

Validation of an ELISA for measurement of fecal cortisol in fishes.

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Abstract

Quantification of glucocorticoid (GC) metabolites in feces has become an established method for the noninvasive assessment of adrenocortical activity. These hormones are frequently determined in plasma samples as parameters of adrenal activity and response to stress. GCs are metabolized and excreted with both intact hormone and their metabolites present in feces. Therefore, the concentration of GCs (or their metabolites) can be measured in excreta. Fecal samples also present the advantages of easy collection, no stress to the animal and elimination of the issue of potentially misleading acute GC spikes. The aim of this study was to determine if an enzyme-linked immunosorbent assay (ELISA) for cortisol was appropriate for monitoring adrenocortical activity in fecal casts of fishes. Performance of the cortisol ELISA was validated by comparison to high performance liquid chromatography (HPLC), which is an established method for measuring free glucocorticoids and glucocorticoid metabolites in feces. Parallelism and sample extraction efficiency were compared for the two methods. Pearson correlation across samples for these methods was 0.996. Results demonstrated that the ELISA was an efficient, sensitive and reliable method for cortisol measurement in fecal extracts, which should permit integration of noninvasive stress monitoring into studies of fish behavior and physiology.

Key Words: cortisol; stress; ELISA; HPLC

1. Introduction

Endocrine activity in an extensive range of species, including fishes, can be significantly altered under stressful circumstances contributed by both natural and anthropogenic factors (Carballo et al., 2005). Fishes subjected to various stressors exhibit primary stress responses similar to mammals, including the release of cortisol from interrenal tissue (equivalent of the mammalian adrenal cortex) (Axelrod and Reisine, 1984). Under chronic stress conditions (e.g., environmental deterioration), long-term mild to moderate cortisol hypersecretion may occur (van den Heuvel et al., 2005). Specific physiological consequences of chronic stress include inhibition of innate immunity (Watanuki et al., 2002), growth (Jentoft et al., 2005) and reproduction (Berg et al., 2004). Quantification of fecal glucocorticoid (GC) metabolites has become a useful method for the noninvasive assessment of adrenocortical activity in temperamental, dangerous or poorly accessible species (Turner et al., 2003, Stead, 2001, Wasser, 2000). While GCs, and especially cortisol, have frequently been determined in plasma as an indicator of stress, blood sampling is itself invasive and stressful. In addition, fecal samples amortize hormone levels across time, eliminating the issue of potentially misleading acute hormonal spikes associated with sampling plasma (Turner et al., 2003). GCs are metabolized and excreted with both intact hormones and their metabolites present in feces (Touma et al., 2003). The main metabolites of glucocorticoids are 17- β -hydroxycorticoids, with a smaller amount (5-10%) metabolized to 17-ketosteroids, and approximately 10% is excreted as free hormone (Chelini et al., 2006). These proportions are relatively stable, making measurement of free hormone a direct and reliable endpoint. The aim of this study was

to determine whether an enzyme-linked immunosorbent assay (ELISA) for cortisol could be applied to measurement of fecal cortisol in fishes. Cortisol monitoring of fishes in both aquaculture and natural settings is a potentially valuable tool. Performance of the cortisol ELISA was validated by comparison to high performance liquid chromatography (HPLC), which has been used for measuring free glucocorticoids and glucocorticoid metabolites in feces of numerous mammalian species (Wasser et al., 2000) and more recently in fishes (Turner et al., 2003). Tests for parallelism and extraction efficiency were used to compare the cortisol ELISA and HPLC in fecal casts of parrotfishes.

2. Materials and Methods

2.1 Animal model

The fish fecal samples used in this study were obtained from adult male and female stoplight and queen parrotfishes (*Sparisoma viride* and *Scarus vetula*, respectively) located within reefs of Great Lamesur Bay, Saint John, US Virgin Islands. These samples were part of a separate study involving fecal collections across several years from fishes inhabiting this bay (Turner et al., 2003). Samples were collected via SCUBA and snorkeling at the same time of day to minimize possible influences of diurnal cortisol concentrations. Since sufficient material was not obtainable from a single fecal cast for the HPLC portion of the study (Turner et al., 2003), each cortisol value was obtained from a pool of samples from 6 different individuals.

2.2 Extraction and Reconstitution

2.2.1 Extraction

Desiccated fecal casts for the ELISA (0.2 g) or HPLC (0.5 g) were mixed in 12 mL spectrophotometric-grade dichloromethane (MeCl₂, Curtin Matheson Scientific, Houston, TX) and shaken for 60 minutes on a motorized shaker (Burrel, Pittsburg, PA) at 25° C and then centrifuged at 3000 rpm for 10 minutes. Ten mL of the liquid layer was combined with 1 mL 1.0 M sodium hydroxide in a glass scintillation vial, shaken, allowed to separate and frozen on dry ice. Eight mL of the MeCl₂ layer was withdrawn, aliquoted (4 mL each) into two 12x75 mm borosilicate glass tubes, evaporated to dryness and stored at -40° C until reconstituted. Dry samples were reconstituted in ethanol and enzyme conjugate (for ELISA) or acetonitrile and distilled water (AcN, for HPLC) for assay (fig. 1). Ethanol was used in the ELISA due to the denaturing effect of AcN on proteins and its propensity to dissolve the plastic microtitre plates.

2.2.2 Reconstitution for ELISA

Extracted cortisol samples (from 0.2 g desiccated fecal material) were mixed with 50 µL ethanol (EtOH) and vortexed for 2 min. Fifty µL increments of aqueous enzyme conjugate were added and vortexed for an additional 2 min. each to give a final volume of 500 µL (see section 2.3). Fifty µL of each sample (in duplicate) was added to the ELISA plate.

2.2.3. Reconstitution for HPLC

Extracted cortisol samples (from 0.5 g desiccated fecal material) were mixed with 100 μL acetonitrile (ACN) and vortexed for 2 min. One hundred μL increments of dH_2O were added and vortexed for an additional 2 min. each to give a final volume of 1000 μL (see section 2.4). A 250 μL aliquot of each sample was loaded onto the chromatography column.

2.3 Cortisol ELISA

A sandwich-type ELISA, modified from methods described by Munro and Lasley (1988), was adapted for determination of fish fecal cortisol. The assay employed a cortisol-horseradish peroxidase ligand and antiserum (Antibody R4866; C.J. Munro, University of California at Davis) and commercial cortisol standards (hydrocortisone reference standard; Sigma-Aldrich Inc., St. Louis, MO). The ELISA was completed in 96-well microtiter plates (Nunc-Immuno, Maxisorp Surface; Fisher Scientific, Pittsburgh, PA) layered 24 hrs beforehand with cortisol 100 antiserum (50 μL /well; diluted 1:8500 in bicarbonate coating buffer; 0.05M $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$, pH 9.6). Plates were sealed tightly with waterproof plate sealer covers and incubated overnight at 4° C. On the day of the assay, the enzyme conjugate (cortisol-3-CMO:HRP) was diluted to 1:20,000 in phosphate buffer (0.1 M PBS containing 0.1% BSA, pH 7.0). Non-bound antibody was removed from the wells of the microtiter plates with wash solution (0.15 M NaCl containing 0.05% v:v Tween 20), blotted and allowed to dry at room temperature. The polyclonal antiserum was raised in rabbits against cortisol-3-carboxymethyloxime, linked to bovine

serum albumin and cross-reacts with cortisol (100%), prednisolone (9.9%), prednisone (6.3%), cortisone (5%) and <1% with androstenedione, androsterone, corticosterone, desoxycorticosterone, 11-desoxycortisol, 21-desoxycortisone, and testosterone (Munro and Lasley, 1988). Fifty μL of phosphate buffer was pipetted across the entire plate followed by 50 μL of cortisol-3-CMO:HRP conjugate solution containing cortisol standards or a reconstituted extracted fecal sample. Stock cortisol standards stored in ethanol were dried under air and reconstituted in 50 μL ethanol and 450 μL diluted enzyme conjugate to give a standard range of 5 to 1000 $\text{pg}/\mu\text{L}$. The dried fecal samples were reconstituted similarly and 50 μL of each sample were pipetted in duplicate for the ELISA. Plates were covered tightly with plate sealer covers and the competitive reaction allowed to proceed for 2 hours. To separate free from bound hormone, the plates were emptied, rinsed 3 times with wash solution, blotted and allowed to dry at room temperature. One hundred μL of freshly prepared substrate solution (0.05 M citrate, 1.6 mM hydrogen peroxide, 0.4mM 2,2-azino-di-3-ethylbenzothiazoline sulfonic acid diammonium salt (ABTS, pH 4.0) was then added to all wells and color was allowed to develop to determine the amount of conjugate (cortisol:HRP) bound to the solid-phase antibody. The color change was stopped after approximately 60 minutes by the addition of 100 μL stop solution (0.15 M hydrofluoric acid containing 0.006 M NaOH and 0.001 M EDTA, pH 3.3). Absorbance was measured at 405 nm with a Bio-Rad Model 550 automatic microtiter plate spectrophotometer (Hercules, CA, USA) and the data were transferred to an interfaced computer for analysis (Gateway model E4300, Irvine, CA). Cortisol concentrations are expressed as nanograms per gram of dry feces (ng/g).

2.4 HPLC

Although details of this procedure for fecal cortisol have been previously reported (Turner et al., 2003), a brief description is provided for the present context. Free cortisol in fish fecals was determined by reverse-phase high performance liquid chromatography (HPLC, Dionex, Sunny vale, CA) utilizing a standard 3.9 x 300 mm C-18 column (Waters, Milford, MA) and a variable wavelength UV detector (Dionex) set at 240 nm. Prior to sample analysis, a water blank was run until the column was free of major peaks and a reference standard containing cortisol (hydrocortisone reference standard; Sigma-Aldrich Inc., St. Louis, MO, 50 μ L in 10% acetonitrile (ACN)/90% water) was run to verify retention times. The flow rate was 1 mL/min and the elution gradient changed from 10% ACN/90% water to 90% ACN/10% water over a period of 25 minutes (ensuring complete separation of sample compounds) and returned to the initial concentration over another 5 minutes. Additionally, the run was continued for 15 minutes at the initial concentration to re-acclimate the column. The standard curve was developed by HPLC runs of duplicate samples of 7 known cortisol concentrations (ranging from 5 pg cortisol/ μ L to 1000 pg cortisol/ μ L).

3. Results

3.1. HPLC

Cortisol was readily detectable and measured by the HPLC analysis and the elution time was 11.91 ± 0.02 minutes. The lower limit for cortisol detection in the HPLC portion of this study was 1.8 ng in a 250 μ L sample loaded onto the chromatographic column. Hormone values are reported as ng cortisol/g dry feces. HPLC standards ranged from 5 to 1000 pg/ μ L in 7 incremental amounts (fig.1). The HPLC-assayed cortisol values ranged from 1118.5 ng per g dry fecal for the 1999 sample to 169.5 ng cortisol per g dry fecal material for the 2003 sample.

3.2 ELISA

A representative ELISA standard curve is shown in figure 2. ELISA-derived cortisol values (ng/g dry fecal) were based on the same concentration as for HPLC with $R^2 = 0.9899$ for the calculated curve (fig. 2). The lower limit using ELISA was 1.0 ng in the usual 50 μ L sample placed in each microplate. ELISA-assayed cortisol values ranged from 1314.2 ng/g dry fecal for the 1999 sample to 210.7 ng/g dry fecal for the 2003 sample.

3.3 ELISA-HPLC Comparison

Fecal cortisol assayed by both HPLC and ELISA across the sampling years 1999 to 2004 yielded nearly identical patterns of cortisol levels (fig. 3). A Pearson correlation coefficient performed on these data was 0.996.

4. Discussion

To date there have been no reports of use of an ELISA for measurement of cortisol in fish feces, as radio immunoassay (RIA) and HPLC are the most commonly used methods.

The present study has shown that fish cortisol can be measured in fecal material by ELISA and this assay exhibits equal reliability and much greater efficiency and sensitivity than HPLC. On a per-mL basis, the ELISA showed 9-fold greater sensitivity than HPLC, which reduces fecal sample weight required for reliable assay (HPLC = 0.5 g, ELISA = 0.2 g). From both per-sample assay time and personnel cost perspectives, the ELISA is more practical and less expensive than HPLC analysis. The time required for a single fecal analysis via HPLC is approximately 45 minutes, whereas an ELISA plate can accommodate up to 96 samples (48 in duplicate) in less than 4 hours. Finally, the equipment cost for ELISA is less than 5% of that for HPLC. We conclude that the fecal-cortisol ELISA validated in this study shows high potential as a sensitive, reliable and cost-effective tool for noninvasive stress assessment in fishes.

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