0.9	1	1.9	3.2	0.06
77	67.2	1.4	0.3	1	0.6	0.7	3.3	0.03
78	303.4	2.2	1.6	10	1.4	5.8	8.8	0.01
79	92.8	1.5	0.3	2.5	0.7	2.3	2.9	0.02
80	219.2	2.5	0.5	7.3	1.1	5	6.5	0.01
81	80	1.8	0.3	2.8	0.7	2.3	2.5	0.03
82	105.5	1.4	0.3	1.2	0.6	0.6	1.1	0.02

Figure 3.20 A representative clinical ratio of dried blood samples (DBS)

A representative profile of seven steroid values measured from eighty-two DBS samples. The clinical ratio was calculated for each sample. Yellow highlighted values are corresponding to samples with cortisol concentration less than 10 ng/ml and clinical ratio is less than 1. Cortisol values and clinical ratios were highlighted in red box.

3.4.3.4 Comparison between serum and DBS samples

To compare the performance of DBS versus liquid serum samples, the seven steroids were measured in both specimen types and the results were compared. A 6 mm diameter (equal to 6 μ l serum) punch size was from DBS and 200 μ l serum sample was used for analysis. We found no difference between the sample types for cortisol, 21-deoxycortisol, 11-deoxycortisol, and 17-OHP concentrations. However, DBS showed very low concentration for DHEA as compared to that in serum samples. For some unknown reasons pregnenolone was not detected in either sample. Androstenedione concentrations were slightly higher in DBS as compared to those in serum samples (Figure 3.21).

Figure 3.21 A representative comparison analysis of six steroids in DBS and serum

Four different samples (A, B, C, and D) were analyzed from DBS and serum to compare steroids levels in both conditions. Each steroid was measured in DBS and serum and plotted in this figure. Steroids concentration (ng/ml) are indicated at the x-axis and shown in green bars (DBS), and red bars (serum). Values represent means $\pm$ S.E.M. for $n = 3$ independent experiments.

4. DISCUSSION

In this study, we aimed at development and validation of a method to simultaneously measure seven steroids (17-hydroxyprogesterone, cortisol, 11-deoxycortisol, 21-deoxycortisol, dehydroepiandrosterone, androstenedione and pregnenolone) using liquid chromatography tandem mass spectrometry (LC-MS/MS) in DBS, which can be used for laboratory diagnosis of congenital adrenal hyperplasia (CAH). Measuring steroids using immunoassay-based methods has been adopted by most clinical laboratories since the 1970s [64]. The principle of the immunoassay is simply based on binding of an antigen to an antibody. The antibody in most cases is developed against the analyte of interest. Automated immunoassay analyzers used by most clinical laboratories provides a convenient and relatively rapid turnaround time (TAT) for the analysis of proteins, steroids, hormones, etc. [65]. However, immunoassays suffer from relatively poor specificity, due to cross reactivity of antibody with molecules with similar structures, and high cost. Low specificity can result in false positive results that may impact the overall management of the patient [66]. The issue of low specificity has encouraged laboratorians to look for a more precise technology for measuring proteins, steroids, etc. Liquid chromatography tandem mass spectrometry (LC-MS/MS) technology was the best choice, as it could provide the high sensitivity and specificity needed for clinical laboratories. This technology became a preferred method in clinical laboratories for detection of inborn errors of metabolism (IEM) in the newborn screening (NBS) programs [67]. Also, LC-MS/MS can perform multiplex measurements in variety of specimens such biological or non-biological samples with different matrices [68]. In particular, LC-MS/MS has been employed in the determination and monitoring of serum steroid levels in patients with endocrine disorders [69, 70]. Although LC-MS/MS has shown superior sensitivity and specificity for measuring steroids, relatively long run time was a reason for not being

recommended as an alternative to immunoassays for measuring 17-OHP [15, 17]. However, recent studies have shown significant improvements in LC-MS/MS methods by shortening the assay run time and measuring several other steroids in addition to 17-OHP that are useful for CAH diagnosis [15, 17, 71]. Indeed, the latter approach improved the positive predictive value of CAH screening from 0.4% with immunoassay to 9.4% with LC-MS/MS [72]. CAH is characterized by dysregulation of the steroidogenesis pathway, including accumulation of 17-OHP, suppression of cortisol production and overproduction of androstenedione and DHEA (a precursor of androstenedione) [73]. In the present study, we also included 11-deoxycortisol and 21-deoxycortisol to differentiate between 21- α hydroxylase deficiency from 11- β hydroxylase deficiency [74]. In addition, pregnenolone was added to the panel to improve the overall diagnosis of CAH because of its significance as a precursor for most of the steroids in the cholesterol pathway [75]. Using this approach we can not only detect CAH caused by 21-hydroxylase deficiency, but also help diagnose most of the remaining cases of CAH that are caused by a deficiency of other enzymes (11 β -hydroxylase, 17 α -hydroxylase, or 3 β -hydroxysteroid dehydrogenase) [76]. Measuring multiple steroids simultaneously can improve CAH diagnosis by calculating clinical ratio. Measuring the clinical ratio (17-OHP+21-deoxycortisol [or androstenedione] divided by cortisol) was adapted by the Centers for Disease Control and Prevention (CDC) and is used to interpret results for the diagnosis of CAH [61]. The mathematical basis of this calculation is based on the observation that concentrations of steroids (i.e., 17-OHP and androstenedione) are commonly high, while concentrations of steroid that are situated downstream the defective enzyme (i.e., cortisol) are relatively low. Thus, the clinical ratio (17-OHP + 21-deoxycortisol [or androstenedione] divided by cortisol) should be equal or higher than one in patients with CAH and below one in otherwise healthy individuals. It is important to note

that by using 21-deoxycortisol or androstenedione we can get more diagnostic information. That is, when 21-deoxycortisol is used one can not only detect CAH, but also differentiates between 21-hydroxylase deficiency and 11-hydroxylase deficiency. Moreover, when one uses androstenedione in the calculation one can detect CAH. Thus, by altering 21-deoxycortisol, we can specifically diagnose the cause of CAH [60, 61].

One of the significant aspects of this work is to use clinical ratio to confirm the diagnosis of CAH. As shown in results section, using just one marker such as 17-OHP cannot confirm CAH diagnosis. This is important in considering the prevalence of CAH in Alberta. There are approximately 60,000 live births each year in Alberta. If we assume 1 in 15,000 children develop CAH, accurate diagnosis of the CAH by measuring the seven steroids and calculating clinical ratio can significantly improve the diagnosis for four children who can benefit early diagnosis, treatment and hopefully normal life [77].

In clinical laboratories, specimens are the most important part of an accurate and successful diagnosis of any disease [78]. Integrity and quality of any specimen depends on its collection and processing which impact the end diagnosis [78]. Patient comfort and all preanalytical variables (factors that impact test results before testing the sample) are also very important and must be considered to achieve the best results. Choosing appropriate specimens is of great importance not only to the patient, but also for accurate diagnosis. For decades liquid specimens (e.g., urine, blood, etc.) have been used for testing in laboratories. There are various limitations related to specimen collection such as patient fear of needles, reactions to needles, the cost for phlebotomy, and demographic distribution of patients (how long they have to travel to the laboratory to give specimens) [79]. DBS sampling can be a good candidate to alleviate most of the complications associated with liquid sample collection [80]. DBS specimen is collected by a heel (for newborn

babies) or finger stick (for adults) to collect a few drops of blood (about 300 μ l to fill the five pre-printed circles on a filter paper), which is an advantage for most CAH patients such as neonates. This approach is relatively less painful compared to venous sampling and easy to collect without the need of a phlebotomist. DBS specimens can simply be put in an envelope and mailed to the laboratory without the need for wet or dry ice and associated cost. Also, DBS are easy to store, mostly without the need for refrigeration. In addition, various reports showed that analytes are stable for a long time in DBS specimens, especially if kept at low temperatures with low humidity. The above mentioned characteristics of DBS make them a cost-effective specimen for laboratories [52, 80-82]. Thus, we chose DBS as the specimen of choice for this study.

First, we aimed to extract steroids from the DBS to achieve the maximum analyte recovery. Organic solvents were used for steroid extraction as it is simple, relatively inexpensive, and suitable to extract the non-polar compounds such as steroids [83]. In order to identify the most efficient extraction solvent, we used various conventional extraction solvents, such as acetonitrile (ACN), methanol, acetone and methyl tert-butyl ether (MTBE). Previous studies achieved successful extraction of steroids using different ratios and/or combinations of these organic solvents. Our data showed that a mixture of methanol and water most efficiently removed the steroids in DBS. In this study a 95% methanol mixture was used as an extraction solvent because it showed a slightly better signal to noise ratio (S/N) for steroid separation as compared to other solvents. Although MTBE and Acetone-ACN solvents have been previously used by other groups to extract steroids from serum and DBS, these solvents were not as sufficient as methanol to extract steroids in DBS [84, 85]. Also, other approaches such as ultrasonication have been used to enhance steroid release from the filter paper during extraction [86]. However, ultrasonication was not efficient to increase steroid release from DBS in our experience.

To determine if there was any cross-contamination (carryover) from the punches during punching out DBS, the tip of the puncher was washed with the extraction solvent and the collected sample was assayed in triplicates. Results showed no carry over.

Optimizing the time required to achieve a maximum recovery for sample extraction was assessed by mixing DBS with the solvent at different time points [0, 2.5, 5, 10, 15 min]. We found that the maximum recovery was obtained at 2.5 min for the steroids. We decided to use 5 min for extraction to ensure all steroids are extracted from DBS.

In addition, we assessed the efficiency of extraction of steroids from DBS and found that 90% or more of steroids were recovered from a single extraction step.

The gas required to dry steroids in organic solvent after extraction was also evaluated. We used Nitrogen (N₂) and air to dry the sample. Nitrogen (N₂) and air are the most commonly used gases in evaporation protocols. We found no significant difference between using either gas, however, we chose nitrogen in our method. Nitrogen is an inert gas. In contrast, air contains oxygen which can cause oxidation of the steroids and may impact eventual measurements of steroids.

To ensure that the reconstituted samples were clean enough (free from fibers or debris coming off filter paper in DBS) for LC-MS/MS analysis, samples were centrifuged prior to injection into LC-MS/MS instrument. Centrifugation by conventional microcentrifuge tubes or ultra-free centrifugal polytetrafluoroethylene (PTFE) membrane filter tubes showed no difference on the mass spectrometry signal intensity. We recommend using ultra-free centrifugal filter tubes to ensure the specimens are free of debris before LC-MS/MS analysis.

Hematocrit has been identified as the major limitation in using DBS because of its potential effect on the analyte quantitation. Samples with high hematocrit are viscous and may spread less

on the filter paper as compared to specimens with normal hematocrit [87]. It has been shown that the hematocrit level in neonates ranges from 42% to 65% and gradually decreases with increasing age [88]. Accordingly, the hematocrit effect on the assay performance was evaluated at different steroid levels using 50 μ l of blood from an adjusted hematocrit of 45, 55 and 65% and the concentration of the seven steroids were assessed at different hematocrits. Hematocrit 55% was used to prepare QC samples in this study because it represents the median range of hematocrit levels in neonates. Our data indicated that there was no clear trend between the blood hematocrit and steroid signal at different hematocrit levels. The effect of hematocrit on DBS on therapeutic drug monitoring (TDM) has been extensively studied by different groups [89-91]. Their results were inconsistent; some groups showed no effects of hematocrit, while other groups found hematocrit impacting their results [91]. Therefore, a quantitative determination of analytes recovery with respect to hematocrit in DBS should be examined as an essential part of method development and validation [92, 93].

In this study, all steroids and their internal standards were detected in positive electrospray ionization (+ESI) mode to achieve the highest signal for each compound without the need of derivatization. However, the main challenge in this approach was the detection of DHEA and pregnenolone due to their low concentrations in serum, poor ionization, and low spectra for product ions [94]. For these latter reasons, most of LC-MS/MS assays have used a derivatization step to measure steroids by LC-MS/MS. We improved our method by optimizing the ionization settings using a very sensitive mass spectrometry (latest version of triple quad mass spectrometry) for best fragmentation of each steroid [70]. Additionally, multiple reaction monitoring (MRM) was applied for highly selective and sensitive detection of the analytes. These conditions enabled us to detect DHEA and pregnenolone along with the other five steroids (cortisol, 17-OHP, 21-

deoxycortisol, 11-deoxycortisol and androstenedione), simultaneously without any additional derivatization step.

Although steroids all have the same core structure (**Figure 4.1**), small differences in the functional groups or the presence of double bonds, results in significantly different fragmentation pattern [95].

Figure 4.1 Steroids core structure

This figure illustrates the carbon atoms and rings of the steroids core structure. This structure consists of seventeen carbon atoms with four integrated rings; three cyclohexane rings (rings A, B and C) and one cyclopentane ring (D ring). Atom numbering is according to the International Union of Pure and Applied Chemistry (IUPAC). Figure adapted from Wikipedia (en.wikipedia.org/wiki/Steroid).

Precursor ions were selected for each steroid in full MS scan mode as protonated ions $[M+H]^+$, except for DHEA and pregnenolone that were selected as $[M+H-H_2O]^+$ [96]. In this case, loss of water (OH group in the 3-position) from the parent structure will produce a precursor ion with -18 Da (largest spectral peak). The product ion transitions were determined from the MS/MS spectra, the most two intense fragments were selected as the quantifier and qualifier transitions, respectively [97]. The MS/MS analysis of all seven steroids was similar to previously published data [98-100]. Our method determined the concentration of cortisol, 11-deoxycortisol, 21-deoxycortisol, 17-OHP, and DHEA, using their deuterated internal standard. Androstenedione has no deuterated internal standard available, so we used isotopically labelled androstenedione- $^{13}C_3$. For pregnenolone, internal standards (deuterated or isotopically labelled) were not readily available when we started this project; thus, it was quantified using isotopically labelled androstenedione- $^{13}C_3$ as internal standard. Recently, a deuterated internal standard for pregnenolone has been released from Cerilliant (Round Rock, TX United States of America) that might be useful for future application.

During the course of developing the best LC gradient method, we optimized different instrumental parameters. The optimization was required to achieve the maximum separation at appropriate resolution for all steroids in a minimum analytical run time. Our data showed that there was a double peak at transition m/z 347.3 that corresponded to the fragments of both 21-deoxycortisol and 11-deoxycortisol. These steroids are isobaric compounds as they possess the same molecular mass [84]. The identical fragments were successfully separated by chromatography allowing us to quantify both steroids individually. Although 17-OHP and DHEA were co-eluted from the column at the same time, we were able to quantify each steroid specifically by its unique MRMs.

We hope that our method will be implemented in clinical laboratories and for that reason we evaluated according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. Analyte specificity was maintained through monitoring quantifier and qualifier ion ratios for each steroid to avoid interferences. Steroid concentrations fell within the linear range of our standard calibration curve. The linearity range for DHEA and pregnenolone was determined to be from 5 ng/ml to 250 ng/ml, the lower limit of quantification (LLOQ) for both steroids was higher than 2.5 ng/ml. Intra-day and Inter-day precision were acceptable with $\%CV \leq 15\%$. Although the maximum $\%CV$ observed in our work might seem high in comparison to those of automated analyzers, it is not uncommon to see $\%CV$ of 15 and higher in mass spectrometer system that used in this study. We think some factors such as low sample volume (the amount of blood in DBS punch is equal to around 6 μ l serum), as well as, source of our QC materials (made from leftover whole blood specimens), might have contributed to the observed $\%CV$. Indeed, when we used QC materials from CDC, that were prepared from large volume of fresh O type blood, our $\%CV$ ranged lower (1.8 to 8.0%). In general, observed $\%CV$ is acceptable, and the out method is appropriate for clinical use.

We have tested our method with two different mass spectrometry ionization sources; APCI and ESI. APCI was routinely used as a “gold-standard” platform to measure steroids. Concentrations of five steroids in identical samples were measured by both methods. Results obtained from both methods (APCI vs. ESI) showed good correlation between the methods. Most current LC-MS/MS assays use ESI as an ionization technique for measuring different molecules, thus, ESI was used in our assay to measure steroids. To further assess the performance of our method, we participated in an external quality control program offered by CDC’s Newborn Screening Quality Assurance Program (NSQAP). This program involves measuring steroids in

five unknown specimens that are provided by NSQAP and reporting the results to be compared to our peer group. CDC uses the generated data to calculate clinical ratio which is calculated by the following formula:

$$\text{Clinical ratio} = \frac{17\text{-OHP} + (21\text{-deoxycortisol [or androstenedione])}{\text{Cortisol}}$$

Clinical ratio of one or greater is diagnostic for CAH. Our results from CDC samples matched those generated by CDC, perfectly.

To further assess the performance of our method, we measured all seven steroids in eighty-two de-identified left over whole blood samples obtained from the Alberta Children's Hospital. Nine samples showed low cortisol levels (< 10 ng/ml) which is one of the criteria seen in patients CAH. However, the calculated clinical ratio for those samples was below one, thus ruling out the diagnosis of CAH. Calculating clinical ratio, as suggested by CDC, is extremely important to provide more meaningful results to clinicians. As mentioned above, very low cortisol levels are seen in patients with CAH; however, low cortisol alone should not be used to diagnose CAH as several other clinical conditions can have low cortisol levels without having CAH. Therefore, using clinical ratio can help in differential diagnosis of CAH. In order to calculate clinical ratio, multiple steroids must be measured. Thus, measuring just one steroid such as 17-OHP (the current accepted marker for CAH) is not enough to calculate clinical ratio. In this work, we chose to simultaneously measure several steroids instead of one (17-OHP) to better help clinicians for their final diagnosis. Although the current calculation of clinical ratio measurements doesn't require all the steroids levels, we hope the new improved clinical ratio in the future uses all seven steroids results to provide much better diagnosis to clinicians. In many circumstances "more is not always better", although in this case we hope it is.

Furthermore, we compared the results for all steroids in DBS samples and liquid serum samples from the same individuals. Four steroids (cortisol, 17-OHP, 21-deoxycortisol and 11-deoxycortisol) results showed a good agreement between DBS and serum [101]. However, DHEA showed a higher concentration in serum compared to DBS. Androstenedione data displayed a slightly higher concentration in DBS compared to serum. The difference in these steroids levels seen in the two different sample types might be due to the difference in matrices (serum vs. whole blood), absence of cells in sera, or difference in protein concentration in the specimens. For unknown reasons, pregnenolone was not detected in either serum or DBS samples. This might be due to the low physiological circulating level(s) of pregnenolone.

In aggregate, we developed an LC-MS/MS method that can accurately and precisely measure seven different steroids in dried blood spots. This method is robust and inexpensive and can be easily implemented in clinical laboratories equipped with LC-MS/MS. The method is validated according to CLSI guidelines and will be implemented in Calgary Laboratory Services in the near future.

The limitation of the assay is the expensive equipment (LC-MS/MS) and the need for skilled technologists to operate, maintain and troubleshoot this sophisticated technology. On average, the initial cost to purchase an LC-MS/MS is approximately \$300,000 plus the renovation cost to prepare the laboratory for its implementation. Obviously, in addition to skilled technologists to run and maintain the instrument, a laboratory scientist with expertise in the field of mass spectrometry and a laboratory director familiar with the field are required to successfully run the instrument, interpret the data and develop new methods. Hopefully, the new generations of mass spectrometers will be more automated and user friendly, so any laboratory can benefit from this magnificent instrument.

5. CONCLUSION AND FUTURE DIRECTIONS

We developed and validated a rapid, sensitive, and specific LC-MS/MS assay for simultaneous measurement of seven steroids in BDS to diagnose CAH. This method not only measures 17-OHP, a marker for CAH, but also measures six additional steroids to calculate the clinical ratio and confirm CAH diagnosis. The use of mass spectrometry in clinical diagnostics has increased in the last 20 years [31, 32]. In particular, LC-MS/MS has been introduced in clinical laboratories to replace the conventional immunoassays used for measuring drugs, steroids, proteins, etc. Using our LC-MS/MS method eliminates shortcomings such as poor specificity and false positive results seen in conventional immunoassays that are currently used in clinical laboratories for measuring steroids. Our method not only improves patient care by producing accurate results, but also eliminate the stress of false diagnosis on patients and their families, as well as, all unnecessary costs associated with mismanagement of the patients. Additionally, this method differentiates between CAH caused by 21-hydroxylase deficiency or 11-hydroxylase deficiency by measuring 21-deoxycortisol and 11-deoxycortisol. An important advantage of this LC-MS/MS method over other LC-MS/MS assays for measuring steroids is our simplified specimen preparation. That is, we eliminated the need for derivatization and the extensive clean-up steps such as extraction and ultrasonication used by other LC-MS/MS methods for steroid analysis. Furthermore, we used dried blood spot (DBS) as the specimen of choice in this study. DBS specimens are very easy to collect without the need for phlebotomist, collect very small volume of blood, appropriate for newborn screening and are more convenient for the patients who fear phlebotomy. Also, DBS are easy to transfer, process and store, and much less expensive compared to conventional liquid specimens. The assay linearity was appropriate for all seven steroids and covered very low, normal, and high concentrations seen in different clinical

conditions. Intra-day and Inter-day precision were acceptable ($CV < 15\%$) for the seven steroids. The assay had good recovery with no carryover and good accuracy. Measuring several steroids simultaneously will provide additional information for management and monitoring differences seen in phenotypic variations of CAH. Further modification of for this method might be useful to use this approach to measure different steroid panels and define other steroidal dysregulation in different endocrine disorders (e.g., Cushing's syndrome, Addison's disease and polycystic ovary syndrome) [102]. Clinical mass spectrometry applications have become more popular in clinical laboratories. However, adapting mass spectrometry requires funding for the initial high cost of the instruments and highly skilled technologists to operate the instrument and skilled laboratory scientist or director to troubleshoot and interpret the results. Once LC-MS/MS technology is successfully implemented in the laboratory, the overall cost for measuring most of the diagnostic markers will be very low in comparison to that of other technologies such as immunoassays. With the introduction of automated specimen processing for LC-MS/MS systems the overall turnaround time will be comparable to that of immunoassays. Mass spectrometry has been adopted in most of the clinical laboratories in western countries. Combination of LC-MS/MS and dried blood spot, will become the method and the specimen of choice for measuring different markers in toxicology, microbiology, endocrinology, and molecular biology in the relatively near future.

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