Development of a New Progesterone Metabolite EIA/ELISA for Non-Invasive Reproductive Monitoring

by Maheeka Embogama, Bobbi O'Hara and Russ Hart, Arbor Assays Inc., Ann Arbor, MI

Introduction

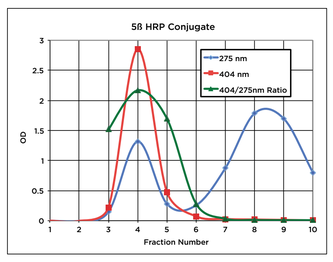
Free and conjugated steroid hormones (estrogens, androgens, and progestogens) can be detected in urine and feces of female animals across reproductive stages. As steroids are passed through the gut they can be transformed to more soluble metabolites, via reduction, hydroxylation and/or converted into water-soluble glucuronides. EIA/ELISA testing of these fecal steroids, specially progesterone metabolites is a convenient method to analyze large numbers of samples both in the laboratory and in field settings to assess reproductive status. However fecal metabolites vary considerably from species to species and it has been suggested that there should be development of specific antibodies and assays for each species. It is obviously a long, expensive and complex task to develop an assay with the correct reactivities to every species fecal metabolite.

In this study we have developed an EIA/ELISA specifically for progesterone metabolites. We conjugated two 3-hydroxyprogesterone derivatives, 5α- and 5β-pregnan-3β (3α)-ol-20-one hemisuccinates, to keyhole limpet hemocyanin (KLH) (for immunization), bovine serum albumin (BSA) for evaluating titers and horseradish peroxidase (HRP) for conjugate use. We discuss the development, evaluation and testing of these antibodies and the ensuing progesterone metabolite EIA/ELISA kit.

Methods

Antibody Generation

5α-pregnan-3β-ol-20-one hemisuccinate and 5β-pregnan-3α-pregnan-3β-ol-20-one hemisuccinate in anhydrous DMF were activated using N-hydroxysuccinimde and dicyclocarbodiimide in DMF overnight at 4°C desiccated. The activated steroids were added in excess to keyhole limpet hemocyanin (KLH) for immunization, BSA for evaluating titers, and HRP for conjugate use, all dissolved in borate buffer, pH 8.5 containing DMF. After 1 hour stirring at room temperature the KLH and BSA conjugates were dialyzed overnight at 4°C against PBS.



The HRP conjugate was purified on an Amersham PD10 column using PBS as the elution buffer. Ten 1mL fractions were collected. Analysis using the 404/275nm optical density ratio was used to identify conjugate fractions. Antibodies were raised to the KLH and BSA conjugates at a facility in Pennsylvania. Three rabbits each were immunized with the KLH 5α- and 5β- conjugates. Antibody titers were assessed by both using BSA-conjugated 5α- and 5β- steroid conjugates and in titer assays using the 5α- and 5β-HRP conjugates. Any positive rabbit bleeds were tested for steroid displacement using Arbor Assays (AAI) Goat anti-rabbit IgG plates, X016-1EA, with AAI Assay Buffer (AB10), X065, Wash Buffer, X007, TMB Substrate, X019, Stop Solution (1M HCl), X020, and the HRP conjugate diluted into AAI Conjugate Diluent, X076.

We choose antibody AAL15, an antibody raised to 5β-pregnan-3α-pregnan-3β-ol-20-one, because of sensitivity along with reactivity to structurally related steroids. AAL15 also showed zero or low reactivity to stress steroids such as cortisol, androgens such as testosterone, estrogens such as estradiol and E1G (data not shown). Rabbit AAL15 is still alive and giving us production bleeds of serum. She was initially immunized in the summer of 2017. She gets boosted and bleed on a regular schedule. AAL15 serum also has quite high titer, approximately 1:40,000 dilution, which ensures long lasting supply.

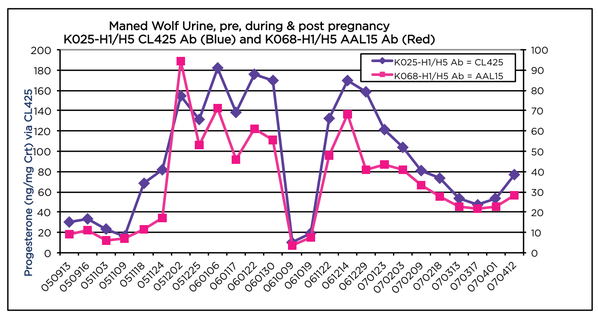
Progesterone Metabolite Assay

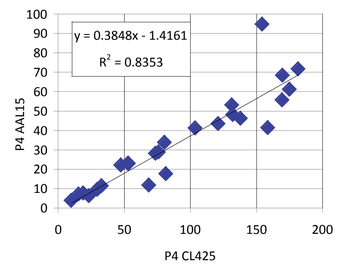
The assay follows our standard template. 50 µL of samples or standards diluted in AB10 are added to the secondary antibody coated plate, a goat anti-rabbit IgG clear plate, X016. 25 µL of conjugate diluted in conjugate diluent, X076 is added and the assay initiated by addition of the rabbit polyclonal antibody. The plate is shaken at RT for 2 hours, followed by washing and addition of 100 µL of TMB substrate, X019. Color generation is carried out at room temperature for 30 minutes when the reaction is terminated by addition of 50 µL stop solution. Generated signal is read at 450 nm. Data was analyzed using a Molecular Devices SoftMax 4PLC template.

Factors tested during research and development and validation were: Alcohol Interference, Cross reactivity; Linearity; Precision, and Sample performance.

Sample Data

Urine samples from a Maned Wolf, (*Chrysocyon brachyurus*) (kind gift of Dr. Rachel Santymire, Lincoln Park Zoo, Chicago, IL) were diluted in the kits assay buffer, AB10, and run in our monoclonal CL425 based assay kit, K025-H1/H5 and the progesterone metabolite kit, K068-H1/H5. Creatinine levels were determined on the urine samples using Arbor Assays Urinary Creatinine kit, K002-H1/H5. The time course of pregnancy and the correlation data are shown below.

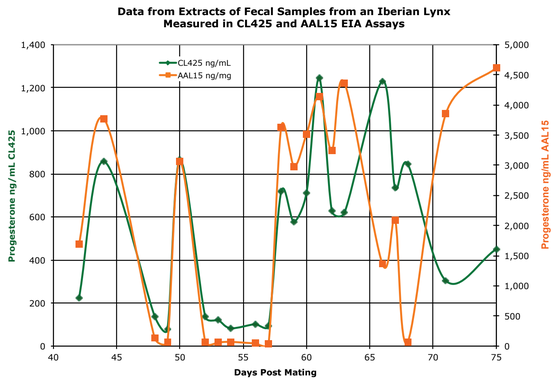




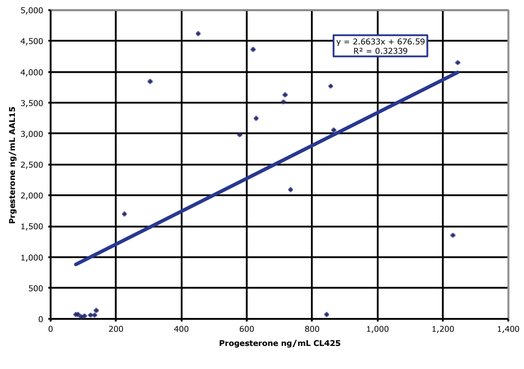
Note the high level of correlation between the measurements with the sample from the Maned Wolf. The measured concentrations are lower with the progesterone metabolite kit, K068-H1/H5, generating about 38.5% of the concentration as our CL425-based assay.

Fecal samples from a pregnant Iberian Lynx, (*Lynx pardinus*) (kind gift of Prof. Martin Dehnhard, Leibniz Institute for Zoo and Wildlife Research, Berlin, Germany) were extracted, concentrated and diluted in assay buffer and run in our monoclonal CL425 based assay kit, K025-H1/H5 and the progesterone metabolite kit, K068-H1/H5. The time course of pregnancy and the correlation data for the Iberian Lynx are shown below.

Time Course from Iberian Lynx Fecal Extracts in K025-H1/H5 and K068-H1/H5

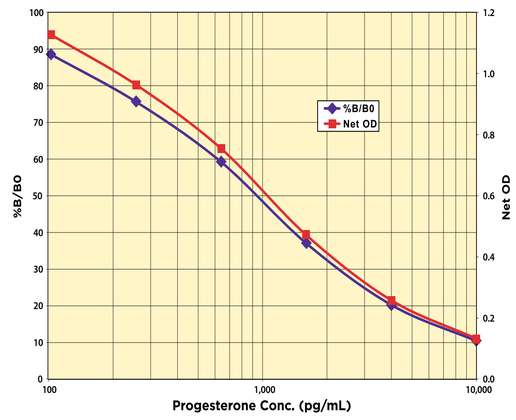


Correlation of Progesterone Measured in CL425 and AAL15 assays on dilutions of fecal extracts from an Iberian Lynx



K068-H1/H5 Assay Validation

K068-H1/H5 Typical Standard Curve



Cross Reactivity, AAL15-CL425 Comparison

Reactant	K068-H1/H5 AAL15 Progesterone Metabolite	K025-H1/H5 CL425 Monoclonal Progesterone
Progesterone	100	100
3β-hydroprogesterone	-	172
3α-hydroprogesterone	-	188
5β-dihydroprogesterone	61.9	-
5α-dihydroprogesterone	56.7	7
Pregnanolone (5β-Pregnan-3α-ol-20-one)	41.2	0.12*
Epiallopregnanolone (5α-pregnan-3β-ol-20-one)	38.3	-
Allopregnanolone	27.3	-
Pregnenolone	17.6	5.9
Epipregnanolone	10.2	-
17α-hydroxyprogesterone	5.7	0.38
11α-hydroxyprogesterone	4.9	147
11β-hydroxyprogesterone	-	2.7
20α-hydroxyprogesterone	0.34	-
Allopregnandiol	0.29	-
Testosterone	0.18	-
Cortisol	<0.1	<0.04
17β-Estradiol	<0.1	-
Corticosterone	<0.1	<0.1
Androstenedione	<0.1	<0.1

** run by Coralie Munro*

Precision

Intra Assay Precision: Three human samples were diluted with Assay Buffer and run in replicates of 20 in an assay. The mean and precision of the calculated Progesterone concentrations were:

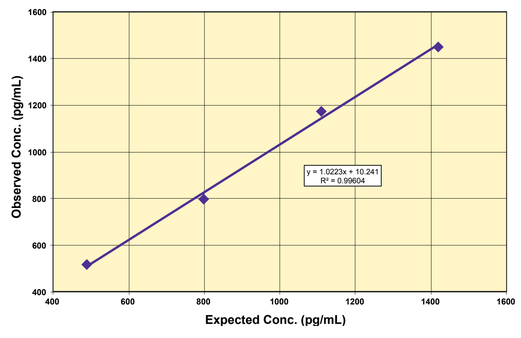
Sample	Progesterone Conc. (pg/mL)	%CV
1	2,377	4.9
2	1,517	7.2
3	710.3	8.0

Inter Assay Precision: Three human samples were diluted with Assay Buffer and run in duplicates in nineteen assays run over multiple days by six operators. The mean and precision of the calculated Progesterone concentrations were:

Sample	Progesterone Conc. (pg/mL)	%CV
1	2,442	6.8
2	1,665	6.6
3	844.6	8.8

Linearity: Linearity was determined by taking two urine samples diluted with Assay Buffer, one with a low diluted progesterone level of 179.2 pg/mL and one with a higher diluted level of 1,729 pg/mL, and mixing them in the ratios given below. The measured concentrations were compared to the expected values based on the ratios used.

Low Urine	High Urine	Expected Conc. (pg/mL)	Observed Conc. (pg/mL)	% Recovery
80%	20%	489.1	519.9	106.3
60%	40%	799.1	797.5	99.8
40%	60%	1,109	1174	105.9
20%	80%	1,419	1451	102.2
			Mean Recovery	**103.6%**



Alcohol Interference: The assay will tolerate up to 5% ethanol in sample volume before any significant reduction in binding is observed.

Summary

We have developed an EIA/ELISA based upon antibodies raised to 5β-Pregnan-3α-ol-20-one. The assay utilizes a polyclonal antibody from a rabbit that we are maintaining and obtaining production quantities of sera from on a scheduled basis. We currently have on hand in Ann Arbor enough polyclonal antibody for over 500,000 plates, so any supply issues will not be a problem for many years.

The assay shows good sensitivity for measuring progesterone and its metabolites in at least 2 widely used sample matrices, urine and fecal extracts. Interestingly for urine samples from a pregnant wolf the commonly used monoclonal CL425 based assay and this new antibody show very similar profiles. Fecal extracts from a felid showed a biphasic pattern about 2 weeks prior to birth. The two assay systems gave good correlation with time from mating when the first 62 days were evaluated with a slope of 4.28 higher concentration with the new antibody than CL425 and a correlation coefficient of 0.894. If the last 13 days of data is included the correlation coefficient drops to 0.323 as the CL425 measured signal drops and the new antibody shows its highest concentration just prior to parturition.