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Enzyme Immunoassay for Plasma Cortisol in Fish

Need for Measuring Cortisol

Plasma cortisol levels in fish are known to rise in response to environmental stressors and during the parr–smolt transformation in salmonids. Measurement of this hormone is important in understanding the stress response of fish and may be helpful in determining optimal release times for migratory salmonids. Traditionally, plasma cortisol in fish was measured by radioimmunoassay (RIA), but increasing costs associated with licensing and the problem of disposal of radioactive material necessitated a search for alternatives. Based on published methods for measuring sex steriods, a competitive solid-phase microtiter enzyme immunoassay (EIA) for measuring plasma cortisol in fish was developed. Enzyme immunoassay has sensitivities, detectibilities, and specificities comparable to RIA. Reproducibility is high for EIA, and the assay requires less expensive equipment and reagents.

Method for Cortisol Enzyme Immunoassay

Corning Easy Wash microtiter plates were coated with Rabbit anti-cortisol antibody (cat. F3-314, lot 345-10-22-80, Endocrine Sciences Products, Calabasas Hills, California). This antibody is supplied lyopholized at a 1:20 dilution. Using a final dilution of 1:16,000 in coating buffer (0.05M carbonate-bicarbonate, pH 9.6, made fresh weekly and stored at 4° C), 150 µl of the antibody was added to each well. Plates were tightly sealed with Pressure Sensitive Film (Falcon 3073, Becton Dickinson, Oxnard, California) and incubated overnight at 4° C. Plates were then washed 5 times with a solution of 0.15 M NaCl containing 0.05% Tween 20 in a microplate washer (Molecular Devices, Menlo Park, California). Plates were dried by inverting them and snapping briskly against a towel.

Enzyme immunoassay buffer (0.1 M phosphate, 0.15 M NaCl, pH 7.0, with 0.1% BSA) was added to the plate at 150 µL/well with a multichannel pipettor. With a Hamilton Microdiluter (Reno, Nevada), 100 µL/well of cortisol-horseradish peroxidase conjugate (a gift from Coralee Munro, University of California, Davis, at 1:500,000 in EIA buffer) was added along with 2.5 µL/well of standard or sample. Standards were made in Ringers' solution and run in quintuplicate, and samples were run in duplicate. Plates were again sealed tightly and incubated overnight at 25° C.

In the morning plates were washed and dried as before. Color development was with 3,3',5,5'-tetramethylbenzidine containing 0.01% hydrogen peroxide (TMB, Kirkegaard & Perry, Gaithersburg, Maryland). The TMB was added at 200 µL/well with a multichannel repeat pipettor as quickly as possible to minimize across-the-plate differences. Each plate was incubated with shaking in a microplate reader (Molecular Devices, Menlo Park, California) until the desired optical density was reached, usually 5–7 min. Then 0.5 M HCl, 50 µL/well, was added to stop the color reaction, and an endpoint reading was taken at 450 nm. Analysis of standards and samples was done on a 4-parameter logistic curve fit (SOFTmax 2.01, Molecular Devices).

Validation of the Assay

Cortisol standards in Ringers' solution and charcoal-stripped plasma were indistinquishable. Subsequently, all standards were made in Ringers' solution. Serial dilutions of three different plasma samples gave displacement curves that were parallel to the standard curve (Fig. 1). Sensitivity, as defined by the dose-response curve, was measurable from 1 ng/mL to 400 ng/mL (Fig. 1). The lower detection limit was 0.30 ng/mL. There was a strong correlation of cortisol

values obtained by EIA and RIA (r = 0.986, n = 36; Fig. 2). Using a pooled plasma sample, the average intra-assay variation was 5.5% (n = 10) and the average inter-assay variation was 8.8% (n = 10).

Testosterone and estradiol showed negligible (less than 1%) cross-reactivity. Cortisone had 1.6, 7.7, and 4.2% cross-reactivity at 10, 100, and 400 ng/mL, respectively (Fig. 3). Cortisone is a breakdown product of cortisol with little biological activity that can interfere with the measurement of cortisol. Endocrine Sciences Products reports similar cross-reactivities for 11-deoxycortisol and corticosterone for this antibody; these two steriods are present in fish plasma at much lower concentrations than cortisol or cortisone. Heat denaturation and ethanol extraction of plasma samples gave mean calculated values of 98 and 87% (n = 9), respectively, compared to untreated plasma. Therefore, EIA allows direct measurement of plasma cortisol in Atlantic salmon (*Salmo salar*).

This cortisol EIA showed good sensitivity and detectability. Compared with RIA, EIA is less costly because of equipment needs, amount of reagents used, licensing and disposal. Although validated here for Atlantic salmon, it should be widely applicable to other species.

Two points of caution should be made regarding the interpretation of results from this EIA. First, because of the rapid response of cortisol to external stressors, special care should be taken in collecting samples. Second, because cortisol is a hormone involved in many physiological processes, knowledgeable interpretation of results should be exercised.

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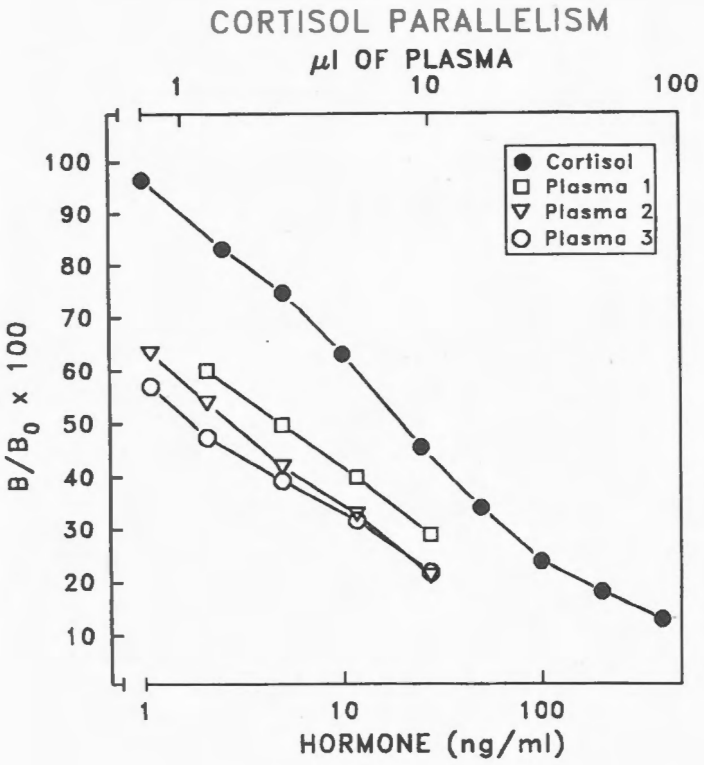

Fig. 1. Displacement curves from serial dilutions of three plasma samples.

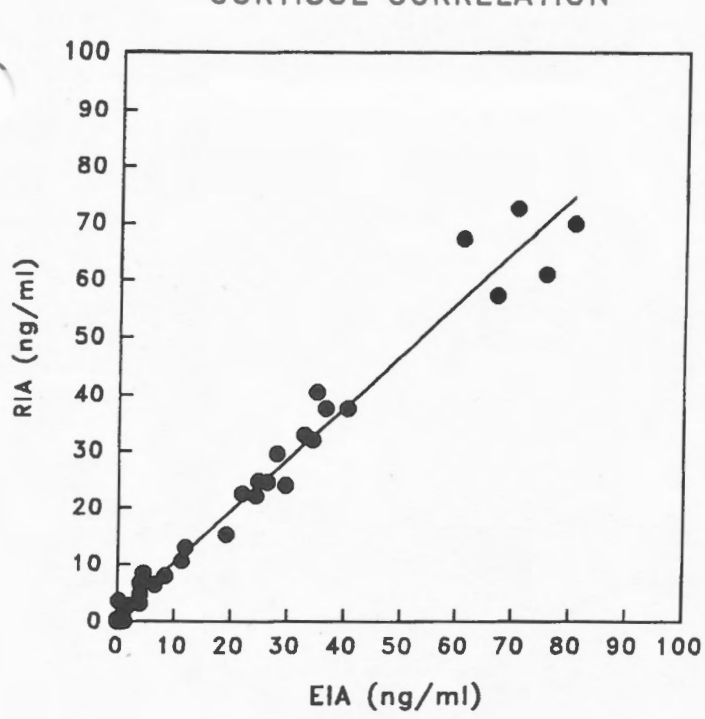

Fig. 2. Correlation of cortisol values from enzyme immunoassay and from radioimmunoassay.

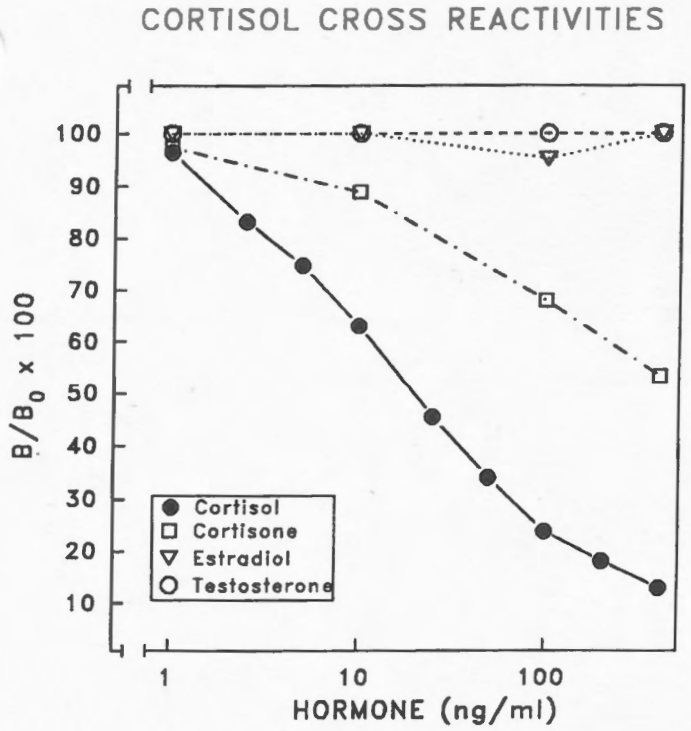

Fig. 3. Cross-reactivity of the steroids testosterone, estradial, and cortisone.