Challenges, Pitfalls and Surprises: Development and Validation of a Monoclonal Antibody for Enzyme Immunoassay of the Steroid 1α-Hydroxycorticosterone In Elasmobranch Species

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Challenges, pitfalls and surprises: development and validation of a monoclonal antibody for enzyme immunoassay of the steroid 1α-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 1α-hydroxycorticosterone (1αOHB) relative to corticosterone (B). Although commercial EIAs are available for B, those tested exhibit only low relative cross-reactivity to 1αOHB (3-5%). To improve measurement of 1αOHB, we developed a monoclonal antibody using a synthesized 1αOHB-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 1αOHB, 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 1αOHB 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 1αOHB in <1 ml (serum) or 1g (interrenal) samples. HPLC analysis revealed the 1αOHB EIA to be superior for measurement of 1αOHB

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

1. Introduction

The corticosteroid 1α, 11β, 21-trihydroxypreg-4-ene-3, 20-dione (1α-hydroxycorticosterone, or 1αOHB) is unique to elasmobranchs and was first isolated in 1966 by Idler and Truscott. Since that time, 1αOHB has been found to be the dominant corticosteroid in many elasmobranch species, and evidence has accrued supporting a dual role for 1αOHB 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 1αOHB in elasmobranch health and physiology, efforts to quantify this unique corticosteroid have largely been unsuccessful.

Early methods for the quantification of 1αOHB 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 1αOHB to 1-dehydrocorticosterone and therefore lacked specificity. Synthesis of 1αOHB 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 1αOHB in elasmobranchs utilized assays and antibodies for corticosterone that were not specific to 1αOHB (e.g. Manire et al., 2007; Rasmussen and Crow, 1993).

In the early 2000s, new methods for the synthesis of purified 1αOHB were developed by the Rimoldi laboratory at the University of Mississippi. This synthetic 1αOHB 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 1αOHB from elasmobranch plasma (Evans et al., 2010). Recently, another successful synthesis of 1αOHB was developed at the University of Ottawa (Wiens et al. 2017) who used HPLC to elucidate the potential role of bacteria in the 1αOHB steroid metabolic pathway. While a combination of HPLC/EIA methods allows for quantification of 1αOHB, it too relies on an antibody specific to corticosterone with low cross-reactivity to 1αOHB. Therefore, we identified a significant need for the development of a specific, rapid and sensitive EIA for 1αOHB.
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-dehydroprogrenenolone acetate oxime (P1510-000); progesterone (P4; Q2600-000)).

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

Purity of synthesized 1α-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 1α-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 1α-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 1α-OH-11-oxo-20-(carbomethoxy) oxime corticosterone (1α.OH-11oxo-20CMO-B).

2.3. 1αOHB 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 1α.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 1α.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 1α-OHB-derivative (see Fig. 1.) prior to primary immunization with the target antigen (1α.OH-11oxo-20CMO-B:PTG). Post-immunization mouse sera and hybridoma supernatants were screened for polyclonal antibody binding of 1α.OH-11oxo-20CMO-B:HRP (1α.OHB HRP conjugate for EIA) using a 96-well plate.
coated with an antibody to capture mouse antibodies and the 1α.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 1α.OH-11oxo-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 1α.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 1α.OHB HRP conjugate. Antibody specificity for 1α.OHB was tested by evaluating the cross-reactivity (relative antigen concentration at 50% B/B0 of the 1α.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 1α.OHB (mouse monoclonal) EIAs. Briefly, plates were coated with 150 μl of 10 μg/ml IgG goat anti-rabbit IgG (GARG; #A009; B EIA); goat anti-mouse IgG (GAMG; #A008; P4 and 1α.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 μl 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 μl standards (B, P4, 1α.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 (1α.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 1α.OHB standards ranged from 61 to 15,000 pg/well.

2.4.1. Optimization of 1α.OHB EIA

2.4.1.1. Equilibrium experiments (competitive EIA): Antisera (1:1,000 to 1:4,000) and 1α.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 1α-OHB standard dose-response curves to maximize sensitivity. A third test reformatted the assay for a comparison of antisera coating volume (150 vs 300 μl 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 1α-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 1α-OHB standards were incubated with the antisera for 0 to 24 hours (0.5, 1, 2, 4, 8, 24 h) before addition of the 1α-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 1α-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 1α-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 ± 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 × 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 1αOHB 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 1αOHB 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 1αOHB 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 μl 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 μl of the sample was injected into a 250 × 4.6 mm Hypersil Gold C18 reverse phase HPLC column (Thermostcientific, Waltham, MA) with a 5 μm 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 μl) or large volume (100 μl) injections (to match the sample injection protocol) of a concentrated steroid panel consisting of 407 μMol (1αOHB) or 25 μMol 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 μl (fractioned samples) or 800 μl (fractioned standards) assay buffer. Percent of total immunoreactivity was quantified using 1αOHB, 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 (1αOHB standard displacement) using the 1αOHB HRP conjugate for the signal. Two IgG₂b clones yielded no binding signal and were discarded. Antibody specificity tests (percent cross-reactivity providing relative antigen concentration at 50% B/B₀ of the 1αOHB standard) completed on the three clones that bound 1αOHB revealed one clone (16D7.F8.) to be superior, with improved binding to 1αOHB (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 1αOHB EIA

3.2.1. Equilibrium experiments (competitive EIA)—A wide range of combinations of 1αOHB antisera (16D7.F8) and 1αOHB HRP conjugate provided sufficient signal to produce satisfactory 1αOHB standard curves, but at low titer and limited sensitivity (≈ 384 pg/well 1αOHB). 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 1αOHB 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 1αOHB 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 1αOHB 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 1αOHB 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× concentration through 1:2 dilution averaged 25.2 ng/ml 1αOHB, see Fig. 3.), whereas the low serum control only approached the lower limit of detection when reconstituted to a 7× 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 μl injection; see Fig. 4. a, b), was used to characterize and evaluate subsequent 100 μl-injections of interrenal (Fig. 4. c) and serum extracts (Fig. 4. d). These results indicated that the highest percent of total immunoreactivity in the 1αOHB EIA was found in a wide band including fractions (fr) 6 through fr8 (6-9 min) representing 1αOHB, 11-DHC, and in smaller amounts, B (but not F, which did not bind to the 1αOHB antisera). Immunoreactivity was observed in fr6 through fr10 (6-11 min) representing 1αOHB, 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 1αOHB EIA suggesting the potential presence of a 1αOHB-immunoreactive metabolite in circulation such as 1αOHB-glucuronide (Idler and Truscott, 1969) more polar (fr5) than native 1αOHB (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 1αOHB 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 1αOHB 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 1αOHB. Cross-reactivity testing revealed that the 1αOHB antisera recognizes the basic B structure and ketones at the C11 position, but not F, nor B molecules with different carbon 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 1αOHB steroid metabolism pathway (and thus potential cross-reactants) in elasmobranchs. Although the 1αOHB monoclonal was more sensitive to 11-DHC than to 1αOHB, 11-DHC is reported in concentrations 1000 times lower than 1αOHB 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 1αOHB HRP conjugate over the synthesized standard or native 1αOHB 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 1αOHB 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 1αOHB metabolic pathway.

In conclusion, development of a monoclonal antisera for EIA of 1 αOHB is an improvement over previous analytical methods used to measure 1αOHB. 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 1αOHB. 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 fractioned with HPLC if needed. Since 1αOHB is highly conserved, being found in a wide variety of elasmobranchs (Idler, 1972), we expect the 1αOHB 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 1αOHB 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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### Highlights

- A monoclonal antisera and EIA for 1α-hydroxycorticosterone (1α.OHB) was developed
- High performance liquid chromatography was used to validate the 1α.OHB EIA
- 1α.OHB can now be rapidly measured in <1ml serum or 1g interrenal tissue
- The 1α.OHB EIA is an improvement from previous analytical methods
- This EIA may help elucidate the role for 1α.OHB in elasmobranch health & physiology
Fig. 1.
Synthesis of the 1α-hydroxycorticosterone (1α.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% CuSO$_4$; vi) KOH/MeOH; vii) TIPS Cl/pyridine; viii) K10 Clay/CHCl$_3$; ix) (PhSe)$_2$/NaBH$_4$/AcOH; x) TBAF/THF. The final product, 1α.OH-11oxo-20-(carbomethoxy) oxime corticosterone (1α.OH-11oxo-20CMO-B) was used in subsequent steps for immunogen preparation for antisera development.
**Fig. 2.**
Antibody specificity of the developed 1α.OHB monoclonal antisera 16D7.F8 evaluating the crossreactivity (relative antigen concentration (ng/ml) at 50% binding (B/B0) of the synthesized 1α.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 1α.OHB monoclonal antisera 16D7.F8 to displace the 1α.OH-11oxo-20CMO-B:HRP conjugate in the 1α.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 1α-hydroxycorticosterone standards (1αOHB; 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 1α.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× concentration in assay buffer.

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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 μl injection of the standards comprised of 1α-hydroxycorticosterone (1αOHB), cortisol (F), 11-dehydrocorticosterone (11-DHC); corticosterone (B), desoxycorticosterone (DOC), and progesterone (P4). Percent total immunoreactivity profiles from 1αOHB (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).