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Challenges, pitfalls and surprises: development and validation of a monoclonal antibody for enzyme immunoassay of the steroid 1 α -hydroxycorticosterone in elasmobranch species

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Abstract

Sharks and rays are popular species used in wildlife ecotourism and aquariums to educate the public on the behavior, ecology and conservation challenges of elasmobranchs. To understand long-term physiological health and welfare under varying social and husbandry conditions, we developed and validated an enzyme immunoassay (EIA) to measure stress/ionoregulatory hormones in managed and semi-free range southern rays (*Hypanus americanus*). Banked serum and interrenal samples from 27 female rays managed at Disney's The Seas with Nemo and Friends[®] and Castaway Cay were used to evaluate measurement of 1 α -hydroxycorticosterone (1 α OHB) relative to corticosterone (B). Although commercial EIAs are available for B, those tested exhibit only low relative cross-reactivity to 1 α OHB (3-5%). To improve measurement of 1 α OHB, we developed a monoclonal antibody using a synthesized 1 α OHB-derivative for evaluation using high-performance liquid chromatography (HPLC) and EIA. Relative displacements of cross-reactant compounds showed that the antibody had good sensitivity for the target antigen 1 α OHB, and low sensitivity to related steroids (desoxycorticosterone and B), but greater sensitivity to 11-dehydrocorticosterone. Tests of competitive vs. noncompetitive EIA formats, reagent titration, and incubation times of the antibody and conjugate were used to optimize sensitivity, repeatability and precision of measured 1 α OHB in standards and samples (4 ng/ml, 90% binding). Tests of sample pre-treatment (pH adjustment) and extraction with varying solvent polarity were used to optimize measurement of 1 α OHB in <1 ml (serum) or 1g (interrenal) samples. HPLC analysis revealed the 1 α OHB EIA to be superior for measurement of 1 α OHB

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compared to use of a B EIA with or without HPLC fractioning. Results may prove useful for extrapolation to guide best practices for 1 α OHB measurement in other elasmobranch species. Improved measurement of stress/ionoregulatory hormones in sharks and rays will be important for many aspects of collection, transport, medical treatment in aquaria and conservation management of these charismatic and ecologically important species.

Keywords

1 α -hydroxycorticosterone; stress; glucocorticoid; mineralocorticoid; HPLC; stingray

1. Introduction

The corticosteroid 1 α , 11 β , 21-trihydroxypregn-4-ene-3, 20-dione (1 α -hydroxycorticosterone, or 1 α OHB) is unique to elasmobranchs and was first isolated in 1966 by Idler and Truscott. Since that time, 1 α OHB has been found to be the dominant corticosteroid in many elasmobranch species, and evidence has accrued supporting a dual role for 1 α OHB in both hydromineral balance (i.e. as a mineralocorticoid) and as part of the endocrine stress axis (i.e. as a glucocorticoid; see Anderson 2012 for review). However, despite growing evidence supporting a crucial role for 1 α OHB in elasmobranch health and physiology, efforts to quantify this unique corticosteroid have largely been unsuccessful.

Early methods for the quantification of 1 α OHB included acid fluorescence (Idler and Truscott, 1968), a double isotope derivative method (Truscott and Idler, 1972), and a derivative-based radioimmunoassay using an antibody against corticosterone and 1-dehydrocorticosterone (Kime, 1977). These first two methods required significant volumes of plasma (>30 mL) and the latter relied on the acid dehydration of 1 α OHB to 1-dehydrocorticosterone and therefore lacked specificity. Synthesis of 1 α OHB for standards and assay/antibody development has proved to be challenging, and initial supplies of the purified steroid synthesized in the early 1970s (Kime, 1972) were rapidly depleted. For the next ~30 years, attempts to quantify 1 α OHB in elasmobranchs utilized assays and antibodies for corticosterone that were not specific to 1 α OHB (e.g. Manire et al., 2007; Rasmussen and Crow, 1993).

In the early 2000s, new methods for the synthesis of purified 1 α OHB were developed by the Rimoldi laboratory at the University of Mississippi. This synthetic 1 α OHB was subsequently used in receptor binding assays (Carroll et al., 2008) as well as validation of a method combining high-performance liquid chromatography (HPLC) fraction isolation with a commercial corticosterone enzyme immunoassay (EIA) for the specific quantification of 1 α OHB from elasmobranch plasma (Evans et al., 2010). Recently, another successful synthesis of 1 α OHB was developed at the University of Ottawa (Wiens et al. 2017) who used HPLC to elucidate the potential role of bacteria in the 1 α OHB steroid metabolic pathway. While a combination of HPLC/EIA methods allows for quantification of 1 α OHB, it too relies on an antibody specific to corticosterone with low cross-reactivity to 1 α OHB. Therefore, we identified a significant need for the development of a specific, rapid and sensitive EIA for 1 α OHB.

2. Methods

2.1. Reagents

All reagents used were HPLC analytical grade quality. Purified steroids used for EIA, HPLC and cross-reactant testing were purchased from Steraloids (Newport, RI; corticosterone (Kendall's compound 'B'; Q1550-000), cortisol (Kendall's compound 'F'; Q3880-000); cortisone (Kendall's compound 'E'; Q2500-000); 11-dehydrocorticosterone (11-DHC; Q3690-000); desoxycorticosterone (DOC; Q3460-000); corticosterone 21-hemisuccinate (B-21-HS; Q1562-000); corticosterone 21-acetate (B-21-OAc; Q1552-000); 16-dehydropregnenolone acetate oxime (P1510-000); progesterone (P4; Q2600-000)).

2.2. 1 α -hydroxycorticosterone (1 α OHB) and 1 α OHB-derivative synthesis

Purity of synthesized 1 α OHB (prepared by Rimoldi, Univ. of Mississippi) was confirmed by LC-MS, proton/carbon NMR, 2D NMR, elemental analyses, and UV-visible spectra (peak absorbance at 242 nm). For antisera development, the goal was to produce a 1 α OHB-derivative that included carbomethoxy oxime (CMO) on the 20-carbon position on the D ring to allow for antibody recognition at the 1- and 11-carbon positions (A and C ring hydroxyls unique to 1 α OHB), while reducing recognition to cortisol and related steroids. Synthesis of the desired molecule was not possible while keeping the hydroxyl at the 11-carbon position. Instead, the final synthesis included an 11-carbon-position ketone (see Fig. 1. ii), producing 1 α OH-11-oxo-20-(carbomethoxy) oxime corticosterone (1 α OH-11oxo-20CMO-B).

2.3. 1 α OHB Antisera development

2.3.1. Preparation of reagents for immunization—Immunogen preparations were conducted according to the method of Tateishi et al. (1980). The 20-hemisuccinate of 11-DHC (11-DHC:20HS; Steraloids Q3700-000) was activated by reaction with N-hydroxysuccinimide (NHS) and dicyclohexylcarbodiimide (DCC) in dimethylformamide (DMF). The activated NHS ester was then added to poly D-glutamic acid, D-lysine (D-GL) HBr salt (Sigma P7658) with 6:4 ratio of amino acids and molecular weight of 20,000 to 50,000 dissolved in 50 mM borate buffer pH8.5, DMF at a ratio of 50:1 steroid:polymer. The mixture was stirred for 1 hour (23°C), quenched (10% lysine in 50 mM borate buffer pH8.5) then purified by collection of 1 ml fractions in 0.1M PBS, pH 7.2 (Amersham PD-10 column). Peak fractions were detected at 280 nm and pooled. The 1 α OH-11oxo-20CMO-B derivative was activated (as above) and coupled to bovine serum albumin (BSA), porcine thyroglobulin (PTG) or peroxidase (HRP). Coupled 11-DHC:20HS:D-GL and 1 α OH-11oxo-20CMO-B:proteins were purified and pooled (as above) and stored at -20°C.

2.3.2. Immunization and monoclonal antibody production—Five BALB/c mice (Charles River Laboratories, Inc.) were pretreated with 11-DHC:20HS:D-GL to reduce immunogenic response and subsequent crossreactivity (Brooks et al., 1993; Tateishi et al., 1980) to the 11-oxo group of the prepared 1 α OHB-derivative (see Fig. 1.) prior to primary immunization with the target antigen (1 α OH-11oxo-20CMO-B:PTG). Post-immunization mouse sera and hybridoma supernatants were screened for polyclonal antibody binding of 1 α OH-11oxo-20CMO-B:HRP (1 α OHB HRP conjugate for EIA) using a 96-well plate

coated with an antibody to capture mouse antibodies and the 1 α OHB HRP conjugate for detection. Two mice showing the highest and most consistent signal were selected for fusion, however, no useful polyclonals were developed at this step. The remaining three mice were immunized with a new preparation of a higher-ratio immunogen of 1 α OH-11 α -oxo-20CMO-B:PTG, and tested for polyclonal signal with a boost/bleed schedule until the binding signal generated reached plateau before selection for fusion.

Positive clones were evaluated for standard displacement potential with 1 α OHB steroid as the standard curve after spectrometric verification of stock concentration (OD 242 nm, extinction coefficient 16,329, FW 364.26) along with the 1 α OHB HRP conjugate. Antibody specificity for 1 α OHB was tested by evaluating the cross-reactivity (relative antigen concentration at 50% B/B₀ of the 1 α OHB standard) of related steroid compounds (B, F, cortisone (E), 11-DHC, DOC, corticosterone 21-hemisuccinate (B-21-HS), corticosterone 21-acetate (B-21-OAc), and 16-dehydropregnenolone acetate oxime) tested as potential cross reactants in the assay with each antibody clone. Positive clones were also tested with corticosterone:3CMO:HRP, cortisone:3CMO:HRP, and cortisol:3CMO:HRP conjugates to assess binding affinity for either conjugate when coupled to carbon-3.

2.4. Enzyme immunoassays (EIA)

Samples were extracted, reconstituted in assay buffer, and run on 96-well microtiter plates in established in-house EIAs (B, P4) using a double-antibody EIA closely following Graham et al (2016) with the following changes as indicated for B (rabbit polyclonal), P4 or 1 α OHB (mouse monoclonal) EIAs. Briefly, plates were coated with 150 μ l of 10 μ g/ml IgG goat anti-rabbit IgG (GARG; #A009; B EIA); goat anti-mouse IgG (GAMG; #A008; P4 and 1 α OHB EIA), Arbor Assays, Ann Arbor, MI) dissolved in coating buffer (10 mM phosphate) and incubated overnight at 23°C. Wells were emptied, then filled with 250 μ l blocking buffer (10 mM phosphate, 0.1% Tween 20, 0.009% sodium azide, 15 mM NaCl, and 1% sucrose in DI H₂O) and incubated overnight. Plates were emptied, placed in a Dry-Keeper desiccator until <20% humidity, then packaged with a desiccant in a heat-sealed pouch and stored at 4°C.

For all assays, duplicate or triplicate 50 μ l standards (B, P4, 1 α OHB), internal serum controls, samples, HRP conjugates and antisera (B EIA: CJM006; P4 EIA: CL425 provided by C. Munro, UC Davis, CA; Cross-reactivities reported for B in Watson et al., 2013; and reported for P4 in Graham et al., 2001) were added to each well except non-specific binding (no antisera). Plates were incubated overnight at 23°C in the dark, then washed and incubated with tetramethylbenzidine for 30 minutes (B and P4 EIA) or 5 to 10 minutes (1 α OHB). Optical density (OD) was quantified with an Emax Plus plate reader (test filter 450 nm, reference filter 650 nm) using SoftMax Pro software (v6.4.2; Molecular Devices LLC, Sunnydale CA). Sensitivity of the B and P4 EIAs were 11.7 pg/well and 6.74 pg/well, respectively. The 1 α OHB standards ranged from 61 to 15,000 pg/well.

2.4.1. Optimization of 1 α OHB EIA

2.4.1.1. Equilibrium experiments (competitive EIA): Antisera (1:1,000 to 1:4,000) and 1 α OHB HRP (1:10,000 to 1:160,000) dilutions were tested in a checkerboard titration and

incubated at 23°C overnight before washing and color reaction to characterize displacement of the HRP conjugate. A second test using successful combinations of the antisera and HRP conjugate was used to characterize and optimize the 1 α OHB standard dose-response curves to maximize sensitivity. A third test reformatted the assay for a comparison of antisera coating volume (150 vs 300 μ l coating volume of goat anti-mouse IgG) that allowed for higher volume of samples to be added to the wells. Dose-response curves, slope, sensitivity and precision were evaluated for each test.

2.4.1.2. Nonequilibrium experiments (non-competitive EIA): In a test for association of the 1 α OHB HRP conjugate with the antisera, the conjugate was allowed to react for 0 to 24 hours (0.5, 1, 2, 4, 8, 24 h) at 23°C before proceeding with the wash and color reaction steps in varying antisera (1:500, 1:1,000, 1:2,000) and conjugate (1:10,000, 1:20,000, 1:30,000) dilutions. In another test with varying dilutions as above, the 1 α OHB standards were incubated with the antisera for 0 to 24 hours (0.5, 1, 2, 4, 8, 24 h) before addition of the 1 α OHB HRP (Yanagishita and Rodbard, 1978), before proceeding with wash and color reaction steps. Tests with positive results (OD 1.0 to 2.0) were selected to assess 1 α OHB standard curves. Curve parameters, OD, slope, CV and sensitivity were assessed to determine optimum reagent concentrations and incubation times to be used in subsequent analyses.

2.6. Animals

Adult female (N=27) *Hypanus americanus*, (Last et al, 2016; formerly *Dasyatis americana*, first described by Hildebrandt and Schroeder in 1928), were used to validate the 1 α OHB antisera. Animals were housed in a 22,000-m³ mixed-species salt water aquarium environment at Walt Disney World's EPCOT "The Seas with Nemo and Friends" (Lake Buena Vista, Florida) or in an open water ocean lagoon at Disney's Castaway Cay, Commonwealth of the Bahamas. At the Seas, water temperature was maintained at 25 \pm 0.5°C. Lighting was provided by 4 banks of lights supplemented with natural light provided by solar tubes and skylights above the tank. Automated lights on timers were set in a gradual sequence to produce a 16:8 hr light:dark cycle.

2.7. Sample collection and processing

Animals were habituated to restraint and had been anesthetized prior to this study. During health assessments, animals were collected into a net stretcher and moved into an anesthesia bath with 55 ppm buffered tricaine methane-sulfonate (MS-222; Argent Chemicals, WA). Once the animal was mildly sedated, a blood sample (20 ml) was drawn dorsally from a vein in the wing (< 10 min) using single-use 21-gauge needles. Samples were allowed to clot at 4°C before centrifugation (791 \times g for 15 min) and partitioned into separate 1.2 ml aliquots prior to storage (-50°C). A low serum control was prepared by thawing and pooling equal volumes from 30 samples from 27 female rays (banked following routine health exams). A high serum control utilized banked serum from a female *H. americanus* collected at post-mortem examination. Immediately prior to this exam, this female had a recent medical history (3 days), loss of appetite, lethargy and other behavior changes. Illness and injury can produce a state of altered homeostasis or allostatic load that has been associated with changes in glucocorticoids (see Romero 2004; Romero et al., 2009; Sapolsky et al., 2000 for

a review), thus this sample was used as a high serum control or “reference sample” (Wosnick et al., 2017) in subsequent tests.

2.7.1. Sample cleanup and extraction optimization—Tested sample extraction methods to maximize 1 α OHB measurement in serum included a) extracted vs non-extracted with acetonitrile (ACN) or dichloromethane (DCM), b) sample pH adjusted (5% by volume 1M NaOH) vs non-adjusted c) room temperature vs ice-bath cooled samples and reagents. All extracted samples were reconstituted in assay buffer (either neat or concentrated) and assayed immediately. Using the optimized EIA and extraction methods for all further analysis, serial dilution of extracted samples were compared to the 1 α OHB standard curve. Parallelism of serial dilution curve slopes were determined by testing the correlation between percent binding values of serial dilutions of the sample relative to the 1 α OHB standard.

2.7.2. Sample processing for HPLC—Serum and interrenal samples for EIA parallelism, HPLC testing, and sample assay utilized the optimized sample extraction method as determined above, and were processed as follows. Serum samples were cooled in an ice-bath and pre-treated with 5% (by volume) of chilled 1N NaOH to minimize extraction of lipids (Bondy et. al., 1956), mixed gently, then immediately extracted in a 6-fold volume of cold DCM, vortexed for 30 s, then separated by centrifugation (3300 rpm; 4°C; 15 min). The organic fraction was collected using a Pasteur pipet, dried down, and reconstituted in 250 μ l methanol for HPLC injection, or diluted with assay buffer for EIA.

Following histological confirmation, banked interrenal tissue from 5 female *H. americanus* collected at post-mortem examination were thawed, combined and homogenized in DI H₂O (1 ml per mg tissue) prior to DCM extraction as per serum samples, above. Approximately 1mg of combined tissue was extracted and reconstituted in methanol for HPLC, or buffer for EIA.

2.8. High performance liquid chromatography (HPLC)

Reverse-phase HPLC was used to separate steroid hormones and metabolites of interest, fractioning samples for subsequent EIA evaluation of immunoreactivity in those fractions. Following the methods of Evans et al. (2010) and Nunez and Trant (1999), two solutions were used to equilibrate a Dionex Ultimate 3000 HPLC (Thermo Scientific; Solution A: 10% methanol and 10% acetonitrile in water; Solution B: 50% methanol and 50% acetonitrile). Following column equilibration in Solution A, 100 μ l of the sample was injected into a 250 \times 4.6 mm Hypersil Gold C18 reverse phase HPLC column (Thermoscientific, Waltham, MA) with a 5 μ m particle size and flow rate of 0.75 ml min⁻¹. Steroids were collected through the column at a linear gradient of 40% to 100% of solution B over 30 minutes. Steroid elution peaks were determined first individually, then from a panel using either small (3 μ l) or large volume (100 μ l) injections (to match the sample injection protocol) of a concentrated steroid panel consisting of 407 μ Mol (1 α OHB) or 25 μ Mol steroid pool (F, 11-DHC, B, DOC, P4) and monitored at 190, 220, 240 and 254 nm. Fractions were collected every 60 s from 1 to 29 minutes into tubes, evaporated, and

reconstituted in 400 μ l (fractioned samples) or 800 μ l (fractioned standards) assay buffer. Percent of total immunoreactivity was quantified using 1 α OHB, B and P4 EIAs.

3. Results

3.1. Antisera development and testing

3.1.1 Immunization and monoclonal antibody production—Three IgG₁ clones produced following the second immunization step yielded positive signals (1 α OHB standard displacement) using the 1 α OHB HRP conjugate for the signal. Two IgG_{2b} clones yielded no binding signal and were discarded. Antibody specificity tests (percent cross-reactivity providing relative antigen concentration at 50% B/B₀ of the 1 α OHB standard) completed on the three clones that bound 1 α OHB revealed one clone (16D7.F8.) to be superior, with improved binding to 1 α OHB (50 to 60 vs 600 ng/ml at 50% B/B₀), and relative insensitivity to E (200 ng/ml), B (310 ng/ml), B-21-OAc (400 ng/ml), B-21-HS (1,275 ng/ml), DOC (3,000 ng/ml), but greater sensitivity to 11-DHC (30 ng/ml; see Fig. 2). Neither cortisol (F), 16-dehydropregnenolone acetate oxime, nor the 3-carbon-position HRP conjugates of cortisol, cortisone or corticosterone bound with antisera 16D7.F8.

3.2. Optimization of 1 α OHB EIA

3.2.1. Equilibrium experiments (competitive EIA)—A wide range of combinations of 1 α OHB antisera (16D7.F8) and 1 α OHB HRP conjugate provided sufficient signal to produce satisfactory 1 α OHB standard curves, but at low titer and limited sensitivity (~ 384 pg/well 1 α OHB). A 1:2,000 antisera dilution and 1:20,000 HRP conjugate dilution was chosen for subsequent evaluation of the standard curve under the remaining test conditions for competitive EIA. Although test conditions altered overall signal strength within acceptable ranges (> 1.0 OD), neither incubation temperature nor increased GAMG coating and EIA reagent volume improved assay sensitivity.

3.2.2. Nonequilibrium experiments (non-competitive EIA)—Association tests determined that the 1 α OHB HRP conjugate bound with sufficient signal (>0.6 OD) within 30 minutes, with increasing OD across all times tested. Incubation overnight did not improve OD signal, but improved 1 α OHB standard curve CVs (<20%). Results of the delayed addition of HRP conjugate tests provided the best results, improving sensitivity to ~200 pg/well. The assay could be run with antisera (1:1,100), standards and samples for a minimum of two hours before addition of the conjugate (1:7,500 for 30 minutes) before washing the plate and proceeding with the color reaction. Triplicate analysis of the standards improved precision and confidence at the lower limit of detection. However, sensitivity and precision were maximized with incubation of the antisera (1:2,000) standards and samples shaking overnight (dark; 23°C) before addition of the HRP conjugate (1:7,500) for 30 min.

3.2.3. Sample cleanup and extraction optimization—Dichloromethane and ACN extraction produced similar concentrations of 1 α OHB in high control serum sample (ranged 25-30 ng/ml in multiple test and EIA conditions). Extraction with DCM was preferred for the ability to use large volumes, and ease of separation of the solvent from the water phase (sample) for efficient evaporation. Lipid interference in the samples was eliminated by

adjustment of pH by pre-treatment with 5% by volume 1M NaOH and the use of chilled samples and solvents, and thus was used for all further analyses and serial dilution validation tests. Serial dilutions of interrenal and high serum control extracts were parallel with the 1 α OHB standard curve and easily measured in a range of dilutions (interrenal: 1:8 through 1:250 dilution averaged 1295.1 ng/g; high serum control: 7 \times concentration through 1:2 dilution averaged 25.2 ng/ml 1 α OHB, see Fig. 3.), whereas the low serum control only approached the lower limit of detection when reconstituted to a 7 \times concentration for assay, measuring 3.5 ng/ml.

3.3. High performance liquid chromatography (HPLC)

Results from the HPLC chromatogram, including peak retention time (RT) and EIA immunoreactivity profile of the HPLC steroid panel (100 μ l injection; see Fig. 4. a, b), was used to characterize and evaluate subsequent 100 μ l-injections of interrenal (Fig. 4. c) and serum extracts (Fig. 4. d). These results indicated that the highest percent of total immunoreactivity in the 1 α OHB EIA was found in a wide band including fractions (fr) 6 through fr8 (6-9 min) representing 1 α OHB, 11-DHC, and in smaller amounts, B (but not F, which did not bind to the 1 α OHB antisera). Immunoreactivity was observed in fr6 through fr10 (6-11 min) representing 1 α OHB, F, 11-DHC and B in the B EIA, and in fr17 through fr19 (17-20 min) in the P4 EIA in a similar manner for the HPLC standards (Fig. 4. a) and interrenal extract (Fig. 4. b). The high serum control (Fig. 4. d) produced 76.6% of the observed immunoreactivity in fr5 through fr7 in the 1 α OHB EIA suggesting the potential presence of a 1 α OHB-immunoreactive metabolite in circulation such as 1 α OHB-glucuronide (Idler and Truscott, 1969) more polar (fr5) than native 1 α OHB (peak RT fr7) that was not observed in the interrenal extract. Conversely, in the B EIA, the high serum control contained only 3.4 % of immunoreactivity within the 1 α OHB fractions (fr5-7), 19.8% in the B-attributable fractions (fr 9–10), and a surprisingly high remainder (71%) in fr11 through fr20 (DOC through P4) indicating that the B antisera used may not be appropriate for 1 α OHB or B measurements in *H. americanus* unless fractioned first with HPLC.

4. Discussion

Here we report the first successful development of a monoclonal antibody for EIA specific to 1 α OHB. Cross-reactivity testing revealed that the 1 α OHB antisera recognizes the basic B structure and ketones at the C11 position, but not F, nor B molecules with different carbon 20/21 groups (HS or CMO), nor B or F with HRP conjugation or other derivatives at carbon-3. Cortisol was not recognized by our antibody, and it was relatively insensitive to B and DOC which are important steps in the 1 α OHB steroid metabolism pathway (and thus potential cross-reactants) in elasmobranchs. Although the 1 α OHB monoclonal was more sensitive to 11-DHC than to 1 α OHB, 11-DHC is reported in concentrations 1000 times lower than 1 α OHB in interrenal incubates (Nunez and Trant, 1999; see Idler 1972 for a review) and was either not detected, or reported at 15 to 30 times lower in circulation in tested elasmobranch species (Truscott and Idler, 1972). Concerns of interference from native 11-DHC, DOC or B in samples can be assessed and mitigated by careful design and selection of HPLC fractions for EIA.

Results from optimization experiments testing competitive vs noncompetitive EIA formats suggest that the antisera has stronger affinity for the 1 α OHB HRP conjugate over the synthesized standard or native 1 α OHB in the sample. However, this could be used to an advantage, with the design of short incubation assays (2-3 hours) for rapid analysis, or long incubation (overnight) noncompetitive EIA formats as needed. However, sensitivity and precision were improved by a sequential non-competitive 24 h EIA format, and limiting time with the HRP conjugate. Analytical validation tests confirmed that DCM extraction of interrenal tissue and serum were parallel with the 1 α OHB standard curve and could be measured in < 1 g tissue or < 1 ml serum. Analysis of HPLC fractions from interrenal extracts produced EIA immunoreactivity profiles implicated in the 1 α OHB metabolic pathway.

In conclusion, development of a monoclonal antisera for EIA of 1 α OHB is an improvement over previous analytical methods used to measure 1 α OHB. Challenges with preparation of the target antigen for the immunogen prompted the need for subtractive immunization, and although its purpose was to increase antisera specificity, it may have contributed to the low titer and sensitivity of the antisera to 1 α OHB. Although the antisera also recognizes 11-DHC, and in lower concentrations, DOC and B, neither are reported in high amounts in circulation in elasmobranchs, and samples can be fractionated with HPLC if needed. Since 1 α OHB is highly conserved, being found in a wide variety of elasmobranchs (Idler, 1972), we expect the 1 α OHB EIA to validate in other species, providing a useful new tool for the study of glucocorticoid and mineralocorticoid function and physiology in elasmobranchs. Improved measurement of 1 α OHB will be invaluable for many aspects of collection, transport and medical treatment, and husbandry in aquaria and conservation management (Hoffmayer and Parsons, 2001; Naples et al., 2012, Renshaw et al., 2012; Skomal and Mandelman 2012).

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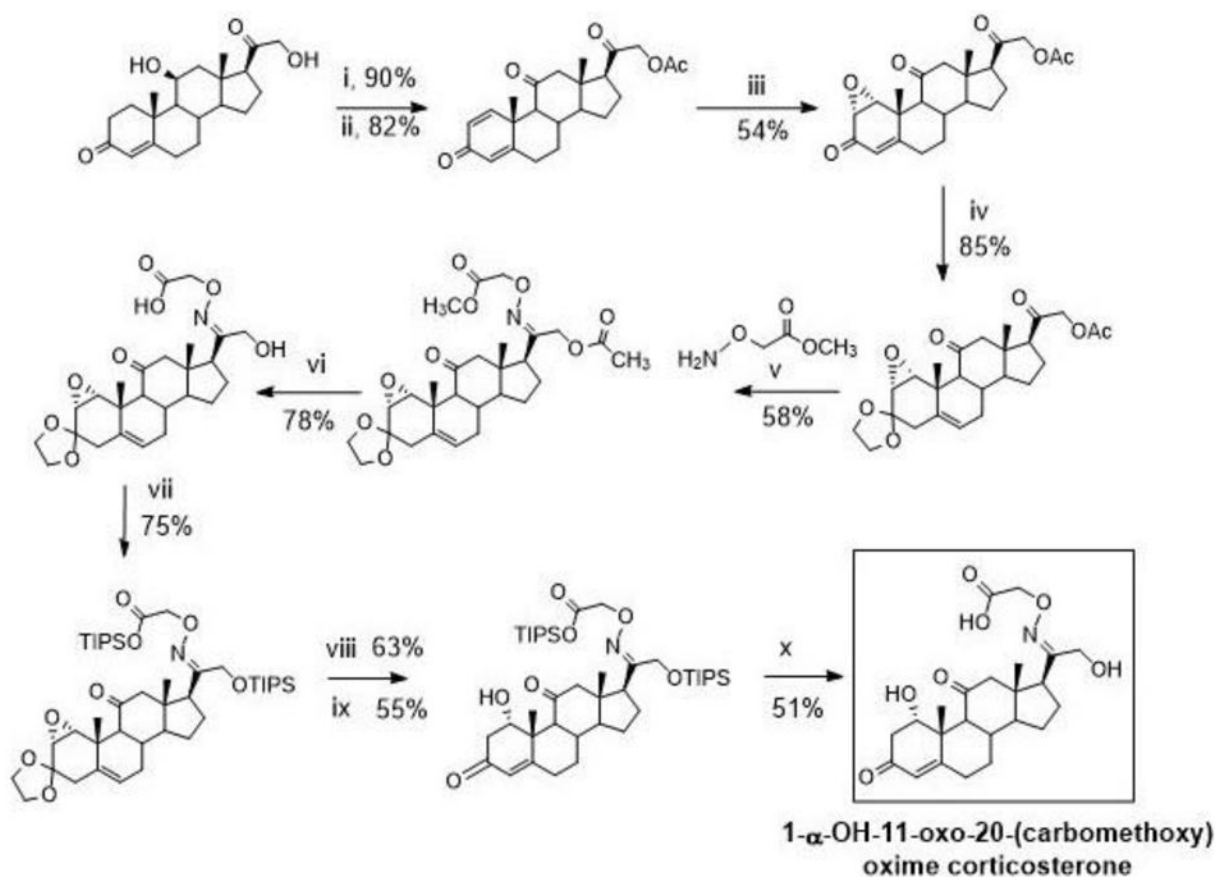
References

- Anderson WG. The endocrinology of 1 α -hydroxycorticosterone in elasmobranch fish: A review. *Comp Biochem Physiol A*. 2012; 162:73–80.
- Carroll SM, Bridgham JT, Thornton JW. Evolution of hormone signaling in elasmobranchs by exploitation of promiscuous receptors. *Mol Biol Evol*. 2008; 25:2643–2652. [PubMed: 18799714]
- Evans AN, Rimoldi JM, Gadepalli RSV, Nunez BS. Adaptation of a corticosterone ELISA to demonstrate sequence-specific effects of angiotensin II peptides and C-type natriuretic peptide on 1 α -hydroxycorticosterone synthesis and steroidogenic mRNAs in the elasmobranch interrenal gland. *J Steroid Biochem Mol Biol*. 2010; 120:149–154. [PubMed: 20236617]
- Graham L, Schwarzenberger F, Mostl E, Galama W, Savage A. A versatile enzyme immunoassay for the determination of progestogens in feces and serum. *Zoo Biol*. 2001; 20:227–236.

- Graham KM, Mylniczeko ND, Burns CM, Bettinger TL, Wheaton CJ. Examining factors that may influence accurate measurement of testosterone in sea turtles. *J Vet Diagn Invest.* 2016; 28:12–19. [PubMed: 26699527]
- Hoffmayer ER, Parsons GR. The physiological response to capture and handling stress in the Atlantic sharpnose shark, *Rhizoprionodon terraenovae*. *Fish Physiol Biochem.* 2001; 25:277–285.
- Idler DR. Steroids in nonmammalian vertebrates. Academic Press, University of California; 1972. 504
- Idler DR, Truscott B. 1 α -hydroxycorticosterone from cartilaginous fish: a new adrenal steroid in blood. *J Fish Res Bd Canada.* 1966; 23:615–619.
- Idler DR, Truscott B. 1 α -hydroxycorticosterone and testosterone in body fluids of a cartilaginous fish (*Raja radiata*). *J Endocrinol.* 1968; 42:165–166.
- Idler DR, Truscott B. Production of 1 α -hydroxycorticosterone in vivo and in vitro by elasmobranchs. *Gen Comp Endocrinol* 2, Supplement. 1969; 2:325–330.
- Kime DE. Measurement of 1 α -hydroxycorticosterone and other corticosteroids in elasmobranch plasma by radioimmunoassay. *Gen Comp Endocrinol.* 1977; 33:344–351. [PubMed: 924124]
- Kime DE. Synthesis of 1 α -hydroxycorticosterone. *J Chem Soc Perkins Trans.* 1972; 1:2371–2374.
- Manire CA, Rasmussen LEL, Maruska KP, Tricas TC. Sex, seasonal, and stress-related variations in elasmobranch corticosterone concentrations. *Comp Biochem Physiol A.* 2007; 148:926–935.
- Naples LM, Mylniczeko ND, Zachariah TT, Wilborn RE, Young FA. Evaluation of critical care blood analytes assessed with a point-of-care portable blood analyzer in wild and aquarium-housed elasmobranchs and the influence of phlebotomy site on results. *J Am Vet Med Assoc.* 2012; 241:117–125. [PubMed: 22720996]
- Nunez S, Trant JM. Regulation of interrenal gland steroidogenesis in the Atlantic stingray (*Dasyatis sabina*). *J Exp Zool.* 1999; 284:517–525. [PubMed: 10469989]
- Rasmussen LEL, Crow GL. Serum corticosterone concentrations in immature captive whitetip reef sharks, *Triaenodon obesus*. *J Exp Zool.* 1993; 267:283–287.
- Renshaw GMC, Kutek AK, Grant GD, Anoopkumar-Dukie S. Forecasting elasmobranch survival following exposure to severe stressors. *Comp Biochem Physiol A Mol Integr Physiol.* 2012; 162:101–112. [PubMed: 21851860]
- Romero LM. Physiological stress in ecology: lessons from biomedical research. *Trends Ecol Evol.* 2004; 19:249–255. [PubMed: 16701264]
- Romero LM, Dickens MJ, Cyr NE. The reactive scope model — A new model integrating homeostasis, allostasis, and stress. *Horm Behav.* 2009; 55:375–389. [PubMed: 19470371]
- Sapolsky RM, Romero LM, Munck AU. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. *Endocr Rev.* 2000; 21:55–89. [PubMed: 10696570]
- Skomal GB, Mandelman JW. The physiological response to anthropogenic stressors in marine elasmobranch fishes: A review with a focus on the secondary response. *Comp Biochem Physiol A Mol Integr Physiol.* 2012; 162:146–155. [PubMed: 22008842]
- Truscott B, Idler DR. Corticosteroids in plasma of elasmobranchs. *Comp Biochem Physiol A.* 1972; 42:41–50. [PubMed: 4402720]
- Watson R, Munro C, Edwards KL, Norton V, Brown JL, Walker SL. Development of a versatile enzyme immunoassay for non-invasive assessment of glucocorticoid metabolites in a diversity of taxonomic species. *Gen Comp Endocrinol.* 2013; 186:16–24. [PubMed: 23462197]
- Wiens J, Ho R, Brassinga AK, Deck CA, Walsh PJ, Ben RN, McClymont K, Charlton T, Evans AN, Anderson WG. Biosynthesis of 1 α -hydroxycorticosterone in the winter skate *Leucoraja ocellata*: evidence to suggest a novel steroidogenic route. *J Fish Biol.* 2017; 91:260–277. [PubMed: 28593636]
- Wosnick N, Bornatowski H, Ferraz C, Afonso A, Sousa Rangel B, Hazin FH, Freire CA. Talking to the dead: using Post-mortem data in the assessment of stress in tiger sharks (*Galeocerdo cuvier*) (Peron and Lesueur, 1822). *Fish Physiol Biochem.* 2017; 43:165–178. [PubMed: 27549099]
- Yanagishita M, Rodbard D. Computerized optimization of radioimmunoassays for hCG and estradiol: An experimental evaluation. *Anal Biochem.* 1978; 88:1–19. [PubMed: 696989]

Highlights

- A monoclonal antisera and EIA for 1 α -hydroxycorticosterone (1 α OHB) was developed
- High performance liquid chromatography was used to validate the 1 α OHB EIA
- 1 α OHB can now be rapidly measured in <1ml serum or 1g interrenal tissue
- The 1 α OHB EIA is an improvement from previous analytical methods
- This EIA may help elucidate the role for 1 α OHB in elasmobranch health & physiology

**Fig. 1.**

Synthesis of the 1 α -hydroxycorticosterone (1 α OHB) derivative (antigen) for antisera development. Starting from corticosterone; i) Acetic anhydride/triethylamine 12 h RT (room temperature); ii) Benzene selenenic acid anhydride/toluene 8 h reflux; iii) DMDO/DCM/acetone 24 h RT; iv) Ethylene glycol/trimethyl orthoformate/PTSA/DCM 2 h reflux; v) Pyridine/10% CuSO₄; vi) KOH/MeOH; vii) TIPSCl/pyridine; viii) K10 Clay/CHCl₃; ix) (PhSe)₂/NaBH₄/AcOH; x) TBAF/THF. The final product, 1 α -OH-11-oxo-20-(carbomethoxy) oxime corticosterone (1 α -OH-11-oxo-20CMO-B) was used in subsequent steps for immunogen preparation for antisera development.

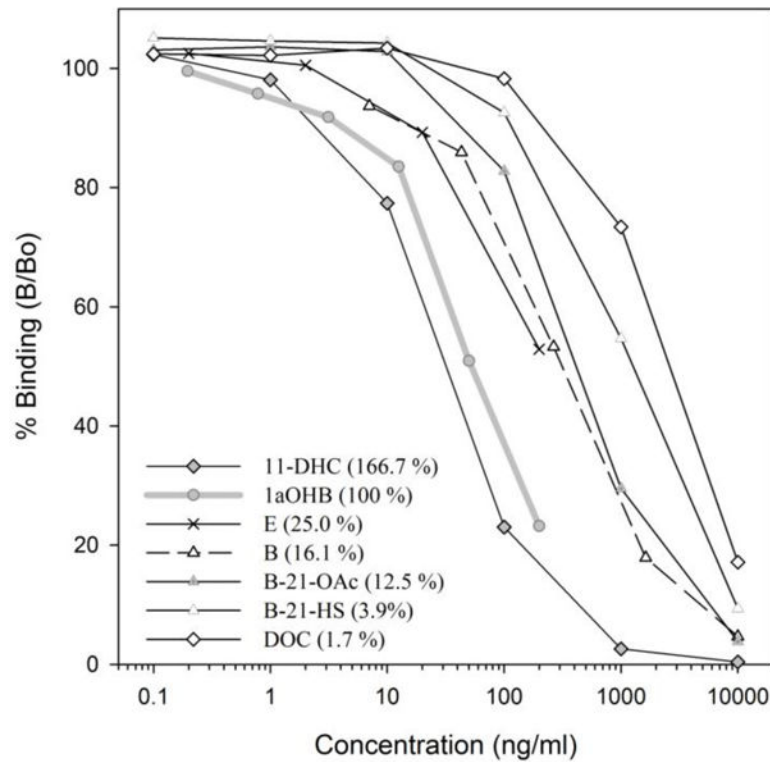


Fig. 2.

Antibody specificity of the developed 1 α OHB monoclonal antisera 16D7.F8 evaluating the crossreactivity (relative antigen concentration (ng/ml) at 50% binding (B/B₀) of the synthesized 1 α OHB standard, designated as 100 %) of structurally-related steroid compounds. Relative percent cross-reactivity (reported in brackets) ranked from highest to lowest were as follows; 11-dehydrocorticosterone (11-DHC), cortisone (E), corticosterone (B), corticosterone 21-acetate (B-21-OAc), corticosterone 21-hemisuccinate (B-21-HS), and desoxycorticosterone (DOC). Neither cortisol (F), 16-dehydropregnenolone acetate oxime, nor the 3-carbon-position HRP conjugates of cortisol (F), cortisone (E), or corticosterone (B) bound with the 1 α OHB monoclonal antisera 16D7.F8 to displace the 1 α OH-11oxo-20CMO-B:HRP conjugate in the 1 α OHB enzyme immunoassay (data not shown).

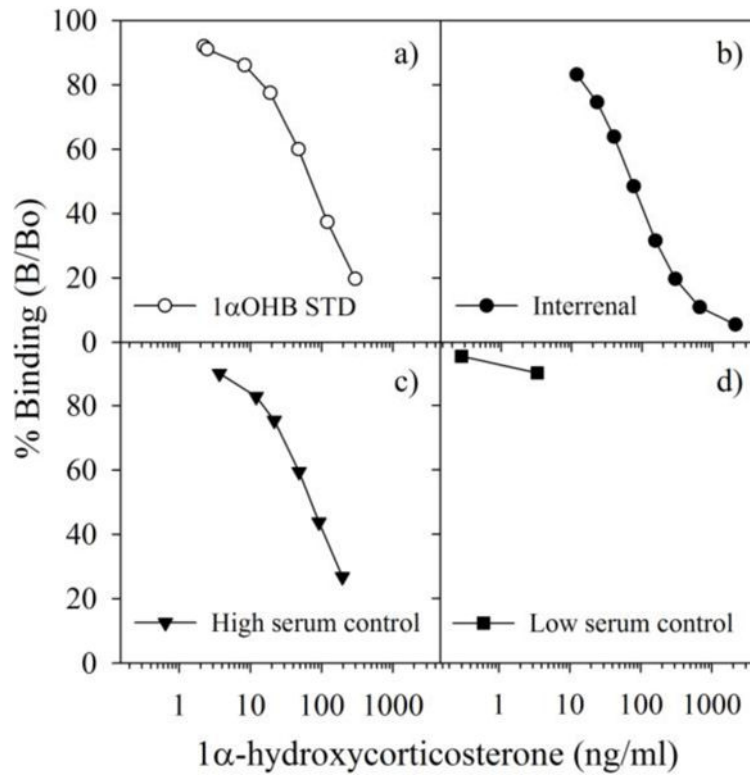


Fig. 3. Parallelism of serial dilutions of b) interrenal extract and c) a high serum control (reference sample) with a) synthesized 1 α -hydroxycorticosterone standards (1 α OHB; range 2.2 to 300 ng/ml). Correlations between percent binding for the interrenal and high serum control samples were significant ($r^2=0.99$) indicating parallelism of slopes. The 1 α OHB concentration in the low serum control (d) was just measurable at the lower limit of detection of the assay when the dried extract was reconstituted to 7 \times concentration in assay buffer.

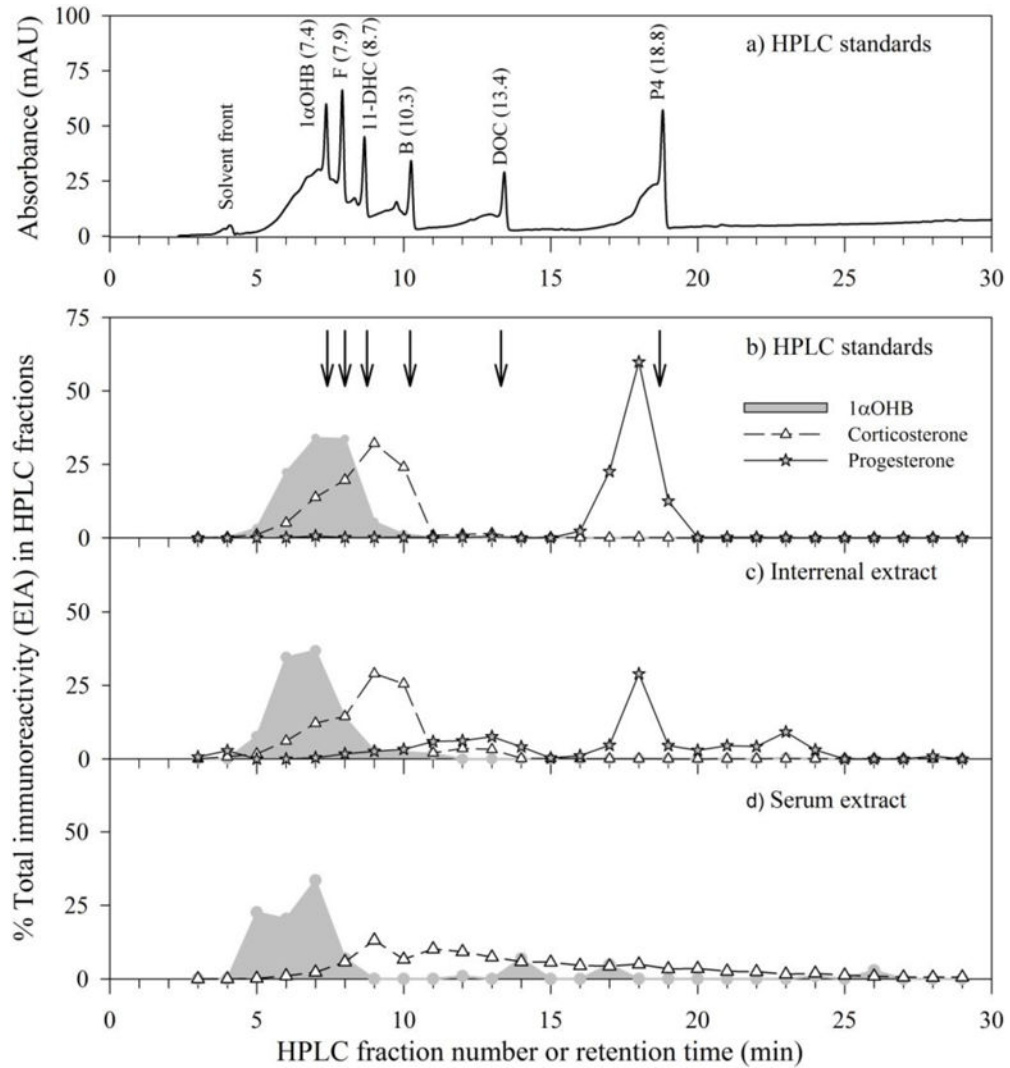


Fig. 4. High performance liquid chromatography (HPLC) absorbance profile chromatogram (mAu) of the steroid standards panel (a) in elution order from highest to lowest polarity. Retention times (brackets indicate peak RT in min) of a 100 μ l injection of the standards comprised of 1 α -hydroxycorticosterone (1 α OHB), cortisol (F), 11-dehydrocorticosterone (11-DHC); corticosterone (B), desoxycorticosterone (DOC), and progesterone (P4). Percent total immunoreactivity profiles from 1 α OHB (grey-shaded fill), B (open triangles) and P4 (grey stars) enzyme immunoassays (EIAs) of HPLC fractions of b) HPLC standard panel (from a), above; downward arrows indicate peak RT), b) dichloromethane- (DCM) extracted *H. americanus* interrenal tissue, and c) DCM-extracted *H. americanus* serum from the high serum control (reference sample; NB: P4 was not assessed in this sample).