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Microscale Serum Extraction Method for the Simultaneous Analysis of Corticosterone and Lipids

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MICROSCALE SERUM EXTRACTION METHOD FOR THE SIMULTANEOUS ANALYSIS OF CORTICOSTERONE AND LIPIDS

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Abstract

Corticosterone is an important steroid for the regulation of metabolism and stress response. Existing methods for the measurement of corticosterone include radioimmunoassay (RIA), enzyme linked immunosorbent assay (ELISA), and liquid chromatography-mass spectrometry (LC-MS). While each of these approaches have their advantages, RIAs use radioactive isotopes that necessitate specially regulated usage and disposal. Furthermore, both ELISAs and RIAs require expensive kits and can only measure a single analyte. In this study, we establish a new sample preparation technique based on a modified Folch extraction that allows for the simultaneous isolation of corticosterone and lipids from serum. The extract is then analyzed by LC-MS. Using only 5 μL of serum, quantification of corticosterone was achieved with coefficients of variation at 3% or less and a detection limit of 0.12 μM. Overall, the results of this study should be beneficial to the measurement of circulating corticosterone and lipids for a variety of studies using small volumes of samples.

Keywords

Corticosterone; Lipidomics; Steroid quantitation; Liquid chromatography / mass spectrometry.

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INTRODUCTION

Corticosterone is the major glucocorticoid produced by mice and is one of the most abundant steroids in mouse serum.[1] Glucocorticoids can elicit different effects on a variety of target organs, typically due to a stress response.[2, 3] Interestingly, corticosterone can affect lipid storage in brown adipose tissue and decreases non-shivering thermogenesis, impacting the thermoregulation capabilities of the animal.[4, 5] Glucocorticoids impact lipid and lipoprotein metabolism *via* both lipolytic and adipogenic effects.[6-9] Furthermore, glucocorticoids can inhibit reproduction through modulation of the hypothalamic-pituitary-gonadal axis and exogenous corticosterone administration to mice decreases uterine receptivity to embryonic implantation.[2, 10] Therefore, to gain a better understanding of the relationships between corticosterone level, reproductive capacity, and lipid metabolism, it would be beneficial to perform simultaneous analysis of corticosterone and lipids within a single sample (e.g. blood serum).

Previous steroid analysis methods, specifically for the quantitation of corticosterone, include radioimmunoassays (RIAs), enzyme-linked immunosorbent assays (ELISAs), and liquid chromatography (LC) or gas chromatography (GC) coupled to mass spectrometry (MS), often including tandem mass spectrometry (MS/MS).[11-17] While these techniques have shown the capability to analyze corticosterone concentrations, each has limitations. RIAs are performed with only 5-10 μL of serum; however, they are limited to the analysis of only one compound and they require the use of radioactive isotopes.[13] ELISAs are also limited to the detection of a single compound and they generally require 50 μL of serum.[14, 15, 18, 19] While LC-MS techniques allow for the analysis of multiple different compounds simultaneously, current approaches can require hundreds of microliters of sample for analysis. This is prohibitive when only small amounts of biological samples can be collected.[20-23]

The current study describes a serum extraction method for the simultaneous analysis of corticosterone and lipids from 5 μL of mouse serum. The generation of this protocol allows for not only the analysis of corticosterone concentration within small sample volumes and

without the need for costly kits, but also allows for the simultaneous analysis of a variety of other steroid hormones and lipid species. More specifically, we show the qualitative analysis of phosphatidylcholine (PC) and lysophosphatidylcholine (LPC) species combined with the quantitative analysis of corticosterone. Overall, this communication provides a new alternative for the measurement of corticosterone in mouse serum that also allows for acquisition of lipid profiles from the same preparation.

EXPERIMENTAL

Chemicals and Solutions. Corticosterone, corticosterone-d4, HPLC grade methanol, HPLC grade water, and chloroform were purchased from Sigma Aldrich (St. Louis, MO). As a safety precaution, all work performed with chloroform was performed in a fume hood. PC 14:0/14:0-d54 was purchased from Avanti Polar Lipids (Alabaster, AL). Corticosterone calibration standards were prepared in 100% HPLC grade water at concentrations of 1.00 µM, 1.50 µM, 2.00 µM, and 2.50 µM. Mobile phases for LC were prepared by adding lithium acetate to methanol and water at a concentration of 50 µM (to encourage the formation of lithium ion adducts and thus improve the ionization efficiency of corticosterone).[24]

Serum Collection. All animal procedures were approved by the University of Nebraska – Lincoln Institutional Animal Care and Use Committee (protocol number 1679). Eight-week old female C57BL/6J mice (Jackson Laboratory; Bar Harbor, ME) were given *ad libitum* access to standard rodent chow (Envigo; Indianapolis, IN) and water. Animals were fasted for twelve hours prior to euthanasia, which was performed using isoflurane overdose and exsanguination. The collected blood was allowed to clot at room temperature and then centrifuged at 1,000 rcf for ten minutes. The resulting serum samples were collected and frozen at -80°C until further analysis.

Serum Extraction. A scheme of the serum extraction protocol is presented in ***Figure 1***. Briefly, 5 µL of serum was spiked with internal standards consisting of corticosterone-d4 and PC 14:0/14:0-d54 at final concentrations of 0.30 µM and 0.10 µM, respectively. Next, 70

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3 μL of HPLC grade water, 100 μL of HPLC grade methanol, and 600 μL of chloroform were added to the mixture. The chloroform volume is high to ensure maximum extraction of the steroids and lipids together, minimizing the partitioning of steroids into the methanol layer. The sample was vortexed for 10 s and incubated at -20°C for 5 min. After incubation, the samples were vortexed for 1 min and centrifuged for 10 min at 13,500 rcf. The chloroform layer was then removed by carefully guiding a 20-200 μL the pipet tip through the aqueous layer and protein pellet and removing only the chloroform layer. The aspirated chloroform phase was then dried in a vacuum centrifuge for 30 min. The sample was then reconstituted in 20 μL of 50% methanol / water and analyzed by LC-MS.

Liquid Chromatography - Mass Spectrometry. LC was performed on a Waters NanoAcquity (Milford, MA) with a BEH C18 column 300 μm x 150 mm. Mobile phase A was HPLC grade water with 50 μM lithium acetate, while mobile phase B was HPLC grade methanol with 50 μM lithium acetate. The following gradient was applied: initially, 55% A and 45% B at 11 μL/min; at 7 min, 53% A and 47% B at 12 μL/min; at 11.5 min, 0% A and 100% B at 12 μL/min; at 24.5 min, 55% A and 45% B at 11 μL/min. The LC run was ended at 25.0 min. The flow rate was increased during the run to decrease the analysis time (the lower flow rate was initially needed to ensure separation of corticosterone from its steroid isomer 11-deoxycortisol). Using standards, we previously confirmed that the LC conditions described above can separate the two isomers above a resolution of 1.5, which allows for the measurement of corticosterone specifically (data not shown). Due to the MS dimension, if other compounds of different mass had coeluted, this would not interfere with the analysis of corticosterone. The LC was coupled to a Waters Synapt G2-S HDMS Q-TOF *via* an electrospray ionization source. All analyses were performed in positive ion mode. The source conditions were set to include a temperature of 80°C, capillary potential of 3.1 kV, and cone potential of 10 V. Data was analyzed using MassLynx 4.1 (Waters) and visualized using Igor Pro 7.0 (WaveMetrics; Lake Oswego, OR) and Sigma Plot 13.1 (Systat, Chicago, IL).

RESULTS

Liquid Chromatography – Mass Spectrometry Analysis. A representative base peak ion chromatogram is presented in ***Figure 2(a)***. The chromatogram exhibits two distinct ranges in retention time containing different analyte classes: one region in which steroids and small non-polar compounds are eluted within the first ~ 10 min of the run, and a second region in which lipids (in this case, primarily phospholipids) are eluted between ~ 10-18 min. Due to the much lower concentrations of steroids compared to lipids, there is a distinct difference in the signal-to-noise ratio in the steroid region as compared to the phospholipid region. Nevertheless, corticosterone was readily detected. The overlaid extracted ion chromatograms for lithiated corticosterone (*m/z* 353.23), several PC lipids, and the corresponding LPC lipids are depicted in ***Figure 2(b)***. The putatively identified PC and LPC lipid compositions are presented in ***Table 1***. These results demonstrate sufficient signal-to-noise ratios to analyze corticosterone and a variety of lipid species from 5 µL of mouse serum.

Corticosterone Quantification. A calibration curve for corticosterone quantification is presented in ***Figure 3***. For this analysis, the peak area of the endogenous corticosterone was normalized to the isotopically labelled corticosterone (d4) peak area. The resulting calibration curve had a coefficient of determination of 0.9999. The limit of detection (LOD) was calculated using the relationship LOD = $3s_b / m$, where s_b is the standard deviation of the y-intercept of the best-fit line and m is the slope of the best-fit line. The LOD for corticosterone was 0.120 µM or ~ 50 ng/mL. Additionally, serum samples from five randomly selected mice were analyzed using this method. Their corresponding corticosterone concentrations are shown in ***Table 2***. These results were found to be in line with previous studies on overnight fasted mice, in which corticosterone levels of approximately 1.3 µM were reported.[25] All samples had a between-day correlation of variation (CV) of 3% or less across three technical replicates. These results suggest that this protocol is suitable for measuring mouse corticosterone levels using samples of low volume.

Impact of Protocol. The results of this method could provide a useful new approach to measure the prominent murine glucocorticoids when only low sample volumes are available. Additionally, small modifications to this method could allow other types of information to be obtained from the same sample. For example, this method produces two other fractions not used in this study: a methanol / water layer and a protein pellet. Previous results from a similar method demonstrated that the protein pellet could be reconstituted and subjected to proteomic analysis, while the methanol layer could be analyzed for polar metabolites.[26] Furthermore, while the goal of this study was to generate a protocol for use of small sample volumes, increasing the volume could allow for analysis of additional steroids with lower abundance.

CONCLUSIONS

Glucocorticoids, specifically corticosterone in rodents, cause numerous physiological effects in a variety of target organs. Corticosterone assists in the regulation of energy metabolism, participates in immune reactions, and elicits the stress response. Steroids, including corticosterone, can be difficult to analyze due to low biological concentrations. Additionally, the ability to analyze corticosterone simultaneously with proteins, lipids, and other metabolites can allow a "multi-omic" analysis when only low volumes of sample are available. Through the protocol presented here, we were able to quantify corticosterone levels with biologically useful detection limits and good reproducibility. This method can be applied to study a variety of questions involving the target analytes and has the potential to deliver additional types of information with less sample than previously described methods.

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FIGURES

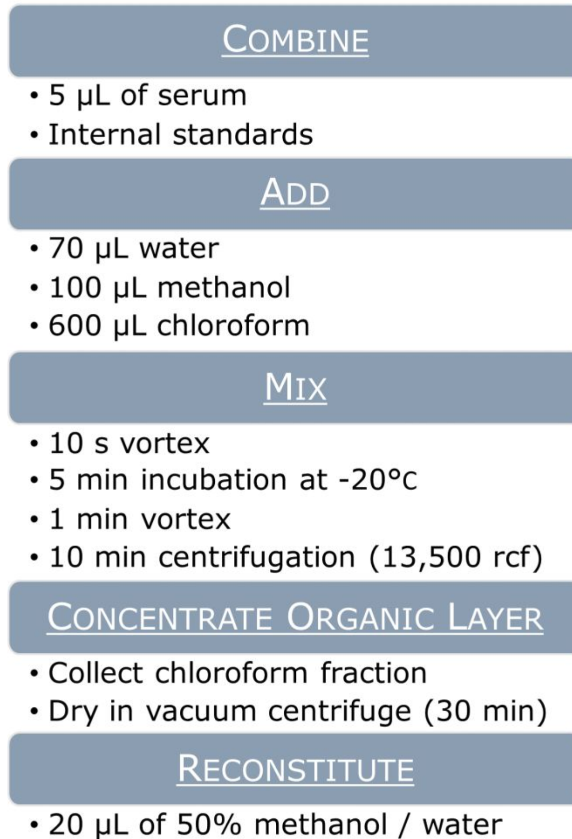

Figure 1. Protocol for microscale extraction of corticosterone and lipids from serum.

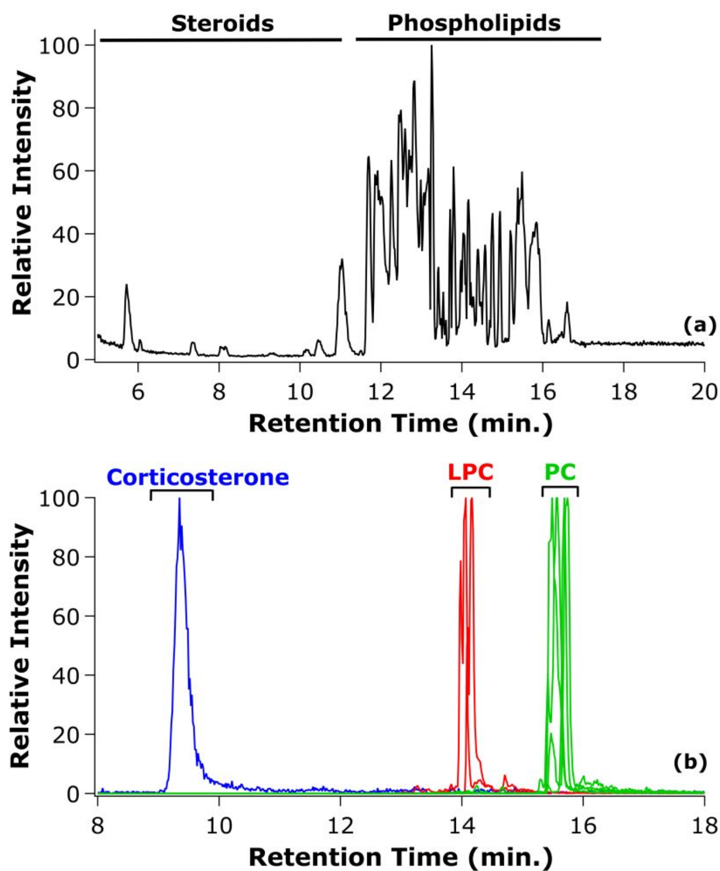

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Figure 2. Base peak ion chromatogram from LC-MS analysis of a mouse serum extract ***(a)*** and extracted ion chromatograms for corticosterone, LPC, and PC lipids ***(b)***.

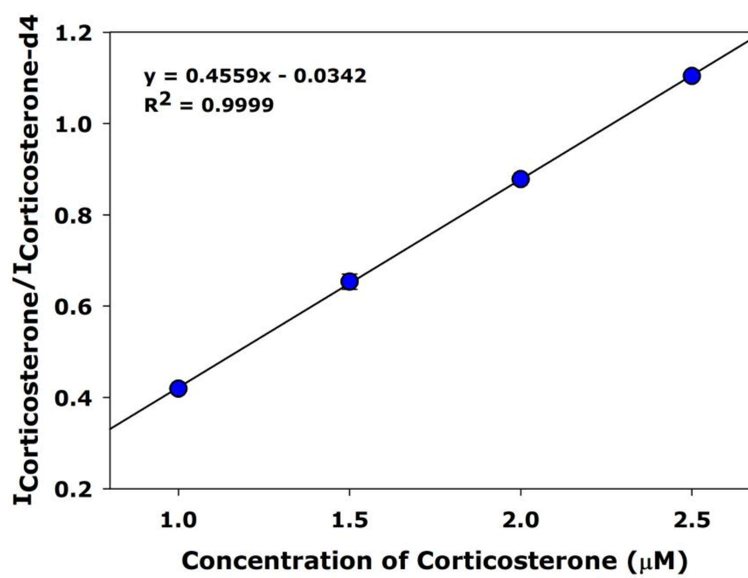

Figure 3. Corticosterone calibration curve with the linear fit equation and coefficient of determination.

Tables

Table 1. Putative compositional identifications for the lipids presented in the extracted ion chromatograms shown in ***Figure 2***. All were identified as lithium ion adducts based on their isotopic patterns.

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m/z	Tentative Identification
502.35	LPC 16:0
526.35	LPC 18:2
764.58	PC 34:2
766.59	PC 34:1
788.58	PC 36:4
790.59	PC 36:3
792.61	PC 36:2

Table 2. Average concentration of corticosterone with standard deviation and coefficient of variation (three technical replicates) for five randomized mouse serum samples.

Sample	Concentration (µM)	SD	CV (%)
1	1.60	0.02	1
2	1.82	0.06	3
3	1.98	0.02	1
4	2.20	0.01	1

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5	2.47	0.06	3

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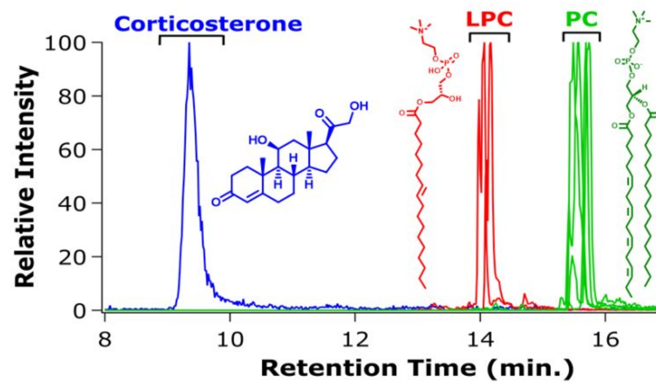

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Table of Contents Textual Abstract.

This report details a serum extraction method for the simultaneous analysis of corticosterone and lipids from 5 μL of serum.