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Stress is experienced all over the world in various forms. It is typically diagnosed through the evaluation of psychological and physical factors. These diagnoses are often time consuming. Stress can negatively impact individual's health and quality of life and treatment for stress is often difficult because stress tolerance levels are different for everyone. Cortisol is a steroid hormone that is released as a response to stress and changes in blood sugar levels. Cortisol is produced naturally in humans and is detectable in blood, urine, saliva, and hair.[1,2]

Liquid chromatography (LC) coupled with mass spectrometry (MS) is a useful analytical technique in biological research. The goal of this study is to develop an LC-MS method that will allow clinical researchers and healthcare professionals to quickly quantify cortisol levels of individuals over a specific timeframe.

Our research focuses on cortisol produced in human hair. The goal is to integrate the methods in the literature and establish a non-invasive method for quantifying cortisol in hair samples. We seek to replace a commercialized immunodetection method for measuring extracted cortisol with an ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) method due to its benefits of higher sensitivity and lower running costs. In the LC-MS method, reversed phase chromatography is used. The eluent from the LC column is ionized by electrospray ionization. The triple quadrupole mass spectrometer is operated in selected reaction monitoring (SRM) mode. A standard

curve was used to determine that the linear dynamic range of the cortisol was 0.80-500 ng/mL.

The long-term goal of this research project is to apply the established LC-MS method to determine the cortisol level in hair and correlate the information to stress level experienced by individuals, in a collaborative research study in the School of Nursing at UNCG.

MASS SPECTROMETRIC DETECTION OF CORTISOL IN HAIR SAMPLES

by

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APPROVAL PAGE

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Date of Final Oral Examination

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CHAPTER I

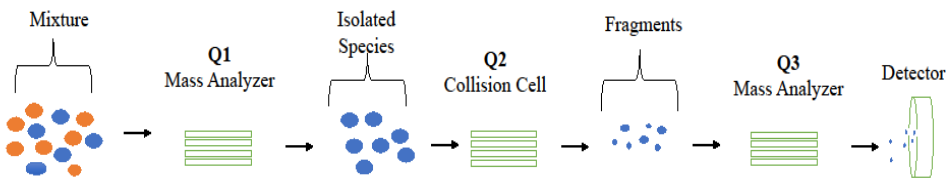
INTRODUCTION

1.1 Mass Spectrometry Overview

Mass spectrometry is an analytical tool that sorts analytes based on their mass to charge ratio. Our research utilizes a liquid chromatography mass spectrometry (LC-MS) method. In the LC-MS method, reversed phase chromatography is used. The eluent from the LC column is ionized by the electrospray ionization technique. Figure 1 highlights the mass spectrometric measurements conducted using selected reaction monitoring (SRM) mode in the triple quadrupole instrument.

SRM is a technique used in triple quadrupole instruments that involves collision-induced dissociation to increase selectivity. The triple quadrupole mass spectrometer consists of three quadrupoles. Quadrupole 1 detects cortisol, quadrupole 2 causes fragmentation, and quadrupole 3 selects fragments.

Figure 1. A Schematic Diagram of a Triple Quadrupole Mass Spectrometer



1.2 Advantages and Disadvantages of Mass Spectrometry for Studying Steroid Hormones

1.2.1 Comparing Other Known Methods

Many methods have been used to quantify cortisol in hair. Two methods are chemiluminescence detection immunosorbent assays (CLIA) and enzyme-linked immunosorbent assays (ELISA).[3] These commonly used approaches were originally designed for cortisol detection and measurements in saliva.[4] However, the specificity of these methods is low and could cause inaccurate determination of cortisol levels.[6]

Other methods commonly used for cortisol quantification in hair samples are high performance liquid chromatography with fluorescence detection (HPLC-FLU) and gas chromatography mass spectrometry (GC-MS). `The HPLC-FLU approach involves pretreatment procedures that are very time consuming and requires large quantities of hair[3]. The GC-MS approach shows good specificity, but is also very time consuming, includes derivatization steps, involves large sample volumes, and has long-throughput times[6].

The proposed method uses selected reaction monitoring (SRM). In SRM mode, precursor ions are selected in the first quadrupole. The ions are fragmented in the second quadrupole and the product ion is selected in the third quadrupole for detection. SRM is more selective due to its ability to do target analysis. Unlike the GC-MS method, our method is 7 minutes long and requires no derivatization. The goal of this thesis research was to develop a sensitive UPLC-MS/MS method for the quantification of cortisol in

human hair. The instrument was operated in the positive mode electrospray ionization (ESI).[6]

1.2.2 History of Cortisol Quantification and Stress Determination

Hans Seyle initially introduced the notion of the stress response in 1936 and categorized it as the moment when a possible threat to the homeostasis of the organism is perceived by the central nervous system.[2] When this happens the hypothalamic-pituitary-adrenal axis (HPAA) is activated and cortisol, shown in Figure 2, is secreted. The response of HPAA is associated with both acute and chronic stress.[2] Measurements of cortisol concentrations are important to research because they share a direct relationship with psychological function. The potential impact of cortisol measurements include clinical, epidemiological and fundamental psychobiological benefits.[3] Therefore, over the last few years, an interest in noninvasive techniques to view stress response has increased.[2]

Figure 2. Molecular Structure and Molecular Mass of Cortisol

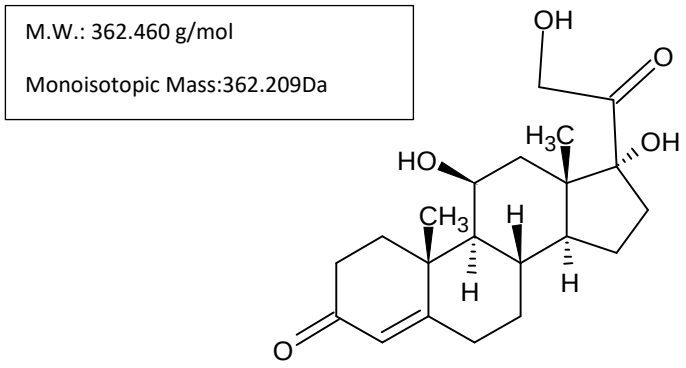


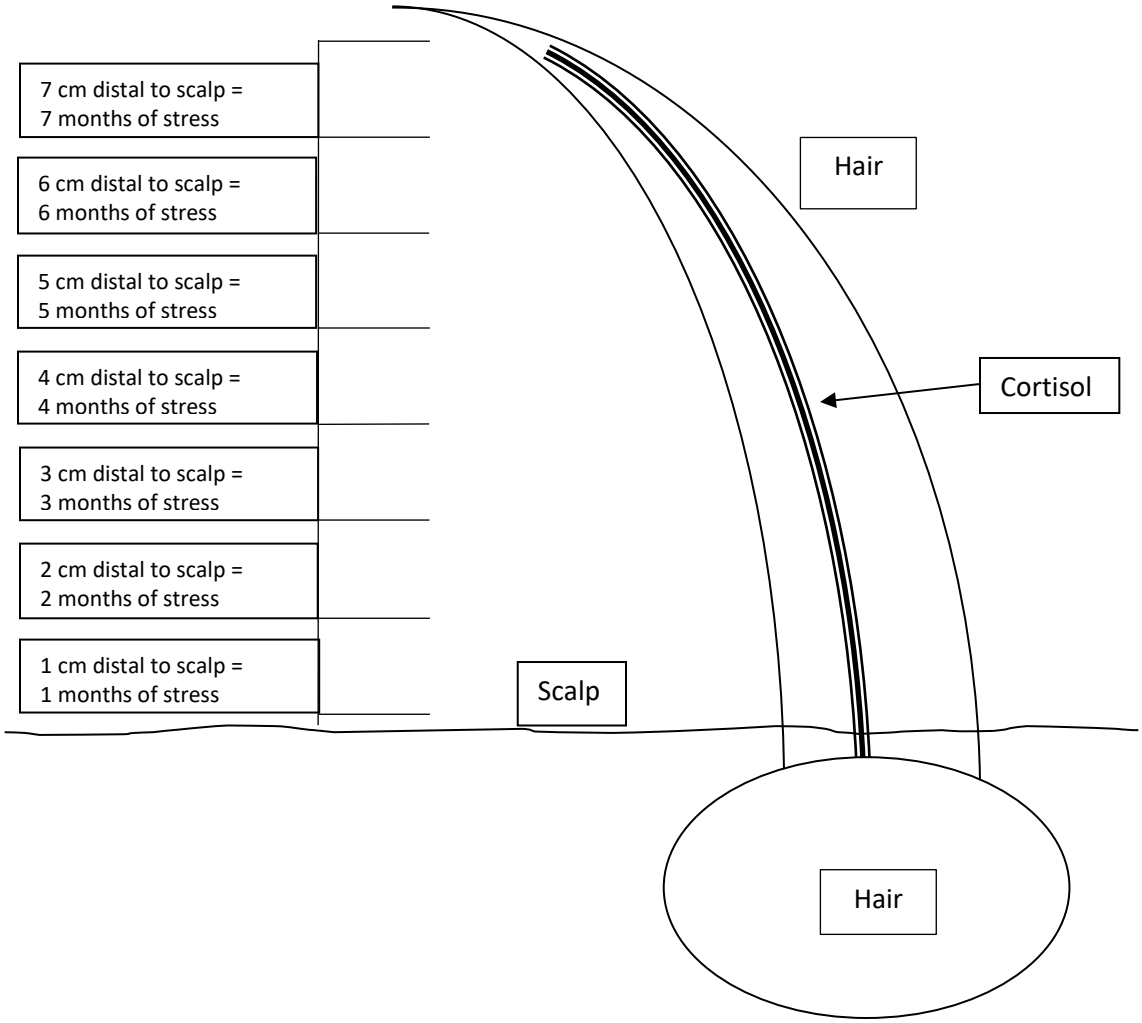
Illustration of the cortisol compound highlighting its molecular weight (M.W.) and monoisotopic mass.

The purpose of this study is to establish an existing method for quantifying cortisol levels in hair within our own facility. Ultimately, we want to use this method to determine stress levels.

Cortisol quantification is an important aspect of clinical research because it is recognized as a biomarker in evaluating stress related illnesses.[4,5] Current sources of cortisol analysis include blood, urine, and salvia.[5] Human hair, however, is becoming the source of interest because it could serve as a non-invasive method for stress analysis[6].

An interest in using hair samples as the non-invasive approach has increased because it offers the benefit of being able to view and measure stress chronologically.[2] Human hair grows an average of approximately 1 cm per month; therefore, the cortisol in a 1 cm segment of hair highlights cortisol exposure of 1 month. Longitudinal studies can be done to detect stress over a period of months to years as shown in Figure 3.

Figure 3. Diagram of Cortisol Production in Hair and Psychological Stress



This diagram highlights hair growth from the scalp in centimeters and illustrates how 1 cm of hair is equivalent to 1 month of stress exposure. [12]

1.3 Specific Goals

1.3.1 Acquire Reference Chromatogram of Cortisol Standard

The goal is to acquire a reproducible chromatogram of the cortisol standard using LC-MS. This was accomplished by diluting the standard with 10% methanol, 90% water, to obtain various concentrations for mass spectrometry analysis. Analysis will be

performed using UPLC and triple quadrupole mass spectrometry. The chromatogram obtained from this experiment will be used as the reference chromatogram in experimental studies.

1.3.2 Evaluate Cortisol Levels in Hair Samples of Healthy Volunteers

The goal is to examine the cortisol levels in the hair samples of the volunteers. Using the reference chromatogram of the cortisol standard, the amount of cortisol present can be determined. Human hair samples from healthy volunteer(s) will be obtained and analyzed using the UPLC-MS/MS. The outcome of these experiments will demonstrate the significance of using UPLC-MS/MS to quantify cortisol in hair samples.

CHAPTER II

ACQUIRE REFERENCE CHROMATOGRAM OF CORTISOL STANDARDS

2.1 Introduction

The goal of this chapter is to review an earlier study and repeat the protocol to obtain a usable chromatographic profile of the cortisol standard using UPLC-MS/MS method. A 1 mg/mL cortisol standard was obtained from Sigma Aldrich. A series of dilutions was used to create a standard curve. Analysis was performed using a Waters Acquity UPLC coupled to a Thermo Fisher Scientific TSQ Quantum Access triple quadrupole mass spectrometer. The chromatograms obtained from these experiments will be used as the reference chromatograms in experimental studies.

2.2 Methods

2.2.1 Instrumentation

Liquid chromatography-mass spectrometry was performed using an Aquity ultra-high performance liquid chromatography (UPLC) system (Waters Corporation) coupled to a TSQ Quantum triple quadrupole mass spectrometer (Thermo Fisher Scientific). Xcalibur software was used to conduct analysis. Solvents used for chemical analyses were purchased from Thermo Fisher Scientific.

2.2.2 Preparation of Sample Dilutions

A standard of cortisol was purchased from Sigma-Aldrich at a concentration of 1 mg/mL in methanol and kept at -40 ºC until usage. The standard was returned to the freezer directly after usage. A 90% optima grade water/ 10% optima grade methanol solution was made and used to dilute the samples to the desired concentration levels. A 10-fold dilution series was used to dilute the standard concentration of 1 mg/mL down to a concentration of 1 µg/mL. Then a series of 2-fold dilutions were used to create a range of concentrations from 0.240 ng/mL and 250 ng/mL. An outline of our experimental approach is shown in Figure 4.

Figure 4. Workflow of Overall Process to Detect Cortisol Using LC-MS

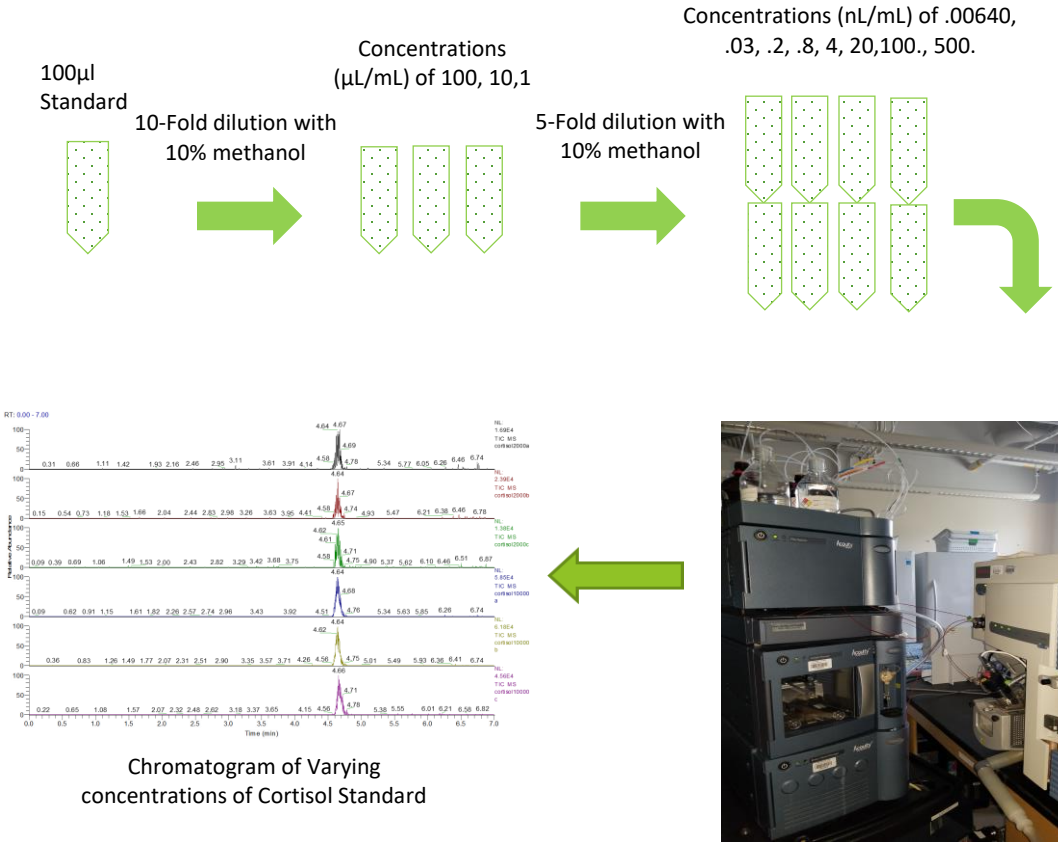
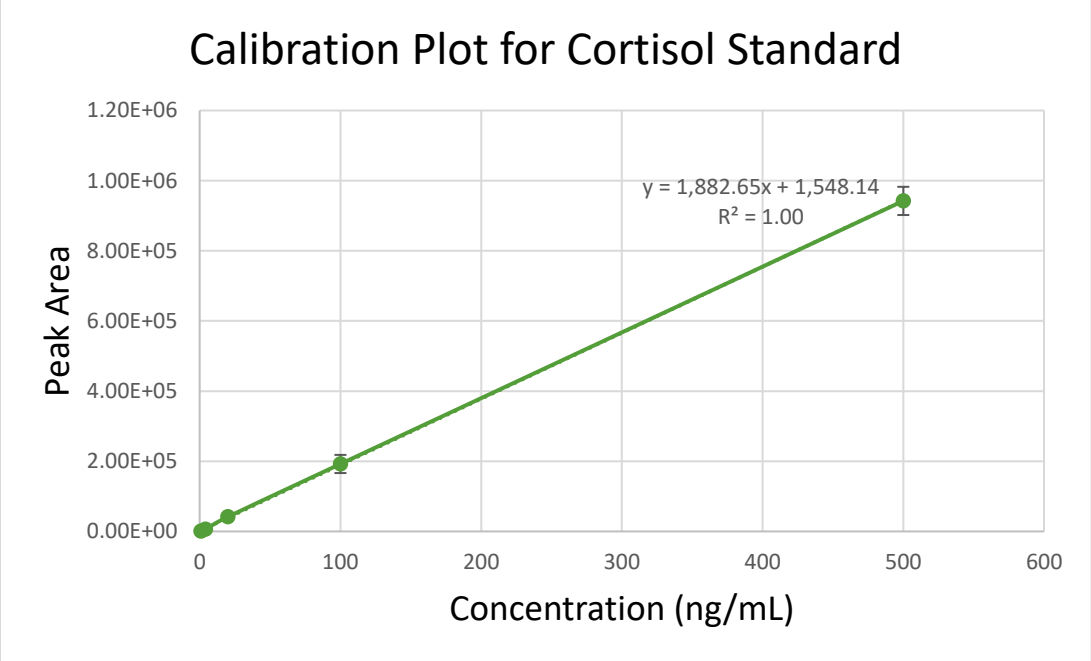


Diagram illustrates the sample preparation and the expected result using our standard cortisol dilutions.

Our preliminary data showed that the cortisol standard can be analyzed using the established method. To determine the lowest concentrations of cortisol that could be detected, a 5-fold dilutions series was used to create a range of concentrations from 0.00640 ng/mL to 500 ng/mL (Figure 5).

Figure 5. Calibration Plot of Cortisol Standard



Calibration plot of cortisol standard resulting from 5-fold dilutions of cortisol over a range of 0.8 ng/mL and 500 ng/mL. The error bars represent the standard deviation of the peak area values and the sample size (n) is equivalent to 5.

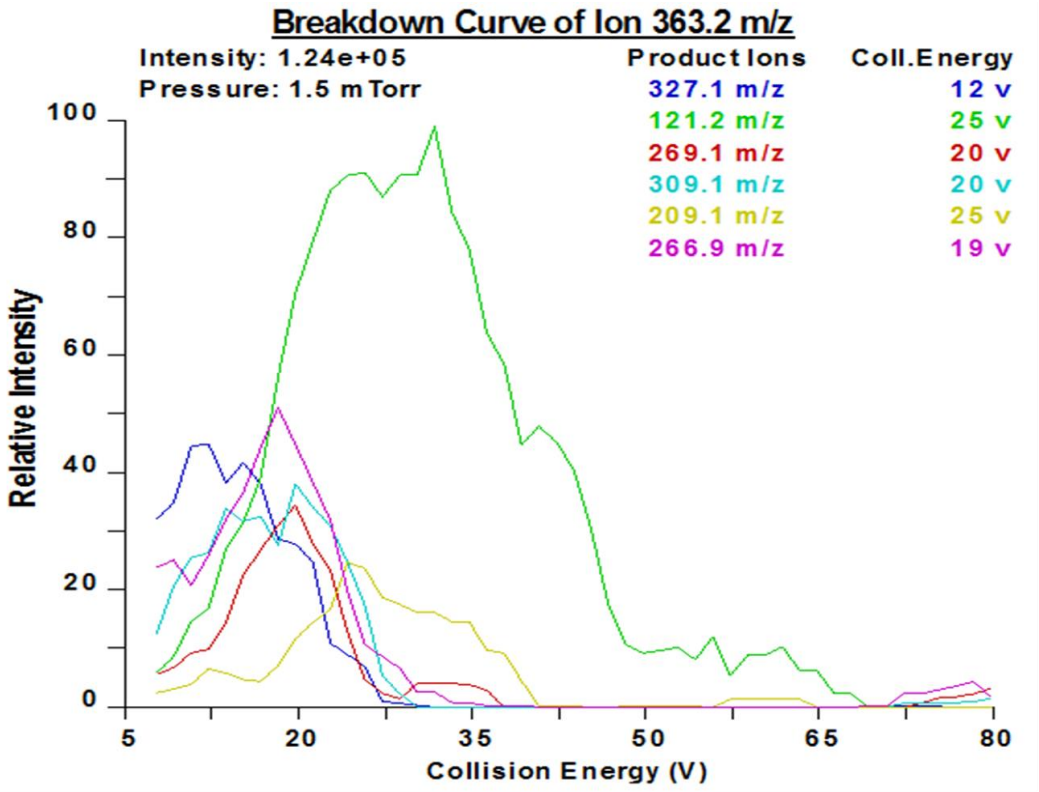
2.2.3 LC-MS/MS Analysis

Cortisol was measured with a LC-MS/MS method. The chromatographic separation was performed on a Waters Acquity UPLC BEH C18 1.7µm, 2.1 x 50mm column with a water and methanol gradient. The Thermo Fisher Scientific TSQ Quantum Access system was equipped with an ESI source operating in positive mode. The UPLC flow rate was set to 0.5mL/min with mobile phase consisting of solvent A, optima LC/MS grade water with 0.1% formic acid, and solvent B, optima LC/MS grade methanol, using the following gradient: Initial – 0.5 min 95% A, 5% B; 0.5-2.5 min 95% A, 5% B; 2.5-5.0 min 60% A, 40% B; 5.0-6.5 min 40% A, 60% B; 6.51-7.0 min 95% A,

5% B. A 10µL injection of sample solution was eluted from a column using a binary solvent system. Peak integration and calculations of concentrations against the standard curve were performed using Xcalibur software.

The mass spectra were collected using a positive mode selected reaction monitoring scan event of the parent mass of cortisol (m/z 363.2). Optimization of fragmentation is shown in Figure 6.

Figure 6. Results from CID Fragmentation of Cortisol



The plot highlights the most abundant fragment ions produced within the cortisol sample of 1 μg/mL concentration. Fragment with m/z ratio of 121 (shown in green) was used as our ion of interest for running our samples in SRM mode because it was the most abundant ion of the ions shown.

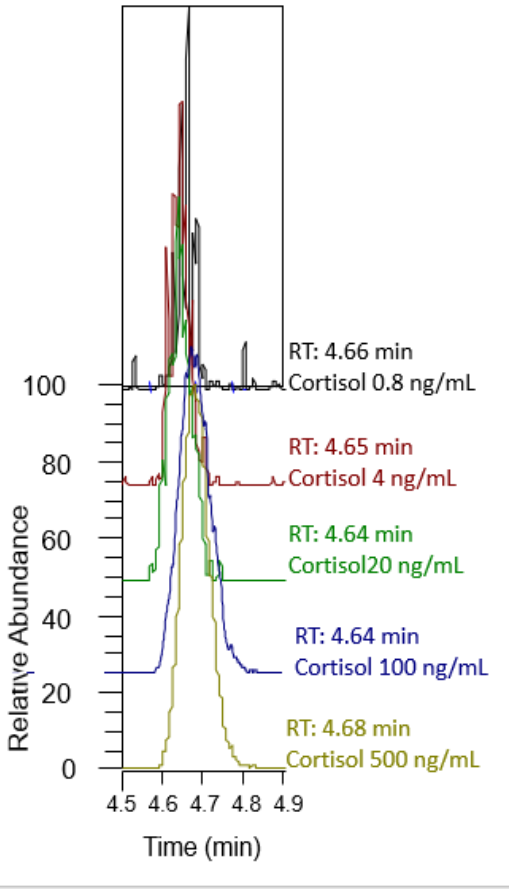
2.3 Results and Discussion

2.3.1 Chromatogram of Standard Cortisol Solution

Our dilutions of the cortisol standard ranging from 0.0064 ng/mL to 500 ng/mL were tested in triplicate on the same day. We were able to determine that our protocol works for our standard solutions and that our data is reproducible. The expected retention time to see our analyte is around 4.6 minutes on the chromatogram. Figure 7 shows our cortisol standard dilutions eluting at 4.6 minutes starting at a concentration of 0.8 ng/mL

to 500 ng/mL.. From the results we have, we were able to develop a standard plot for the analysis of cortisol.

Figure 7. Chromatograms of Varying Concentrations of Cortisol Standard

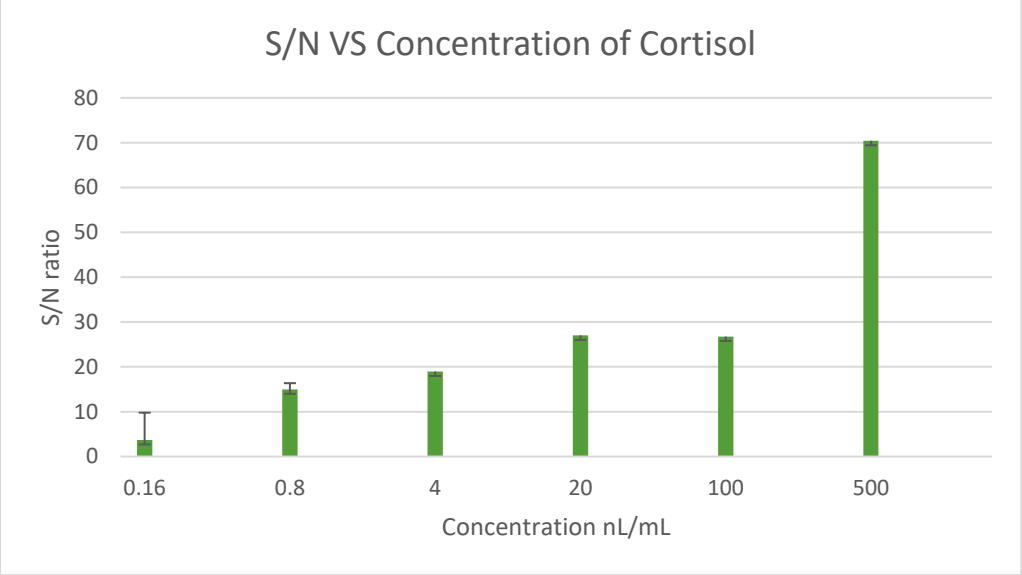


One representative chromatogram resulting from each cortisol standard concentrations of 0.8 ng/mL, 4 ng/mL, 20ng/mL, 100ng/mL, and 500 ng/mL in triplicate run on same day.

2.3.2 Reproducibility of Method

We analyzed each standard sample at varying concentrations using the UPLC-MS/MS method and constructed a calibration plot. Assessment of the reproducibility of the method was conducted on the 5-fold dilution. Analysis of the samples were completed in triplicate on three different days. The signal to noise ratio was calculated to determine our limit of detection (LOD) and limit of quantification (LOQ). The LOD for this method were estimated to be 0.16 ng/mL and the LOQ for this method was determined to be 0.8 ng/mL respectively, shown in Figure 8.

Figure 8. Plot of Signal/Noise Ratio Compared to the Varying Concentration Levels of Cortisol.



Plot of signal/noise ratio compared to the varying concentration levels. The graph excludes concentrations below 0.8 ng/mL. We can see that the S/N ratio is greater than 10. Therefore, we can see that the LOQ is 0.8 ng/mL.

A linear range of 0.8 ng/mL and 500 ng/mL was obtained for the method. This method was successfully applied to the standard cortisol solution purchased from Sigma-Aldrich. This demonstrated that UPLC-MS/MS can be used for the determination and quantification of cortisol. Intra-day and Inter-day analyses were performed on the cortisol standard and are shown in Table 1. According to the intermediate precision data in Table 1, concentrations of 4 ng/mL and higher are reproducible on different days. However, the lower concentration of 0.8 ng/mL yielded an intermediate precision value of 132%; which is greater than 100%. This is due to having an abnormally low concentration value for one of the two days concentration measurements were performed. Because of this large value, we must say that the concentration of 0.8 ng/mL is not reproducible at this time.

Table 1. Precision Data for Cortisol Standard

Theoretical concentration (ng/mL)	Measured concentration[a] (ng/mL)	Residuals[b] (%)	Repeatability[c] (%)	Intermediate precision[d] (%)
0.800	0.782	2.25%	9.67%	132.%
4.00	3.26	18.5%	13.7%	19.7%
20.0	20.2	1%	1.35%	9.41%
1.00×10^2	1.01×10^2	1%	5.29%	6.03%

[a]The measured concentration is an average of back-calculated concentration of cortisol obtained from triplicate analyses on two different days.

[b]Measured concentration-theoretical concentration)/ theoretical concentration x 100.

[c]Repeatability is expressed as the percentage relative standard deviation for triplicate analyses conducted on a single day.

[d]Intermediate precision is expressed as the percentage relative standard deviation of the three back-calculated cortisol concentrations (each on average of triplicate measurements) determined on two separate days.

2.4 Conclusion

We present a LC-MS/MS based method for cortisol quantification. Using this method, we were able to quantitatively measure the cortisol standard at various concentrations in SRM mode. In SRM mode we used the most abundant fragment ion (m/z of 121.2), which had the most representation of scans across the peaks and thus the best sensitivity for our analyte.

In addition, the mobile phase and solvents are important to our study and can present potential problems if the wrong solvents are used. We tried different columns such as the Waters Acquity UPLC T3 1.8µm, 2.1 x 50mm column suggested by prior studies that we were attempting to replicate from the literature.[7] Our elution gradient began with a 0% methanol and 100% water. Due to availability and complications, we switched our column to a Waters Acquity UPLC BEH C18 1.7µm, 2.1 x 50mm column in which we must consider stability. A C18 BEH (Ethylene Bridged Hybird) column is less effective in 100% aqueous solutions.[8] This type of column is more effective in organic solutions. Therefore, we switched to an elution gradient that began with 5% methanol and 95% water. For the same reasons, we also changed the solvent used to dilute the samples from 100% UPLC grade methanol to solution containing 10% of UPLC grade methanol and 90% of UPLC grade water.

CHAPTER III

EVALUATE MEASUREMENTS OF CORTISOL LEVELS IN HEALTHY VOLUNTEER HAIR SAMPLES

3.1 Introduction

The goal of this chapter is to determine cortisol levels in human hair samples from healthy volunteers through UPLC-MS/MS methodology. Healthy volunteers are characterized as adults between the ages of 18 and 50. The average concentration of cortisol in each of the volunteers used in this study and the amount of cortisol in picogram per milligram of hair used is highlighted in Table 2. Based on the results of the cortisol standard plot obtained in our earlier work, we expect that we can identify and compare levels of cortisol secretions in hair samples. The outcome of these experiments will demonstrate the suitability of using UPLC-MS/MS to quantify cortisol in hair samples.

Table 2. Results of Volunteers.

Person	Avg. Conc. (ng/mL)	Cortisol in pg per mg of hair
C	6.63	51.8
E	15. 6	120

Persons A, B and D are not shown due to availability and undetectability.

3.2 Methods

3.2.1 Sample Collection

Hair was sampled from male volunteers ranging from ages 18-50 years old. The entire length of hair to be sampled was secured with a rubber band and the hair cut from the posterior vertex. Afterwards, we used a ruler to measure 3 cm from the first cut and cut again to obtain the 3 cm sample. The sample was stored in a 15 mL polypropylene centrifuge tube at -20 ºC until we were ready to use it.

3.2.2 Sample Washing and Drying

Prior to use, the hair was washed with 5 mL of LC-MS grade isopropanol at room temperature, followed by repeated inversion for 3 minutes using a rotator. The isopropanol was drained into a waste container. Washing steps were repeated once and the hair samples were allowed to dry in an incubator at 52 ℃. The samples were weighed every 2 hours until constant weight was achieved, indicating dryness. It takes approximately 7 to 8 hrs for the hair to dry at this temperature.

3.2.3 Sample Preparation and Cortisol Extraction

Once the sample dried, the hair was cut into small pieces on a weigh boat and added to a preweighed 2 mL microcentrifuge tube with sterile forceps. Up to 60 mg of hair was added to each 2 mL microcentrifuge tube. Afterwards, 1.5 mL of methanol was added to the microcentrifuge tube, the tube was capped, and the sample was incubated for 18 hrs. at room temperature with constant inversion using the rotator. The samples were centrifuged at 10,000 rpm for 5 min at room temperature and 1 mL of clear supernatant was transferred to a clean 1.5 mL microcentrifuge tube.

3.2.4 Solvent Evaporation and Sample Reconstitution

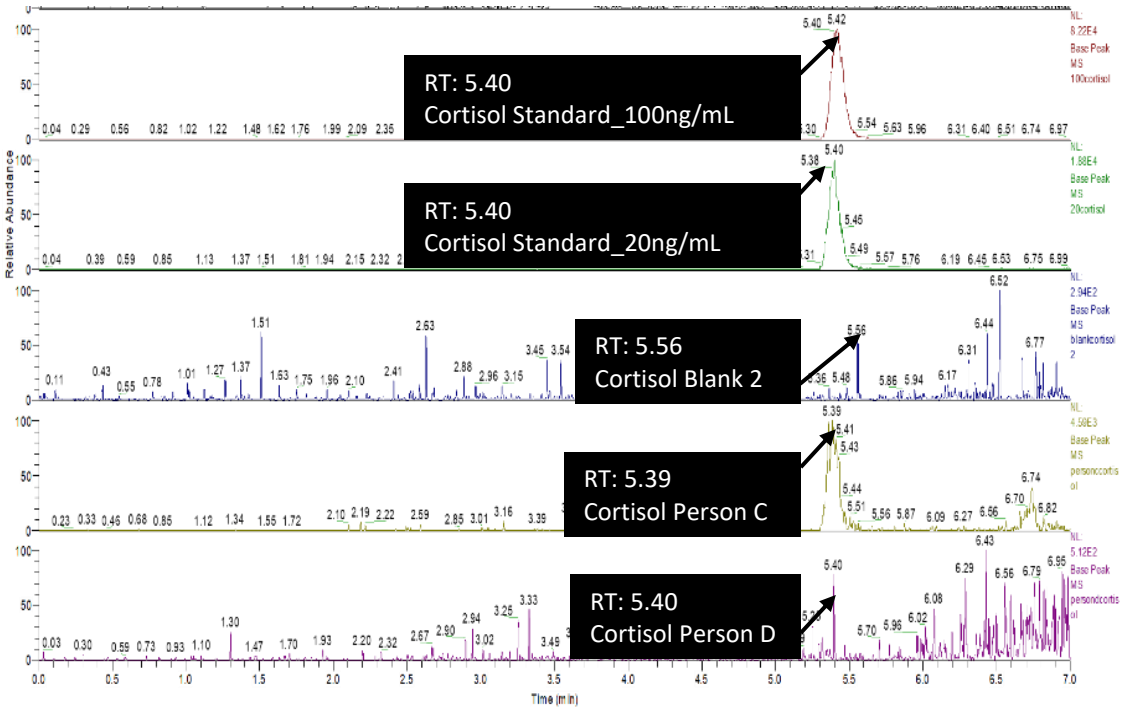
The methanol was evaporated by a Speedvac vacuum evaporator, until completely dry. The dry residue was resuspended in 200 µL 90:10 Optima grade water: Optima grade methanol and immediately used or frozen at -20ºC for later analysis.

3.3 Results and Discussion

3.3.1 Chromatogram of Cortisol from Human Hair

Our samples from person C and person D were tested along with the cortisol standard dilutions made on the same day. Analysis was conducted, and we were able to determine that our protocol was able to measure cortisol extracted from the hair sample. We expected to see cortisol at around 4.6 minutes on the chromatogram. Figure 9 shows the expected cortisol peak from person C, coming out at around 5.4 minutes, which is in line with our cortisol standard ran during the same analysis. This shift in retention time from 4.6 minutes is due to the column that was used, which contained a guard that was not present in the early stages of the experiments. However, we can see that cortisol from person D was undetectable at this time.

Figure 9. Results of Cortisol Standard, Blank(s), and Human Hair Samples



Chromatograms resulting from cortisol standard of 100 ng/mL (shown in red) and 20 ng/mL (shown in green), blank (shown in blue), human hair sample (person C) of unknown concentration (shown in yellow), and human hair sample (person D) of unknown concentration (shown in pink).

3.3.2 Use of Isopropanol in the Washing Step

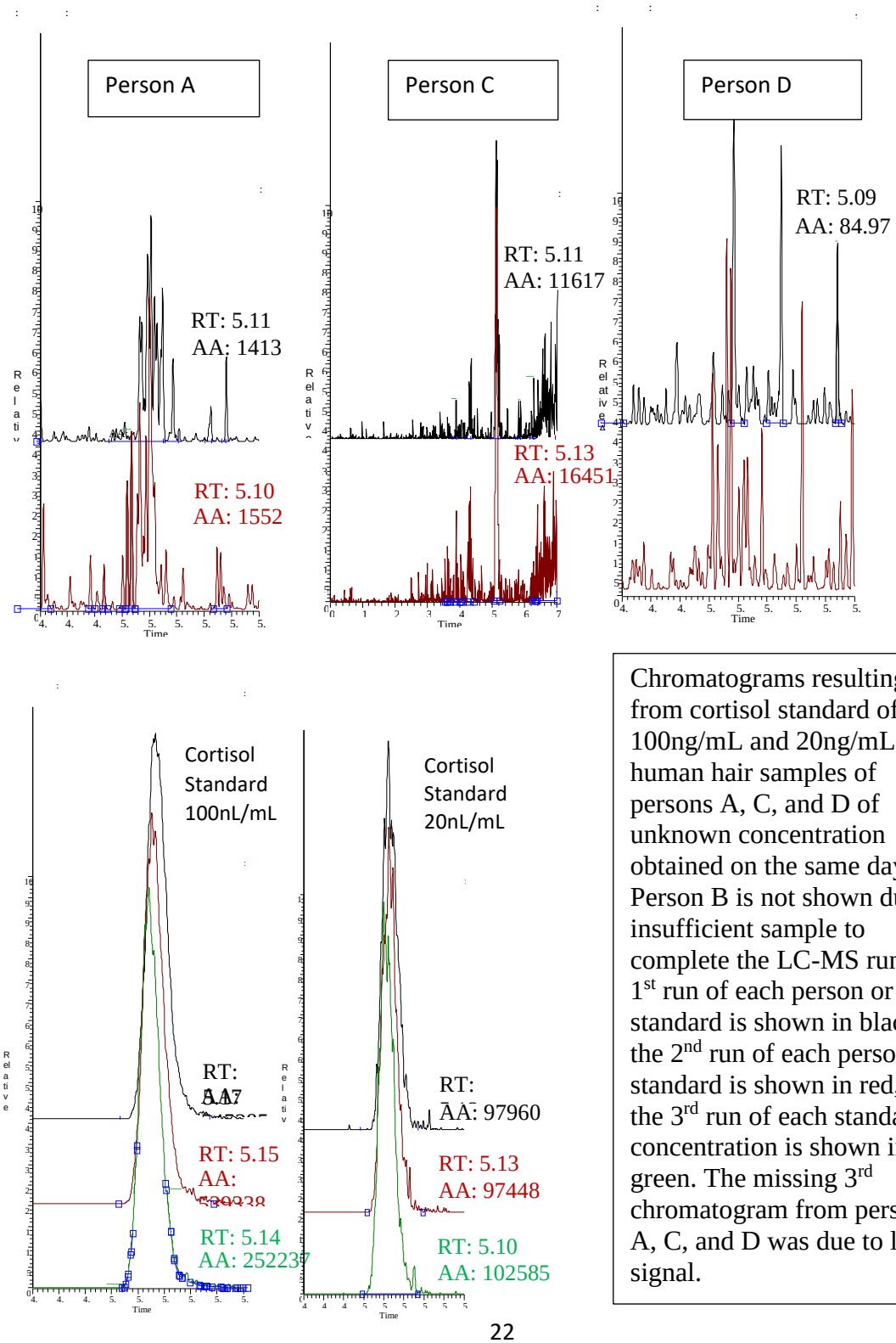
Isopropanol is a wash solvent commonly used to remove external contaminates involved with hair samples.[13] Cortisol is found within the hair shaft both internally and externally; however, we aim to focus on the internal hair shaft cortisol. Often the external contaminates, including sweat and lipids, contain additional cortisol and if not removed can alter our results by increasing the amount of cortisol detected from our UPLC-MS/MS method.[13] Therefore, using isopropanol to do a couple of short washes to the hair

samples is beneficial to our study because it removes these contaminants without penetrating the hair samples and lowering the internal cortisol levels.[13]

3.3.3 Reproducibility of Method for Hair Samples

In the initial analysis of persons C and D, the results revealed that the sample from person C contained a component that matched the retention time for the cortisol standard. The results also revealed that cortisol levels were undetectable in person D, possibly due to preparation. To confirm our results, check the reproducibility of our chromatogram, and check for potential error in the preparation steps, we cut more hair samples from volunteers A, C, and D. Our samples from persons A, C and D were tested along with the cortisol standard dilutions made on the same day in triplicate. We analyzed different concentrations of the cortisol standard and the analytes using the UPLC-MS/MS method. The results from this are shown in Figure 10. Assessment of the reproducibility of the chromatogram was confirmed in run 1 and 2 of each sample. We used 100 µl of each human hair sample extraction for triplicate injections. Each run lasted 7 minutes and had a flow rate of 0.5 mL/min. The peak area values for persons A, C, and D showed that the results for person C had a much larger peak area value than persons A or D. This signifies that person C has a higher level of cortisol than persons A and D at this time. The chromatogram for person D revealed no peak at the expected retention time again. We can confirm that the initial run of person D was not due to an error in preparation.

Figure 10. Analysis of Human Hair Samples and Cortisol Standard



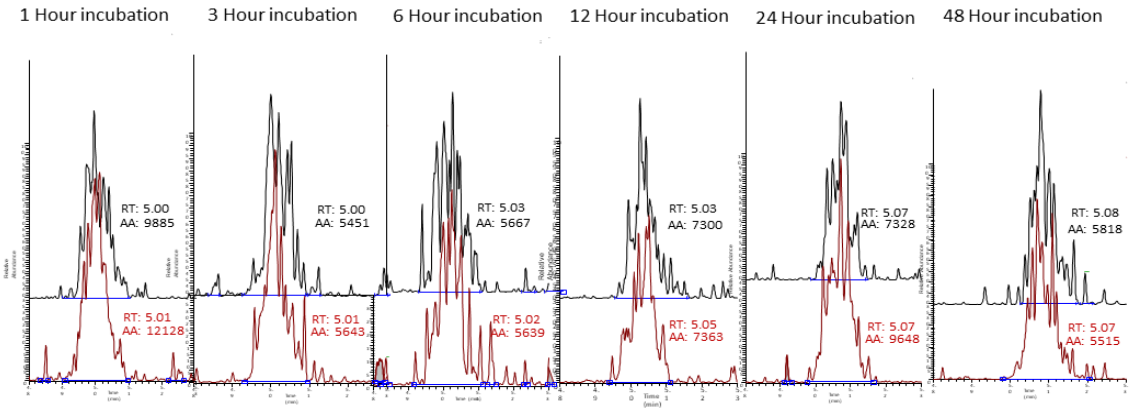
Chromatograms resulting from cortisol standard of 100ng/mL and 20ng/mL and human hair samples of persons A, C, and D of unknown concentration obtained on the same day. Person B is not shown due to insufficient sample to complete the LC-MS run. The 1st run of each person or standard is shown in black, the 2nd run of each person or standard is shown in red, and the 3rd run of each standard concentration is shown in green. The missing 3rd chromatogram from persons A, C, and D was due to low signal.

3.3.4 Necessity of Incubating the Hair Samples in Methanol

Incubation of the subjects' hair samples in methanol is essential to obtaining the most cortisol possible because methanol penetrates the hair shaft and extracts cortisol from the internal hair shaft.[13] The use of methanol instead of other solvents is vital to our study because cortisol and other steroid hormones have been determined to be more soluble in alcohols of lower molecular weight compared to those of higher molecular weight.[13] In the sample cutting and cortisol extraction step of our protocol, we used methanol to incubate our hair samples over time.

In addition to the importance of using methanol during incubation, we wanted to know if the amount of time the hair sample incubated in the methanol would be a factor. In previous studies, most individuals incubated their sample for approximately 18-hours. We looked at the hair sample of person C over a 48-hour time span in increments of 1-hour, 3-hour, 6-hour, 12-hour, and 48-hour to see if we observed a difference. The results of our test are shown below in Figure 11. As you can see, at each time increment the retention time in which our analyte came out was approximately the same. However, our signals were highest during the 12-hour and 24-hour increments. Therefore, we concluded incubating our sample for approximately 18 hours is sufficient to receive the best signal.

Figure 11. Chromatogram of Varying Incubation Time for Person C



Chromatogram resulting from incubation of human hair sample of person C of unknown concentration over a 48 hour time period run on same day. The 1st run of each incubation hour is shown in black and the 2nd run of each incubation hour is shown in red. The 3rd run of each incubation hour is not shown. (Note: 100 µL was used for each sample).

3.3.5 Percent Recovery Experiment

A recovery experiment was conducted with hair sample from person E. The recovery experiment is important because it helps validate the efficiency of the method. An 800 µL extract from person E was available. A total of 600 µL of the extract was divided equally into 6 micro-centrifuge tubes and labelled with numbers 1 through 6. The other 200 µL of the extract was placed in a micro-centrifuge tube and labelled with the number 7. Known amounts of 100 ng/mL of cortisol standard and known amounts of diluent (90:10 optima grade water: optima grade methanol) were added to each tube to reach a total volume of 200 µL in each tube as shown in Table 3. All 7 samples were analyzed in triplicate using the UPLC-MS/MS method.

Table 3 Percent Recovery Data Part A.

Tube Number	Amount of Extract (µL)	Amount of Standard (µL)	Amount of diluent (µL)	Known spiked concentration added (ng/mL)
1	100	15	85	7.5
2	100	30	70	15
3	100	45	55	22.5
4	100	60	40	30
5	100	75	15	37.5
6	100	0	100	0
7	200	0	0	0

The table shows the additions of the cortisol standard (100ng/mL) and the diluent to each tube to conduct the recovery experiment.

The concentration of each sample was determined using the previous calibration curve shown in Figure 5. The concentration of cortisol was calculated from each replicated measurement and averaged out for each tube. The average concentration of cortisol in each tube was used as the determined spiked concentration value in Figure 12. The average concentration of cortisol in tube 6 shown in Table 4 was used as the raw concentration value in Figure 12 because it contained only the extract. The amount of cortisol in each tube shown in Table 4 was determined by taking the amount of 100 ng/mL standard shown in Table 3, converting the value to milliliters, and multiplying it by 100 ng/mL. The known spiked concentration for each tube shown in Table 4 was determined by dividing the amount of cortisol in each tube by the total volume in each tube. The standard deviation of the triplicate analysis for each tube was calculated as shown in Table 4. The percent recovery value was calculated using the equation in Figure 12 and the values in Tables 3 and 4 for each tube. The results from each percent recovery

calculation are shown in Table 4. An example of the percent recovery calculation is shown in Figure 12.

Figure 12. Equation and Example Calculation for Percent Recovery

$$\frac{(\text{Determined spiked concentration} - \text{Raw concentration of the sample})}{(\text{Known spiked concentration added})} \times 100$$

Example Calculation:

Tube 1:

15 uL x 1 mL/1000 uL = .015 mL x 100 ng/mL =1.5 ng of cortisol

1.5 ng of cortisol / total volume of 200 uL =7.5 ng/mL cortisol

Using equation:

((24.802-8.22) / 7.5) x 100 = 221 % recovery

Table 4. Percent Recovery Data Part B.

Tube	Peak Area at 121 m/z	Measured Conc. Of Cortisol (ng/mL)	Avg. Conc. (ng/mL)	Amount of Cortisol Added (ng)	Expected Conc. (ng/mL)	Percent Recovery
1	18514	9.01	24.8	1.50 ng	7.50	221%
	57377	29.7				
	68836	35.7				
2	65398	33.9	42.9	3.00ng	15.0	231%
	93787	49.0				
	87568	45.7				
3	98181	51.3	61.0	4.50ng	22.5	234%
	132075	69.3				
	119050	62.4				
4	88320	48.1	63.2	6.00 ng	30.0	183%
	148995	78.3				
	124478	65.3				
5	238125	126	124	7.50 ng	37.5	309%
	256766	136				
	211111	111				
6 (Raw Conc.)	16771	8.09	8.22	N/A	N/A	N/A
	19985	9.79				
	14284	6.77				
7 (personE)	29971	15.1	15.6	N/A	N/A	N/A
	35328	17.9				
	27375	13.7				

The table highlights the results of the percent recovery experiment and the average concentration values of analytes.

When calculating the percent recovery, the expected values are generally below or slightly above 100%. Our percent recovery values shown in Table 4 are too high, ranging from 180% to 310%. We conclude that our percent recovery values do not seem reasonable and that they may be the result of a few possible errors, which may include instrument fluctuations between the analysis of the calibration curve and the samples. For our analysis we used a calibration curve generated on 06/27/2017 while our samples weren't analyzed until 07/13/2018. The response for our 100 ng/mL standard was 4.38E4 on 06/27/2017, but increased to 1.08E5 on 07/13/2018 proving the propensity for our instrument to fluctuate.

Therefore, we decided to analyze the data further to understand the percent recovery values. Because of the instrument fluctuations, we decided to use a normalization factor to normalize our data. The normalization factor was calculated using the instrument response for our 100 ng/mL standard in conjunction with the prior standard plots. The normalization values are shown in Table 5 and the calculations shown in Figure 13.

The normalized concentration of cortisol in each tube was calculated using the normalization factor and the average concentration value of each tube shown in Figure 13. The normalized concentration value for tube 6 shown in Table 6 was used as the raw concentration value in Figure 12. The known spiked concentration for each tube remained the same. The percent recovery value was calculated using the equation in Figure 12 and the values in Tables 3 and 6 for each tube. The results from each normalized percent recovery calculation are shown in Table 6. An example of the percent

recovery calculation is shown in Figure 13. Because of the value changes resulting from our normalized data, we used the normalized concentration value for person E in the place of the average concentration value to determine the amount of cortisol in pg per mg of hair. The amount of cortisol in the hair of person was determined to be 45.2 pg/mg .

Table 5. Normalization Factor Values.

Calibration Plot Equation	Sample	Peak Area	Avg. Peak Area	Avg. Conc. (ng/mL)	Normalization Factor
y = 1882.7x + 1548.1	100 ng/mL	499867	504000	267	2.67
		505152			
		506382			
y = 1228.2x – 4746.4	100 ng/mL	499867	504000	414	4.14
		505152			
		506382			

The table highlights the calculated values used to determine the normalization factor from the 100 ng/mL standard concentration analyzed on the same day as the percent recovery experiment.

Figure 13. Normalization Factor and Normalized Percent Recovery Calculations

1st: Calibration PlotCurve Equation:
y = 1882.7x + 1548.1
(Avg. Peak Area) = 1882.7 (Avg. Conc.) + 1548.1
Using equation:
(504000) =1882.7 (x) + 1548.1
x = 267 ng/mL
Normalization Factor = Avg. Conc./100
Normalization Factor = 267/100
Normalization Factor =2.67

2nd: Calibration Plot Equation (Most Recent):
y = 1228.2x – 4746.4
(Avg. Peak Area) = 1228.2 (Avg. Conc.) – 4746.4
Using equation:
(504000) =1228.2 (x) – 4746.4
x = 414 ng/mL
Normalization Factor = Avg. Conc./100
Normalization Factor = 414/100
Normalization Factor = 4.14

Example Calculations for Tube 1 using the 1st Calibration Plot Equation:

Normalized Avg. Concentration = Avg. Conc. / Normalization Factor
Normalized Avg. Concentration = 24.8 ng/mL /2.67
Normalized Avg. Concentration = 9.30 ng/mL

Percent Recovery = ((Determined spiked concentration-raw concentration of the sample)/ (Known Spiked concentration added)) x 100%.
Percent Recovery = ((9.30-3.08) / 7.5) x 100
Percent Recovery = 82.9%

Table 6. Normalized Percent Recovery Data A.

Tube	Area	Avg. Peak Area	Measured Avg. Conc. (ng/mL)	Normalized Avg. Conc. (ng/mL)	Percent Recovery
1	18514	48200	24.802	9.30	82.9%
	57377				
	68836				
2	65398	82300	42.866	16.1	86.7%
	93787				
	87568				
3	98181	116000	61.023	22.9	88.0%
	132075				
	119050				
4	88320	121000	63.233	23.7	68.7%
	148995				
	124478				
5	238125	235000	124.18	46.5	116%
	256766				
	211111				
6 (Raw Conc.)	16771	17000	8.2150	3.08	N/A
	19985				
	14284				
7 (personE)	29971	30900	15.586	5.84	N/A
	35328				
	27375				

The table highlights the results of the normalized percent recovery data using the first calibration plot (Normalization factor of 2.67).

Table 7. Normalized Percent Recovery Data B.

Tube	Area	Avg. Peak Area	Measured Avg. Conc. (ng/mL)	Normalized Avg. Conc. (ng/mL)	Percent Recovery
1	18514	48200	43.1	10.4	81.7%
	57377				
	68836				
2	65398	82300	70.9	17.1	85.5%
	93787				
	87568				
3	98181	116000	98.3	23.7	86.35%
	132075				
	119050				
4	88320	121000	102	24.6	67.8%
	148995				
	124478				
5	238125	235000	195	47.1	114%
	256766				
	211111				
6 (Raw Conc.)	16771	17000	17.7	4.27	N/A
	19985				
	14284				
7 (personE)	29971	30900	29.0	7.00	N/A
	35328				
	27375				

The table highlights the results of the normalized percent recovery data using the second calibration plot (Normalization factor of 4.14).

Our percent recovery values from our normalized data shown in Table 6 were within the expected range, ranging from 80% to 120%, except for one value being at approximately 70%. To further validate our calculations, we used the equation from the most recent calibration plot shown in Figure 13, to perform a second set of calculations

and got very similar results as shown in Table 7. We conclude that after normalizing our percent recovery data, our values are reasonable. Although this is not the proper approach for regular use of the method, the treatment with the two different calibration plots that produced similar percent recovery data was done to explain the abnormally high percent recovery values.

In the future, we plan to repeat the percent recovery experiment. We also plan to make a calibration plot on the same day of our experiment. In doing so this will eliminate errors due to instrument fluctuations and the use of old calibration plots.

3.4 Conclusion

Currently we believe that time and temperature is a potential problem for our hair samples. Though we were able to confirm that our hair samples are completely dry at approximately 7 hrs. at a temperature of 52 ℃ based on the weight of the hair samples from start to finish, we are not sure if the temperature at which we dry the hair at is safe for all types of hair. The hair sample from Person D was dried for approximately 9 hrs. During the drying process, we noticed that person D's hair started breaking apart and becoming more brittle after 7 hrs. of drying. However, we continued to dry the hair because the weight of the hair was continuing to drop, not considering that the dropping weight could have been due to loss of the hair sample in between transfers of hair from the weigh boat dish to the microcentrifuge tube. Another reason for the difference in the chromatograms of person C compared to person D can be contributed to the fact that the sample weight from person C was 30.24 mg; whereas, the sample weight for person D was 24.40 mg. Our protocol calls for up to 60 mg of hair, we believe that sample weight

may be a factor in how much cortisol is detected during analysis and using at least 30 mg, but not more than 60 mg per sample will be beneficial to the study as it progresses.

Regarding the shift in retention time shown in Figure 9 of our analyte, we believe that this shift could be due to two possible reasons. The first reason is that there could be a problem with the instrument. The second, most likely reason is the fact that the column we used had a column guard on it that was not present before, which too could shift our expected retention time.

There are potential problems associated with evaluating stress levels in a healthy volunteer through cortisol levels in hair samples. This is simply due to the fact that the concept of health and/or stress can be defined very differently. Sample collection could present a possible problem because if the hairs are not cut from the posterior region of the head or cut too close to the scalp.[1] Other factors such as hair growth rate, sex, age, hair color, and environmental exposure are examples of challenges to this study.[2] The concept of gathering a healthy volunteer is debatable because in society today we must come to common ground as to what we as researchers on this particular study define as healthy. Healthy to one individual could be unhealthy compared to another. One of the most difficult parts of this study was the hair grinding step because after the hair is washed, it becomes very sticky and is very hard to maintain. We did not use a bead beater as used by others in prior reports to grind our sample because it was not available at our facility. We tried several processes to grind the hair including using a mortar and pestle and freezing the hair in a microcentrifuge tube in -80 ℃. We also tried placing a hair sample in a microcentrifuge tube with sterilized chrome beads and vortexing the tube to imitate a

bead beater. In addition to the chrome beads, we used sterilized screws to see if they would aid in grinding the hair. However, with these processes the hair remained intact. Cutting the hair into fine pieces worked best for our experiments.

CHAPTER IV

CONCLUSION

4.1. Conclusion

The overall goal of this study was to establish a non-invasive LC-MS method to detect and quantify cortisol in hair samples in our own mass spectrometry facility. The end result was used to compare the methods in the literature and to decide if this LC-MS method would be as good as the existing commercialized method for cortisol detection. The linear range of detection was analyzed and the recovery experiment was used to validate the findings. Confirmation of cortisol identification was achieved through MS-MS analysis.

The hair samples obtained for this study were cut from healthy volunteers between the ages of 18-45 years. For the purposes of our study, we defined healthy as an individual having no known preexisting health conditions. In determining if an individual is healthy we also considered factors such as employment and current activities.

4.2. Future Considerations

This project can be expanded to analyze hair samples of healthy individuals that has been exposed to UV radiation as opposed to having applied heat and color.[1] Studies have shown that sensitivity improved when the ionization was changed from positive to negative. Therefore, it would be beneficial to investigate different ionization methods to compare to the present method. The bulk of our study is based on the output of a triple

quadrupole mass spectrometer. It would be interesting to investigate other systems such as the time-of-flight (TOF) and Orbitrap due to the fact that they also offer good sensitivity or more precise mass measurements.[3]

Ultimately, we hope to use these measurements to test and standardize stress levels. We plan to use our current methodology to help analyze the stress levels of students here at UNCG, with an emphasis on the students taking organic chemistry, which is often recognized as the most difficult subject. We also plan to partner with the military to help evaluate and diagnose the stress levels of soldiers and military personnel to be able to prevent the onset or slow down the progression of post-traumatic stress syndrome and/or other health complications associated with such a career.

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