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Short Communication

Analysis of steroids in songbird plasma and brain by coupling solid phase extraction to radioimmunoassay

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Abstract

It is a common practice to extract steroids from plasma, serum, or tissue samples prior to steroid measurement by radioimmunoassay (RIA) or enzyme immunoassay (EIA). Steroid extraction is critical because it can remove substances that interfere with the RIA or EIA. Steroid extraction is commonly achieved using organic solvents, such as diethyl ether or dichloromethane. However, organic solvent extractions can suffer from low recovery, imprecise recovery, or incomplete removal of assay interference. Here, we describe validations of a simple protocol to extract steroids (e.g., dehydroepiandrosterone, corticosterone, and estradiol) from avian plasma, serum, and brain tissue using solid phase extraction (SPE) with commercially available C_{18} columns. We compare various methods for (1) eluting steroids from columns, (2) drying eluates, and (3) resuspending dried eluates prior to RIA. The SPE method yields high and consistent recoveries. The SPE method also effectively separates steroids from interfering substances, even when extracting steroids from lipid-rich plasma and brain tissue. These data indicate that SPE is superior to organic solvent extraction on several measures. SPE should be broadly useful for extracting steroids from plasma or tissue samples.

Keywords: Corticosterone; Dehydroepiandrosterone; DHEA; Estradiol; Neurosteroid; Songbird; RIA; EIA; Song sparrow; European starling; Zebra finch; Avian; Yolk; Bird; Chicken; Stress; Hormone; C18; Sample preparation; Steroid extraction; Lipid

1. Introduction

Analyzing steroid levels via immunoassay is a very common method to determine endocrine status. For example, plasma levels of corticosterone and sex steroids are often measured to evaluate stress physiology (Newman and Soma, 2006; Romero et al., 2006) or reproductive state in songbirds and other species (Williams et al., 2005). In addition to circulating steroids in the plasma, there is accumulating evidence of local steroid synthesis in the brain and other organs (Baulieu, 1997; Soma et al., 2004; Soma et al., 2005, 2006; Demas et al., 2007). Thus it is critical to measure steroid levels within target tissues.

When measuring steroids, especially in tissues, sample preparation prior to immunoassays is a crucial step in the analytical process and has a direct impact on the accuracy and precision of the assay (Chard, 1995). A major obstacle in analyzing steroids in tissues is that lipids, such as free fatty acids and triglycerides, can interfere with immunoassays (Rash et al., 1980; Jawad et al., 1981). In particular, brain tissue is rich in lipids, and measuring steroids in egg yolk is also complicated by the high lipid content (Schwabl, 1995). Extremely lipemic plasma samples can also be a problem, especially in animals with a lipid-rich diet, such as seabirds (Speake et al., 1999), carnivores, and nestling birds. Female birds that are depositing yolk into eggs can also have high levels of plasma lipids. Thus,

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separating steroids in plasma and tissue samples from lipids and other sources of assay interference can be important for accurate and reliable measurements.

Traditionally, organic solvents (e.g., dichloromethane (DCM), diethyl ether, hexane) have been used to extract steroids from samples (Samuels, 1949). However, many variables must be considered when choosing an appropriate extraction technique; these include the steroid being measured, study species, sex, season, reproductive state, and sample type (tissue, plasma and serum). Organic solvent extraction may be appropriate for plasma samples with low lipid content (Soma and Wingfield, 2001) or for immunoassays that require very small amounts of plasma. However, for plasma samples or tissue samples with high lipid content, organic solvent extraction can yield low recoveries (O'Grady, 1968), variable recoveries (Fuqua et al., 1995), or incomplete removal of interfering substances. Importantly, the effectiveness of organic solvent extraction should be determined empirically on a case-by-case basis. Another consideration is that organic solvents used for steroid extraction are often highly reactive or extremely flammable. For example, DCM is an organochlorine with carcinogenic properties, and diethyl ether has high volatility and a low ignition point.

Solid phase extraction (SPE) is an alternative to traditional organic solvent extractions (Thurman and Mills, 1998; Telepchak et al., 2004). Previous studies using SPE report high and reliable analyte recovery and more effective removal of interfering substances (Lee and Goeger, 1998). SPE is relatively straightforward to set up in a laboratory and the materials are commercially available. In SPE, a solid sorbent material, typically an alkyl bonded silica, is packed into a column. Extracting relatively non-polar compounds (such as steroids) from a polar matrix (such as water) requires a solid sorbent containing non-polar functional groups of octadecyl (C_{18}) bonded silicas. Steroids bond with C_{18} groups on the sorbent and are extracted from the sample matrix. Steroids are then eluted from the column.

Here we describe validations of SPE to isolate steroids from avian plasma and brain. We address two primary issues: (1) optimization of steroid recovery using radiolabeled and radioinert steroids and (2) removal of substances that interfere with radioimmunoassay (RIA). Our experiments focus on songbirds, which are widely studied for a variety of research questions about hormonal and environmental influences on physiology and behavior (Goodson et al., 2005b). To our knowledge, measurement of steroid levels in songbird brain has not been previously described.

2. Materials and methods

2.1. Subjects

Plasma pools were created from (1) wild nestling European starlings (*Sturnis vulgaris*), (2) captive nestling zebra finches (*Taeniopygia guttata*), and (3) wild adult song sparrows (*Melospiza melodia*) in non-breeding condition. Plasma from nestling European starlings appeared lipemic upon visual inspection. Brain tissue was collected from wild nestling European starlings, captive adult zebra finches, and wild adult male song sparrows in non-breeding condition. Commercially available chicken serum (Sigma C-540) was used for preliminary validations. Animal use protocols were approved by the Animal Care Committee at the University of British Columbia and conform to the guidelines promulgated by the Canadian Council on Animal Care.

2.2. Reagents

Radioinert dehydroepiandrosterone (DHEA), corticosterone (CORT), and 17β-estradiol (E_2) were purchased from Steraloids (Newport, Rhode Island). Radiolabeled steroids were purchased from Perkin-Elmer (^{3}H-DHEA, NET814; ^{3}H-E_2, NET517). HPLC-grade methanol (MeOH) and HPLC-grade dichloromethane (DCM) were purchased from Fisher Scientific. Absolute ethanol (EtOH) was obtained from the UBC Chemistry Department.

2.3. SPE protocol

The SPE protocol involves seven major steps: (1) Solvation, columns are primed with ethanol; (2) Equilibration, water is passed through columns to prepare sorbent for sample loading; (3) Sample loading, samples in aqueous matrix are passed through the columns; (4) Interference elution, water is passed through columns to wash out interfering polar substances; (5) Sample elution, steroids are eluted from columns with a small amount of eluant into 7 mL glass vials; (6) Drying, eluates are dried; and (7) Resuspension, dried eluates are resuspended in assay buffer.

For SPE, we used a 24-place vacuum manifold (United Chemical Technologies, UCTVMF024GL, approx $930 cdn), non-endcapped C_{18} columns (500 mg C_{18} material, 6 mL column volume, UCTCUC18156, approx. $2 cdn/column), and polytetrafluoroethylene tips. We systematically manipulated several variables after sample loading to evaluate effects on (1) steroid recovery and (2) removal of assay interference.

To measure steroid recovery, plasma and brain samples were spiked with known quantities of tritiated steroids. A known amount of tritiated steroid was added to samples prior to loading samples onto columns. To evaluate loss of tritiated steroids during the process, aliquots were counted in a liquid scintillation counter (Beckman Coulter LS-6500) for 2 min. After initial studies using chicken serum spiked with ^{3}H-DHEA or ^{3}H-E_2, the protocol was applied to song sparrow brain tissue spiked with either ^{3}H-DHEA or ^{3}H-E_2. In addition, steroid recovery was evaluated by spiking samples with radioinert steroids prior to extraction and measuring extracted samples using RIA. By comparing unspiked and spiked samples, we were able to calculate recovery of exogenous radioinert steroids.

2.4. Eluting solvent

Choosing the appropriate solvent for elution is important in maximizing extraction efficiency. We compared several solvents (5 mL, all HPLC-grade): methanol, 90% MeOH (9:1 MeOH:dH_2O), and DCM. These solvents are common in SPE (MeOH) or traditional organic solvent extraction methods (DCM).

2.5. Drying method

Following sample elution, the eluates are dried prior to resuspension in assay buffer. We compared recovery efficiency when eluates were dried under air or nitrogen (N_2). To dry eluates under air, the vacuum manifold was used to pull a steady stream of air through the columns and over the eluates at room temperature. To dry eluates under N_2, samples were placed in a water bath at 38 °C under a steady stream of medical-grade N_2.

2.6. Resuspension volume

Resuspending dried eluates prior to immunoassay is an important step that greatly affects recovery. Steroids have low solubility in aqueous buffers and may adhere to glass vials after drying. To determine whether

the volume of resuspension buffer affects recovery, two volumes of phosphate buffered saline with gelatin (PBSG) were added to dried eluates: 220 µl or 440 µl. These volumes are sufficient for one or two immunoassays depending on expected steroid concentrations and assay sensitivity.

2.7. Ethanol in resuspension

We also determined whether EtOH aids in resuspending steroids. Using high-grade absolute ethanol, we compared the effects of 0, 5, and 10% EtOH in the resuspension buffer on recovery of radiolabeled steroids from dried eluates. The absolute EtOH was added directly to the bottom of the glass vials containing dried eluates, the vials were vortexed briefly, the assay buffer was added, and the vials were vortexed (2 min).

An important concern is whether EtOH in the resuspension buffer affects the immunoassay (e.g., by affecting the primary or secondary antibodies). To address this issue, we determined whether 5 or 10% EtOH had an effect on the measured concentrations of known amounts of steroids.

2.8. Resuspension mixing method

We determined whether a manual vortexer (VWR, 58816-121) or a rack shaker (IKA Vibrax VWR basic) affected recovery of radiolabeled steroids. Resuspension buffer was added to dried eluates, vials were either vortexed (4 min) or shaken (60 min), stored at 4 °C overnight, and either vortexed (4 min) or shaken (60 min) again.

2.9. Radioimmunassays

We examined recovery of exogenous radioinert DHEA, CORT and E_2 via RIA. Known quantities of radioinert DHEA, CORT or E_2 were added to samples prior to SPE. We calculated recovery by comparing the quantity of steroids in unspiked and spiked samples.

To determine whether SPE effectively removed substances that interfere with the RIAs, we serially diluted plasma and brain samples after SPE. If interfering substances are removed, then serially diluted samples should be parallel to the standard curve, when comparing the percentage of tracer bound (Chard, 1995). To measure DHEA, we used a specific double antibody RIA (DSL 8900, Diagnostic Systems Laboratories, Webster, TX). The DHEA RIA was modified according to Granger et al. (1999) to increase sensitivity and has been previously used for songbird plasma (Goodson et al., 2005a; Soma, 2006). To measure E_2, we used a specific double antibody RIA (DSL 39100, Diagnostic Systems Laboratories, Webster, TX) which was modified according to Shirtcliff et al. (2000). To measure CORT, we used a specific double antibody RIA (ImmuChem 07-120103, MP Biomedicals, Orangeburg, NY) which was modified according to Washburn et al. (2002).

2.10. Statistical analysis

To determine parallelism between serial dilutions of plasma and brain tissue with the standard curve, we tested equality of slopes using an analysis of covariance (ANCOVA). A lack of significant interaction between serial dilution and standard curve indicates that the line slopes are similar. If the interaction term is significant, then the slopes of the serially diluted sample and the standard curve are not parallel. We used JMP IN 5.1 (SAS, Cary, NC) for linear models and Sigma Stat 3.0 (Jandel Scientific, San Rafael, CA) for between group comparisons. We considered test results significant for $p < 0.05$. Means ± standard errors represent central tendency and variability, respectively.

3. Results

3.1. Recovery of radiolabeled steroids

The highest recoveries of radiolabeled DHEA and E_2 from chicken serum were obtained when samples were eluted with 90% MeOH and then resuspended in buffer with 10% EtOH, although the difference between 5 and 10% EtOH in resuspension buffer was slight (Table 1). Resuspending dried extracts in either 220 µl or 440 µl resuspension matrix did not alter the recovery of ^{3}H-DHEA (Table 1). Drying eluates under N_2 at 38 °C, particularly with 5 or 10% EtOH in the resuspension buffer, significantly increased recovery of radiolabeled DHEA and E_2 (Table 2). Resuspending dried eluates with either a manual vortexer or automatic shaker did not affect recovery of radiolabeled DHEA when EtOH was added to the resuspension buffer (5% EtOH: t-test, $t = -0.49$, $p = 0.64$; 10%

Table 2
Recovery of ^{3}H-DHEA and ^{3}H-E_2 from chicken serum extracts dried under N_2 or under air after solid phase extraction

% Ethanol in resuspension	N_2 at 38 °C		Air at 22 °C	
	DHEA	E_2	DHEA	E_2
0	45.2 ± 2.2	65.9 ± 1.6	50.0 ± 3.3	50.0 ± 1.7
5	72.8 ± 1.9	73.9 ± 2.1	56.2 ± 2.9	53.2 ± 1.5
10	80.3 ± 2.2	80.6 ± 0.7	60.2 ± 1.0	58.2 ± 1.0

Note: Data are means ± SEM and $n = 4$ for each group.

Table 1
Recovery of ^{3}H-DHEA and ^{3}H-E_2 from chicken serum using solid phase extraction

Steroid: eluant	Resuspenion volume (µl)	% Ethanol in resuspension		
		0	5	10
DHEA: 90% methanol	220	45.3 ± 2.2	72.8 ± 1.9	80.0 ± 2.2
	440	65.8 ± 1.6	72.7 ± 6.2	81.2 ± 3.0
DHEA: 100% methanol	220	70.9 ± 2.6	70.7 ± 2.2	78.1 ± 0.1
	440	65.5 ± 1.0	67.3 ± 5.0	70.6 ± 7.5
DHEA: dichloromethane	220	40.3 ± 2.9	67.3 ± 0.8	65.1 ± 1.7
	440	37.8 ± 3.6	62.1 ± 3.4	68.5 ± 1.4
Estradiol: 90% methanol	220	65.9 ± 1.6	73.9 ± 2.1	80.6 ± 0.7
Estradiol: dichloromethane	220	46.6 ± 1.3	52.1 ± 0.9	56.2 ± 1.1

Note: Data are means ± SEM and $n = 4$ for each group.

EtOH: t-test, $t = -0.13$, $p = 0.90$; data not shown). However, if no EtOH was added to the resuspension buffer, recovery increased significantly from $46 \pm 1.4\%$ to $56 \pm 3.9\%$ with the rack shaker (t-test, $t = -2.51$, $p = 0.04$).

Lastly, highest recovery of radiolabeled DHEA from songbird brain tissue was $68.9 \pm 0.8\%$ and was achieved with 5% EtOH in resuspension buffer (ANOVA, $F_{2,11} = 4.27$, $p = 0.05$). Highest recovery of radiolabeled E_2 from songbird brain tissue was $66.6 \pm 2.4\%$ and was unaffected by percentage of EtOH in the resuspension buffer (ANOVA, $F_{2,11} = 0.007$, $p = 0.9$).

3.2. Recovery of radioinert steroids

We also determined recovery of known amounts of radioinert steroids that were added to samples prior to SPE and then measured via RIA ($n = 4$ replicates for each trial). Except where stated otherwise, 5% EtOH was used in the resuspension of dried eluates. For DHEA, recovery of 50 pg DHEA added to chicken serum was 113% with 5% EtOH in resuspension and 120% with 10% EtOH in resuspension. Recovery of 50 pg DHEA added to nestling starling plasma was 104%. Recovery of 25 pg DHEA added to 0.5 mg song sparrow brain tissue was 80% and recovery of 50 pg DHEA added to 1 mg song sparrow brain tissue was 72%.

Recovery of 0.5 pg 17β-E_2 added to nestling starling plasma was 107%. Recovery of 1 pg 17β-E_2 added to nestling starling brain tissue was 62%.

For CORT, recovery of 25 pg added to nestling starling plasma and brain tissue were 86% and 71%, respectively.

We also determined the effect of absolute EtOH in the resuspension buffer on RIAs. For DHEA RIA, 5% and 10% EtOH in the resuspension buffer did not affect the concentration of DHEA in known controls (Fig. 1). Similarly, for CORT or E_2 RIA, when compared with 0%, 5% EtOH in the resuspension buffer did not affect the concentration of CORT or E_2 in known controls (data not shown).

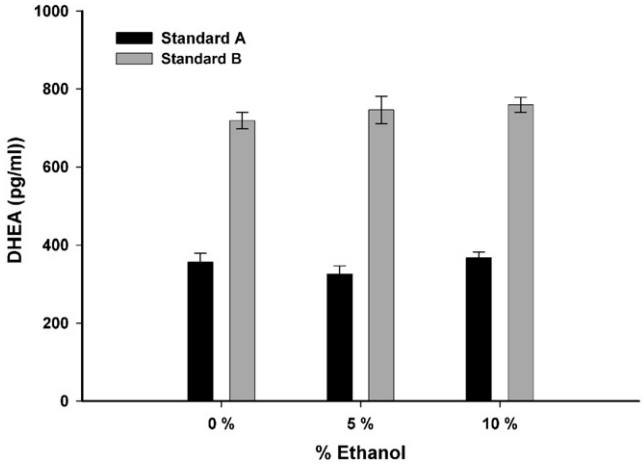

Fig. 1. Adding absolute EtOH to the resuspension buffer (either 5 or 10% of total resuspension volume) did not change the concentration of known DHEA standards measured with RIA. Data are means ± standard error.

3.3. Removal of interfering substances

To determine whether SPE effectively removed interfering substances, such as lipids, from plasma and brain samples, we compared serially diluted samples and standard curves. For DHEA, we examined plasma and brain tissue that were not extracted by either organic solvents or SPE. Without extraction, serially diluted song sparrow plasma and brain tissue yielded displacement curves that were not parallel to the standard curve (data not shown; Plasma: ANCOVA, $R^2 = 0.96$, significant interaction, $F_{2,15} = 102.31$, $p = 0.007$; Brain Tissue: ANCOVA, $R^2 = 0.98$, significant interaction, $F_{2,13} = 244.98$, $p < 0.0001$). Indeed, the serially diluted brain tissue yielded a flat displacement curve. Second, we examined the effects of DCM extraction. With DCM extraction, serial dilutions of nestling zebra finch plasma, nestling European starling plasma, and song sparrow brain tissue yielded displacement curves that were not parallel to the DHEA standard curve (Fig. 2a, Brain Tissue: $R^2 = 0.96$, significant interaction, $F_{2,13} = 28.87$, $p = 0.0002$; Plasma: $F_{2,13} =$ ANCOVA, $R^2 = 0.97$, significant interaction, $F_{2,18} = 22.18$, $p < 0.0001$). Third, we evaluated the efficacy of other organic solvent extractions. Song sparrow plasma extracted with diethyl ether or hexane/ethyl acetate (9:1) also yielded displacement curves that were not parallel to the DHEA standard curve (data not shown; ANCOVA, $R^2 = 0.96$, significant interaction, $F_{3,20} = 13.87$, $p = 0.0004$).

In contrast, with SPE, serially diluted nestling European starling plasma and song sparrow brain tissue yielded displacement curves that were parallel to the DHEA standard curve (Fig. 2b, Brain Tissue: $R^2 = 0.98$, no significant interaction, $F_{2,13} = 0.58$, $p = 0.46$; Plasma: ANCOVA, $R^2 = 0.98$, no significant interaction; $F_{2,10} = 0.04$, $p = 0.84$). Similarly, for E_2 RIA, serially diluted adult zebra finch brain extracted with SPE was parallel to the E_2 standard curve (Fig. 2c, ANCOVA, $R^2 = 0.56$, no significant interaction, $F_{1,8} = 4.3$, $p = 0.1$). Lastly, for CORT RIA, serially diluted adult song sparrow brain extracted with SPE was parallel to the CORT standard curve (Fig. 2d, ANCOVA, $R^2 = 0.99$, no significant interaction, $F_{1,10} = 3.84$, $p = 0.10$).

4. Discussion

Extraction of steroids from lipid-rich samples has been an obstacle for analyzing steroids in lipemic plasma and tissue. These results indicate that solid phase extraction (SPE) is a suitable method for extracting steroids from lipid-rich avian plasma and brain samples. SPE is a straightforward procedure that can easily be established in a laboratory (Fig. 3). SPE yields relatively high and consistent recovery of radiolabeled and radioinert steroids. After SPE, serially diluted plasma and brain samples yield displacement curves that are parallel to the standard curve. Interestingly, steroid levels in songbird brain have not been previously described. This method provides a reliable way to quantify steroids in small tissues or tissues that are rich in interfering substances.

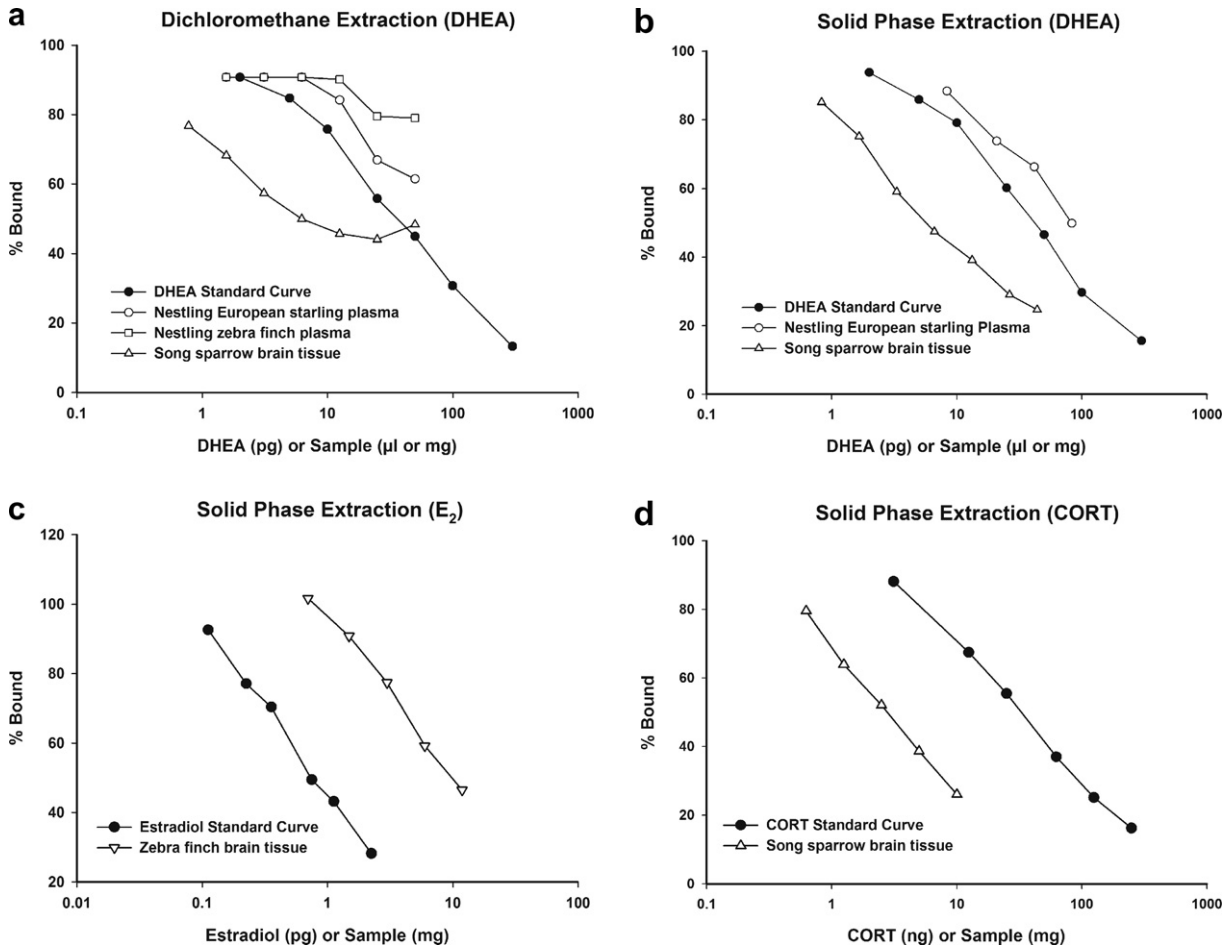

Fig. 2. Solid phase extraction (SPE), but not dichloromethane (DCM) extraction, effectively removed substances that interfere with RIA. (a) Serially diluted samples of nestling European starling plasma, nestling zebra finch plasma and song sparrow brain tissue extracted with DCM are not parallel to DHEA standard curve. For DCM extraction, steroids were extracted with 3 mL of HPLC-grade DCM (twice) and extracts were dried under N_2 and resuspended in assay buffer. (b) Serially diluted samples of nestling European starling plasma and song sparrow brain tissue extracted with SPE are parallel to the DHEA standard curve. (c) Serially diluted zebra finch brain tissue extracted with SPE is parallel to the E_2 standard curve. (d) Serially diluted song sparrow brain tissue extracted with SPE is parallel to the CORT standard curve.

Extraction techniques can be examined using radiolabeled steroids, serially diluted samples, and radioinert steroids. Adding known quantities of radiolabeled steroids to samples prior to extraction provides information on recovery, but does not indicate if the extraction removes substances that interfere with immunoassay. Examining parallelism between serially diluted samples and a standard curve reveals the ability of the extraction to remove interfering substances, but does not indicate recovery efficiency. Serial dilutions are also useful in determining an appropriate sample volume (Chard, 1995). Lastly, calculating recovery of known amounts of radioinert steroids added prior to extraction and measured with immunoassay provides information on both recovery and removal of interfering substances.

Eluting the steroids from C_{18} columns with 90% HPLC-grade MeOH in deionized water yields the highest recovery of radiolabeled steroids. Methanol forms hydrogen bonds with the silica surface and breaks the van der Waals interactions between the steroids and the C_{18} material. Thus, it is an effective solvent for eluting non-polar analytes, such as steroids. Adjusting the solvent can affect which analytes are eluted. For example, eluting with a lower percentage of MeOH in water can specifically elute sulfated steroids, but not unconjugated steroids (Liere et al., 2004). Other non-polar solvents, such as acetonitrile and ethyl acetate, may also work well for non-polar, lipophilic analytes and should be examined in future studies.

Drying the eluates is necessary prior to resuspension in assay buffer. Drying the eluates under a steady stream of medical-grade N_2 yields significantly higher recovery of radiolabeled steroids than drying under a stream of air. Importantly, the N_2 is free of possible contaminating substances that may be present in air, and inert N_2 is less likely to cause steroid oxidation. Furthermore, drying under N_2 at 38 °C is much faster than drying at room temperature. Additionally, eluates can be dried using a vacuum concentrator, and future studies will examine this method.

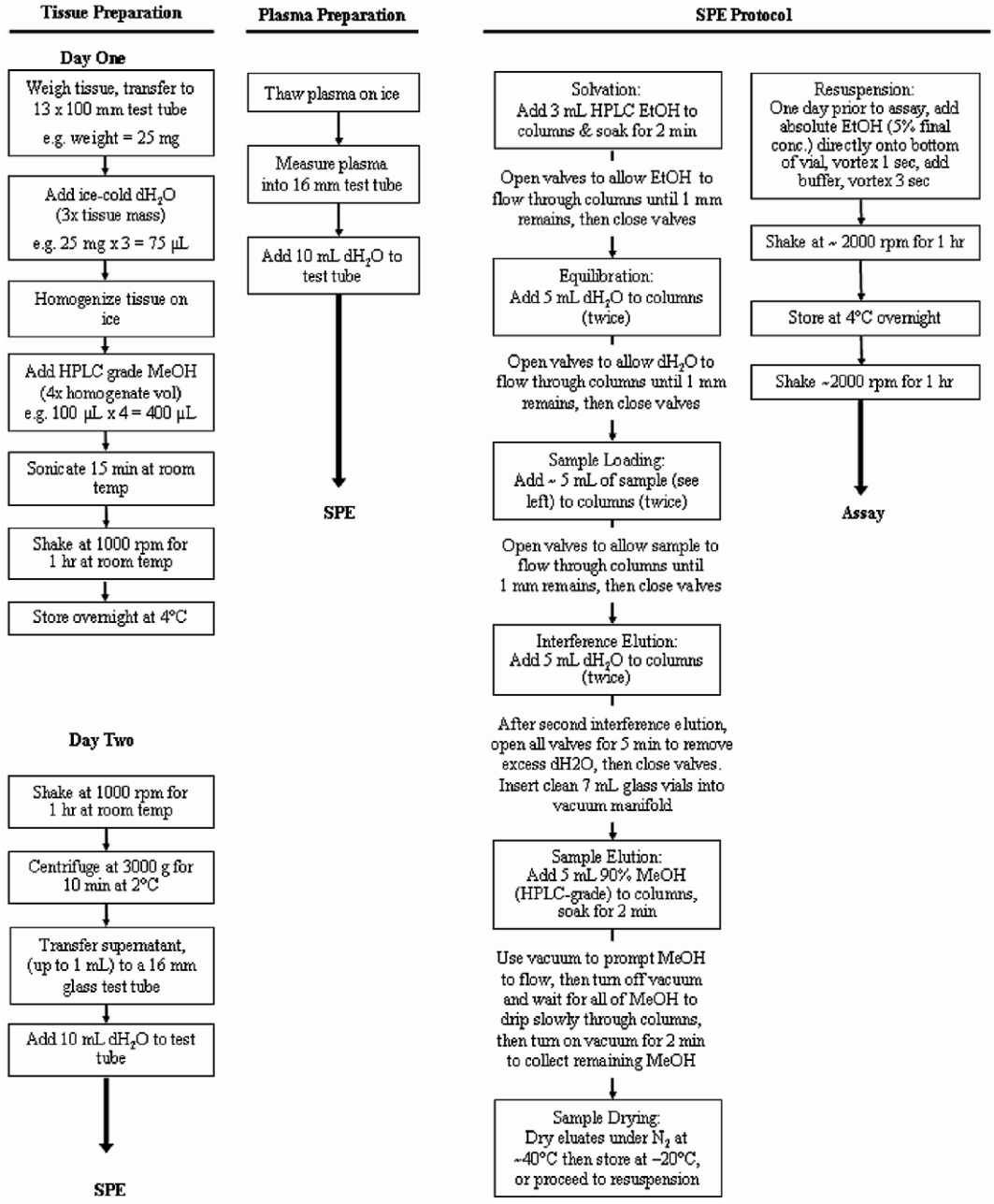

Fig. 3. Summary of solid phase extraction protocol for tissue and plasma samples. C_{18} columns should be closed prior to addition of liquids for all steps. After liquid has been gently added to the column, columns are opened and liquid is allowed to drip into collecting vessel. If necessary, vacuum pressure may be used to begin dripping, but dripping should be continued without vacuum pressure to ensure a slow and constant drip rate. During SPE, it is crucial that a small amount of liquid (approximately 1 mm above packing material) remains in the column between steps unless otherwise stated (for details see Thurman and Mills (1998)).

After drying, dried eluates can be stored at −20 °C prior to resuspension in assay buffer. An addition of absolute EtOH in the resuspension, either 5 or 10%, significantly improves recovery of radiolabeled steroids. The EtOH is added directly to the dried eluates at the bottom of the vials and then brought up to final volume with assay buffer. In our laboratories, we routinely use 5% EtOH in resuspension buffer, which does not affect the DHEA, E_2 or CORT RIAs in our laboratories. However, the effect of EtOH should be determined for each RIA or EIA used.

These data also demonstrate that SPE effectively reduces assay interference in songbird samples for measurement of DHEA, E2, and CORT. For other analytes, it is possible that a second interference elution with dilute MeOH or another eluant will be important to further reduce assay interference (e.g., Vallee et al., 2000). Further work on the interference elution(s) is a goal for future experiments.

While SPE can separate unconjugated steroids from sulfated steroids (Liere et al., 2004), SPE has typically not been used to separate various unconjugated steroids from

each other. In this regard, SPE C_{18} columns generally have been used differently than Celite chromatography columns, which can separate several unconjugated steroids (Wingfield and Farner, 1975; Soma et al., 1999). Note that C_{18} columns can be used with HPLC to separate unconjugated steroids, but HPLC C_{18} columns are much longer than SPE C_{18} columns and thus have greater separating ability. Whether SPE C_{18} columns can be used to separate unconjugated steroids from each other is an important question for future studies.

These data demonstrate that SPE is useful prior to RIA, but SPE is also useful prior to EIA (Acosta et al., 2000; Love et al., 2005; Williams et al., 2005), high-performance liquid chromatography (Hojo et al., 2004; Ziegler and Wittwer, 2005), and gas chromatograph–mass spectrometry (Mensah-Nyagan et al., 1998; Vallee et al., 2000). SPE is an attractive alternative to traditional organic solvent extractions, because recoveries are high and consistent, and removal of assay interference is more complete. The equipment and columns are commercially available, and set-up is straightforward. Also, it may be possible to reduce the cost of SPE by washing and reusing the C18 columns.

These data highlight the importance of empirically determining how to prepare samples prior to steroid measurement. This should be done on a case-by-case basis. Many variables can affect the choice of sample preparation technique: the steroid measured, study species, sex, season, reproductive state, and sample type (e.g., tissue, plasma, serum). First, in some cases, no extraction may be needed. For example, Washburn et al. (2002) demonstrate that CORT can be measured in avian plasma without extraction, when using a particular CORT RIA. Second, in other cases, an organic solvent extraction may be sufficient. For example, Wingfield and Farner (1975) demonstrate that T can be measured in avian plasma after DCM extraction, especially when DCM extraction is followed by Celite column chromatography. In addition, Tsutsui and Yamazaki (1995) measured pregnenolone and DHEA in Japanese quail brain after ethyl acetate extraction. Third, in other cases, SPE may be required. The present results indicate that SPE is more effective than organic solvent extractions for removal of interfering substances from songbird brain and lipemic plasma. Migues et al. (2002) measured DHEA in chicken brain after SPE, but to our knowledge, the present report is the first examination of steroid levels in the brain of songbirds, an important model system in neuroendocrinology. Also, to compare steroid levels in tissues and plasma, both types of samples should be processed similarly. That is, if SPE is required for tissue but not plasma samples, both types of samples should be prepared using SPE to avoid a confound. SPE should prove useful for a variety of researchers interested in measuring steroids in plasma, serum, brain, yolk, feces, urine, and whole body homogenates from a wide range of organisms.

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References

Acosta, T.J., Ozawa, T., Kobayashi, S., Hayashi, K., Ohtani, M., Kraetzl, W.D., Sato, K., Schams, D., Miyamoto, A., 2000. Periovulatory changes in the local release of vasoactive peptides, prostaglandin $F_{2\alpha}$, and steroid hormones from bovine mature follicles in vivo. Biol. Reprod. 63, 1253–1261.

Baulieu, E.E., 1997. Neurosteroids of the nervous system, by the nervous system, for the nervous system. Recent Prog. Horm. Res. 53, 1–32.

Chard, T., 1995. An Introduction to Radioimmunoassay and Related Techniques. Elsevier, New York.

Demas, G.E., Cooper, M.A., Albers, H.E., Soma, K.K., 2007. Novel mechanisms underlying neuroendocrine regulation of aggression: a synthesis of rodent, avian and primate studies. In: Blaustein, J.D. (Ed.), Behavioral Neurochemistry and Neuroendocrinology. Handbook of Neurochemistry and Molecular Neurobiology, vol.21, Springer, pp. 337–372.

Fuqua, J.S., Sher, E.S., Migeon, C.J., Berkovitz, G.D., 1995. Assay of plasma testosterone during the first six months of life: importance of chromatographic purification of steroids. Clin Chem. 41, 1146–1149.

Goodson, J.L., Evans, A.K., Soma, K.K., 2005a. Neural responses to aggressive challenge correlate with behavior in nonbreeding sparrows. Neuro. Report 16, 1719–1723.

Goodson, J.L., Saldanha, C.J., Hahn, T.P., Soma, K.K., 2005b. Recent advances in behavioral neuroendocrinology: insights from studies on birds. Horm. Behav. 48, 461–473.

Granger, D.A., Schwartz, E.B., Booth, A., Curran, M., Zakaria, D., 1999. Assessing dehydroepiandrosterone in saliva: a simple radioimmunoassay for use in studies of children, adolescents and adults. Psychoneuroendocrinology 24, 567–579.

Hojo, Y., Hattori, T., Enami, T., Furukawa, A., Suzuki, K., Ishii, H., Mukai, H., Morrison, J.H., Janssen, W.G.M., Kominami, S., Harada, N., Kimoto, T., Kawato, S., 2004. Adult male rat hippocampus synthesizes estradiol from pregnenolone by cytochromes P45017α and P450 aromatase localized in neurons. Proc. Natl. Acad. Sci. 101, 865–870.

Jawad, M.J., Wilson, E.A., Rayburn, W.F., 1981. Effect of serum lipids on radioimmunoassays of unconjugated estriol in serum. Clin. Chem. 27, 280–285.

Lee, C., Goeger, D.E., 1998. Interference of 6β-Hydroxycortisol in the quantification of urinary free cortisol by immunoassay and its elimination by solid phase extraction. Clin. Biochem. 31, 229–233.

Liere, P., Pianos, A., Eychenee, B., Cambourg, A., Liu, S., Griffiths, W., Schumacher, M., Sjovall, J., Baulieu, E.E., 2004. Novel lipoidal derivatives of pregnenolone and dehydroepiandrosterone and absence of their sulfated counterparts in rodent brain. J. Lipid Res. 45, 2287–2302.

Love, O.P., Chin, E.H., Wynne-Edwards, K.E., Williams, T.D., 2005. Stress hormones: a link between maternal condition and sex-biased reproductive investment. Am. Nat. 166, 751–766.

Mensah-Nyagan, G.A., Do-Rego, J.L., Beaujean, D., Feuilloley, M., Marcual, A., Lange, C., Pelletier, G., Vaudry, H., 1998. Biosynthesis

of neuroandrogens in the frog brain. Ann. NY Acad. Sci. 839, 400–402.

Migues, P.V., Johnston, A.N.B., Rose, S.P.R., 2002. Dehydroepiandrosterone and its sulphate enhance memory retention in day-old chicks. Neuroscience 109, 243–251.

Newman, A.E.M., Soma, K.K., 2006. Neural secretion of DHEA and corticosterone during acute stress? Abstr. Soc. Neurosci. Program No. 269.16.

O'Grady, J.E., 1968. The determination of oestradiol and oestrone in the plasma of the domestic fowl by a method involving the use of labeled derivatives. Biochem. J. 106, 77–86.

Rash, J.M., Jerkunica, I., Sgoutas, D.S., 1980. Lipid interference in steroid radioimmunoassay. Clin. Chem. 26, 84–88.

Romero, L.M., Cyr, N.E., Romero, R.C., 2006. Corticosterone responses change seasonally in free-living house sparrows (*Passer domesticus*). Gen. Comp. Endocrinol. 149, 58–65.

Samuels, L.T., 1949. Metabolism of steroids by tissues. I. Determination of testosterone and related steroids in tissue extracts. J. Biol. Chem. 168, 471–475.

Schwabl, H., 1995. Yolk is a source of maternal testosterone for developing birds. Proc. Natl. Acad. Sci. USA 90, 11446–11450.

Shirtcliff, E.A., Granger, D.A., Schwartz, E.B., Curran, M.J., Booth, A., Overman, W.H., 2000. Assessing estradiol in biobehavioral studies using saliva and blood spots: simple radioimmunoassay protocols, reliability, and comparative validity. Horm. Behav. 38, 137–147.

Soma, K.K., 2006. Testosterone and aggression: Berthold, birds and beyond. J. Neuroendocrinol. 18, 543–551.

Soma, K.K., Alday, N.A., Hau, M., Schlinger, B.A., 2004. Dehydroepiandrosterone metabolism by 3β-hydroxysteroid dehydrogenase/Δ5–Δ4 isomerase in adult zebra finch brain: sex difference and rapid effect of stress. Endocrinology 145, 1668–1677.

Soma, K.K., Sinchak, K., Lakhter, A., Schlinger, B.A., Micevych, P.E., 2005. Neurosteroids and female reproduction: estrogen increases 3β-HSD mRNA and activity in rat hypothalamus. Endocrinology 146, 4386–4390.

Soma, K.K., Sullivan, K., Wingfield, J.C., 1999. Combined aromatase inhibitor and antiandrogen treatment decreases territorial aggression in a wild songbird during the nonbreeding season. Gen. Comp. Endocrinol. 115, 442–453.

Soma, K.K., Wingfield, J.C., 2001. Dehydroepiandrosterone in songbird plasma: seasonal regulation and relationship to territorial aggression. Gen. Comp. Endocrinol. 123, 144–155.

Speake, B.K., Decrock, F., Surai, P.F., Groscolas, R., 1999. Fatty acid composition of the adipose tissue and yolk lipids of a bird with a marine-based diet, the emperor penquin (*Aptenodytes fosteri*). Lipids 34, 283–290.

Telepchak, M.J., August, T.F., Chaney, G., 2004. Forensic and Clinical Applications of Solid Phase Extraction. Humana Press, New Jersey.

Thurman, E.M., Mills, M.S., 1998. Solid-Phase Extraction: Principles and Practice. John Wiley & Sons Inc., New York.

Tsutsui, K., Yamazaki, T., 1995. Avian neurosteroids. I. Pregnenolone biosynthesis in the quail brain. Brain Res. 678, 1–9.

Vallee, M., Rivera, J.D., Koob, G.F., Purdy, R.H., Fitzgerald, R.L., 2000. Quantification of neurosteroids in rat plasma and brain following swim stress and allopregnanolone administration using negative chemical ionization gas chromatography/mass spectrometry. Anal. Biochem. 287, 153–166.

Washburn, B.E., Morris, D.L., Millspaugh, J.J., Faaborg, J., Schulz, J.H., 2002. Using a commercially available radioimmunoassay to quantify corticosterone in avian plasma. The Condor 104, 558–563.

Williams, T.D., Ames, C.E., Kiparissis, Y., Wynne-Edwards, K.E., 2005. Laying-sequence-specific variation in yolk oestrogen levels, and relationship to plasma oestrogen in female zebra finches (*Taeniopygia guttata*). Proc. Biol. Sci. 272, 173–177.

Wingfield, J.C., Farner, D.S., 1975. The determination of five steroids in avian plasma by radioimmunoassay and competitive protein-binding. Steroids 26, 311–321.

Ziegler, T.E., Wittwer, D.J., 2005. Fecal steroid research in the field and laboratory: improved methods for storage, transport, processing, and analysis. Am. J. Primatol. 67, 159–174.