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2ND TIER ASSAY FOR THE DETECTION OF CONGENITAL ADRENAL HYPERPLASIA BY VIRGINIA'S NEWBORN SCREENING LABORATORY: STEROID PROFILE BY HPLC-MS/MS

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

By

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Abbreviations

°C	degrees Celsius
11-DOC	11-deoxycortisol
17-OHP	17α-hydroxyprogesterone
21-DOC	21-deoxycortisol
3β-HSD	3β-hydroxysteroid dehydrogenase
4-AD	androstenedione
ACMG	American College of Medical Genetics
ACTH	adrenocorticotropic hormone
ARUP®	Associated Regional and University Pathologists, Inc.
CAD	collision assisted dissociation
CAH	congenital adrenal hyperplasia
CDC	Centers for Disease Control and Prevention
CE	collision energy
CLIA	Clinical Laboratory Improvement Amendments
CORT	cortisol
cps	counts per second
CV	coefficient of variation
CXP	collision cell exit potential
DBS	dried blood spot
DELFIA	dissociation-enhanced lanthanide fluorescence immunoassay
DP	declustering potential
EP	entrance potential
ES	electrospray
FIA	flow injection analysis
FWHM	full width at half maximum
GC-MS	gas chromatography-mass spectrometry
GS1	nebulizer gas

GS2	heater gas
GSP™	Genetic Screening Processor
HPLC	high performance liquid chromatography
HPLC-MS/MS	high performance liquid chromatography-tandem mass spectrometry
in	inch
LOQ	lower limit of quantitation
m/z	mass-to-charge ratio
MCA	multi-channel analysis
min	minute
mL	milliliter
mm	millimeter
MRM	multiple reaction monitoring
msec	millisecond
MS/MS	tandem mass spectrometry
NBS	newborn screening
NC CAH	non-classic CAH
ng	nanogram
NSQAP	Newborn Screening Quality Assurance Program
PEEK	polyetheretherketone
PKU	phenylketonuria
Q1	quadrupole 1
Q2	quadrupole 2 (collision cell)
Q3	quadrupole 3
QC	quality control
rpm	revolutions per minute
Rs	peak resolution
RUSP	Recommended Uniform Screening Panel
S/N	signal-to-noise ratio

SACHDNC	Secretary's Advisory Committee on Heritable Disorders in Newborns and Children
SV CAH	simple virilizing CAH
SW CAH	salt-wasting CAH
UHPLC	ultra-high pressure liquid chromatography
UNSAT	unsatisfactory
V	volt
x g	times gravity
µg	microgram
µL	microliter
µm	micrometer

Abstract

Congenital Adrenal Hyperplasia (CAH) encompasses several disorders related to disruptions in the adrenal steroid production pathway. These disruptions may cause virilization of the external female sex organs, incorrect gender assignment, precocious puberty, and in the most severe form, may cause life-threatening salt wasting and adrenal crisis if not detected and treated early in the newborn period.

17α-Hydroxyprogesterone (17-OHP) is the primary target for immunofluorescence detection of CAH from dried blood spots in newborn screening (NBS). Unfortunately, current immunoassay techniques for the detection of CAH suffer from high false positive rates. The primary factors contributing to false positive determinations can include the natural increase of 17-OHP due to stress stimuli as well as cross-reactivity of the immunoassay antibody with other hormones and endogenous compounds in blood.

Analysis of the adrenal steroid profile and corresponding analyte ratios using high performance liquid chromatography (HPLC) or ultra-high pressure liquid chromatography (UHPLC) combined with tandem mass spectrometry (MS/MS) has been shown to be a sensitive and selective technique for the significant reduction of the false positive reporting rate for CAH in newborn screening.

In working toward optimization, validation, and implementation of an HPLC-MS/MS steroid profile for use by Virginia's Newborn Screening laboratory as a 2nd tier analysis for CAH screening, a commercially-available core-shell HPLC column with a biphenyl stationary phase was determined to offer adequate retention and selectivity to achieve baseline resolution of isobaric target analytes under rapid reversed phase gradient conditions. Method linearity, precision, and accuracy were assessed using enriched dried blood spot materials. Double-blinded analyses of over 300 newborn dried blood spot specimens were used to determine clinical sensitivity and specificity of the assay, which is projected to

substantially reduce the false positive reporting rate for CAH screening while meeting target sample turnaround times.

CHAPTER 1: INTRODUCTION

1.1 Newborn screening

Newborn screening (NBS) is widely described as a public health success and owes its roots to Dr. Robert Guthrie, who, in the late 1950s, modified a bacterial growth inhibition assay to measure phenylalanine as a biochemical marker for detection of phenylketonuria (PKU).[1,2] PKU is a devastating metabolic disorder causing an inability to metabolize the amino acid phenylalanine to tyrosine, which can lead to severe mental disabilities due to metabolic crisis if not detected and treated early. [3] By the early 1960s, Guthrie and Susi showed that the assay was well-suited for screening of large newborn populations using a dried blood spot matrix.[1] In the years that followed, NBS for PKU was adopted by public health laboratories in all fifty states as well as many countries around the world.[4] As additional disorders proved to be good candidates for screening and as analytical technologies advanced, NBS expanded to encompass dozens of disorders. The main mission of NBS is to utilize population-wide screening of all infants in order to offer the best chance of identifying rare inherited disorders as early as possible, ideally before the onset of symptoms. This early detection offers the best possibility for immediate intervention and improved outcomes.

Most NBS tests continue to utilize the dried blood spot (DBS) matrix, which involves dropping capillary blood, usually from a heel stick of the infant, onto specialized filter paper and allowing the blood to dry. Samples are ideally collected when the newborn is between one and two days old, allowing enough time for biomarkers to build up in the blood of individuals affected by the disorders, but not waiting so long as to significantly delay testing.[5,6] The DBS sample is minimally invasive to collect

and is easy to package and transport to the testing laboratory, whether by commercial carrier or courier service. Once at the laboratory, paper punches containing dried blood are removed from the blood spots for analysis.

As the number of candidate disorders for NBS grew, various analytical technologies were adopted by screening laboratories to accommodate testing. Screening methodologies are ideally rapid, economical, and have adequate sensitivity to differentiate affected individuals from the normal population. Beyond bacterial growth inhibition assays, one of the earliest technologies adopted for NBS was the immunoassay. [7] During the 1990s and early 2000s, flow injection analysis-electrospray ionization-tandem mass spectrometry began being incorporated by NBS laboratories as a method to rapidly screen for several metabolic disorders simultaneously from a single blood spot punch.[4,8]

While NBS was eventually adopted by all fifty states, there were often wide inconsistencies between states regarding which conditions were screened and timelines for implementation. In the early 2000s, in an effort to solidify national guidelines regarding exactly which conditions should be the primary targets for newborn screening, an expert panel from the American College of Medical Genetics (ACMG) provided the Recommended Uniform Screening Panel (RUSP) of primary and secondary conditions as top candidates for mandated universal NBS.[9] The original recommended panel included 29 core disorders and 25 secondary disorders. The process also established evaluation criteria of data related to screening methodologies, costs, possible treatments, and outcomes related to universal screening for consideration of future candidate disorders for inclusion on the RUSP. The current RUSP, now evaluated and maintained by the Secretary's Advisory Committee on Heritable Disorders in Newborns and Children (SACHDNC) under the Secretary of Health and Human Services, contains 35 core disorders and 26 secondary disorders.[10]

Many of the disorders commonly targeted by NBS involve reduced or eliminated function of enzymes. [3,5,11] The two most common biochemical approaches to screen for deficient enzyme activity involve direct or indirect analysis. In direct analysis, enzyme activity is measured, usually through interaction with a specific enzyme substrate to produce a reaction target that is then measured by the assay. The amount of target produced over a specific time period allows for calculation of enzyme activity rate. The indirect approach involves detection and quantitation of biomarkers, usually the buildup of precursors, proximal to the target enzyme.

NBS laboratories, in consultation with medical specialists or advisory committees, continually strive to reach a balance in trying to hone methods and cutoffs to avoid missing individuals affected by a disorder with maintaining the lowest possible false positive rate. False positive results occur when the analytical result for a specimen falls outside of the anticipated normal range for a given analyte, prompting collection of another specimen or referral for diagnostic testing, when the individual is not affected with the disorder. While less detrimental than a missed diagnosis, false positive results can cause a myriad of issues within the NBS system as well as to families that are impacted by the screening results. False positive results may cause added stress to new parents as they worry whether their child has a potentially serious condition and face the added costs associated with diagnostic testing. False positives may add stress and caseload to medical specialists tasked with clinical follow-up of babies identified through screening. Reporting a high number of false positives may also diminish confidence in the overall validity of the screening assay as a complacency may develop among physicians that receive many reports of positive screening results that rarely, if ever, pan out to be a true diagnosed case. False positives also strain NBS laboratories and follow-up programs as they create more samples for the laboratory to run as well as more cases that must be tracked within the follow-up system. [12–14]

Congenital adrenal hyperplasia (CAH) is among the core RUSP disorders screened by all US states and territories.[10,15] Primary screening methods for CAH rely on immunoassays targeting a single

biomarker, 17α-hydroxyprogesterone (17-OHP). [5,7,16] Immunoassays screening for elevated levels of 17-OHP suffer from high false positive rates, often 1-3 %, even when assay cutoffs are adjusted to correct for common influential factors such as low birth weight or gestational age.[17,18] 17-OHP becomes elevated under times of increased stress, as is often the case with severely premature, low birth weight, or sick newborns. Adrenal immaturity may also play a role in elevation of 17-OHP.[18,19] Additionally, immunoassay antibodies for 17-OHP show cross reactivity for other endogenous steroids and precursors, some of which are at much higher concentrations in the blood stream than 17-OHP.[20] All of these factors contribute to high false positive rates for CAH screening based on immunoassays targeting 17-OHP.

1.2 Congenital adrenal hyperplasia

1.2.1. Steroidogenesis in the adrenal glands

Glucocorticoid and mineralocorticoid steroid production in the adrenal glands is regulated by adrenocorticotropic hormone (ACTH) produced in the pituitary gland of the brain.[11] Under normal function, production of the glucocorticoid cortisol serves as a feedback loop to the process. Steroids are synthesized from cholesterol via a series of cleavage, dehydrogenation, and hydroxylation reactions facilitated by five enzymes. As indicated in Figure 1, the production of the glucocorticoid cortisol involves side chain cleavage of cholesterol between carbons 20 and 22 to form pregnenolone. Next, pregnenolone is hydroxylated at carbon 17 by 17α-hydroxylase to form 17α-hydroxypregnenolone. Dehydrogenation of oxygen at carbon 3 by 3β-hydroxysteroid dehydrogenase (3β-HSD) yields 17-OHP. 17-OHP is then further hydroxylated by 21-hydroxylase to form 11-deoxycortisol (11-DOC). Finally, 11-DOC is hydroxylated by 11β-hydroxylase to form cortisol.[21]

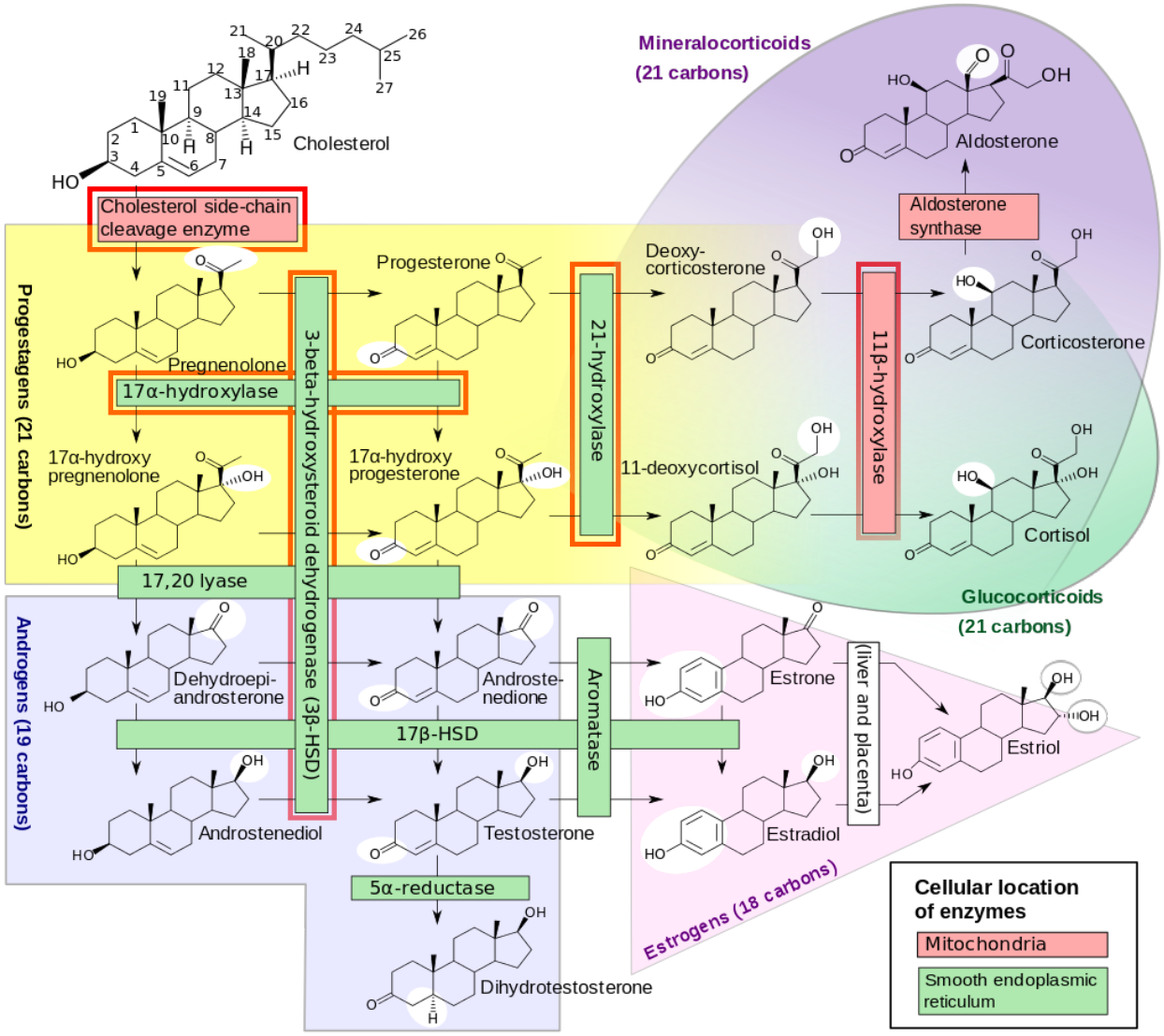

Figure 1. Schematic of human steroidogenesis, Reprinted from WikiJournal of Medicine 2014 – Congenital adrenal hyperplasia, Retrieved September 25, 2019, from https://en.wikipedia.org/wiki/Congenital_adrenal_hyperplasia#/media/File:Steroidogenesis.svg. Reformatted to include red boxes around enzymes associated with congenital adrenal hyperplasia under Creative Commons License CC BY-SA 3.0.

In addition to the biosynthesis pathways depicted in Figure 1, an additional steroid, 21-deoxycortisol (21-DOC) may be produced by hydroxylation of excess 17-OHP by 11β-hydroxylase when 21-hydroxylase activity is reduced or absent, as depicted in Figure 2.[22]

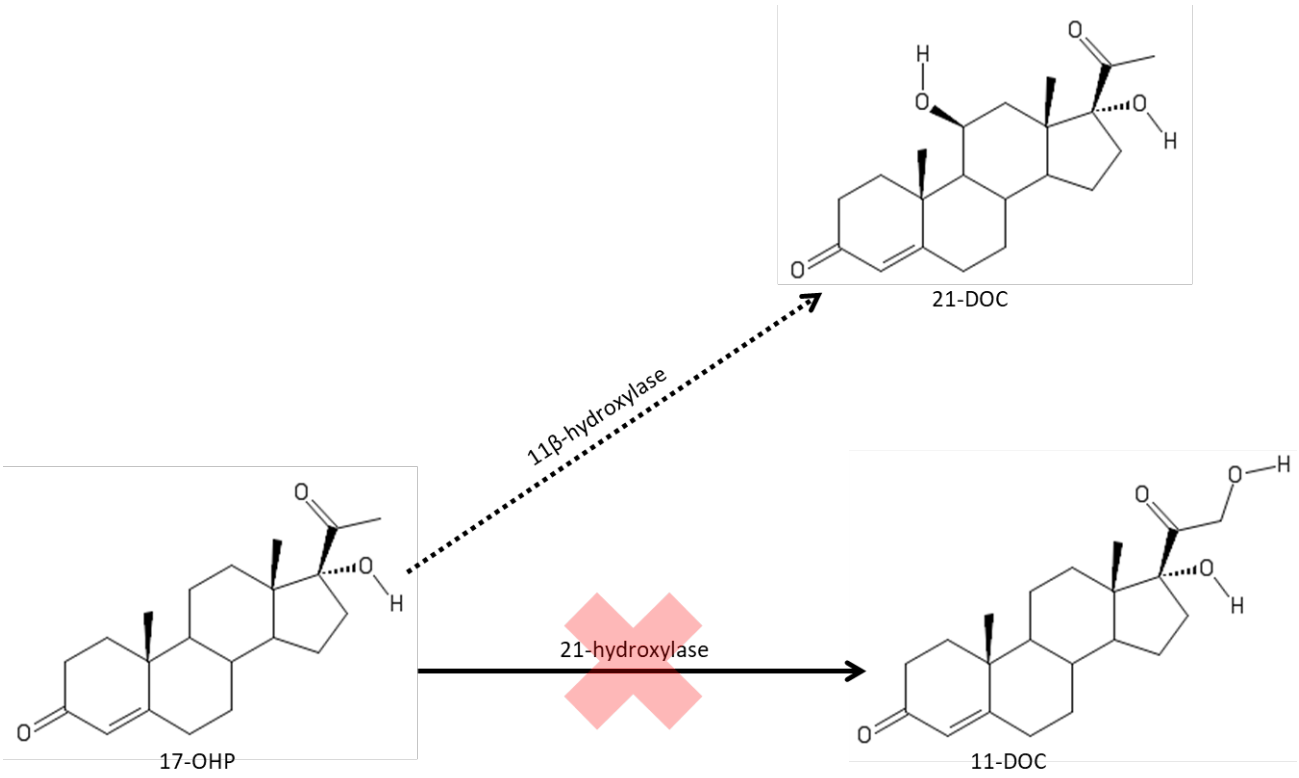

Figure 2. Schematic of enzymatic hydroxylation of 17-OHP to 21-DOC via 11β-hydroxylase in the absence of residual 21-hydroxylase function.

1.2.2. Pathophysiology

CAH is a group of disorders caused by genetic mutations leading to reduced or eliminated activity of one of the five enzymes along the glucocorticoid and mineralocorticoid production pathway in the adrenal glands, outlined in red in Figure 1.[11,21] The most common cause of CAH is deficiency of the 21-hydroxylase enzyme which is responsible for approximately 90-95 % of CAH cases.[11,16,23] Because 21-hydroxylase deficiency is far more common than other forms of CAH, there is strong justification for selecting the precursor to the 21-hydroxylase enzyme, 17-OHP, as the primary target for newborn screening assays. CAH is characterized into classic and non-classic forms. Classic forms of CAH are the most severe, and often present with symptoms at birth such as in-utero virilization of external genitals which may even lead to incorrect gender assignment of severely virilized females at birth. Classic CAH is

also characterized by low cortisol production relative to elevated levels of steroid precursors and androgens within the blood. If untreated, classic forms of CAH may also lead to precocious puberty, accelerated skeletal aging, and shortened adult stature. Classic CAH is further subdivided into two forms: salt-wasting and simple virilizing. The most severe, salt-wasting form of CAH accounts for between two-thirds to three-quarters of classic CAH cases, while simple virilizing CAH accounts for the remaining quarter to one-third of cases. Salt-wasting CAH may cause life-threatening shock, dehydration, and adrenal crisis within the first days or weeks of life.[23] Simple virilizing CAH has moderate severity, as it can be associated with significant androgen production and therefore virilization of the external genitals, however it does not cause salt-wasting crisis. Incidence rates for classic CAH are reported to be between 1:12,000 to 1:15,000 live births but can show wide variability among certain subpopulations.[16,23] Non-classic CAH shows a later age at onset and is often diagnosed after the newborn period and may lead to precocious puberty, accelerated skeletal aging, and shortened stature. Some cases of non-classic CAH do not require treatment. Up to 5 % of CAH cases are caused by deficiencies in enzymes other than 21-hydroxylase, the most common being 11β -hydroxylase.[23]

1.2.3. Treatment

Treatments for CAH can involve life-long administration of glucocorticoids and mineralocorticoids to replace deficient cortisol and aldosterone and to reduce androgen production. Salt supplementation may also be necessary for patients with the salt wasting form of classic CAH.[16,23] Severely virilized females may undergo reconstructive surgery of the external genitals.[24,25]

1.2.4. Primary methodology for newborn screening

Currently, the Virginia NBS laboratory screens DBS specimens from all babies born in the Commonwealth for CAH utilizing a solid phase time-resolved fluorescence immunoassay method on the PerkinElmer Genetic Screening Processor (GSP™) targeting 17-OHP.[26] As indicated in Figure 3, the assay

works by competitive binding between sample 17-OHP and europium-labeled 17-OHP with rabbit 17-OHP antibodies in solution. The rabbit 17-OHP antibodies are bound to antibodies fixed to the walls of the well plate. After washing away unbound material, a dissociation-enhanced lanthanide fluorescence immunoassay (DELFIA) inducer is added to cleave the europium ions from the labeled 17-OHP molecules. The DELFIA inducer forms a fluorescent chelate with the freed europium molecules in solution and this fluorescence is measured.[27] The measured fluorescence signal is inversely proportional to 17-OHP concentration within the sample.[26,27]

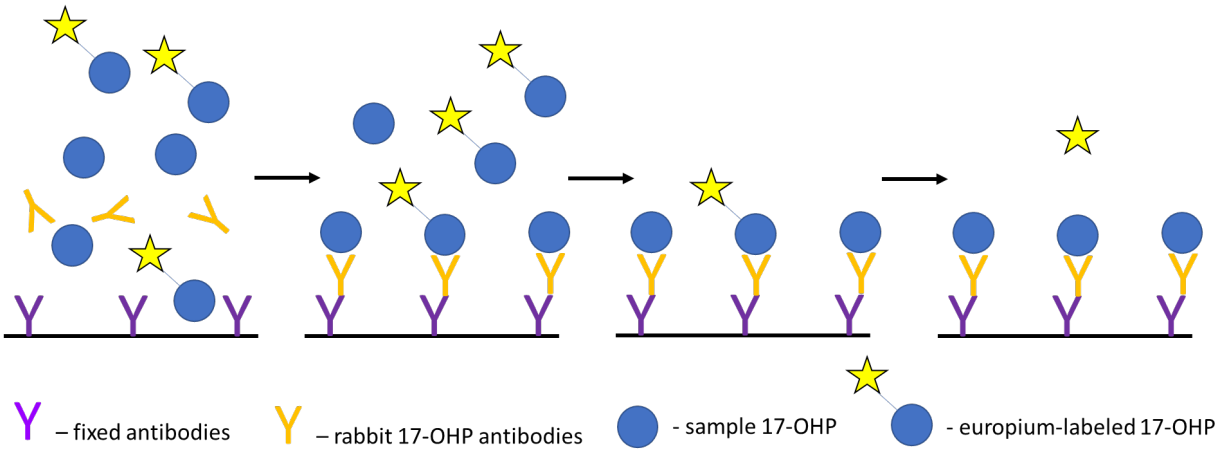

Figure 3. Representative schematic of competitive binding fluorescence immunoassay. Adapted from https://www.perkinelmer.com/lab-products-and-services/application-support-knowledgebase/delfia/delfia-trf-assays.html

When using only the immunoassay method for CAH screening, the NBS laboratory employs replicate DBS analysis and birthweight-adjusted cutoffs for interpretation of CAH screening results. A single initial DBS punch is analyzed from each DBS specimen received by the laboratory using the immunoassay targeting 17-OHP. Any specimen showing 17-OHP greater than or equal to 20 ng/mL serum (or the top 3% of specimens from analysis of each 96 well plate, whichever number of specimens is greater) is repeated by the immunoassay using duplicate DBS punches from the same DBS specimen. All specimens that do not require replicate analysis are reported as within normal limits for the CAH

screening test. For samples requiring replicate analysis, 17-OHP concentration results are compared to the birthweight-adjusted cutoffs depicted in Table 1. Due to stress related elevations of 17-OHP in the premature population, low birthweight newborns have the highest relative 17-OHP cutoffs as compared with higher birthweight newborns. Upon comparison to birthweight-adjusted 17-OHP cutoffs, a determination is made for each DBS specimen to indicate whether the results were reported as within normal limits, abnormal, or critical. Results reported as within normal limits do not require additional testing or interventions. Abnormal results trigger the request for a repeat DBS specimen to be drawn from the infant and returned to the NBS laboratory as soon as possible for confirmatory testing. Critical results require immediate referral of the infant to the Virginia Department of Health NBS follow-up team and requests for the primary care physician to consult with a pediatric endocrinologist as well as to order diagnostic tests to determine if CAH is present.

Table 1. Birthweight-adjusted 17-OHP cutoffs employed when using the immunoassay alone for CAH screening.

Birthweight Category	Normal 17-OHP (ng/mL serum)	Abnormal 17-OHP (ng/mL serum)	Critical 17-OHP (ng/mL serum)
< 1250 g	< 55	≥ 55 & < 65	≥ 65
1250 – 1749 g	< 40	≥ 40 & < 60	≥ 60
1750 – 2249 g	< 35	≥ 35 & < 45	≥ 45
≥ 2250 g	< 25	≥ 25 & < 45	≥ 45

1.2.5. Issue with primary screening methodology

Due to natural, stress-related, elevations of 17-OHP in newborns caused by prematurity, low birth weight or illness, as well as cross-reactivity of the immunoassay antibodies with interfering compounds, a disproportionately high number of false positive results are reported.[28–33] Adjusting assay cutoff algorithms to include consideration of factors such as birth weight or gestational age are commonly used and have been shown to reduce false positive reporting rates based upon 17-OHP

immunoassay screening, especially among severely premature or low birth weight infants [17], however, false positive rates still remain high relative to many other NBS assays.

1.2.6. Need for 2nd tier analysis

Supplemental 2nd tier analyses of dried blood spot specimens have been shown to reduce false positive rates for CAH in newborn screening programs.[28–33] Second tier assays involve performing additional analysis using the same DBS specimen, without the need to request additional samples. Though sometimes more expensive or time-consuming than 1st tier analyses, 2nd tier assays are often employed to offer improved specificity over 1st tier methods.[12] 2nd tier biochemical screening for CAH involves detection and quantitation of multiple analytes along the adrenal steroidogenesis pathway. By accurately quantitating cortisol along with steroid precursors and androgen, the screening laboratory is able to establish a more comprehensive evaluation of the enzyme function within the adrenal glands and ratios may be used to better differentiate affected cases of CAH, where cortisol production will often be significantly reduced, from unaffected individuals with elevated concentration of steroid precursor unrelated to CAH.

Table 2 depicts concentration ranges for cortisol, steroid precursors, and androstenedione within blood specimens from newborns and infants unaffected by CAH reported to the Human Metabolome Database.[34] References are for human blood and do not necessarily represent analysis of dried blood spots.

Table 2. Blood concentration ranges of cortisol and steroid precursors within newborns and infants unaffected by CAH reported to the Human Metabolome Database.[34]

Analyte	Concentration range in unaffected newborns and infants (ng/mL serum)
17-OHP	0.14 – 30
21-DOC	0.69 – 5.5
11-DOC	< 42
4-AD	0.40 – 8.0
CORT	9.4 – 1200

1.3 LC-MS/MS methods for 2nd tier CAH screening

1.3.1. Reported approaches

The most frequent reports of methodologies employed for 2nd tier CAH analysis in newborn screening involve liquid chromatography with detection by tandem mass spectrometry. Most NBS laboratories are familiar with tandem mass spectrometers as they are commonly utilized to accommodate routine screening for amino acid and acylcarnitine indicators of various metabolic disorders, usually via flow injection analysis (FIA).[4,8] If redundant instrumentation is available, the ability to perform 2nd tier analysis may be possible with slight modifications to existing equipment in order to incorporate liquid chromatography into the analysis. Earlier reported alternatives for detection and quantification of a panel of steroids for CAH diagnosis involve analysis by gas chromatography-mass spectrometry (GC-MS).[35–37] GC-MS methods offer improved specificity over immunoassays, however they often involve complicated sample preparation or derivatization steps as well as longer analysis times, making GC-MS assays less than ideal for high throughput NBS laboratories.

In 2004, Lacey et al. reported a 2nd tier screening assay for CAH that utilized high performance liquid chromatography with tandem mass spectrometry (HPLC-MS/MS) for the detection and quantification of 17-OHP, androstenedione (4-AD), and cortisol from NBS dried blood spots.[28] The assay utilized a 4.8 mm DBS punch and aqueous elution of the blood followed by repeated liquid-liquid extraction using diethyl ether. The extracts were then dried and reconstituted in solvent phase appropriate for reversed phase HPLC. The chromatographic program utilized reversed phase gradient separations on a C_{18} column. The gradient profile was 12 min long in order to fully elute a compound released by the filter paper matrix. The assay used a single internal standard, stable isotope-labeled 17-OHP-D_8. In addition to determination of 17-OHP concentrations, the authors reported that an additional

metric involving calculation of the ratio of the sum of peak areas for 17-OHP plus 4-AD divided by the peak area for cortisol offered added specificity to further reduce false positive results.

In 2007, Janzen et al. from the NBS program in Hanover, Germany, reported an HPLC-MS/MS assay for DBS screening that targeted an expanded panel of steroid markers, including 21-DOC and 11-DOC with a rapid 6 min analysis time per injection.[30] Their use of a 20 mm length C_{18} HPLC column allowed for a short gradient profile. In 2008 the same group reported an expansion of the assay to include quantitation of testosterone and dihydrotestosterone, which are not typically produced in significant amounts within the adrenal glands but are more commonly produced in the gonads. [31,38] They reported the use of two stable isotope-labeled internal standards, 17-OHP-D_8 and cortisol-D_2. The authors reported an algorithm that utilized 21-DOC as the primary marker for detection of CAH due to 21-hydroxylase deficiency, however, other reports suggest that 21-DOC may not always elevate in the absence of significant ACTH stimulation and therefore may not be detected in all cases.[39,40] It is therefore recommended that 21-DOC be used as a secondary marker for CAH screening. By targeting 11-DOC within the assay, the same group reported detection of rare cases of CAH due to 11β-hydroxylase deficiency through newborn screening.

In 2011, the California NBS program reported an HPLC-MS/MS assay for 2nd tier screening of CAH using a C_{18} column with an approximate 8.5 min analysis time per injection.[29] The assay was optimized for detection and quantitation of 17-OHP, 4-AD, cortisol, and secondary analytes 21-DOC and 11-DOC. The inclusion of secondary analytes with the screening algorithm was reported to reduce the impact of the 2nd tier assay by maintaining too many false positive results for CAH screening.

Multiple publications report the use of ultra-high performance liquid chromatography (UHPLC) pumps and columns with sub-2 µm silica particles for the stationary phase support.[32,41] This setup may allow for more rapid and efficient separations than standard HPLC, but can come at an additional cost

for specialized equipment. In 2008, ARUP® Laboratories, in collaboration with the Utah NBS program, reported a 2nd tier CAH screening assay utilizing UHPLC reversed phase separations on a C_{18} column at a run time of approximately 4 min per injection.[32] The Utah report also established the use of a 2nd tier CAH algorithm cutoff based solely on elevated clinical ratio. This was due to results from a prospective study wherein the DBS sample from one newborn affected by CAH showed a 17-OHP concentration within normal limits by the 2nd tier screening assay, however showed a highly elevated clinical ratio. This particular infant had received corticosteroid treatment to combat breathing issues prior to collection of the DBS specimen, possibly leading to reduced levels of 17-OHP. The use of a separate cutoff defined solely by the clinical ratio of concentrations of 17-OHP and 4-AD relative to cortisol is reported to serve as a safeguard in order to detect cases of individuals with CAH whose analyte profiles might be masked by receiving corticosteroid treatment prior to collection. The Utah study also reported use of stable isotope-labeled internal standards for each target compound, which is reported to improve precision and accuracy of calculated concentrations.[33]

In 2015, an LC-MS/MS assay of steroids from DBS was reportedly used to study reference ranges within the population of South Korea.[42] They reported use of a C_{18} stationary phase ligand on a superficially porous (core-shell) stationary phase support. The assay was able to achieve separation of seven steroids, including isobaric species, however the analysis time was reported at 20 min per injection.

Biphenyl stationary phase ligands have been reported to show improved separation of various steroids, including isobaric species.[43] While serum diagnostic assays have been reported, no reports using biphenyl stationary phase ligand for DBS analysis in NBS were found.

1.3.2. Chromatographic resolution of isobars

Chromatographic separation is often necessary when attempting to accurately quantitate isobaric compounds in biochemical analysis using detection by tandem mass spectrometry. Isobars are molecules with the same molecular weight but different structure. If the molecules share the same precursor mass-to-charge ratio (m/z), but significantly differ either in structure or constituent atoms, differentiation by MS/MS experiments may still be possible if a product ion specific to each isobar is able to be isolated and detected. Isobars that share a precursor m/z and also fragment to similar product ions are not able to be differentiated by the mass spectrometer and must first be resolved into distinct analyte bands on-column so they reach the ionization source at different times.

In the case of molecules related to steroidogenesis in the adrenal glands, several isobaric compounds are constitutional isomers that share similar structure, varied only in the positioning of hydroxy groups. In positive electrospray ionization mode, 17-OHP and deoxycorticosterone share the same precursor m/z of 331. These species are usually easily resolved chromatographically. For LC-MS/MS assays that target only 17-OHP, 4-AD, and cortisol, isobaric interferences are not typically of issue. Expanded assays that target 21-DOC or 11-DOC pose a more difficult challenge. 21-DOC, 11-DOC, and another molecule, corticosterone share the same precursor m/z of 347. These three isobars are often more difficult to resolve chromatographically. Some of the reported assays offer poor resolution of isobars.[30,31] Others employ ultra-high performance liquid chromatography (UHPLC) or longer HPLC gradients to adequately resolve isobars. [28,29,32,41,42] Additionally, some of the assays reporting separation and quantitation of 21-DOC and 11-DOC make no mention of corticosterone, which has been shown to elute between 21-DOC and 11-DOC during separations on C_{18} columns. Columns based on a biphenyl stationary phase ligand have been shown to efficiently separate steroids from plasma matrix,[43] but have not been reported as being utilized in NBS 2nd tier assays of DBS.

1.3.3. Reduction of false positive results

The main impact of 2nd tier CAH screening is reduction of false positive NBS reporting. False positive results may cause undue burden on the newborns and their families, the healthcare system, and the NBS system. An overabundance of false positive results may also lead to reduced credibility of screening results. Primary screening assays for CAH only target one molecule, 17-OHP. Immunoassays targeting 17-OHP show cross reactivity for other endogenous compounds commonly found, often at much higher concentrations, in biological matrices. Additionally, 17-OHP is significantly elevated when newborns experience stress due to illness, prematurity, or prolonged delivery. LC-MS/MS 2nd tier analysis offers improved specificity by providing a more comprehensive evaluation of the enzyme system impacted by CAH. 2nd tier LC-MS/MS assays for CAH offer a more specific quantitative value of 17-OHP concentration by differentiating the molecule from endogenous interferences. Quantitation of molecules downstream of the enzymes most often affected by CAH allows for determination of healthy adrenal function relative to true cases of CAH.

1.3.4. Challenges of reported approaches

Some of the reported studies note false negative results. Of greatest concern are false negative results for salt-wasting or simple virilizing cases. Many of the missed cases are attributable to the 1st tier 17-OHP results for the DBS specimen failing to cross analyte cutoffs that trigger 2nd tier analysis. Most 2nd tier assays screening for CAH take into account clinical ratios of precursor analytes such as 17-OHP, 4-AD, or 21-DOC relative to the final product of enzymatic steroidogenesis, cortisol, to aid in differentiation between affected individuals and individuals with normal enzyme function. Cases missed by 2nd tier analysis often show elevated results for 17-OHP but do not show an elevated ratio of precursor analytes relative to cortisol concentration and therefore were not reported as abnormal. In cases of moderate or mild forms of CAH, enough residual enzyme activity may be present to generate

enough cortisol to push ratios lower, resulting in a false negative interpretation. NBS programs must continually look retrospectively at cases successfully diagnosed based on screening algorithms as well as any cases missed by the screening algorithm in order to refine cutoffs or processes to ensure the best possible outcomes. Non-classic CAH is thought to have a much higher prevalence than classic CAH, with some estimates above 1% in some populations, but typically does not result in virilization at birth and is not reliably detected by NBS. Second tier analysis is not anticipated to improve detection rates for non-classic CAH and may, in fact, rule out as normal some cases of non-classic CAH detected by elevated 17-OHP levels alone. Other challenges to reported 2nd tier assays include poor resolution of isobars or long HPLC gradient runs in order to achieve adequate resolution of target compounds.

1.3.5. Importance of timeliness in CAH screening

The most severe, salt-wasting form of CAH can lead to life-threatening adrenal crisis as early as the first few weeks of life. It is therefore imperative that newborn screening for CAH occur quickly. This is especially important for identification of males with CAH, as there may be no physical symptoms of CAH evident at birth, whereas virilized females will present with ambiguous genitalia at birth. CAH is among the NBS conditions considered to be time-critical. Current national timeliness guidelines recommend that all NBS programs aim to report presumptive positive results for time-critical NBS conditions at no later than five days of life.[6] This gives the greatest opportunity to get newborns under care and treatment before the onset of life-threatening symptoms. To achieve this goal, emphasis is placed on timely collection of NBS specimens, rapid and reliable transport to the testing laboratory, and that screening methods and algorithms occur with as little delay as possible while maintaining high-quality results.

1.4 Project goals

The primary objectives for this work were development of a 2nd tier CAH screening assay using HPLC-MS/MS and validation of the method for implementation within Virginia's NBS laboratory. The validated assay should utilize existing instrumentation available within the laboratory with minimal modifications, should offer acceptable sensitivity for the most severe forms of CAH while greatly reducing false positive reporting, and should offer rapid throughput to enable timely reporting of results. Another aim of this work was to improve upon reported HPLC conditions to achieve baseline resolution of isobaric interferences while maintaining a rapid chromatographic separation.

Several steps were involved in the systematic optimization of the method and subsequent validation. First, electrospray ionization and fragmentation parameters in the tandem mass spectrometer were optimized for each target compound, each stable isotope-labeled internal standard, as well as know isobaric interferences. Next, chromatographic conditions were optimized to select stationary phase ligand and gradient conditions. HPLC column temperature settings were also optimized. Next, DBS extraction and sample preparation conditions were optimized to ensure adequate sensitivity and reproducibility. Finally, matrix-matched DBS calibration, quality control, and method validation materials were produced. Following optimization, method validation experiments were conducted to provide documented evidence that the assay would consistently provide accurate data when placed into full production.

CHAPTER 2: METHOD DEVELOPMENT

Method development experiments followed a systematic approach for obtaining optimal instrument parameters for analysis of dried blood spots for targeted steroid markers. The primary goals of the method development process were to utilize existing instrumentation within the NBS laboratory with minimal modifications and to achieve a rapid analysis time that would accommodate a high throughput workflow while maintaining acceptable sensitivity for target compounds and optimal separation of known isobaric interferences.

2.1 MS/MS compound optimization using direct infusion

Direct infusion experiments were performed using a syringe pump set to a flow rate of 10 µL/min. The infusion syringe was connected directly to the electrospray ionization source via a length of PEEK tubing.

2.1.1. Infusion stock preparation

Analytical standards of 17-OHP, cortisol, 4-AD, 11-DOC, and corticosterone, each at 1.0 mg/mL, were diluted to 1.0 µg/mL in methanol as intermediate stocks. Each intermediate stock was further diluted to 200 ng/mL in 50:50 methanol: water with 0.1% formic acid to produce individual infusion standards.

Analytical standards of 21-DOC, deoxycorticosterone, cortisol-D_4, 17-OHP-D_8, 21-DOC-D_8, and 11-DOC-D_5, each at 100 µg/mL in methanol, were diluted to 200 ng/mL in 50:50 methanol: water with 0.1% formic acid to produce individual infusion standards.

An analytical standard of 4-AD-$^{13}C_3$ at 100 µg/mL acetonitrile was diluted to 200 ng/mL in 50:50 methanol: water with 0.1% formic acid to produce an individual infusion standard.

2.1.2. Precursor ion isolation

A Sciex API 4000 (Framingham, MA, USA) triple quadrupole mass spectrometer equipped with an electrospray ionization source was utilized for all mass spectrometry experiments. To isolate the precursor ion for each analyte and internal standard, each 200 ng/mL infusion standard was individually introduced into the electrospray ionization source in positive ionization mode using the same ionization parameters: electrospray (ES) voltage 5500 V, nebulizer gas (GS1) 19 arbitrary units, declustering potential (DP) 60 V, entrance potential (EP) 10 V. Scanning only quadrupole 1 (Q1), the $[M+H]^+$ precursor ion mass-to-charge ratio (m/z) was isolated for 10 Multi Channel Analysis (MCA) events. Precursor ions for each analyte are noted in Table 3, and structures are depicted in Figure 4.

Table 3. Precursor ions

Analyte	Precursor ion (m/z)
4-AD	287.1
4-AD-$^{13}C_3$	290.2
17-OHP	331.3
17-OHP-D_8	339.2
Deoxycorticosterone	331.3
21-DOC	347.1
21-DOC-D_8	355.2
11-DOC	347.1
11-DOC-D_5	352.1
Corticosterone	347.3
Cortisol	363.1
Cortisol-D_4	367.1

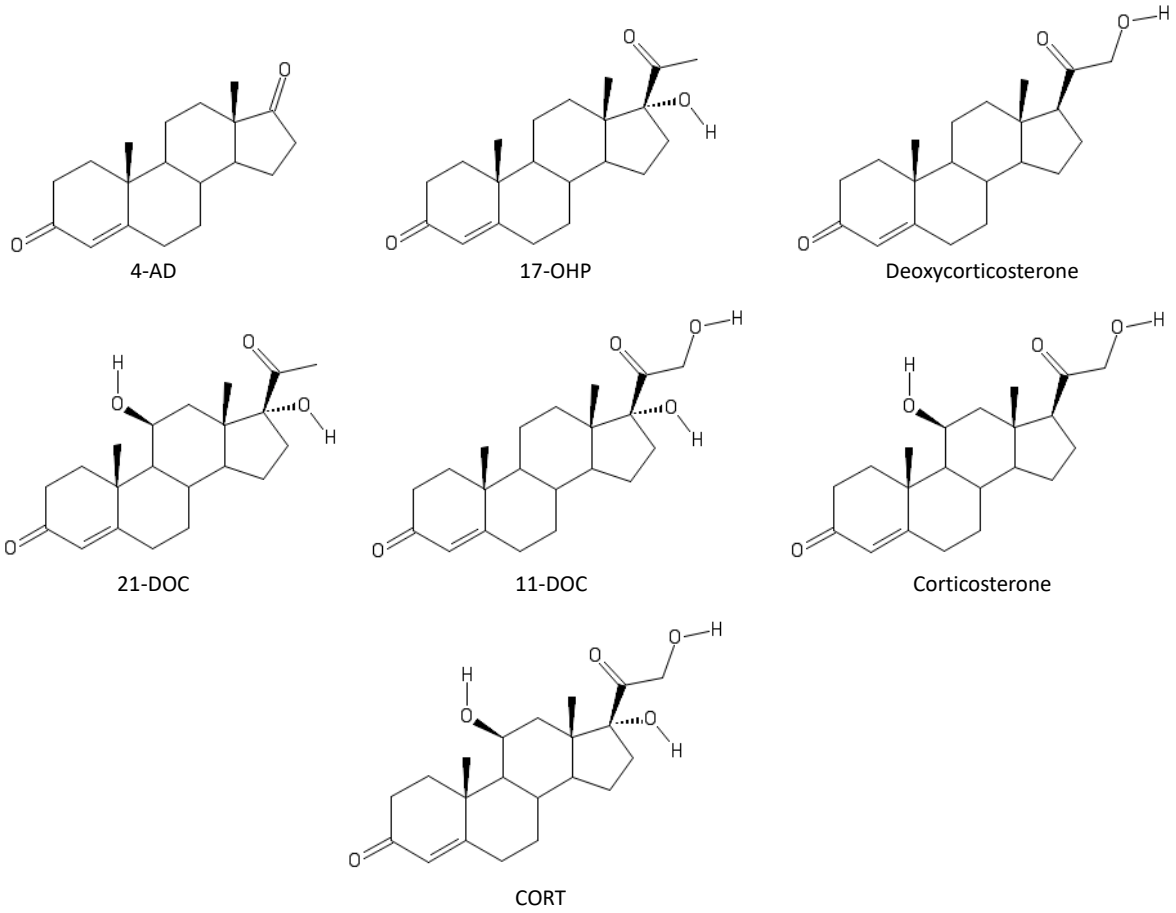

Figure 4. Chemical structures of target analytes and isobaric interferences.

2.1.3. Product ion scanning

Following isolation of precursor ions using Q1 scans in positive ion mode, a product ion profile was identified for each analyte using the product ion scan function. Each 200 ng/mL infusion standard was individually introduced into the electrospray ionization source in positive ionization mode using the same ionization parameters: electrospray (ES) voltage 5500 V, nebulizer gas (GS1) 19 arbitrary units, declustering potential (DP) 60 V, entrance potential (EP) 10 V, collision energy (CE) 25 V, collision cell exit potential (CXP) 7 V. In product ion scan mode, the precursor ion for each analyte was entered for isolation in Q1 and fragmentation in the collision cell (Q2). Q3 was then scanned over a large mass range

to allow for detection of possible product ion m/z values. The four to six most abundant product ions for each analyte, indicated in Table 4 were selected for further compound optimization.

Table 4. Most abundant product ions

Analyte	Product Ions (m/z)
4-AD	97.0, 109.0, 251.0, 269.1
4-AD-$^{13}C_3$	100.0, 112.0, 126.0, 271.9
17-OHP	97.0, 109.0, 295.1, 313.0
17-OHP-D_8	100.0, 113.2, 175.1, 321.0
Deoxycorticosterone	97.1, 109.2, 295.1, 313.2
21-DOC	121.1, 175.1, 269.2, 311.1, 329.1
21-DOC-D_8	125.0, 180.1, 275.1, 319.0
11-DOC	97.0, 109.0, 328.9, 311.0, 299.0
11-DOC-D_5	100.0, 112.8, 316.2, 334.1
Corticosterone	96.9, 120.8, 135.0, 293.0, 311.0, 329.2
Cortisol	96.8, 120.9, 267.0, 309.0, 326.9
Cortisol-D_4	121.0, 313.2, 330.9, 349.2

Additionally, a more comprehensive study of product ions was initiated for isobaric compounds to determine if any unique product ions might be identified for each compound to aid with differentiation by the mass spectrometer alone. 11-DOC, 21-DOC, corticosterone, 17-OHP, and deoxycorticosterone were evaluated using product ion scans at collision energies between 5 V and 50 V at 5 V intervals. The product ion profiles for 11-DOC, 21-DOC, and corticosterone were compared to determine if any unique product ions were observed. Figure 5 shows representative product ion scans for 11-DOC, 21-DOC, and corticosterone at collision energy of 25 V. The isolated precursor mass-to-charge ratio was 347 m/z for all three compounds. Similarly, the product ion profiles for 17-OHP and deoxycorticosterone were compared. No unique product ions at sufficient abundance for quantitation were identified, reinforcing the need to achieve baseline chromatographic resolution of isobars prior to detection by mass spectrometry.

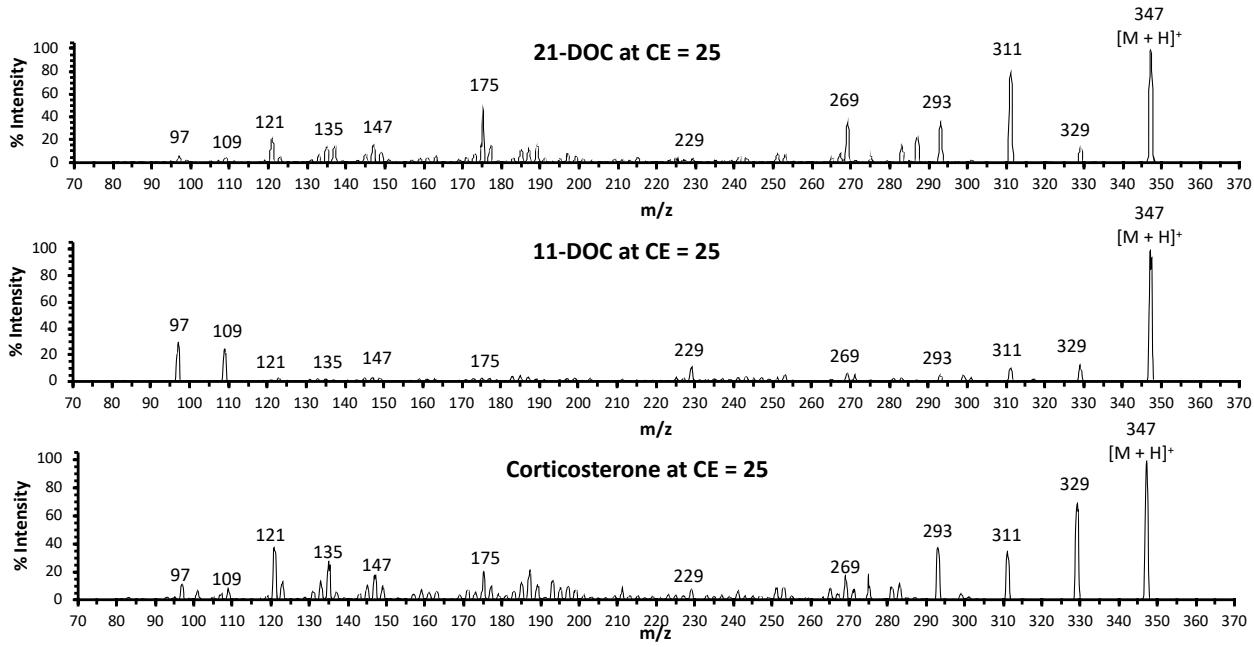

Figure 5. Representative product ion spectra of isobaric compounds. Isolated precursor ion = 347 m/z. Collision energy = 25 V.

2.1.4. Compound optimization

Isolation and fragmentation conditions of potential target product ions identified for each analyte, listed in Table 4, were further optimized using compound optimization features within the instrument software. The isolation and fragmentation parameters that were optimized for each ion transition were declustering potential (DP), collision energy (CE), and collision cell exit potential (CXP). For each analyte, a standard solution at 200 ng/mL concentration was directly infused into the electrospray source using a syringe pump. The precursor-to-product ion transitions specific to that analyte were entered into the software. The software then systematically ramped through each parameter, selecting as optimal the parameter setting that yielded the greatest detector signal for the ion transition. This process was repeated for all analytes and internal standards for all candidate ion transitions. Results of the compound optimizations were used when assigning multiple reaction monitoring (MRM) parameters for the full instrument acquisition method, explained in Chapter 3.

2.2 Electrospray ionization source parameter optimization using flow injection analysis

Electrospray ionization source parameter optimization was used to determine optimal electrospray source settings that were not compound specific and was performed using flow injection analysis (FIA) features within the instrument software compound optimization environment. An acquisition method incorporating optimized MRM parameters for each target analyte, internal standard, and potential isobaric interference was created and a short (1 min) isocratic HPLC run at 500 µl/min flow rate was added to the method parameters. The autosampler was set to load 5 µL into the injection loop for each injection. In FIA, a small volume of sample was introduced into a flow of mobile phase before moving directly to the electrospray ionization source, without separation on-column. The standard used for FIA optimization was made by mixing equal volumes of the 200 ng/mL infusion standards for each analyte, internal standard, and isobaric interference, yielding a final concentration for all compounds of 16.7 ng/mL in 50:50 methanol: water with 0.1% formic acid. With each injection in the FIA experiment, the setting for a single source parameter is changed. Each parameter setting was analyzed in duplicate and results for all ion transitions were compared to assess which set point for each parameter yielded the greatest overall signal across all analyte transitions. Source parameters that were optimized include: electrospray voltage, nebulizer gas (GS1), heater gas (GS2), desolvation temperature, and curtain gas. The setpoints tested for each parameter are summarized in Table 5. The collision assisted dissociation (CAD) gas parameter was set to 6 for all experiments.

Table 5. Electrospray ionization source parameter optimization FIA test points

Parameter	Test Points
Electrospray positive voltage (V)	5500, 5000, 4500, 4000
GS1 (arbitrary units)	50, 45, 40, 35
GS2 (arbitrary units)	40, 35, 30, 25
Desolvation temperature (°C)	550, 500, 450, 400
Curtain gas (arbitrary units)	45, 40, 35, 30

FIA source parameter optimization was performed using two mobile phase constituents. Mobile phase A was water with 0.1% formic acid. Mobile phase B was methanol with 0.1% formic acid. FIA experiments were originally performed with isocratic mobile phase settings at 70% B and were then later repeated with isocratic mobile phase settings at 50% B. Optimized source parameter settings at each isocratic mobile phase formulation are summarized in Table 6. Because early chromatographic optimization experiments with C_{18} stationary phase ligand indicated that the earliest eluting target compounds would elute near 50% B, optimized source parameters at 50% B mobile phase conditions were carried through remaining experiments.

Table 6. Optimized electrospray ionization parameters from FIA experiments

Parameter	70% B	50% B
Electrospray positive voltage (V)	5000	4000
GS1 (arbitrary units)	40	45
GS2 (arbitrary units)	40	40
Desolvation temperature (°C)	500	550
Curtain gas (arbitrary units)	35	45

2.3 Chromatographic optimization

Among the goals for development of an LC-MS/MS assay for 2nd tier screening of CAH were to utilize existing instrumentation within the laboratory, making as few modifications as possible, and to achieve an analysis run time that would accommodate high-throughput analysis and the most rapid possible turnaround times. Existing instrumentation within the newborn screening laboratory included Prominence LC-20AD pumps (Shimadzu, Kyoto, Japan), which have a maximum pressure limit of 400 bar (5801 psi). While ultra-high pressure liquid chromatography (UHPLC) has been shown to effectively separate the isobars associated with CAH analysis, the 400 bar pressure limit of the LC-20AD pumps is

not compatible with traditional UHPLC backpressures utilizing fully-porous stationary phase support materials.[44]

HPLC columns with superficially porous stationary phase support materials were, therefore, investigated. Superficially porous stationary phase supports consist of an inner sphere of solid silica with a thin layer of porous silica deposited on the surface.[45] Columns packed with superficially porous stationary phase support can allow for the use of smaller particle sizes and higher mobile phase flow rates than would typically be possible at backpressures within acceptable limits of HPLC equipment. It was predicted that some of the benefits of UHPLC analysis could be attained without the need to purchase UHPLC equipment.

HPLC columns from the Kinetex™ line from Phenomenex (Torrance, CA, USA) utilize superficially porous stationary phase support material and were incorporated into this study. Two stationary phase ligands were analyzed to assess viability as a column option for 2nd tier CAH analysis. The Kinetex™ C18 column was selected as a comparison to columns previously reported in the literature for 2nd tier CAH analysis.[28,30–32,44] The Kinetex™ Biphenyl column was selected to determine if the biphenyl stationary phase ligand offered a more effective separation of isobaric compounds.[43,46] The column dimensions selected for analysis were 2.6 µm particle size, 50 mm length, and 3.0 mm column internal diameter.

2.3.1. C_{18} stationary phase ligand

A Kinetex™ C18, 2.6 µm, 50 x 3.0 mm HPLC column provided by Phenomenex (Torrance, CA, USA) was installed in-line between the autosampler injection valve and the electrospray ionization source. The column was kept at room temperature and subjected to a mobile phase gradient at a flow rate of 500 µL/min. Mobile phase constituent A consisted of water with 0.1% formic acid. Mobile phase constituent B consisted of methanol with 0.1% formic acid. A mixed analytical standard, consisting of all target analytes, internal standards, and isobaric interferences, each at a concentration of 16.7 ng/mL in

50:50 methanol: water with 0.1% formic acid was used for chromatography optimization studies. Of greatest concern was the resolution of isobaric compounds from one another and the overall chromatographic run time. The resolutions of the three isobars, 21-DOC, 11-DOC, and corticosterone (precursor m/z = 347.1) and isobars 17-OHP and deoxycorticosterone (precursor m/z = 331.3) were calculated using Equation 1:

Equation 1. Chromatographic resolution equation

$$R_s = 1.18 \times \left(\frac{t_{R2} - t_{R1}}{w_{0.5h1} + w_{0.5h2}}\right)$$

R_s is peak resolution, t_{R1} is the retention time for peak 1, t_{R2} is the retention time for peak 2, $w_{0.5h1}$ is the full width at half maximum (FWHM) for peak 1, and $w_{0.5h2}$ is the FWHM for peak 2.

An initial exploratory gradient, depicted in Table 7, was used to evaluate the resolution capability of the column. Under these gradient conditions, the column showed adequate resolution of 17-OHP and deoxycorticosterone, R_s = 2.75. However, 21-DOC was not fully resolved from its isobars, R_s = 1.11, and there was coelution between 11-DOC and corticosterone, shown in Figure 6.

Table 7. Initial exploratory gradient for C_{18} column

Time (min)	%A	%B
0.01	40	60
1.00	30	70
3.50	25	75
4.00	5	95
4.50	5	95
4.60	40	60
5.00	40	60

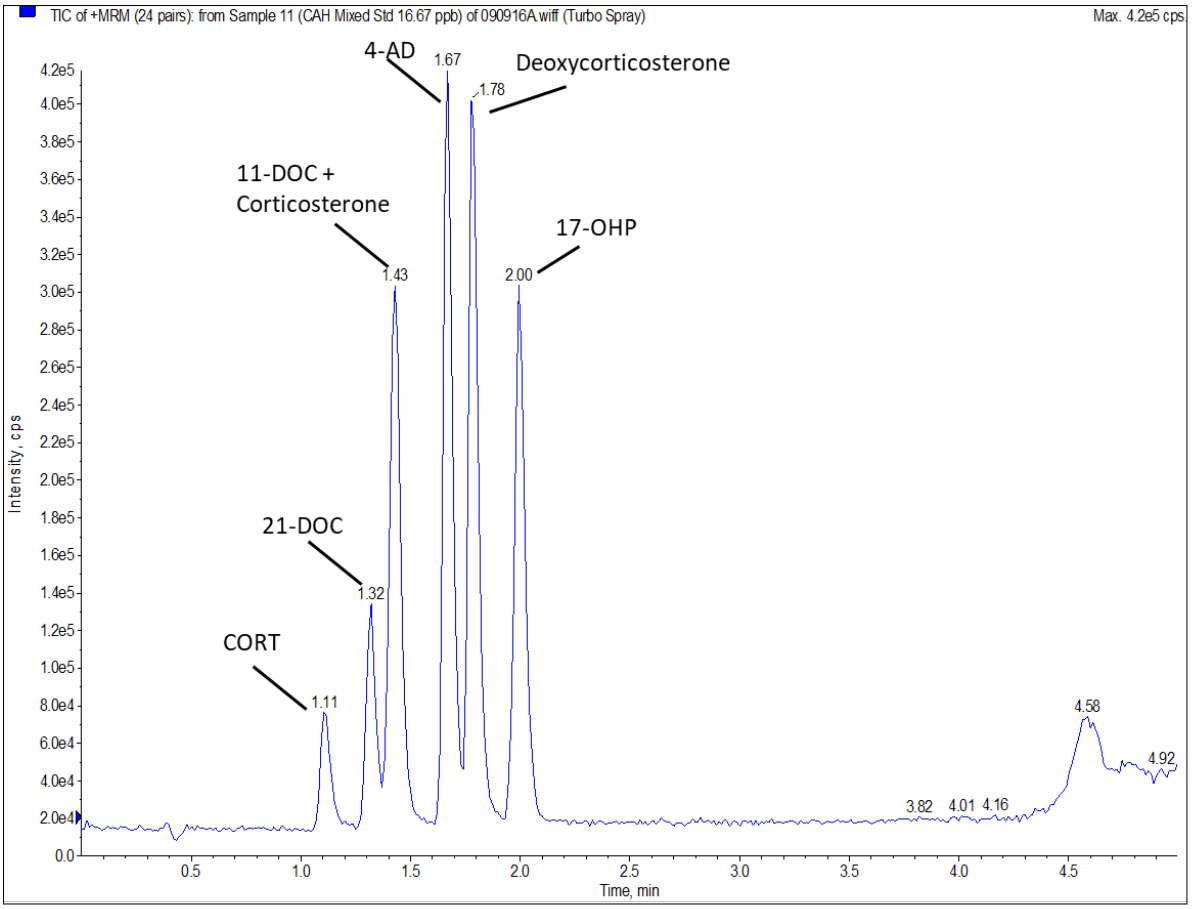

Figure 6. Representative chromatographic profile of C_{18} column using initial exploratory gradient described in Table 7 depicted by the total ion chromatogram for all MRM transitions.

The mobile phase gradient was then extended, indicated in Table 8, incorporating a smaller slope of increase in mobile phase B over time in an attempt to allow for longer retention of isobars for increased resolution. While resolution of 17-OHP and deoxycorticosterone was improved at R_s = 4.62, the longer gradient failed to adequately separate 11-DOC from corticosterone. 21-DOC was fully resolved from 11-DOC and corticosterone using the long gradient, R_s = 2.11. Figure 7 shows a representative chromatogram of C_{18} separations using the long gradient depicted by the total ion chromatogram for all MRM transitions.

Table 8. Long gradient for C_{18} separations

Time (min)	%A	%B
0.01	40	60
1.00	40	60
8.50	25	75
9.00	5	95
9.50	5	95
9.60	40	60
10.50	40	60

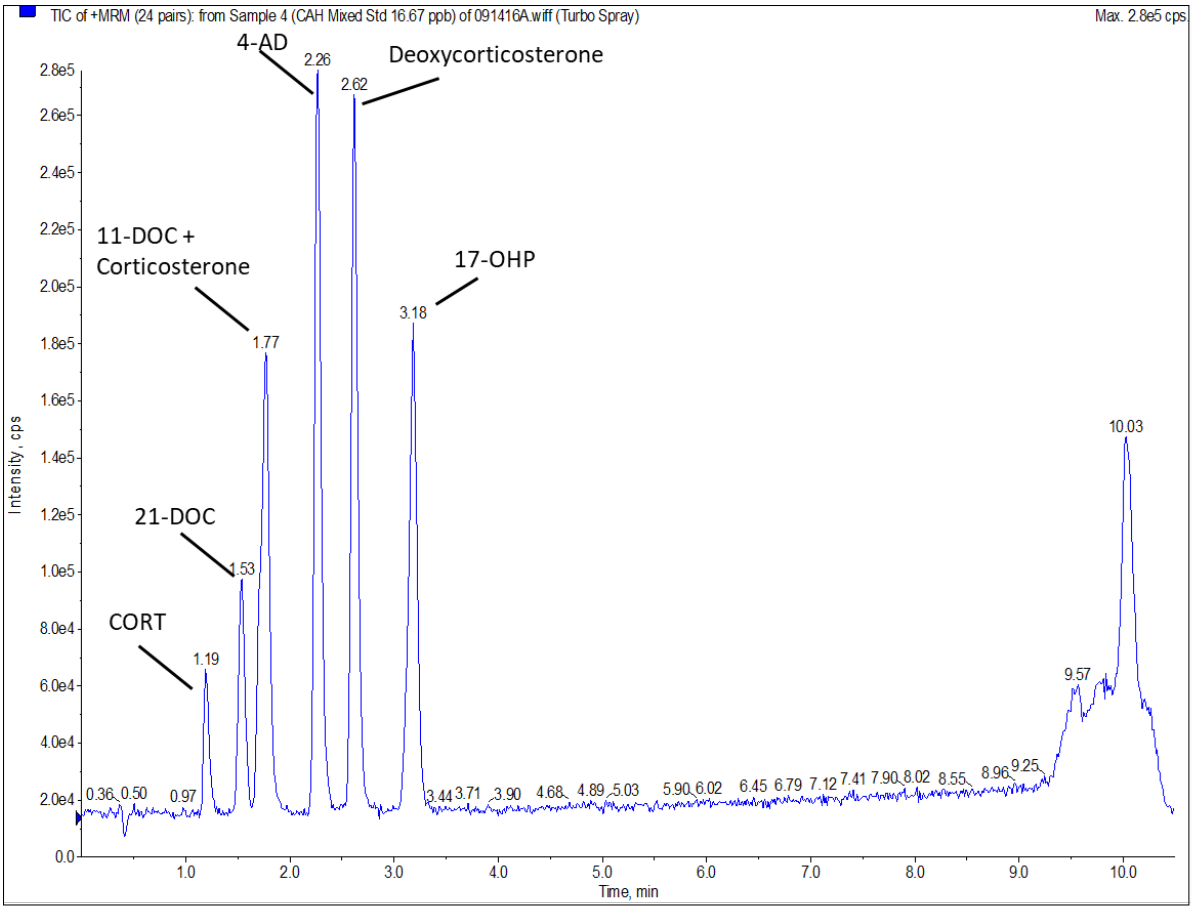

Figure 7. Representative chromatographic profile of C_{18} separations using long gradient described in Table 8 depicted by the total ion chromatogram for all MRM transitions.

As indicated by the initial exploratory and long gradients, the analytes of interest were eluting from the C_{18} column quite early in the gradient profile, before reaching high organic concentrations. Therefore, a series of gradients were explored that employed a long isocratic hold at the beginning of

the gradient profile. Baseline resolution of 21-DOC, corticosterone, and 11-DOC was achieved with a 5 min isocratic hold at 50% B followed by ramp up to 95%, as indicated in Table 9. Figure 8 is a representative chromatogram of analyte separations using the long initial isocratic hold depicted by the total ion chromatogram for all MRM transitions. Though baseline resolution was achieved between 21-DOC and corticosterone, R_s = 2.53, and between corticosterone and 11-DOC, R_s = 2.24, the drawbacks to this gradient profile were band broadening and lower sensitivity for the compounds that elute within the isocratic hold region. Additionally, the long run time of over 10 min was not conducive to high-throughput analysis.

Table 9. Gradient with long initial isocratic hold for C_{18} separations

Time (min)	%A	%B
0.01	50	50
5.00	50	50
8.50	25	75
9.00	5	95
9.50	5	95
9.60	50	50
10.50	50	50

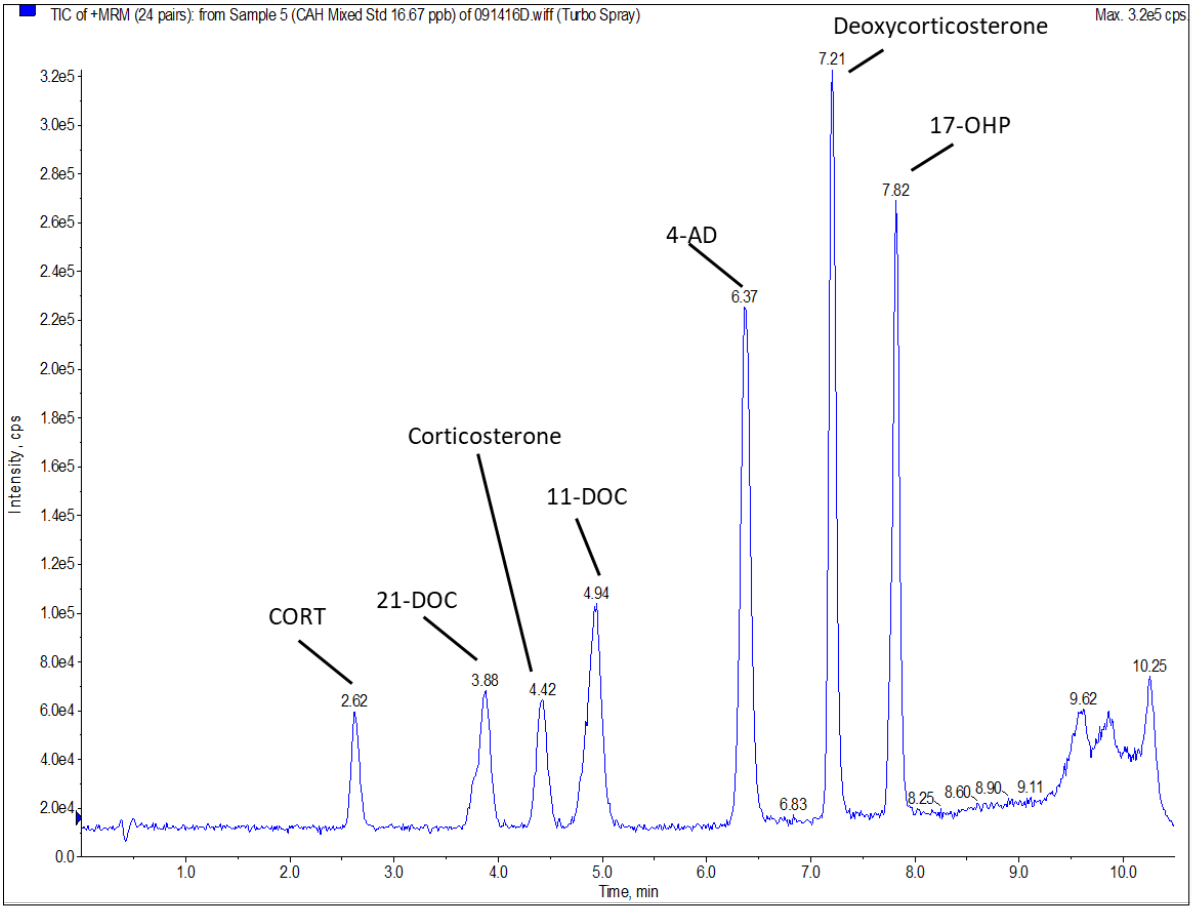

Figure 8. Representative chromatographic profile of C_{18} separations using gradient with long initial isocratic hold at 50% B described in Table 9 depicted by the total ion chromatogram for all MRM transitions.

Finally, adjustments were made to the gradient profile to incorporate an isocratic hold at the beginning of the run but shorten the overall run time closer to 5 min per run. Unfortunately, none of the attempted gradient modifications were able to achieve a shorter run time while maintaining baseline resolution of isobars.

2.3.2. Biphenyl stationary phase ligand

A Kinetex™ Biphenyl, 2.6 µm, 50 x 3.0 mm HPLC column provided by Phenomenex (Torrance, CA, USA) was installed in-line between the autosampler injection valve and the electrospray ionization source. The column was kept at room temperature and subjected to a mobile phase gradient at a flow rate of 500 µL/min. Mobile phase constituent A consisted of water with 0.1% formic acid. Mobile phase constituent B consisted of methanol with 0.1% formic acid. A mixed analytical standard, consisting of all target analytes, internal standards, and isobaric interferences, each at a concentration of 16.7 ng/mL in 50:50 methanol: water with 0.1% formic acid was used for chromatography optimization studies.

A long gradient, summarized in Table 10, was employed initially in order to establish retention characteristics of the biphenyl stationary phase ligand. As indicated in Figure 9, all compounds were well retained on the column and baseline resolution was achieved for all isobars. 11-DOC and corticosterone were identified as the critical pair of closest eluting isobars. Baseline resolution, R_s = 2.08, of the critical pair was achieved under these conditions. 4-AD and deoxycorticosterone coelute on the biphenyl column, however these compounds are easily differentiated by the mass spectrometer due to differing precursor ions.

Table 10. Long gradient for biphenyl separations

Time (min)	%A	%B
0.01	80	20
8.00	5	95
8.50	5	95
8.51	80	20
10.00	80	20

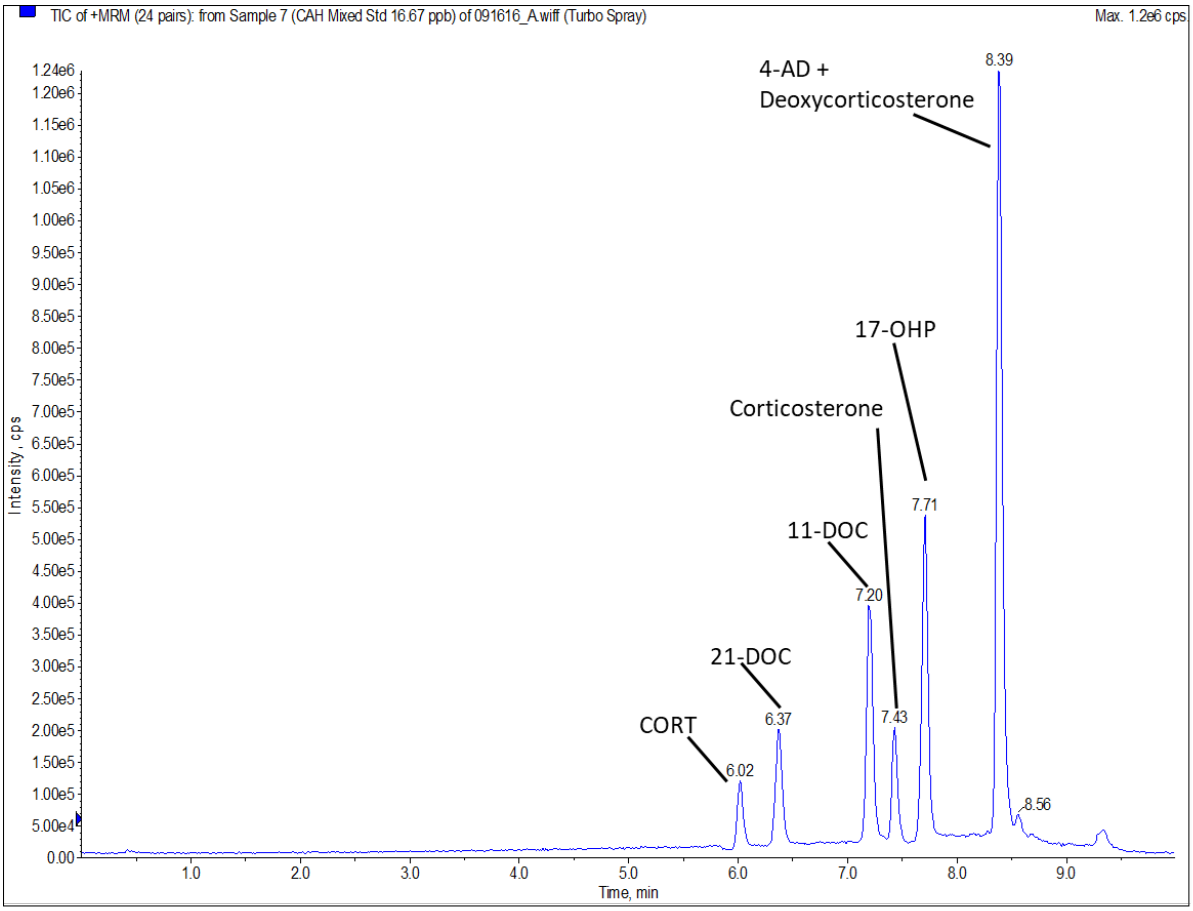

Figure 9. Representative chromatographic profile of biphenyl separations using long gradient described in Table 10 depicted by the total ion chromatogram for all MRM transitions.

The gradient was then modified to shorten the overall run time while attempting to maintain baseline resolution of the critical pair. As indicated in Table 11, the shortened gradient began with 50% mobile phase B and ramped to 95% B over 4.5 min. The overall runtime was reduced to 6 min and adequate resolution was maintained. Figure 10 shows a representative chromatogram of biphenyl separations using the shortened gradient depicted by the total ion chromatogram for all MRM transitions. Baseline resolution of the critical pair was maintained, R_s = 2.21, under these modified gradient conditions.

Table 11. Shortened gradient for biphenyl separations

Time (min)	%A	%B
0.01	50	50
4.50	5	95
5.00	5	95
5.01	50	50
6.00	50	50

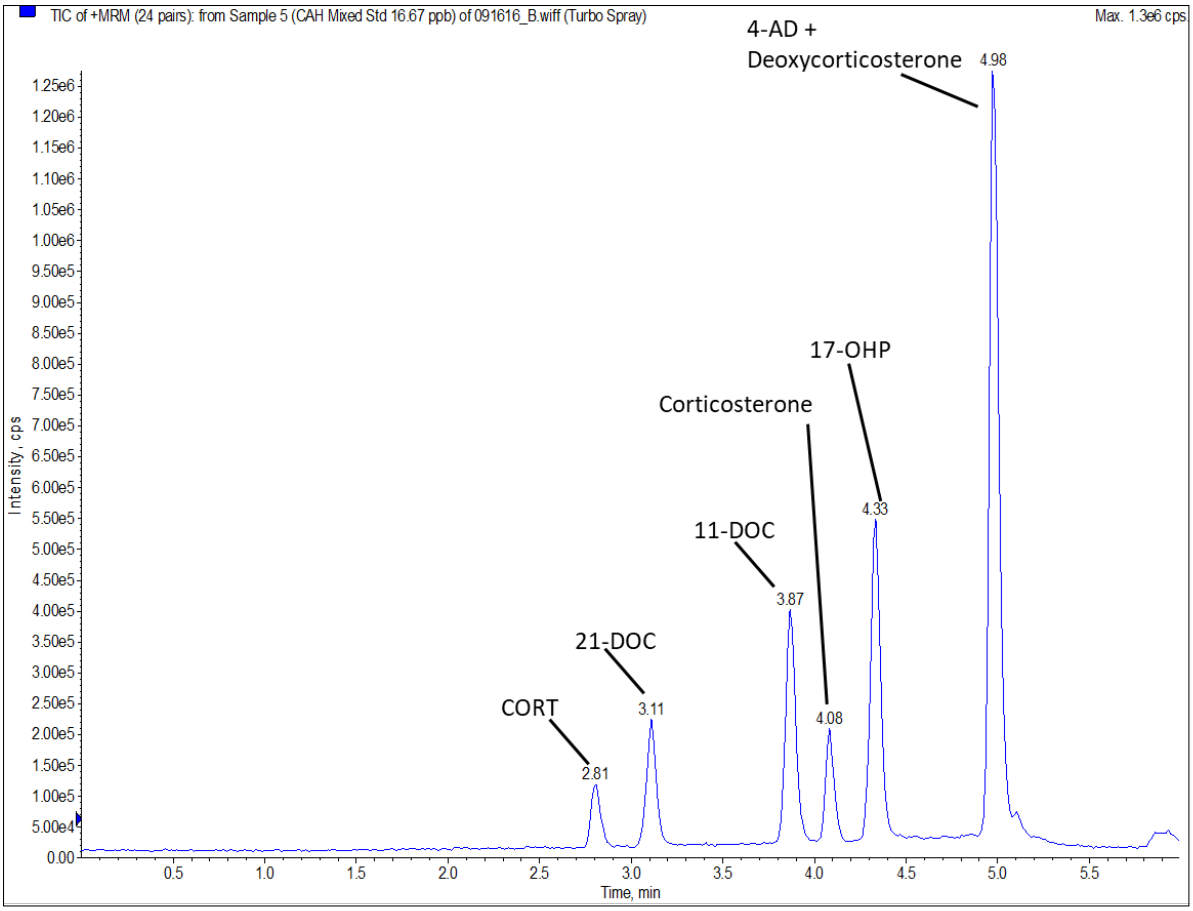

Figure 10. Representative chromatographic profile of biphenyl separations using shortened gradient described in Table 11 depicted by the total ion chromatogram for all MRM transitions.

The biphenyl stationary phase ligand achieved better resolution of isobaric compounds within a shorter run time compared to the C_{18} stationary phase ligand. The biphenyl column was therefore selected for further optimization and validation.

2.3.3. Column temperature optimization

After selection of biphenyl as the optimal stationary phase ligand, HPLC column temperature optimization was performed. By using a column heater, a more consistent column temperature is maintained as compared to ambient room conditions and higher column temperatures can allow for faster separations at lower backpressure. Replicate injections (N = 5) of a mixed analytical standard, consisting of all target analytes, internal standards, and isobaric interferences, each at a concentration of 16.7 ng/mL in 50:50 methanol: water with 0.1% formic acid were performed under the same mobile phase gradient conditions, varying only the temperature of the HPLC column heater. Experiments were performed at room temperature (21), 30, 40, 50, and 60 °C. Results for retention time, peak width, and resolution of the critical isobaric pair were compared at all temperatures.

All data metrics were acceptable at each of the comparative column temperatures. A column temperature of 50 °C was selected due to faster elution as compared to room temperature which maintaining baseline separation of the critical pair. Additionally, a column temperature of 60 °C was avoided due to this being the maximum allowable column temperature to avoid significant column degradation as indicated by the manufacturer. Using the optimized column temperature of 50 °C, the biphenyl gradient conditions were further modified to the final conditions presented in Table 12, with a 4.5 min run time. The increased retention of the target compounds on the biphenyl stationary phase ligand as compared to the C_{18} stationary phase ligand also allowed for the mobile phase gradient profile to be further modified to begin with a higher (65%) organic concentration in the mobile phase, which allowed for a shorted overall gradient profile as well as faster equilibration back to initial conditions following elution of the last compounds from the column. Figure 11 shows a representative chromatogram of biphenyl separations using the shortened gradient depicted by the total ion chromatogram for all MRM transitions.

Table 12. Fully optimized mobile phase gradient conditions.

Time (min)	A%	B%	Flow Rate (µL/min)
0.00	35	65	500
3.00	5	95	500
3.50	5	95	500
3.51	35	65	500
4.50	35	65	500

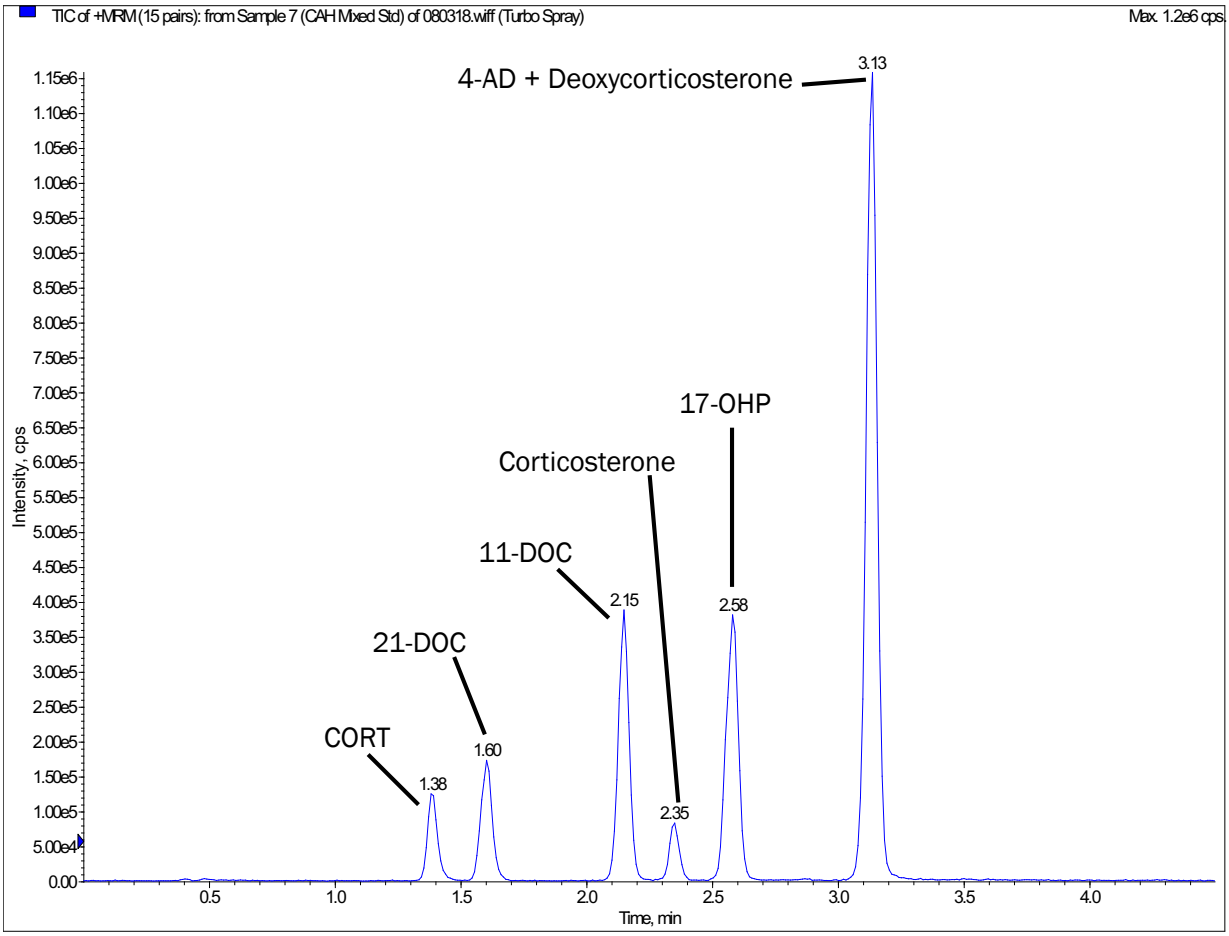

Figure 11. Representative chromatographic profile of biphenyl separations using fully optimized gradient conditions and heated column as described in Table 12, depicted by the total ion chromatogram for all MRM transitions.

2.4 DBS extraction optimization

2.4.1. DBS Extraction Solution formulation

Based upon extraction solution formulations reported in the literature,[28,30,32,41] initial DBS extraction experiments were performed using a formulation consisting of 80:20 acetonitrile: water and a non-aqueous formulation consisting of 50:50 acetonitrile: methanol. Each formulation was enriched to 2 ng/mL of each stable isotope-labeled internal standard. DBS Quality Control (QC) materials provided by the Newborn Screening Quality Assurance Program at the Centers for Disease Control and Prevention (CDC NSQAP) were extracted and analyzed by the optimized LC-MS/MS method. The formulation of 80:20 acetonitrile: water showed better signal during comparative analysis and was selected for further optimization (data not shown).

Formulations of DBS Extraction Solution containing 70:30 acetonitrile: water, 80:20 acetonitrile: water, and 90:10 acetonitrile: water were prepared, each enriched to 2 ng/mL of each stable isotope-labeled internal standard. DBS QC materials from CDC NSQAP were extracted and analyzed using the optimized LC-MS/MS method. 80:20 acetonitrile: water with 2 ng/mL of each internal standard was selected as the final DBS Extraction Solution formulation.

2.4.2. DBS extraction procedure optimization

The DBS extraction procedure was optimized using DBS QC materials from CDC NSQAP. DBS extraction steps mostly follow those previously reported in the literature.[30,32,41] DBS eluate transfer volume and final reconstitution volume began at 150 µL and 80 µL, respectively but were optimized to 175 µL and 50 µL, respectively in order to maximize assay signal-to-noise at low analyte concentrations. The plate drying time was optimized to 20 min to reach complete dryness. It was determined that the final reconstituted volume needed to be transferred to a low-volume 96-well plate in order to enable a

higher liquid column height in each well to ensure the autosampler needle can reproducibly draw the full 20 µL injection volume from each extract well. Plate centrifugation was utilized to help remove residual particulate matter from extraction of DBS punches as well as to remove air bubbles from the low-volume 96-well plate prior to loading extracts on the instrument.

2.4.3. Optimization of number of DBS punches per well

2nd tier CAH methods reported in the literature often report the use of large DBS punches, 4.8 mm or 6.0 mm in diameter, in order to ensure an adequate volume of blood within the extract to reach assay sensitivity requirements.[28,30–32] Large DBS punches, however, can create logistical problems within the NBS laboratory. The ability to punch large DBS punches may be limited by the area available within a dried blood spot. This can be particularly problematic for 2nd tier analyses as DBS have often already received several punches for 1st tier analysis prior to 2nd tier testing being initiated. Similarly, removing a large DBS punch from a specimen limits the area of dried blood available for subsequent analysis. Additionally, many NBS assays utilize 3.2 mm (1/8 in) punches, yielding approximately 3.1 µL blood per punch. Because this is the most common punch size, most automated or semi-automated punching equipment is preconfigured with 3.2 mm punch heads. Moving to a larger punch size may require installation of a different punch head. For these reasons, the NBS laboratory opted to utilize a 3.2 mm punch size for 2nd tier CAH analysis.

Initial method optimization work was performed using two 3.2 mm DBS punches per well, yielding approximately 6.2 µL blood per well. Low concentration DBS materials enriched by the NBS laboratory were subsequently used to determine if additional punches were needed to achieve acceptable signal-to-noise at the lower limit of quantitation for the assay. By a comparative study, it was determined that three 3.2 mm punches per well, yielding approximately 9.3 µL blood per well, were

necessary to reliably achieve acceptable signal-to-noise at low analyte concentrations. Subsequently, the assay was validated using three 3.2 mm DBS punches per well.

2.5 Summary of method development

The method development process yielded comprehensive method parameters that achieved the main goals for the assay. The core-shell biphenyl column allowed for baseline resolution of isobars utilizing a rapid gradient profile at system backpressures amenable to HPLC equipment. The only major modification to the existing HPLC-MS/MS instrumentation was addition of a column heater. Following optimization of the method parameters, the assay needed to be validated prior to full implementation for analysis of patient DBS specimens.

CHAPTER 3: EXPERIMENTAL

3.1 Chemicals and reagents

Cortisol, 17-OHP, 4-AD, 21-DOC, 11-DOC, corticosterone, and 11-deoxycorticosterone (deoxycorticosterone) analytical standards as well as cortisol-D_4, 17-OHP-D_8, 4-AD-$^{13}C_3$, 21-DOC-D_8, and 11-DOC-D_5 isotope-labeled internal standards were purchased from Cerilliant Corporation (Round Rock, TX, USA). Optima LC-MS grade water, Optima LC-MS grade methanol, and Optima LC-MS grade acetonitrile were purchased from Fisher Scientific (Pittsburg, PA, USA). Formic acid was purchased from Acros Organics (Fairlawn, NJ, USA). Washed packed human red blood cells and whole blood were purchased from ZenBio (Research Triangle Park, NC, USA). Double charcoal-stripped defibrinated human plasma was purchased from SeraCare (Milford, MA, USA). Unbuffered sterile saline was purchased from Moltox Molecular Technology, Inc. (Boone, NC, USA).

3.2 Materials and equipment

Pools of hematocrit-adjusted steroid-depleted human blood used for dried blood spot calibrator and quality control specimen production were prepared by washing red blood cells and combining with double charcoal-stripped defibrinated human plasma. Units of washed packed human red blood cells or human whole blood were aliquoted to 50 mL polypropylene conical tubes purchased from Becton Dickinson (Franklin Lakes, NJ, USA). An equal volume of sterile saline was added to each tube and mixed

using an open-air rocker purchased from Fisher Scientific (Pittsburg, PA, USA). The tubes were then centrifuged using an Allegra X-14R centrifuge by Beckman Coulter, Inc. (Brea, CA, USA). The plasma or saline fraction was removed from each tube using an EV320 Evac Waste System by Argos Technologies, Inc. (Vernon Hills, IL, USA). Blood units were washed and least three times. Hematocrit levels were evaluated using a HemataStat II hematocrit centrifuge from EKF Diagnostics (Stanbio Laboratory, Boerne, TX, USA).

A Wallac DBS Puncher from PerkinElmer (Waltham, MA, USA) was used to punch 3.2 mm discs from dried blood spots into 96-well round bottom polypropylene plates. All pipetting steps were carried out using single and multichannel Finnpipette F2 adjustable-volume pipettes with Finntip polypropylene tips from ThermoFisher Scientific (Waltham, MA, USA). Centrifugation of 96-well plates was carried out using an ST-16 centrifuge with M-20 microplate rotor from ThermoFisher Scientific (Waltham, MA, USA). A digital microplate shaker from ThermoFisher Scientific (Waltham, MA, USA) was utilized to agitate microplates during sample extraction. An Evaporex EVX192 plate dryer from Apricot Designs (Covina, CA, USA) was utilized for plate drying steps. Round-bottom polypropylene 96-well plates from Corning (Salt Lake City, UT, USA), V-bottom polypropylene NUNC 96-well plates from Thermo Scientific (Rochester, NY, USA), and low-volume V-bottom polypropylene 96-well plates from Abgene (Portsmouth, NH, USA) were utilized for sample preparation and analysis.

3.3 Stock solution and working solution preparation

Analytical standards in 1 mL aliquots each of cortisol, 17-OHP, 11-DOC, and corticosterone were purchased from Cerilliant Corporation (Round Rock, TX, USA) as stock solutions at 1 mg/mL in methanol. Analytical standards in 1 mL aliquots each of 21-DOC and deoxycorticosterone were purchased from Cerilliant as stock solutions at 100 µg/mL methanol. Analytical standards in 1 mL aliquots of 4-AD were

purchased from Cerilliant as stock solution at 1 mg/mL in acetonitrile. Analytical standards in 1 mL aliquots each of cortisol-D_4, 17-OHP-D_8, 21-DOC-D_8, and 11-DOC-D_5 stable isotope-labeled internal standards were purchased from Cerilliant as stock solutions at 100 µg/mL in methanol. Analytical standards in 1 mL aliquots of 4-AD-$^{13}C_3$ stable isotope-labeled internal standard were purchased from Cerilliant as stock solution at 100 µg/mL in acetonitrile.

Each of the 1 mg/mL analyte stock solutions was diluted to 1 µg/mL in methanol to prepare individual intermediate stocks. Individual intermediate stocks of all un-labeled compounds were also prepared at 5 µg/mL in methanol.

For mass spectrometry infusion experiments, individual stocks of all analytes were prepared at 200 ng/mL in 50:50 methanol: water with 0.1% formic acid. Stable isotope-labeled 100 µg/mL internal standard stocks were combined in equal volumes, mixed, and then were divided into 50 µL aliquots at a concentration of 20 µg/mL each. Aliquots were sealed in individual screw-cap vials and stored in freezer (-20 $\pm$ 5°C) until use. DBS Extraction Solution was prepared by adding the full contents of one mixed internal standard vial to 500 mL of 80:20 acetonitrile: water, to yield a final internal standard concentration of 2 ng/mL. Reconstitution Solution was prepared at 50:50 methanol: water with 0.1% formic acid.

For preparation of dried blood spot calibration and quality control materials, a mixed analyte stock containing cortisol, 17-OHP, 4-AD, 21-DOC, and 11-DOC was prepared in methanol at a concentration of 25 µg/mL of each analyte. This mixed analyte stock was further diluted to prepare intermediate stocks at 4 µg/mL and 0.25 µg/mL, both in methanol.

3.4 Preparation of hematocrit-adjusted dried blood spot calibration standards, quality control standards, and validation study standards

Dried blood spots (DBS) were prepared for use as calibration standards, quality control standards, and materials specific to the linearity, limit of quantitation, and interference studies for method validation. The prepared DBS were manufactured by enriching hematocrit-adjusted human blood pools with standard concentrations of target analytes, spotting the blood on filter paper cards and allowing them to dry before storing in a freezer (-20 ± 5 °C) with desiccant.

Hematocrit-adjusted human blood pools, depleted of target steroids and precursors, were prepared by repeated washing of purchased units of human red blood cells or human whole blood to yield washed packed red blood cells. Red blood cells or whole blood were thoroughly mixed with equal volumes of unbuffered sterile saline and centrifuged at 3000 rpm, under refrigeration at approximately 4 °C. Vacuum aspiration then was used to remove as much of the plasma, saline, and buffy coat fractions as possible. The washing, centrifugation, and vacuum aspiration process was repeated three times for each blood unit. The washed packed red blood cells then were mixed with defibrinated, charcoal-stripped human plasma, mimicking human serum, to reach a desired hematocrit level of 50 ± 1%. Hematocrit levels were verified using a hematocrit centrifuge. Additional volumes of washed packed red blood cells or defibrinated, charcoal-stripped plasma were added to each blood pool to adjust the hematocrit level up or down, respectively. Once the desired hematocrit level was reached, the blood pools were ready for enrichment with target compounds.

Class A volumetric glassware was used to prepare 25 mL volumes of calibrator and quality control standards. To prepare calibration standards at concentrations of 1, 2, and 5 ng/mL serum, a predetermined volume of 0.25 µg/mL mixed analyte stock was added to each volumetric flask. An additional predetermined volume of methanol was added to each volumetric flask in order to keep the solvent volume in each flask identical. Each flask was then filled to volume with hematocrit-adjusted

steroid-depleted blood, capped, and thoroughly mixed by inversion. Similarly, to produce calibration standards at concentrations of 20, 100, and 500 ng/mL serum, a predetermined volume of 25 µg/mL mixed analyte stock was added to each volumetric flask followed by a predetermined volume of methanol. Each flask was then filled to volume with hematocrit-adjusted steroid-depleted blood, capped, and thoroughly mixed by inversion. Low, medium, and high concentration quality control standards were prepared by adding a predetermined volume of 4 µg/mL mixed analyte stock to each volumetric flask followed by a predetermined volume of methanol. Each flask was then filled to volume with hematocrit-adjusted steroid-depleted blood, capped, and thoroughly mixed by inversion. Additionally, a flask containing only an equivalent volume of methanol as the calibrator and quality control standards was filled to volume with hematocrit-adjusted steroid-depleted blood, capped, and thoroughly mixed by inversion to serve as a matrix blank. All calibrator and quality control materials were filtered through glass wool prior to spotting on filter paper.

In addition to calibrator and quality control standards, enriched blood materials specific to method validation activities were prepared in 2 mL volumes prior to being spotted on filter paper. Low concentration enriched blood standards were prepared at concentrations of 0.25 and 0.50 ng/mL serum using the 0.25 µg/mL mixed analyte stock and equivalent volumes of additional methanol. These materials were used to evaluate the lower limit of quantitation (LOQ) for the assay. A high concentration enriched blood standard was prepared at 1000 ng/mL serum using the 25 µg/mL mixed analyte stock and equivalent volume of methanol. The 1000 ng/mL serum standard was used for linearity and carryover experiments. For interference studies, 2 mL volumes of hematocrit-adjusted steroid-depleted blood were enriched with varying concentrations of target analytes as well as known isobaric interferences. The concentration scheme for the interference study materials is provided in Table 13. The critical pair of isobars, 11-DOC and corticosterone, were enriched at opposing concentrations to determine whether varying concentrations of the non-target analyte corticosterone showed any

unfavorable influence on linear response or calculated concentrations of the target analyte 11-DOC. Additionally, by enriching high concentrations of corticosterone with low concentrations of 11-DOC the ability of the instrument software peak identification and integration algorithm to correctly assign the 11-DOC peak was evaluated. Deoxycorticosterone and 4-AD were enriched at opposing concentrations because these compounds co-elute from the HPLC column under the optimized chromatographic conditions for the assay. The influence of varying concentrations of the non-target compound deoxycorticosterone on the linear response or calculated concentration of the target compound 4-AD was evaluated.

Table 13. DBS enrichment concentrations for interference study. All concentrations are ng/mL serum.

Sample	[CORT]	[21-DOC]	[11-DOC]	[Corticosterone]	[17-OHP]	[4-AD]	[Deoxycorticosterone]
A	250	25	0	500	50	10	100
B	500	10	5	250	100	25	50
C	0	5	10	100	250	50	25
D	5	0	25	50	500	100	10
E	10	500	50	25	0	250	5
F	25	250	100	10	5	500	0
G	50	100	250	5	10	0	500
H	100	50	500	0	25	5	250

All enriched blood solutions were filtered through glass wool (where volumes allowed) and then spotted on Whatman® 903 filter paper using a digital repeater pipette set to a dispense volume of 75 µL per blood spot. Spotted filter paper cards were allowed to dry at room conditions before being stored in a freezer with desiccant until analysis. In all, over 3000 dried blood spots were produced for method validation experiments.

3.5 HPLC parameters

HPLC equipment used for method development and validation experiments included: Shimadzu pumps LC-20AD, system controller CBM-20A, solvent degasser DGU-20A_3 (Shimadzu, Kyoto, Japan). An HTC PAL autosampler from CTC Analytics (Zwingen, Switzerland) was used to introduce samples into the mobile phase flow. An EchoTherm CO20 column heater from Torrey Pines Scientific (Carlsbad, CA, USA) was used. A Kinetex Biphenyl column (3.0 x 50 mm, 2.6 µm) with Biphenyl SecurityGuard ULTRA guard cartridges from Phenomenex (Torrance, CA, USA) was used for reversed phase liquid chromatography. A binary gradient using two LC-20AD pumps was carried out with mobile phase A consisting of water with 0.1% formic acid and mobile phase B consisting of methanol with 0.1% formic acid. Gradient conditions are summarized in Table 12 in section 2.3.3 (page 35), and a typical chromatogram is shown in Figure 11 in section 2.3.3 (page 35). The mobile phase flow rate for routine separations was 500 µL/min. The column temperature was maintained at 50 °C and sample plates were maintained in the autosampler at room conditions. Autosampler syringe and injection valve wash cycles between injections consisted of four cycles of 100 µL 50:50 methanol: water with 0.1% formic acid. The autosampler injection volume was set to 20 µL.

The HPLC column was slowly equilibrated each day using a modified version of the routine method. The column equilibration method utilized the same initial gradient conditions, however the flow rate began at 50 µL/min and was slowly increased to 500 µL/min over a period of 10 minutes.

3.6 Mass spectrometer parameters

Tandem mass spectrometry was carried out using a Sciex API 4000 (Framingham, MA, USA) triple quadrupole mass spectrometer operated in positive electrospray ionization mode with multiple

reaction monitoring (MRM) of target analytes and stable isotope-labeled internal standards. Optimization of ionization and MRM parameters was performed by direct infusion of 200 ng/mL stock solutions using a syringe pump at a flow rate of 10 µL/min as described in Chapter 2. Common mass spectrometer parameters, optimized for maximum sensitivity and shared by all target analytes and internal standards, include: ion spray voltage 4000 V, desolvation temperature 550 °C, collision assisted dissociation (CAD) gas pressure 6.00 arbitrary units, nebulizer gas (GS1) flow 45 arbitrary units, heater gas (GS2) flow 40 arbitrary units, curtain gas flow 45 arbitrary units, entrance potential (EP) 10 V. Table 14 shows optimized, compound-specific MRM parameters.

Table 14. Multiple Reaction Monitoring (MRM) parameters.

Analyte	Q1 Precursor Ion (m/z)	Q3 Product Ion (m/z)	Dwell Time (msec)	DP (V)	CE (V)	CXP (V)
4-AD_Q	287.10	109.00	25	86	35	8
4-AD_C	287.10	97.10	50	86	51	6
4-AD_IS	290.20	112.00	15	91	35	8
17-OHP_Q	331.30	109.00	50	96	39	8
17-OHP_C	331.30	97.10	50	96	61	6
17-OHP_IS	339.20	100.00	15	101	43	8
11-DOC_Q	347.10	109.00	25	96	41	8
11-DOC_C	347.10	97.00	25	96	43	6
11-DOC_IS	352.10	112.80	15	96	47	8
21-DOC_Q	347.10	311.10	50	96	25	16
21-DOC_C	347.10	175.10	50	96	29	12
21-DOC_IS	355.20	319.00	15	101	23	20
CORT_Q	363.10	120.90	50	96	35	8
CORT_C	363.10	326.90	50	96	23	20
CORT_IS	367.10	121.00	15	60	52	12

DP, declustering potential; CE, collision energy; CXP, collision cell exit potential; _Q, quantitation ion transition; _C, confirmation ion transition, _IS, stable isotope-labeled internal standard ion transition

3.7 Sample preparation parameters

Dried blood spots from patient specimens, calibrators, and quality control materials were analyzed in duplicate on each 96-well plate and were prepared for LC-MS/MS analysis using the same protocol. Three 3.2 mm discs were punched from the dried blood spot into a single well of a round-bottom 96-well plate. DBS Extraction Solution, 200 µL, was added to each well containing DBS punches. The plate was covered with aluminum foil and placed on a plate shaker at 300 rpm for 30 minutes. Following the shaking period, plates were centrifuged at 1500 x g for 10 minutes. Following

centrifugation, 175 µL of supernatant was transferred to a V-bottom NUNC 96-well plate. The DBS punches were discarded. The NUNC 96-well plate containing supernatant was placed on a plate dryer and was concentrated to dryness under heated air. The dried wells were reconstituted to 50 µL using Reconstitution Solution. The plate was covered with foil and placed on a plate shaker at 300 rpm for 5 minutes. The entire reconstituted volume was then transferred to a low-volume V-bottom plate. The low-volume 96-well plate was covered with foil and centrifuged at 1500 x g for 2 minutes to remove any bubbles. Following centrifugation, the plate was transferred to the autosampler for LC-MS/MS analysis.

3.8 Method validation experiments

Following HPLC, tandem mass spectrometry, and sample preparation parameter optimizations, the full method was evaluated using several criteria to determine expected performance and reliability prior to full implementation within the Newborn Screening Laboratory. The Newborn Screening Laboratory is part of Virginia's Public Health Laboratory and is accredited and audited under the Clinical Laboratory Improvement Amendments (CLIA). Method validation experiments were performed to meet the criteria under CLIA for evaluation of a laboratory-developed test.

3.8.1. Matrix effects

To study matrix effects, a tee union was installed in between the outlet end of the HPLC column and the inlet for the electrospray ionization source. An infusion line was connected to the third port of the tee union. A syringe containing a 200 ng/mL infusion solution of all target analytes and stable isotope-labeled internal standard compounds was connected to the infusion line. Using a syringe pump set to a flow rate of 5 µL/minute, the infusion solution was pumped into the HPLC column eluent and mixed before being introduced to the electrospray ionization source.

Matrix contributions to ionization suppression or enhancement were measured by studying the multiple reaction monitoring (MRM) signal for each of the target analytes and internal standards at the mass spectrometer. Through constant flow of the infusion solution, a flat, steady MRM signal is anticipated for each analyte trace if no matrix effects are present. As matrix components are introduced to the system and eluted through the HPLC column, dips in MRM signal indicate ionization suppression due to matrix effects while increases in MRM signal indicate ionization enhancement.

This experiment was conducted while introducing only solvent into the system (no matrix), introducing extracts of filter paper (no blood matrix), and introducing extracted dried blood spot matrix in order to compare ionization effects based upon the various components.

3.8.2. Carryover

Carryover effects were studied by analyzing replicates (N = 3) of DBS extracts containing high (1000 ng/mL serum) concentrations of target compounds followed immediately by analysis of DBS extracts of unenriched (blank) blood matrix. The DBS blank analyses were scrutinized to determine how much quantifiable signal of each target compound was detectable in the DBS blanks. The experiments were repeated for two assays.

Additionally, to test whether carryover was occurring due to over-retention or poor elution of target compounds from the HPLC column, replicates of high concentration (1000 ng/mL serum) DBS extracts were analyzed. Immediately following the final high concentration replicate, the mobile phase gradient program was repeated without injection of any new extracted material from the autosampler (no syringe). The run was scrutinized to determine if any quantifiable signal was present for any of the target compounds based upon elution of over-retained materials from the HPLC column.

3.8.3. Interfering or co-eluting substances

DBS materials were enriched with pre-determined concentrations of potentially interfering isobaric compounds. One set of isobaric compounds that were studied included 21-deoxycortisol (21-

DOC), 11-deoxycortisol (11-DOC), and corticosterone, all sharing the sample precursor ion mass-to-charge ratio of 347.10 m/z. 21-DOC and 11-DOC are target analytes for the assay whereas corticosterone is a possible interferent. A second set of isobaric compounds that were studied included 17-OHP and deoxycorticosterone, both sharing the same precursor mass-to-charge ratio of 331.30 m/z. 17-OHP is a target analyte of the assay and deoxycorticosterone is a possible interferent. Deoxycorticosterone also co-elutes with androstenedione (4-AD), a target compound for the assay.

Replicates of the enriched DBS materials were analyzed to study whether linear response of the target compounds was maintained in the presence of varying concentrations of isobaric or co-eluting interferents. Additionally, the peak resolution was calculated between the closest eluting pair of isobaric compounds, 11-DOC and corticosterone, considered the critical pair for HPLC separation, to ensure that baseline resolution (> 1.5) was achieved in order to avoid potential influence on concentration calculations.

3.8.4. Linearity and lower limit of quantitation (LOQ)

Linearity was assessed by plotting replicate (N=12) internal calibration responses of DBS materials enriched with analyte concentrations ranging from 0.25 ng/mL serum up to 1000 ng/mL serum. The plot of the replicates was compared to linear and polynomial fit models to determine across what concentration range the assay showed adequate linearity for each analyte. Linearity was considered acceptable when the mean of the replicate responses did not deviate more than 15% from the linear fit model at each concentration level.

Lower limit of quantitation (LOQ) was assessed by replicate analyses (N=12) of DBS materials with decreasing concentrations of each target compound. Two metrics were utilized to determine the LOQ: signal-to-noise ratio and replicate reproducibility (%CV). The LOQ for each target compound was

determined as the concentration at which analyte response exceeds a 10:1 signal-to-noise ratio and replicate reproducibility of less than 20% CV.

3.8.5. HPLC column batch-to-batch reproducibility

In order to evaluate anticipated reproducibility of HPLC columns as they were replaced over time, columns from different production lots were compared. A total of six Phenomenex Kinetex Biphenyl, 2.6 µm, 50 x 3.00 mm HPLC columns were used for the comparison study. The columns were from three different production lots, two columns from each lot.

Replicate injections of unextracted standard solution (N=5 injections per column) containing target analytes, stable isotope-labeled internal standard compounds, and potential isobaric interferences were analyzed for column comparison. Metrics for peak area, retention time, peak width, and resolution of critical pair (11-DOC from corticosterone) were compared across column lots.

Multiple extracts of DBS QC (low, medium, high) were pooled and repeatedly analyzed (N = 5 replicates per QC level per column) on all six columns to compare calculated concentrations, peak areas, peak widths, and analyte retention times.

3.8.6. Recovery

Analyte recoveries were calculated using replicated analyses (N=10) of DBS QC material provided by the CDC NSQAP.[33] Internal calibration was performed to determine calculated analyte concentrations using Equation 2:

Equation 2. Internal calibration equation

$$C_{analyte} = \frac{\left(I_{analyte} \times V_{ext} \times C_{IS}\right)}{\left(I_{IS} \times V_{BS}\right)}$$

$C_{analyte}$ is analyte concentration, $I_{analyte}$ is analyte response intensity, V_{ext} is the extract volume, C_{IS} is the internal standard concentration, I_{IS} is the internal standard response intensity, and V_{BS} is the volume of serum in the DBS extract. By utilizing internal calibration, raw recoveries for each analyte were able to be obtained.

3.8.7. Accuracy and precision

Method accuracy and precision were evaluated both inter- and intra-run using replicate analysis of DBS QC materials. Inter-run precision and accuracy data included 52 replicates each of three levels of QC (low, medium, high) utilizing three analysts with 26 analytical runs over a period of 15 days. Each analytical run contained two replicates of each QC and no more than two analytical runs were completed per day. Intra-run precision and accuracy were evaluated utilizing 25 replicates of each DBS QC level (low, medium, high) within a single analytical run.

3.8.8. Extract stability

Extract stability was evaluated by preparing pools of DBS extracts, including blanks, calibrators, QC, and NSQAP materials. Pooled extracts then were divided among seven microtiter plates and covered with foil. One plate was analyzed on the day of extraction (Day 0). The remaining six plates were divided into two subgroups of three plates each. One subgroup was stored at room conditions while the other subgroup was stored under refrigeration (1-5°C). At one, two, and seven days post-extraction, one plate from each subgroup was analyzed to compare metrics such as calculated analyte concentrations and analyte peak area.

3.8.9. Ruggedness

Multiple variable factors were introduced into the study wherever practical in order to ensure overall assay ruggedness. Three analysts performed extractions, instrument analysis and data review throughout the validation. Precision, accuracy, and clinical sensitivity/specificity analyses were performed over a period of 16 days, by three analysts, with no more than two assays with two QC replicates each being run on any given day. Multiple lots of the various solvents and HPLC columns were utilized throughout the validation study. Mobile phase and DBS Extraction Solution preparations were made by multiple analysts throughout the study.

3.8.10. Clinical sensitivity and specificity

Double-blinded de-identified NBS specimens were analyzed in duplicate using the proposed HPLC-MS/MS assay. Results of the patient sample analyses were evaluated using a provisional cutoff algorithm to establish an interpretation of either within normal limits, abnormal (requiring request of a repeat specimen), critical (requiring immediate referral to follow-up), or unsatisfactory (consistent results were not able to be obtained). The 1st tier 17-OHP results and clinical outcomes for each patient specimen were unknown to the three individuals participating in the validation study prior to analysis and interpretation using the provisional cutoff algorithm.

CHAPTER 4: RESULTS AND DISCUSSION

The optimized LC-MS/MS method needed to be validated prior to being moved into production within the laboratory and being used to report patient results. The method was validated as a laboratory-developed test for implementation and audit under the Clinical Laboratory Improvement Amendments (CLIA).[47] Method performance and characteristics that were validated include matrix effects, carryover, potential interfering substances, linearity, lower limit of quantitation, HPLC column reproducibility, accuracy and precision, clinical sensitivity and specificity, recovery, extract stability, and ruggedness.

4.1 Matrix effects

Matrix effects leading to ionization suppression or enhancement were studied by infusing a mixed standard of all target analytes and internal standard compounds into the HPLC column eluent prior to ionization at the electrospray ionization source and running the mobile phase gradient profile. Experiments were conducted while injecting only solvent onto the column (no matrix), injecting extracts of filter paper (no blood matrix), and injecting extracted, unenriched dried blood spot matrix in order to compare ionization effects based upon the various matrix components. Figure 12 shows overlaid representative total ion chromatograms of all MRM transitions of matrix effect experiments comparing

ionization suppression when solvent (black trace) or dried blood spot extract (blue trace) are injected on-column. A representative total ion chromatogram of all MRM transitions of the typical analyte elution profile is overlaid (red trace) to compare ionization suppression regions relative to analyte retention times. Analysis with only solvent showed the least ionization suppression while ionization suppression effects increased with the introduction of extracted filter paper and still further with the introduction of extracted blood matrix. In all cases, initial severe ionization suppression occurs as unretained compounds elute from the HPLC column and ionization signal recovers across the course of the gradient HPLC run. The use of stable isotope-labelled internal standard compounds for quantitation of all target compounds helps to mitigate ionization suppression effects as these compounds co-elute with their respective target analytes.

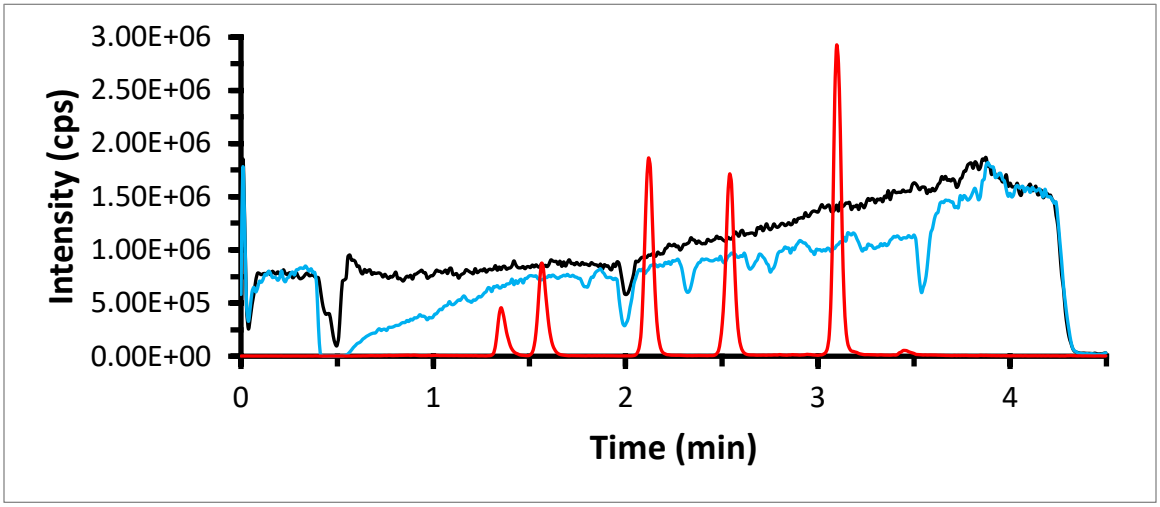

Figure 12. Representative total ion chromatograms of all MRM transitions of matrix effect experiments comparing ionization suppression when injecting solvent (black trace) or dried blood spot extract (blue trace). The overlaid red trace shows typical analyte elution profile.

4.2 Carryover

Experiments were conducted to determine full system carryover as well as carryover due only to overloading or over-retention of target analytes on the HPLC column. To test for full system carryover,

three replicates of DBS extracts containing concentrations of 1000 ng/mL serum of cortisol, 21-DOC, 11-DOC, 17-OHP, and 4-AD were analyzed. Immediately following the final high concentration replicate, DBS extracts of unenriched blood matrix were analyzed. Figure 13 shows a total ion chromatogram of all MRM transitions of high concentration DBS extract analysis as well as a total ion chromatogram of all MRM transitions of unenriched DBS extract that immediately followed. Internal standard ion traces are removed from the unenriched DBS extract chromatogram as the internal standard peaks give the illusion of carryover. HPLC column overloading or over-retention was tested by analyzing three replicates containing concentrations of 1000 ng/mL serum of cortisol, 21-DOC, 11-DOC, 17-OHP, and 4-AD followed immediately by repeating the mobile phase gradient profile with no injection from the autosampler. Figure 14 shows a total ion chromatogram of all MRM transitions of a high concentration DBS extract analysis as well as a total ion chromatogram of all MRM transitions from a repeat of the mobile phase gradient profile without injection from the autosampler.

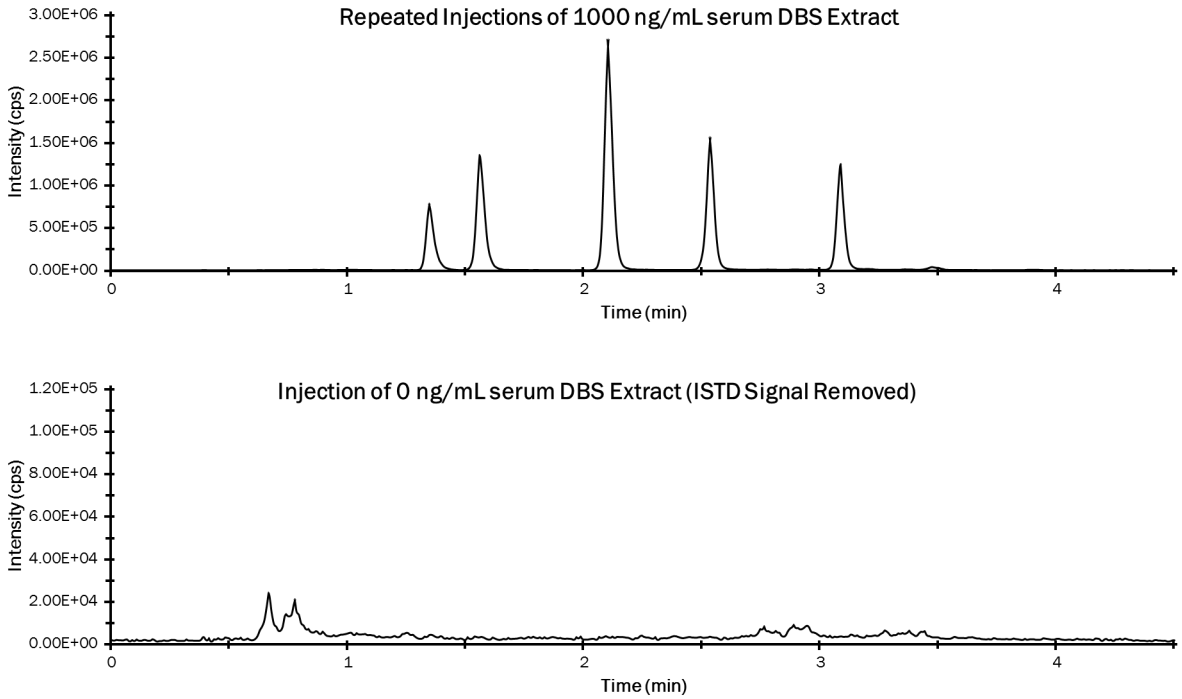

Figure 13. Representative total ion chromatogram of all MRM transitions from repeated analysis of high concentration DBS extracts (top) and total ion chromatogram of all MRM transitions from analysis of unenriched DBS extract with internal standard traces removed (bottom).

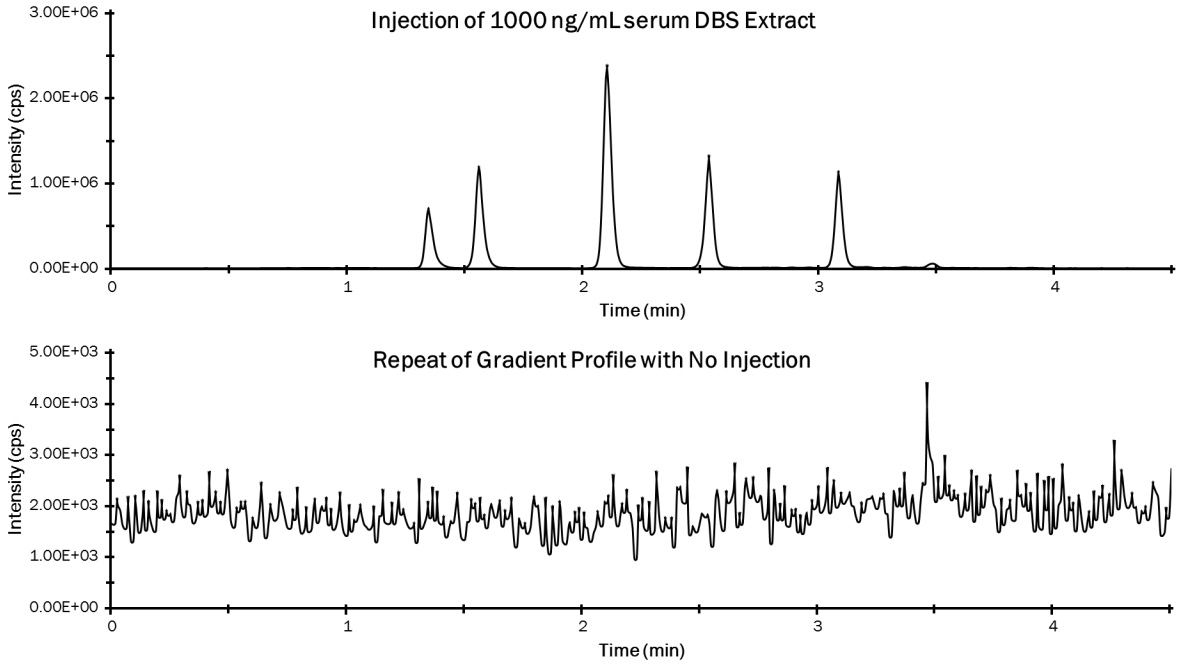

Figure 14. Representative total ion chromatogram of all MRM transitions of repeated analysis of high concentration DBS extracts (top) and total ion chromatogram of all MRM transitions of repeat of mobile phase gradient profile with no injection from autosampler (bottom).

Both carryover experiments showed acceptable results as no detectable carryover was observed. Calculated concentrations for analytes within the DBS blank extracts were below reportable limits and were in line with typical analysis of DBS blanks. No discernable peaks were detected to indicate over-retention on the HPLC column. The high concentration DBS extracts used in carryover experiments exceed the anticipated analyte concentrations within most patient specimens analyzed by this assay.

4.3 Interfering or co-eluting substances

During repeated analysis of DBS extracts from materials enriched with varying concentrations of potential isobaric interferences, all isobaric compounds were adequately resolved under the optimized

HPLC gradient conditions using the biphenyl stationary phase ligand. The critical pair of 11-DOC and corticosterone showed baseline resolution in all experiments. The co-elution of deoxycorticosterone with 4-AD did not negatively impact the ability to properly quantify 4-AD using the assay, given the fact that 4-AD and deoxycorticosterone have different precursor-to-product ion transitions and are therefore able to be differentiated by the mass spectrometer.

An example of baseline resolution of the critical pair, 11-DOC and corticosterone, from a DBS extract containing low concentration (5 ng/mL serum) of the 11-DOC target analyte and high concentration (250 ng/mL serum) of the potential interference, corticosterone, is shown in Figure 15. By achieving baseline resolution, the instrument peak detection and integration software was able to correctly identify the target compound, regardless of the concentration difference between the target and the interference.

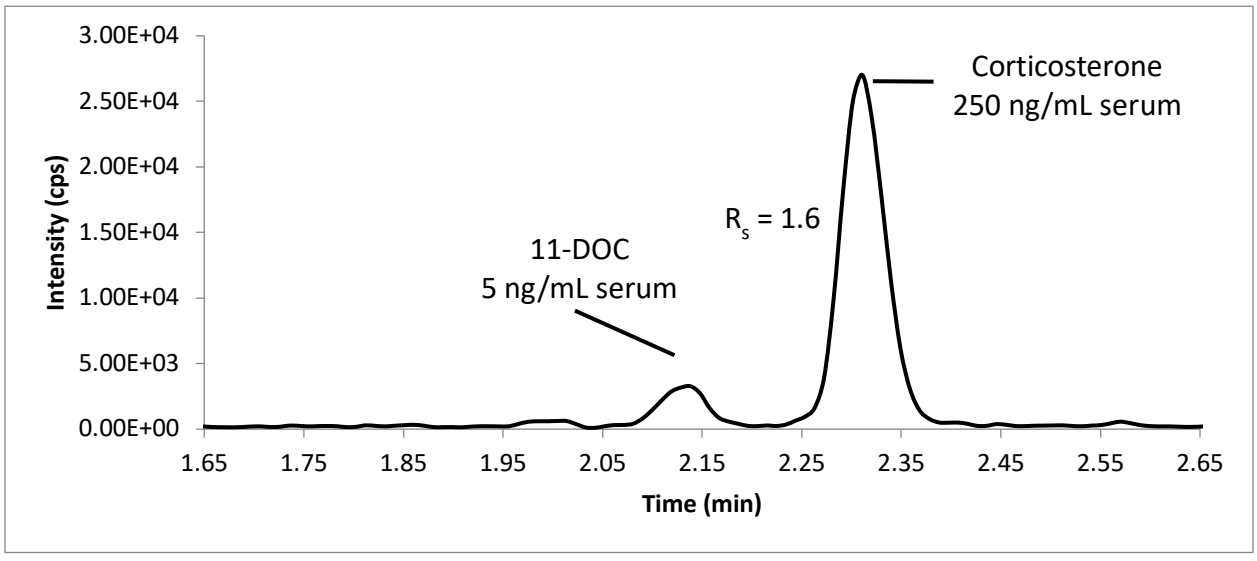

Figure 15. Representative extracted ion chromatogram example of baseline resolution of critical pair from interference study. Even at much lower concentration within the sample, the 11-DOC peak was successfully identified and integrated by the quantitation software. The extracted ion trace depicted is the quantitation ion transition for 11-DOC (347.10 → 109.00 m/z).

4.4 Linearity and lower limit of quantitation (LOQ)

Using enriched dried blood spot calibration materials, internal calibration responses were plotted against expected concentrations for all five target compounds with varying concentration ranges including 0.25 to 1000 ng/mL serum, 0.5 to 1000 ng/mL serum, 1 to 1000 ng/mL serum, 2 to 1000 ng/mL serum, 2 to 500 ng/mL serum, 1 to 500 ng/mL serum, 0.5 to 500 ng/mL serum, and 0.25 to 500 ng/mL serum. For all five target compounds, the widest concentration range over which acceptable linearity was maintained was between 0.25 ng/mL serum and 500 ng/mL serum. No weighting factor was applied to the linearity study. Figure 16 shows cortisol linear response between 0.25 and 500 ng/mL serum. Figure 17 shows 21-DOC linear response between 0.25 and 500 ng/mL serum. Figure 18 shows 11-DOC linear response between 0.25 and 500 ng/mL serum. Figure 19 shows 17-OHP linear response between 0.25 and 500 ng/mL serum. Figure 20 shows 4-AD linear response between 0.25 and 500 ng/mL serum.

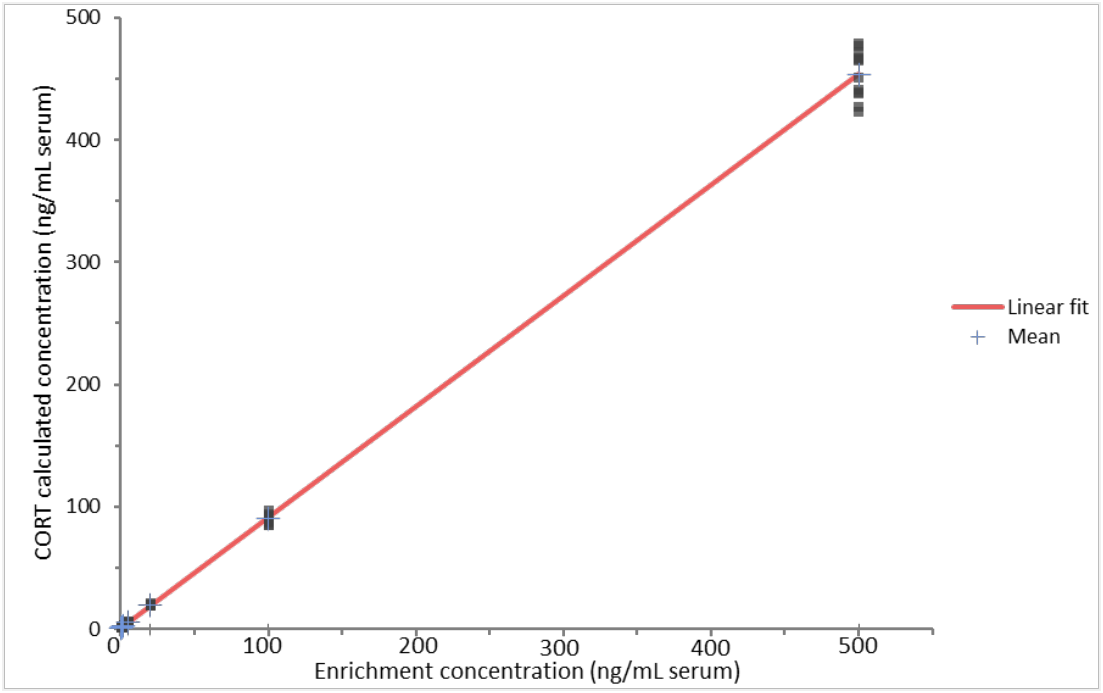

Figure 16. Linearity plot for cortisol of enriched vs. calculated concentration for DBS analysis using internal calibration. Linear fit equation: y = 0.91x + 0.72; R^2 = 0.9979

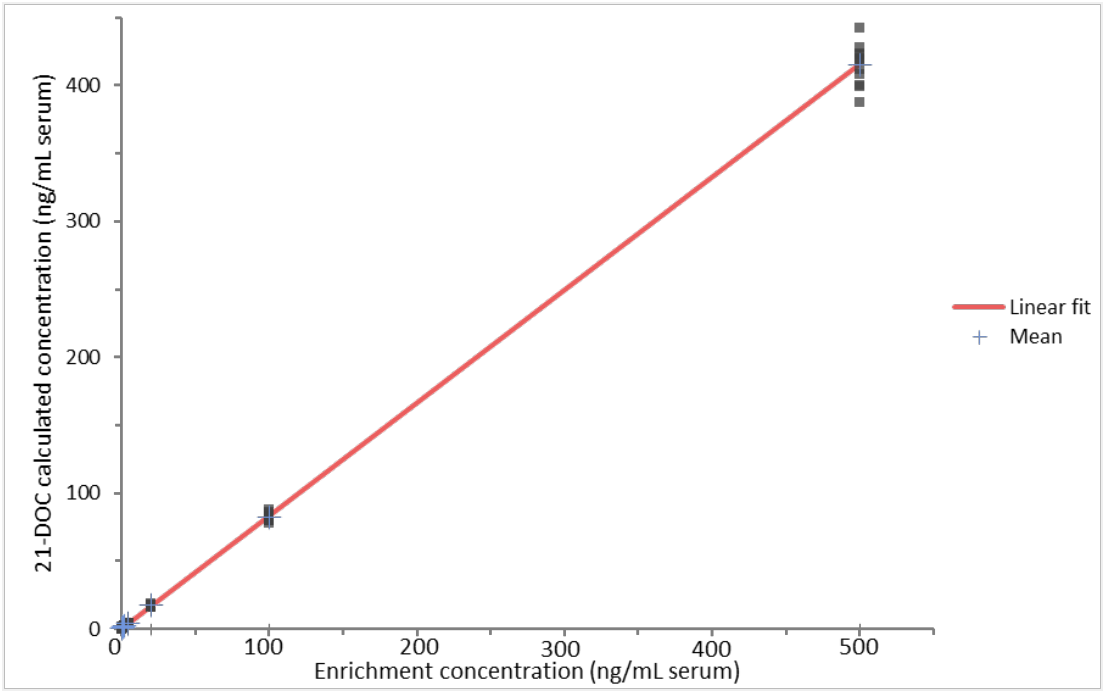

Figure 17. Linearity plot for 21-DOC of enriched vs. calculated concentration for DBS analysis using internal calibration. Linear fit equation: y = 0.83x + 0.25; R^2 = 0.9986

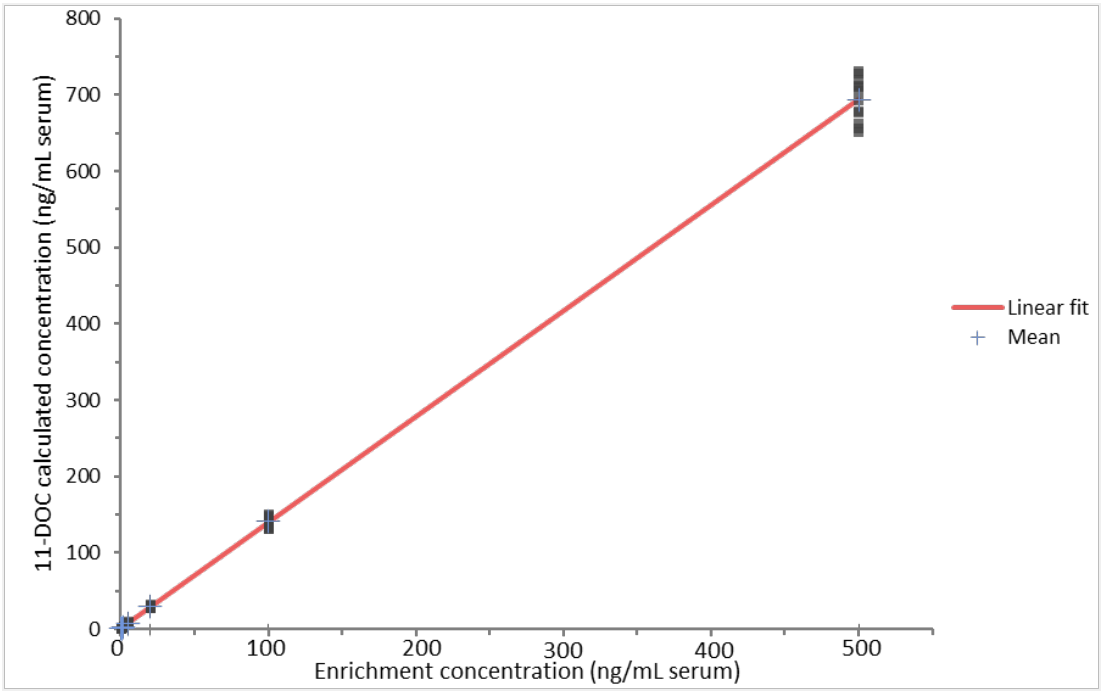

Figure 18. Linearity plot for 11-DOC of enriched vs. calculated concentration for DBS analysis using internal calibration. Linear fit equation: y = 1.39x + 0.84; R^2 = 0.9981

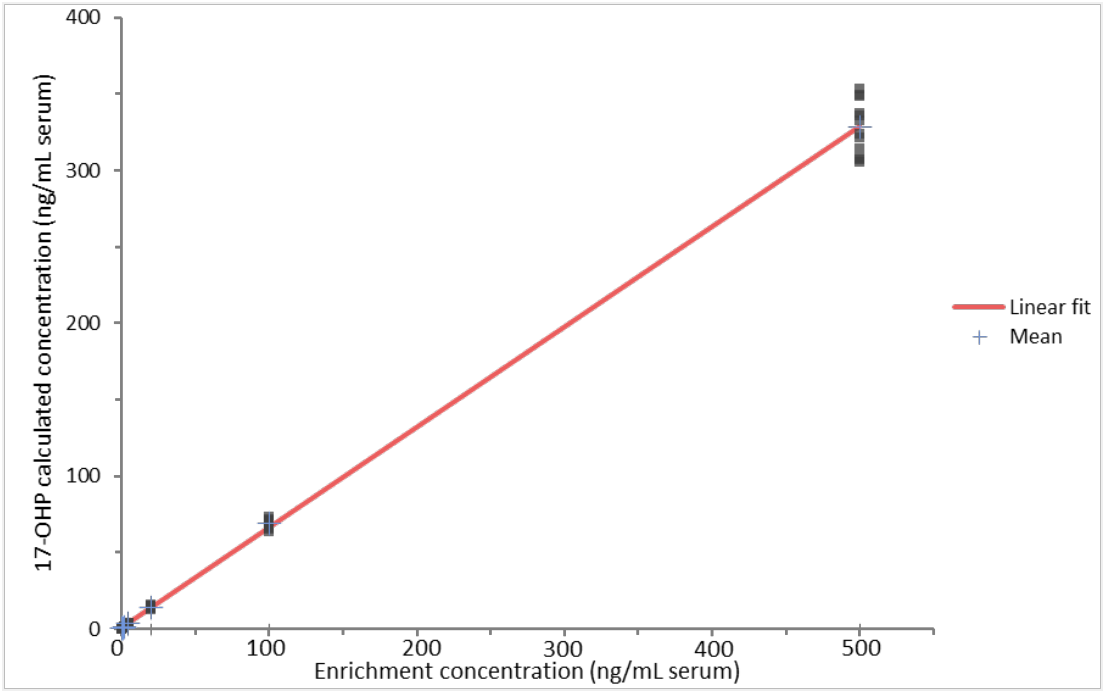

Figure 19. Linearity plot for 17-OHP of enriched vs. calculated concentration for DBS analysis using internal calibration. Linear fit equation: y = 0.66x + 0.73; R^2 = 0.9968

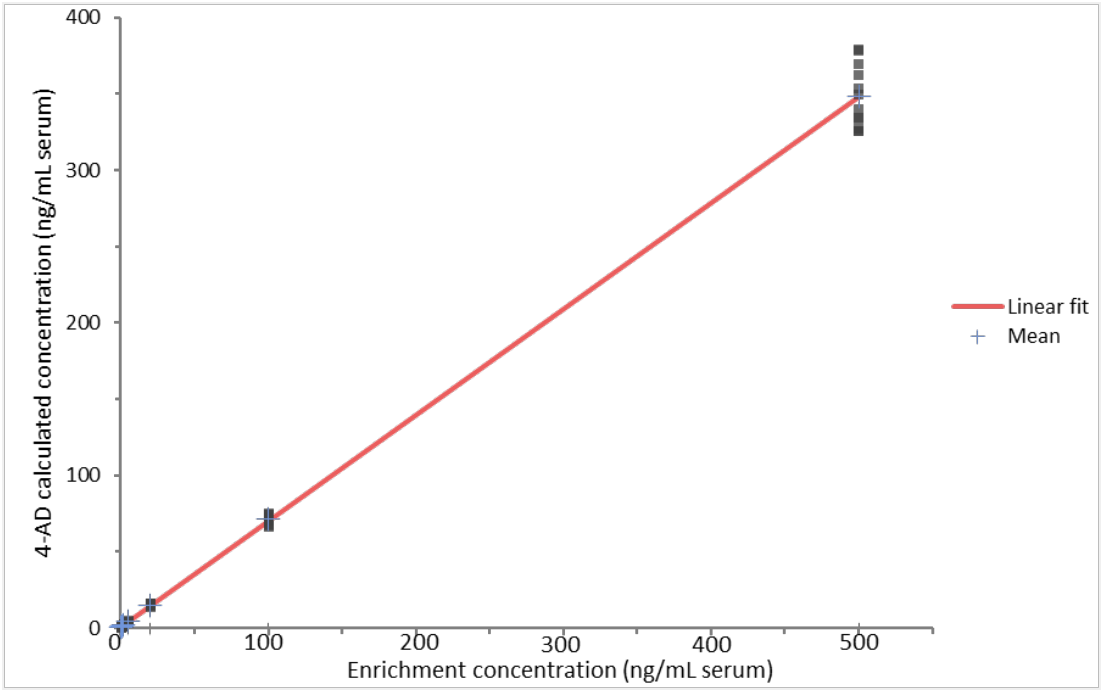

Figure 20. Linearity plot for 4-AD of enriched vs. calculated concentration for DBS analysis using internal calibration. Linear fit equation: y = 0.70x + 0.77; R^2 = 0.9962

Lower limit of quantitation (LOQ) was determined for each of the five target analytes by evaluating replicate analyses of dried blood spot extracts with concentrations as low as 0.25 ng/mL serum and by using two criteria. The signal-to-noise ratio (S/N) of each analyte peak and coefficient of variation (CV) for internal calibration concentration calculations of replicates were used to determine the LOQ for each analyte. The LOQ for each analyte was determined as the lowest concentration at which replicate analyses yielded a signal-to-noise ratio $\geq$ 10:1 and CV $\leq$ 20%.

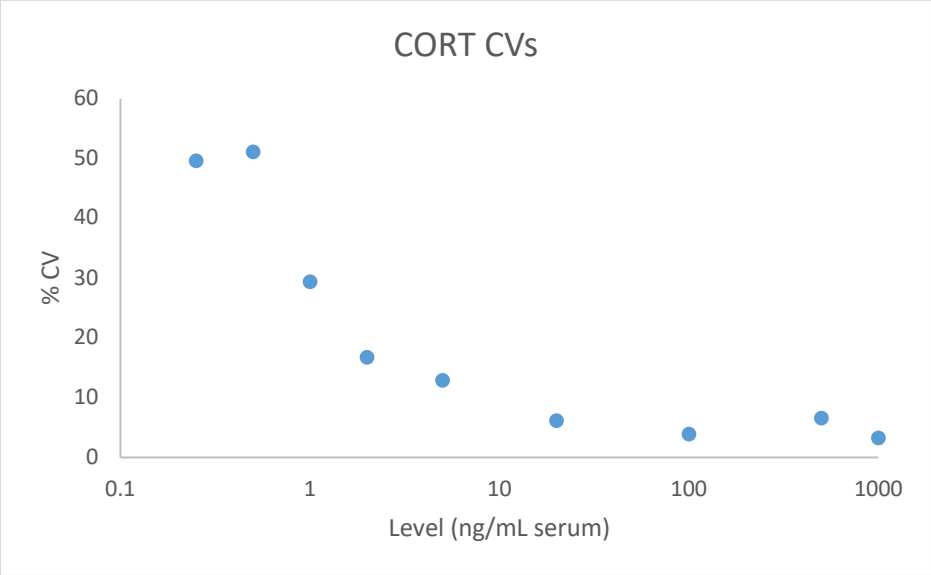

Figure 21. Coefficients of variation for cortisol for replicate analyses of DBS extracts.

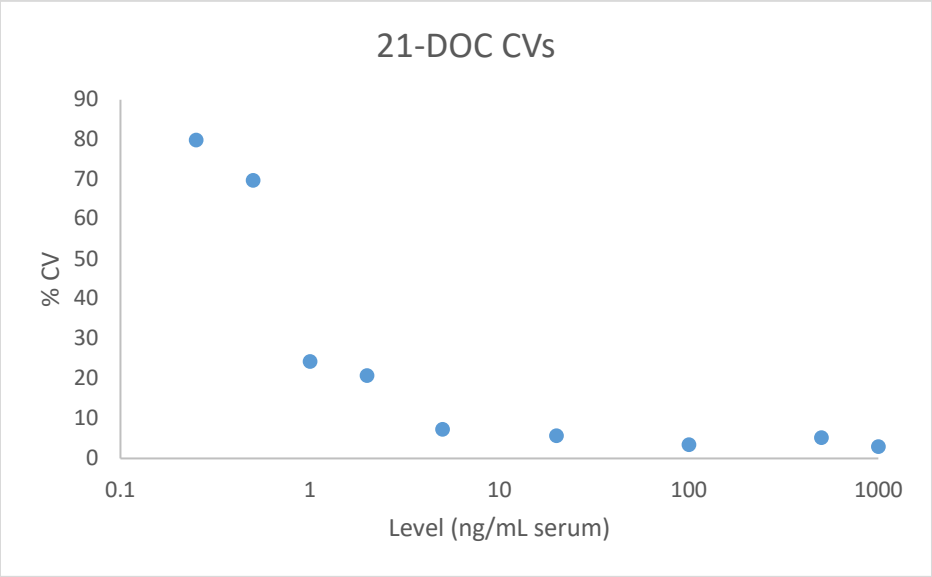

Figure 22. Coefficients of variation for 21-DOC for replicate analyses of DBS extracts.

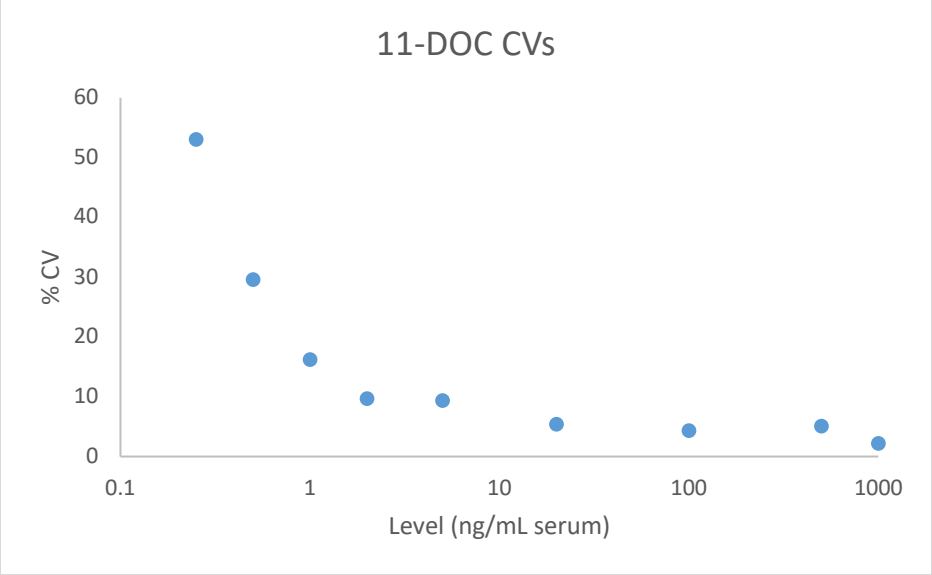

Figure 23. Coefficients of variation for 11-DOC for replicate analyses of DBS extracts.

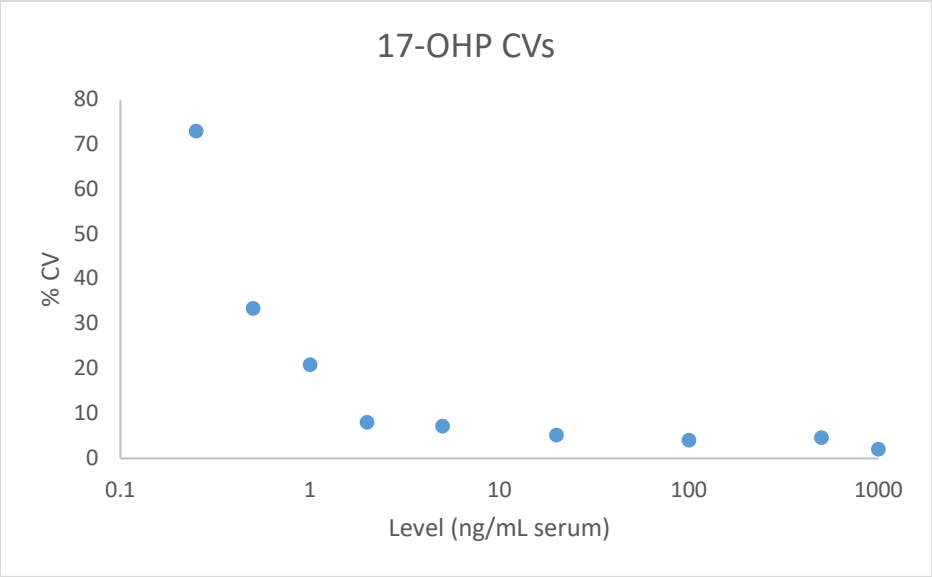

Figure 24. Coefficients of variation for 17-OHP for replicate analyses of DBS extracts.

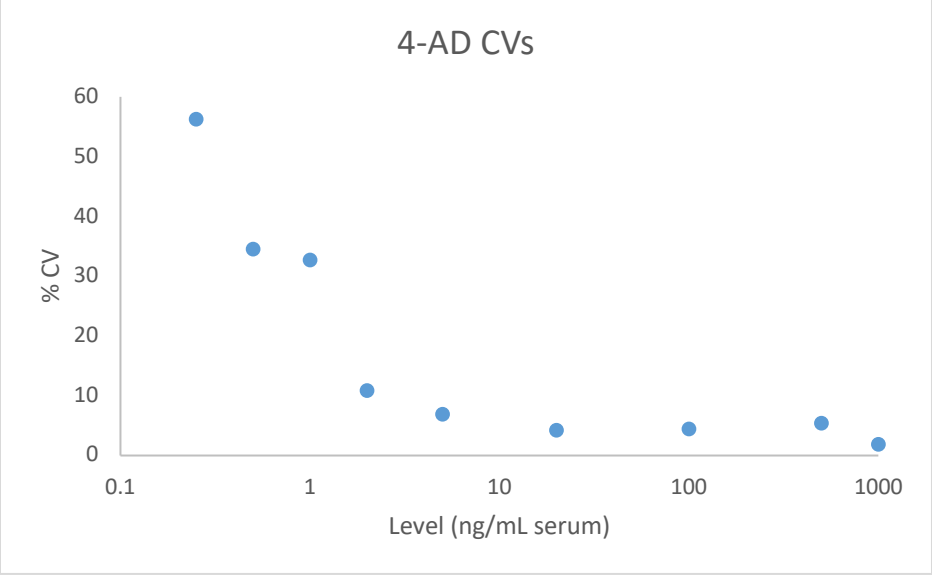

Figure 25. Coefficients of variation for 4-AD for replicate analyses of DBS extracts.

LOQ determinations using both S/N and CV criteria are summarized in Table 15. Based upon LOQ findings as well as the concentration range over which all five target compounds showed acceptable linearity, and in order to utilize identical calibrators for all five compounds, calibration curves consisting of DBS enriched with concentrations ranging between 2 to 500 ng/mL serum were used for

subsequent analyses. A weighting factor of 1/x was used for generation of calibration curves for patient sample analysis.

Table 15. LOQ determinations based upon signal-to-noise ratio and reproducibility of replicates.

	Lower Limit of Quantitation (LOQ), ng/mL serum	
Analyte	**Signal-to-Noise ≥ 10:1**	**CV ≤ 20%**
CORT	2.00	2.00
21-DOC	2.00	2.00
11-DOC	0.50	1.00
17-OHP	1.00	1.00
4-AD	0.50	2.00

4.5 HPLC column batch-to-batch reproducibility

Six HPLC columns, two columns from each of three production lots, summarized in Table 16, were utilized for batch-to-batch comparison to evaluate column reproducibility and reliability.

Table 16. Summary of columns used for HPLC column batch-to-batch reproducibility evaluation.

Column Number	Production Lot	Serial Number
1	5715-0055	H17-379453
2	5715-0056	H17-293218
3	5715-0057	H17-317696
4	5715-0055	H17-379454
5	5715-0056	H17-293219
6	5715-0057	H17-317698

Following replicate (N = 5) analyses of an unextracted standard solution containing the five target analytes, five isotope-labeled internal standards, as well as corticosterone and deoxycorticosterone as possible isobaric interferences, metrics for peak area, retention time, peak width, and resolution of critical pair (11-DOC from corticosterone) were compared across column lots and showed excellent reproducibility across all metrics, not exceeding overall variability of 5% CV for

peak area, analyte retention time, or peak width (using full width at half maximum, FWHM) across all columns. Additionally, all six columns showed acceptable baseline resolution > 1.5 of the critical pair (11-DOC and corticosterone).

Additionally, extracts of DBS QC material were pooled and then replicates (N = 5) were analyzed using each HPLC column. It should be noted that, while the calculated concentrations for the column with serial number H17-379453 were comparable to the other columns in the study, the pooled DBS extraction volume ran low when testing this column, therefore the DBS QC data from this column are potentially unreliable and were excluded from comparison analysis. Comparison metrics from the pooled DBS extracts were acceptable across the remaining five columns in the study.

Because acceptable retention, resolution, and reproducibility were observed across and within production lots, using both analytical standards as well as pooled DBS extracts, the NBS laboratory is able to predict with high confidence that the assay should continue to perform in a reproducible manner as HPLC columns are periodically replaced.

4.6 Recovery

Analyte recoveries were determined through replicate (N=10) analyses of DBS QC materials provided by the CDC NSQAP. Internal calibration was performed using Equation 2 to obtain calculated concentrations for each analyte within the specimen without influence from a calibration curve. Recoveries are summarized in Table 17.

Table 17. Analyte recoveries.

		Recovery (%)				
Sample	Enrichment (ng/mL serum)	CORT	21-DOC	11-DOC	17-OHP	4-AD
C1811	50	107	76	153	71	76
D1811	100	101	78	151	74	76
E1811	500	110	80	151	72	77

Analyte recoveries for CORT, 21-DOC, and 4-AD were within ± 25% of the reported enrichment level. 17-OHP recoveries were slightly lower, between 71% and 74%, though the recoveries are consistent across all concentration levels. Recoveries below 100% are likely due to failure to fully elute the analyte from the filter paper of the dried blood spot matrix during the extraction process. The recoveries for 11-DOC are much higher (>150%), however they are consistent across all concentration levels. It is unknown at this time why the recovery for 11-DOC was so high, however the use of matrix-matched dried blood spot calibration curves for quantitation of patient specimens corrects for high or low recoveries of each analyte as is indicated by the method accuracy data presented in section 4.7. It should be noted that an additional QC sample from CDC NSQAP, B1811, was analyzed, however data from the B1811 level were excluded from recovery analysis because the concentration values obtained by the NBS laboratory, as well as the consensus mean reported to the CDC from other laboratories, were far lower than the reported enrichment level for cortisol.

4.7 Accuracy and precision

Accuracy was evaluated using calculated QC concentration relative to the established mean concentration for each QC level. Concentrations were calculated using internal standard DBS calibration

curves. The low and high analyte recoveries reported in section 4.6 were corrected for by use of calibration curves. Precision was evaluated using the coefficient of variation of replicates. Inter-run accuracy and precision were evaluated using results for DBS QC materials at low, medium, and high concentration, yielding a total of 52 replicates at each QC level. It should be noted that one additional analytical run was excluded from analysis for all analytes due to a high outlier for 17-OHP at the low QC level. Inter-run accuracy and precision are summarized in Table 18 and Table 19, respectively. Intra-run precision and accuracy were evaluated using 25 replicates of each QC level within a single analytical run. Intra-run accuracy and precision are summarized in Table 18 and Table 20, respectively.

Table 18. Inter- and intra-run accuracy.

Inter- and Intra-run Accuracy (%)						
	QC Low (8 ng/mL serum enrichment)		QC Medium (40 ng/mL serum enrichment)		QC High (200 ng/mL serum enrichment)	
Analyte	Inter-run (N=52)	Intra-run (N=25)	Inter-run (N=52)	Intra-run (N=25)	Inter-run (N=52)	Intra-run (N=25)
CORT	103	99	97	102	102	97
21-DOC	98	102	94	100	100	99
11-DOC	99	106	95	98	100	94
17-OHP	99	101	93	102	98	99
4-AD	97	103	96	104	100	101

Table 19. Inter-run precision.

Inter-run Precision (N=52)									
	QC Low (ng/mL serum)			QC Med (ng/mL serum)			QC High (ng/mL serum)		
Analyte	Mean	SD	%CV	Mean	SD	%CV	Mean	SD	%CV
CORT	10.93	0.80	7.34	47.4	2.2	4.63	258	11	4.33
21-DOC	8.4	1.0	12.3	40.7	2.0	4.97	226	10	4.45
11-DOC	8.81	0.76	8.64	44.0	1.9	4.24	238	10	4.11
17-OHP	9.55	0.70	7.36	47.2	2.3	4.82	253	10	4.04
4-AD	9.34	0.67	7.14	47.0	1.9	4.08	253	11	4.44

Table 20. Intra-run precision.

Intra-run Precision (N=25)									
	QC Low (ng/mL serum)			QC Med (ng/mL serum)			QC High (ng/mL serum)		
Analyte	Mean	SD	%CV	Mean	SD	%CV	Mean	SD	%CV
CORT	10.90	0.55	5.08	48.2	2.5	5.10	250	11	4.53
21-DOC	8.48	0.87	10.2	41.1	2.0	4.97	222	10	4.51
11-DOC	9.46	0.63	6.62	43.9	1.8	4.17	227.8	7.5	3.31
17-OHP	9.82	0.46	4.70	48.5	2.5	5.07	248.9	9.3	3.74
4-AD	9.55	0.42	4.43	48.8	1.9	3.99	254.7	8.6	3.38

The method showed acceptable inter- and intra-run accuracy, well within 100 $\pm$ 20% for all analytes. Inter-run precision was acceptable as all analytes were below 20% CV for replicates. Similarly, intra-run precision was acceptable for all analytes, as CV of replicates was below 15% in all cases.

4.8 Extract stability

DBS extracts showed acceptable reproducibility for calculated concentration, at less than 20% CV for all analytes at one, two, and seven days post-extraction, whether stored under refrigeration or at room conditions. It should be noted, however, that both plates tested at seven days post-extraction required reconstitution prior to analysis due to evaporative loss. For this reason, the storage of plates for later analysis is not recommended beyond two days post-extraction.

4.9 Ruggedness

As many variable factors were introduced into the validation study as were practical. Three analysts performed extractions, instrument analysis and data review throughout the validation. Precision, accuracy, and clinical sensitivity/specificity analyses were performed over a period of 16 days,

by three analysts, with no more than two assays with two QC replicates each being run on any given day. Multiple lots of the various solvents and HPLC columns were utilized throughout the validation study. Mobile phase and DBS extraction solution preparations were performed by multiple analysts throughout the study. As evidenced by the precision and accuracy data, the proposed method performs consistently and is sufficiently rugged given reasonable variation among factors such as personnel and consumable reagents.

4.10 Clinical sensitivity and specificity

A cohort of 344 double-blinded de-identified DBS specimens previously analyzed and reported by Virginia's Newborn Screening Laboratory using the 1st tier time-resolved immunofluorescence assay for 17-OHP, were analyzed using the 2nd tier LC-MS/MS assay over a period of 16 days, comprising 27 analytical runs, using three different analysts. The original results for each sample and clinical status of each patient were unknown to the analysts during analysis and interpretation against the provisional cutoff algorithm. The cohort of de-identified DBS specimens included samples from individuals diagnosed with various forms of CAH, samples shown to produce false positive 17-OHP results by time-resolved immunofluorescence (including samples reported as critical, abnormal, or unsatisfactory), and samples that were within normal limits for 17-OHP by time-resolved immunofluorescence. Samples were considered to be false positive for 17-OHP by time-resolved immunofluorescence if a subsequent sample from the same patient was analyzed by the Newborn Screening Laboratory and found to be within normal limits for 17-OHP by time-resolved immunofluorescence. Where possible, the first specimen from each patient was analyzed in order to evaluate how the 2nd tier assay would perform based on the original specimen received by the laboratory. Clinical sensitivity was assessed by evaluating the effectiveness of the 2nd tier LC-MS/MS assay at identifying cases of various forms of CAH. Clinical

specificity was assessed by determining the effectiveness of the 2nd tier LC-MS/MS assay at reducing false positive results compared with 17-OHP analysis by time-resolved immunofluorescence alone. A summary of the 1st tier screening outcomes for the de-identified patient specimens analyzed for the validation study are included in Table 21.

Table 21. 1st tier screening outcomes for de-identified DBS analyzed during method validation study.

Sample Type	Number of Specimens	Comment
True Positive	16	9 x Salt Wasting (SW) CAH 2 x Simple Virilizing (SV) CAH 5 x Non-classic (NC) CAH
False Positive	118	1st tier 17-OHP Interpretations: 13 x Critical[a] 67 x Abnormal[b] 38 x UNSAT[c]
True Negative	210	
Total	**344**	

[a] Critical interpretation triggers immediate referral for follow-up evaluation and diagnostic testing

[b] Abnormal interpretation triggers request for a repeat DBS specimen to be collected for confirmatory analysis by the NBS laboratory

[c] UNSAT (unsatisfactory) interpretation indicates that valid results were unable to be obtained and a repeat DBS specimen is requested by the NBS laboratory

The provisional cutoff algorithm used for the validation study is provided in Table 22. Per the provisional validation cutoff algorithm, 17-OHP concentration was evaluated in conjunction with the clinical ratio: ([17-OHP] + [4-AD]) / [CORT], which can serve to greatly reduce the number of false positive results reported based upon elevated 17-OHP alone.[28,29,32,33] Additionally, the algorithm incorporates a mechanism for referring, as critical, samples that show a markedly elevated clinical ratio, regardless of 17-OHP concentration. This mechanism may act as a safeguard for 2nd tier analysis of DBS specimens from individuals with CAH that received steroid treatment prior to collection of the DBS specimen.[32]

Table 22. Provisional cutoff algorithm used for 2nd tier LC-MS/MS assay validation study.

Analyte	Abnormal Cutoff (Request Repeat Specimen)	Critical Cutoff (Immediate referral to Follow-up)	Comments
Clinical Ratio: ([17-OHP] + [4-AD]) / [CORT]	1.00*	3.50	Ratio above abnormal cutoff only reported as positive when 17-OHP is >= 12.00 ng/mL serum. Ratio above critical cutoff reported regardless of 17-OHP concentration
17-OHP	N/A	12.00 ng/mL serum*	17-OHP above cutoff is only reported as positive when ratio is >= 1.00
21-DOC	6.00 ng/mL serum	12.00 ng/mL serum	
11-DOC	6.00 ng/mL serum	12.00 ng/mL serum	
*** Group critical ONLY when ratio is >= 1.00 AND 17-OHP is >= 12.00**			

Of the 344 double-blinded patient specimens analyzed, 210 had previously screened as within normal limits by the 1st tier 17-OHP assay, 118 were reported as false positive by the 1st tier 17-OHP assay (including critical, abnormal, and unsatisfactory), and 16 samples were from individuals diagnosed with various forms of CAH. Of the 16 samples from individuals diagnosed with various forms of CAH, nine specimens were from patients diagnosed with the most severe, classic salt-wasting form of CAH, five specimens were from patients diagnosed with a less severe later onset, non-classic form of CAH, and two specimens were from patients diagnosed with the moderate simple virilizing form of CAH. It should be noted that one specimen from a diagnosed case of CAH was originally thought to be associated with a diagnosis of simple-virilizing CAH during the blinded validation study, however, was determined to be a non-classic CAH diagnosis after completion of the validation study.

From the cohort of 210 patient specimens that previously screened as within normal limits by the 1st tier assay, 208 were interpreted as within normal limits by the 2nd tier assay. Two specimens were deemed to be unsatisfactory due to inconsistent results by the 2nd tier assay, requiring a request for repeat NBS specimens, therefore these two results are classified as false positives by 2nd tier.

From the cohort of 118 patient specimens that were shown to be false positives by the 1st tier assay, meaning that a subsequent specimen from each patient was received and analyzed by the 1st tier assay and was found to be within normal limits, 88 specimens were deemed to be within normal limits by the 2nd tier assay. 30 specimens remained as false positives by the 2nd tier assay based upon the provisional validation cutoff algorithm. Of these 30 remaining false positives, 26 were deemed to be abnormal or critical based solely upon results obtained for the 11-DOC analyte. Four of the remaining false positives exhibited results similar to those expected for severe CAH cases, showing elevated 17-OHP with an elevated clinical ratio ([17-OHP] + [4-AD]) / [CORT] and therefore remained as false positives. All four specimens were collected from premature infants.

From the cohort of 16 patient specimens from individuals diagnosed with various forms of 21-hydroxylase CAH, eight of nine specimens from severe classic salt-wasting cases of CAH were identified as critical by the provisional cutoff algorithm. One of five cases of later onset, non-classic CAH was identified as critical by the provisional cutoff algorithm. One of two cases of moderate simple-virilizing CAH was identified as critical by the provisional cutoff algorithm.

Without adjustment, the provisional cutoff algorithm used for the validation resulted in greater than 70% net reduction in false positive results for CAH screening. The provisional cutoff algorithm was sufficient to identify most cases of salt-wasting CAH, however, most notably, the provisional cutoff scheme failed to identify one salt-wasting case of CAH, failed to identify one simple-virilizing case of CAH, and resulted in a larger than expected number of false positive results due solely to the 11-DOC cutoffs. Based

on these findings, adjustments to the cutoff algorithm were proposed that would ensure 100% sensitivity for all salt-wasting and simple-virilizing CAH cases and raise the 11-DOC cutoffs to avoid unnecessary false positive results based solely upon the 11-DOC analyte. The 17-OHP and clinical ratio critical cutoffs were lowered to 8.00 ng/mL serum and 2.00, respectively. The 11-DOC analyte is meant to be indicative of cases of CAH caused by 11β-hydroxylase deficiency, however, the cohort of DBS specimens analyzed for the validation study did not include any known cases of CAH caused by 11β-hydroxylase deficiency. All instances of elevated 11-DOC in the validation study are presumed to be false positives. Therefore, percentiles were calculated in order to adjust the 11-DOC cutoffs to further limit the number of reported false positive results while remaining at a conservative level to avoid missing potential true cases of CAH due to 11β-hydroxylase deficiency. The 11-DOC abnormal and critical cutoffs were raised to 20.50 ng/mL serum and 28.42 ng/mL serum, respectively. These adjusted 11-DOC cutoff values represent the 99.5 and 99.9 percentiles, respectively and remain well below 11-DOC concentrations detected in newborn screening specimens from diagnosed cases of CAH due to 11β-hydroxylase deficiency reported in the literature.[24,31] The proposed adjustments to the cutoff algorithm are depicted in Table 23.

Table 23. Proposed go-live cutoff algorithm.

Analyte	Abnormal Cutoff (Request Repeat Specimen)	Critical Cutoff (Immediate referral to Follow-up)	Comments
Clinical Ratio: ([17-OHP] + [4-AD]) / [CORT]	1.00*	2.00	Ratio above abnormal cutoff only reported as positive when 17-OHP is >= 8.00 ng/mL serum. Ratio above critical cutoff is reported regardless of 17-OHP concentration
17-OHP	N/A	8.00 ng/mL serum*	17-OHP above cutoff is only reported as positive when ratio is >= 1.00
21-DOC	6.00 ng/mL serum	12.00 ng/mL serum	
11-DOC	20.50 ng/mL serum	28.42 ng/mL serum	
*** Group critical when ratio is >= 1.00 AND 17-OHP is >= 8.00 ng/mL serum**			

Re-evaluation of de-identified specimen results against the proposed cutoff algorithm yielded a greater than 90% net reduction in false positive results for CAH screening, while maintaining 100% sensitivity to salt-wasting and simple-virilizing CAH. The false positive results that remained based upon the updated algorithm were all from specimens collected from premature infants.

CHAPTER 5: CONCLUSIONS

An HPLC-MS/MS assay for 2nd tier dried blood spot screening of CAH was developed, optimized, and validated for a concentration range of 2 to 500 ng/mL serum. The method showed acceptable results for precision, accuracy, linearity, and ruggedness. The proven batch-to-batch reproducibility of HPLC columns provides assurance that data will remain consistent, even as columns are replaced. The incorporation of a reversed-phase chromatographic method utilizing an HPLC column with a superficially porous stationary phase support and a biphenyl stationary phase ligand enabled crucial baseline resolution of isobaric compounds, while allowing for rapid separation (5 minutes injection-to-injection) without the need for ultra-high pressure liquid chromatography (UHPLC) equipment. These chromatographic parameters offer significant improvement over other HPLC methods reported in the literature and are more readily adaptable to existing technologies within many newborn screening laboratories. A comparison of pros and cons of both the immunoassay-only (1-tier) algorithm as compared to the new 2-tier approach are summarized in Table 24.

Table 24. Pro-Con comparison of 1-tier vs. 2-tier approached to CAH screening

CAH Screening Workflow	Pro	Con
1-tier, immunoassay-only, with birthweight-adjusted cutoffs	• Rapid: ~3.5 hr to process full 96 well plate • Simple • Semi-automated • Inexpensive	• High false-positive rate (~2% of specimens tested) • Limited information: 17-OHP only
2-tier, immunoassay for 17-OHP followed by HPLC-MS/MS steroid panel	• Significantly reduces false-positive reporting rate • More comprehensive: more information to physicians • Meets target turn-around times • Eliminate need for birthweight-adjusted cutoffs	• More expensive per sample • Slower: ~10.5 hr to process full 96 well plate • Complex data review • Unable to eliminate all false positives

The 2nd tier assay showed greatly improved clinical specificity when compared to the 1st tier time-resolved immunofluorescence assay for 17-OHP alone and is projected to significantly reduce the false positive rate for CAH screening. Based upon the cohort of DBS specimens analyzed as part of the clinical sensitivity and specificity study, there were only eight false positive specimens using the updated 2nd tier CAH cutoff algorithm compared to 118 false positives (critical, abnormal, or unsatisfactory designations) from the same cohort of DBS specimens using only the immunoassay for 17-OHP, even when utilizing birthweight-adjusted cutoffs. This represents greater than 90% reduction in total false positives from the study. Based upon the analysis of de-identified DBS specimens from newborns diagnosed with various forms of CAH using the provisional cutoff algorithm for method validation, the 2nd tier assay showed clinical sensitivity for most cases (eight of nine) of the most severe classic salt-wasting form of CAH. Proposed updates and improvements to the cutoff algorithm will ensure 100% sensitivity for classic salt-wasting and simple-virilizing CAH for this group of specimens. The 2nd tier assay is not predicted to achieve 100% sensitivity for less severe non-classic form of CAH. Similar outcomes are documented in the literature.[29,48,49]

Under the proposed workflow to incorporate the validated 2nd tier LC-MS/MS assay into CAH screening, the NBS laboratory will eliminate confirmation testing, in duplicate, by the 1st tier assay. Instead, any specimens showing elevated 17-OHP by 1st tier fluorescence immunoassay will be forwarded directly to the 2nd tier analysis. The NBS laboratory will, therefore, analyze a higher proportion of samples per year by the 2nd tier assay than other NBS programs which employ 2nd tier assays for CAH screening, however, the laboratory will be better positioned to maintain an approximate two-day turnaround time for CAH screening results. This anticipated turnaround time will enable the laboratory to continue to report time-critical CAH results within nationally recommended guidelines.[6]

Several parallel endeavors beyond method development and validation were initiated in order to move toward full implementation of 2nd tier CAH screening within Virginia's Newborn Screening Laboratory. One effort included coordination with software developers for design and testing of laboratory information management system integration for sample tracking, resulting, and reporting of the 2nd tier CAH screening workflow. Additionally, input and feedback were solicited from pediatric endocrinologists on the screening workflow and cutoff algorithm to ensure that the 2nd tier assay will meet program goals without detrimental impacts to the screening process and clinical outcomes. A cohort of additional laboratory personnel were trained to perform the assay in order to allow for 7 day-per-week, 365 day-per-year operation. A second LC-MS/MS instrument is also being validated to serve in a backup capacity to ensure that 2nd tier analysis is able to be performed each day, even if the primary instrument is down for maintenance or repairs.

The 2nd tier CAH screening assay was moved into production by the Virginia NBS program on October 2, 2019. During the first month of testing, out of 8993 samples received for NBS analysis, 570 samples were analyzed by the 2nd tier CAH screening method. Of the 570 samples analyzed by the 2nd tier CAH screening method, 23 were reported as critical and were referred to the Virginia Department of Health for follow up. Four samples were reported as abnormal prompting request of an additional DBS

specimen. A total of nine samples were deemed unsatisfactory due to lack of adequate sample quantity or inconsistent analytical results and an additional DBS specimen was requested. The remaining 534 specimens were deemed to be within normal limits by the 2nd tier assay. One case whose DBS sample was identified as critical by the 2nd tier assay received a diagnosis of the classic salt-wasting form of CAH. Nine samples reported as critical or abnormal by the 2nd tier assay have received a subsequent sample from the same infant that was within normal limits by the 2nd tier assay, indicating that the critical or abnormal results reported for the original samples were likely false positive. Outcomes for the remaining critical or abnormal specimens are pending.

Following full implementation of the 2nd tier CAH screening assay, continual monitoring of screening outcomes will help to inform quality improvement activities as well as any necessary changes to the assay workflow or cutoff algorithm in order to achieve the greatest possible reduction to the false positive reporting rate for CAH screening, while avoiding missed cases of salt-wasting or simple-virilizing CAH.

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CURRICULUM VITAE

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Education

B.S. Chemistry, 2005, North Carolina State University, Raleigh NC

M.S. Chemistry, 2019, Virginia Commonwealth University, Richmond VA

Work Experience

Job Title: Scientist II - Principal Scientist, Newborn Screening Laboratory, 2015-2019

Location: Virginia Division of Consolidated Laboratory Services, Richmond VA

Duties: Responsible for investigation, optimization, validation, and implementation of dried blood spot screening and confirmatory assays for the detection of metabolic and endocrine disorders in neonates. Evaluation, validation, and implementation of FDA-cleared kit for dried blood spot screening for lysosomal storage disorders. Work with IT development teams to establish requirements for integration of screening assays into Laboratory Information Management System and perform user acceptance testing prior to implementation. LC-MS/MS technical leader, responsible for evaluation and validation of new instrumentation, training of personnel, and continual quality improvement. Write technical reports and standard operating procedures.

Job Title: Scientist II – Scientist Senior, Chemical Terrorism Response and Preparedness Laboratory, 2010-2015

Location: Virginia Division of Consolidated Laboratory Services, Richmond VA

Duties: Responsible for quantitative analysis using LC-MS/MS, GC-MS, and ICP-MS for clinical and environmental specimens. Maintained chain-of-custody for evidentiary samples. Performed individual and collaborative method development, optimization, and validation projects and worked with grant lead to coordinate projects for other scientists. Technical leader responsible for review and interpretation of quantitative analytical data and training of laboratory personnel. Trained first responders statewide regarding proper sample collection, chain-of-custody procedures, and laboratory

response capabilities. Wrote and reviewed technical standard operating procedures. Responsible for review of QA/QC data and procedures.

Job Title: Scientist I, Chemical Terrorism Response and Preparedness Laboratory, 2006-2010

Location: Virginia Division of Consolidated Laboratory Services, Richmond VA

Duties: Responsible for quantitative analysis using LC-MS/MS and GC-MS for clinical and environmental specimens. Maintained chain-of-custody for evidentiary samples. Assisted with development and implementation of automated 96-well plate extraction protocols. Maintained stockpile of laboratory consumables for coverage during emergency surge capacity events. Oversaw preparation and distribution of Environmental Crimes Task Force field sampling kits to first responders. Trained first responders statewide regarding proper sample collection, chain-of-custody procedures, and laboratory response capabilities.

Job Title: Contractor (Kelly Scientific), Chemical Terrorism Response and Preparedness Laboratory, 2005-2006

Location: Virginia Division of Consolidated Laboratory Services, Richmond VA

Duties: Responsible for supply ordering, assembly, quality checks, and shipment of Environmental Crimes Task Force field sampling kits to first responders. Maintained database of all chain-of-custody evidentiary samples analyzed by the laboratory group. Completed training for one method utilizing LC-MS/MS analysis of clinical specimens. Received training and performed analysis of unknown environmental specimens using GC-MS.

Publications

1. Guiseppi-Elie, A., Taylor, S., Lingerfelt, L., **Nixon, C.**, Georgiana, R., Kim, J., Smith, S., Mangrum. B., Farrell, N. Studies of the Interaction of Platinum Drugs with DNA Using Oligonucleotide Microarrays. *Macromolecular Symposia* **2006**, 235, 115-120. DOI: 10.1002/masy.200650314

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4. Reddy, M.K., **Nixon, C.**, Wyatt, S.A., Croley, T.R. A robust high-throughput sample preparation and liquid chromatography/tandem mass spectrometry method for the quantitation of β-lyase metabolites of sulfur mustard as 1,1'-sulfonylbis-[2-(methylthio)ethane] in human urine. *Rapid Commun. Mass Spectrom.* **2013**, 27, 1128-1134. DOI: 10.1002/rcm.6541