THESIS

DEVELOPMENT OF A DIRECT (NON-EXTRACTED) ENZYME IMMUNOASSAY FOR MEASUREMENT OF SERUM PROGESTERONE LEVELS IN MARES

Submitted by

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WE HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER OUR SUPERVISION BY RYAN MICHAEL BROOKS ENTITLED DEVELOPMENT OF A DIRECT (NON-EXTRACTED) ENZYME IMMUNOASSAY FOR MEASUREMENT OF SERUM PROGESTERONE LEVELS IN MARES BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE.

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ABSTRACT OF THESIS

DEVELOPMENT OF A DIRECT (NON-EXTRACTED) ENZYME IMMUNOASSAY FOR MEASUREMENT OF SERUM PROGESTERONE LEVELS IN MARES

Progesterone (P4) is a steroid hormone produced by the corpus luteum of the ovary and the placenta of the mare. Progesterone is required for the maintenance of pregnancy and an assessment of endogenous concentration would be useful in many diagnostic applications related to equine breeding management. The overall objective of this study was to develop and validate a direct enzyme-linked immunosorbent assay (ELISA) for the measurement of P4 in serum of in the mare. The specific aims were as follows: 1) to develop a quantitative and sensitive progesterone assay that could be used for non-extracted equine serum or plasma, and 2) to convert the ELISA from a 96-well plate format to a single cuvette system to allow quantification by a commercially available spectrophotometer. Significant events in the successful development of the ELISA included the use of purified anti-progesterone antibody, heterologous combination of antibody and conjugate, use of TMB substrate, and methodology to avoid organic solvent extraction. It was determined that by lowering the volume of serum used in the assay and lowering the pH of the serum, the need for extraction could be avoided. The overall correlation between ELISA of non-extracted serum and radioimmunoassay (RIA) of extracted serum was high (r = 0.81); and the correlation between ELISA and RIA for

progesterone concentrations less than 5.0 ng/ml, the range most important for clinical diagnosis, was even greater (r = 0.91). The direct ELISA assay has great potential for use in the equine breeding industry as it will allow for diagnostic tests to determine the adequacy of corpus luteum function in a pregnant mare, presence or absence of luteal tissue, and assessment of the end of seasonal transition.

Key words: Progesterone, mare, ELISA, microplate

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CHAPTER 1

REVIEW OF LITERATURE

Overview of Progesterone

Progesterone is a C_{21} steroid hormone derived from a precursor, cholesterol. Cholesterol can be derived from acetate in the liver or by de novo synthesis in the smooth endoplasmic reticulum in steroidogenic tissues. Free cholesterol is transported from the outer mitochondrial membrane to the inner mitochondrial membrane via steroid acute regulatory protein (StAR). Once inside the mitochondria, cholesterol is acted on by cytochrome P450 side-chain cleavage enzyme, yielding pregnenolone, the first step in all steroid biosynthesis (16). Pregnenolone is then transformed to progesterone by the enzyme 3β-hydroxy-Δ^5-steroid dehydrogenase (3β-HSD)(35). The chemical structure and pathway for biosynthesis of progesterone are shown in Figures 1.1 and 1.2, respectively.

Progesterone is secreted by the corpus luteum (CL) of the ovaries, placenta, and potentially the adrenal glands. Production of progesterone by the CL is supported primarily by luteinizing hormone (LH) secreted from the anterior pituitary gland. The main source of progesterone is the corpus luteum. This primary source of progesterone changes during pregnancy in the mare as the fetal-placental unit begins producing progestins around days 50 to 70 (48). Serum concentrations of progesterone are high,

(> 10 ng/ml), for about the first 150 days of gestation due to production by both primary and secondary corpora lutea (13). Progesterone is nearly non-detectable in maternal blood after 200 days of gestation although it is present in the placenta and fetal circulation (52). Pregnancy is maintained in late gestation by various progestins, including 5α-DHP, 3β-5P, ββ-diol, 20α-5P and βα-diol (13,24).

Progesterone has multiple functions in the mare. Production of this hormone is required for the maintenance of pregnancy. Progesterone produced from the corpus luteum that formed after ovulation is responsible for uterine secretions and physical embryo-uterine interactions such as embryo mobility, fixation, and orientation (48). In addition, both estrogen and progesterone are key components to positive- and negative-feedback loops that control the release of prolactin, FSH, and LH from the anterior pituitary as well as various other reproductive hormones (35). The LH pulse-generating system is negatively impacted by progesterone in the mare.

Progesterone is metabolized very rapidly in the mare, primarily to 5α-pregnane derivatives. Progesterone is almost completely metabolized in one passage through the GI mucosa and liver (3). The metabolites are primarily excreted in the urine (20). Metabolic clearance rates of progesterone were not significantly different in anestrous, diestrous, ovariectomized, pregnant, or lactating mares (15).

Only about 1-3% of progesterone is free (ie. unbound) in the blood of mares. A majority of progesterone is bound to cortisol binding globulin (CBG) and albumin carrier proteins (5). The proportion of progesterone bound to CBG and albumin is somewhat controversial and may be species specific. In humans, it is reported that 80% of progesterone is bound to albumin and 18% to transcortin (CBG), while the remaining 2%

is free (51). Once steroid hormones enter into the bloodstream via endogenous secretion or exogenous administration, they immediately interact with several plasma steroid-binding proteins. Albumin is a high-abundance protein with low steroid-binding affinity and specificity whereas CBG is a low abundance protein with high steroid-binding affinity and specificity. Plasma concentrations of CBG are much lower than that of albumin but CBG plays an important role in binding and transporting biologically active steroids in the blood. Cortisol binding globulin has a single steroid binding site with which it can bind either progesterone or cortisol with high affinity (17). Understanding the relationship between CBG and progesterone is of extreme importance when developing an ELISA as it is necessary to remove these carrier proteins or dissociate progesterone from the carrier protein to accurately measure total progesterone concentrations. This can be accomplished in several ways including organic solvent extraction, displacing progesterone by protein binding agents such as 8-anilino-1-naphthalene sulphonic acid (ANS), or salicylate, by proteolytic enzymes, low pH, or heat treatment.

Sources of Progesterone

Progesterone is the primary hormone produced by the corpus luteum. The ovary contains follicles and/or a corpus luteum at various stages of the estrous cycle. The follicle is composed of both granulosa and thecal cells which proliferate and hypertrophy following ovulation to form the corpus luteum. After ovulation, granulosa cells become luteinized and conversion of progesterone to 17α-hydroxyprogesterone is reduced resulting in progesterone becoming the primary secretory product. Granulosa and theca

cells are needed for normal steroidogenesis (35). Luteal cells have abundant 3β-HSD activity which is required for the conversion of pregnenolone to progesterone. The placenta is also a source of progesterone but is discussed in further detail in sources of progesterone during pregnancy.

Estrous Cycle

The estrous cycle of the mare is defined as the period from one ovulation to the subsequent ovulation. The estrous cycle averages 21 days in length. The period of sexual receptivity is denoted as estrus and physiologically coincides primarily with the follicular phase. Diestrus is the period in which a mare rejects the sexual advances of the stallion and coincides physiologically with the luteal phase (35).

Behavioral estrus lasts about 4-7 days in the mare and is stimulated by rising levels of estradiol-17β produced by a developing dominant follicle and the absence of progesterone. Estrus is a period when the mare is sexually receptive to the stallion and the genital tract is prepared to accept and transport spermatozoa to the oviducts for fertilization (4,35). Ovulation, or release of the mature oocyte, occurs approximately 24 to 48 hours before the end of estrus. Note that some mares will not show typical behavioral signs associated with estrus (ie. silent heat) though they have elevated estradiol, low progesterone and develop a dominant follicle that will eventually ovulate.

Diestrous behavior in the mare lasts approximately 14 to 15 days and is controlled by the hormone progesterone produced by the corpus luteum. The mare is not receptive to the stallion during diestrus and the uterus is prepared to accept and nurture the

conceptus. Diestrus ends with the regression of the corpus luteum, or luteolysis, and the start of the next follicular phase (4,35).

Progesterone concentrations vary depending on the day of cycle. During estrus, serum progesterone concentrations are at baseline levels, which are less than 1 ng/ml (48). Progesterone concentrations begin to rise 12 to 24 hours after ovulation and reach peak levels within 6 days after ovulation (15,35). Concentrations of progesterone average 6-10 ng/ml between days 5 and 14 after ovulation (1) then decline dramatically on day 14 or 15 when luteolysis occurs secondary to prostaglandin $F_{2\alpha}$ release from the endometrium. Figure 1.3 illustrates the typical profiles of serum progesterone and $PGF_{2\alpha}$ throughout the equine estrous cycle. Progesterone is responsible for inhibiting estrous behavior, closure of the cervix, and preparing the uterus for subsequent support of a pregnancy. Progesterone dominates over the effects of estrogen and inhibits secretion of LH, but not of FSH, and therefore does not inhibit follicular development. This is characterized by the fact that during the luteal phase when progesterone concentrations are high, follicles continue to develop and sometimes even ovulate (35).

Pregnancy

Progesterone is essential for the maintenance of pregnancy in the mare. Among other roles, progesterone induces the endometrium to conform to invasion of microcotyledons and gaseous exchange demands of the placenta, to stimulate endometrial glands to secrete "uterine milk" for the nourishment of the embryo, and to maintain quiescence of the myometrium (2,52). The mechanism used to ensure high blood

concentrations of progesterone in the pregnant mare is different than other domestic species.

There are three sources of progestins throughout pregnancy in the mare. The initial corpus luteum that formed after the ovulation that resulted in the conceptus is solely responsible for the production of progesterone until approximately day 40. The progesterone secreted from the primary corpus luteum increases until about day 8 post ovulation at which point levels slowly begin to decrease until day 28 to 30. It is thought that the 1°CL may undergo partial regression though it does not completely regress until much later in gestation, contrary to initial belief. The 1°CL regresses along with the 2°CL around 180 to 220 days of gestation (34,53). It has been suggested that CL regression occurs as a result of the loss of the luteotropin equine chorionic gonadotropin (eCG). This is most likely not the case as eCG is nearly nondetectable at 120 days of gestation, long before the regression of primary and secondary corpora lutea (35).

Secondary corpora lutea form around day 40 of gestation and are a supplementary source of progesterone in the pregnant mare (48). They result in a dramatic increase in concentrations of serum progesterone. The formation of 2°CL coincides with the development of endometrial cups and subsequent secretion of equine chorionic gonadotropin (eCG), which has a luteotropic role. The number of 2°CL increase until approximately 140 days; however, the quantity of 2°CL varies greatly from mare to mare (35). There are two types of 2°CL, those arising from ovulation and those from luteinization without ovulation. Some refer to corpora lutea resulting from ovulation as secondary and those resulting from luteinization of anovulatory follicles as accessory (48). It is still unknown as to the proportion that results from ovulations compared to

anovulatory luteinization although formation of 2°CL from ovulation is much more likely to occur between days 40 to 70 than after day 70 (35). A majority of 2°CL are nearly identical in physical characteristics when compared to the 1°CL (53). All 2°CL regress around 180 to 220 days (53).

Ovariectomy of pregnant mares prior to day 50 resulted in pregnancy loss in all mares. Ovariectomy of mares between 50 to 70 days of gestation resulted in 45% of mares losing their pregnancy. None of the mares ovariectomized on day 140 or 210 were affected in terms of pregnancy status (25,35). A study performed by Hinrichs et al. (1987) used ovariectomized mares for embryo transfer and placed mares on progesterone treatment through 100 days of gestation. All ovariectomized mares maintained pregnancy after progesterone treatment was discontinued at 100 days (22). Therefore, it appears that the placenta of the mare produces progesterone and various metabolites and is the main source of progesterone starting in mid-gestation though it begins to produce measurable levels around days 50 to 70 (24,48,50). The placenta becomes the main source of progesterone production between days 70 and 100 (2,48). Additionally, administration of prostaglandin $F_{2\alpha}$ to pregnant mares removed luteal progesterone and caused loss of pregnancy (27). Furthermore, ovariectomized mares treated with altrenogest, a synthetic progestin, resulted in maintenance of pregnancy (21). As shown with ovariectomized mares, pregnancy continued in 67% of mares ovariectomized on day 70, and at 140 and 210 days of gestation, the ovaries were no longer needed to support pregnancy as the all mares maintained pregnancy (25). The placenta, which is the source of progestins at this point, is in direct and intimate contact with the principal target tissue, the endometrium (2). There is an absence of 3β-HSD in the fetal gonads indicating that

progesterone was not being secreted by the fetus as this enzyme is necessary for the conversion of pregnenolone to progesterone (15). However, in the last month of gestation, the enlarging fetal adrenal gland secretes significant amounts of pregnenolone which is utilized by the placenta to synthesize progestagens and explains the steep rise in progestagen concentrations in the 4 to 6 weeks prior to parturition, after which they fall sharply (2).

Progesterone concentrations peak at 10-20 ng/ml from 60 to 90 days of gestation and stay relatively constant until days 120 to 150. Progesterone concentrations reached the lowest point between 240 to 300 days (35). Figure 1.4 shows the concentrations of progesterone during pregnancy in mares. Several other progestagens are very high in concentration and active throughout pregnancy in the mare (24). Progesterone and its metabolites are rapidly dispersed in both maternal and fetal circulation (36). Progesterone gradually increased during the last 30 days of gestation and peaked 5 days prior to parturition.

Causes of low progesterone

Low progesterone is of great concern in the pregnant mare. There are two main classifications that exist: 1) the absence of adequate endogenous progesterone secretion due to factors that affect the function of the CL, and 2) the inability of "normal" circulating progesterone levels to maintain pregnancy. Examples of the first include accidental $PGF_{2\alpha}$ administration, luteolysis resulting from endotoxemia, and failure of maternal recognition of pregnancy. Examples of the second include mares that consistently lose their pregnancy yet have normal serum progesterone levels, placentitis,

and impending abortion due to stress in late gestation. Low luteal progesterone production may arise from a variety of mechanisms including primary corpus luteum insufficiency, luteolysis due to uterine inflammation (endometritis) and subsequent release of $PGF_{2\alpha}$, failure of mechanisms responsible for maternal recognition of pregnancy followed by luteolysis, luteolysis due to systemic endotoxemia, and stress. Luteal insufficiency or inadequate production of progesterone by the corpus luteum has been proposed as a cause of early pregnancy loss; however, there is very limited evidence to support primary luteal insufficiency as the true cause (2,9). In a study conducted by Irvine et al. (1990), it was found that out of 17 mares that exhibited early pregnancy loss, only one was associated with low progesterone levels in maternal circulation (26).

Importance of measuring progesterone

Measuring progesterone in the normal cycle is of interest to determine the phase of the estrous cycle (ie. estrus versus diestrus). This can be extremely beneficial if one is unable to ultrasound a mare or if a mare has "silent heats." Plasma progesterone levels increase significantly within the first 12 hours of ovulation and are > 2.0 ng/ml 48 hours after ovulation (44). Evaluation of serial serum samples can be used to determine the day of ovulation.

There are several pathologic reproductive conditions that arise in the mare. These include persistent anovulatory follicles, luteinized anovulatory follicles, questionable ovulations, granulosa cell tumors and many more. In a study performed at Colorado State Univeristy on mares with ovulation failure, serum progesterone concentrations were below 1 ng/ml in 14.3% of mares. These mares had persistent anovulatory follicles

(PAF), which were characterized by the prolonged presence of a follicle with an absence of echogenic particles in the follicular lumen. In contrast, 85% of anovulatory mares had elevated progesterone levels. These mares had luteinized anovulatory follicles (LAF) characterized by a progressive increase in echogenicity of the follicular lumen. Being able to assess the progesterone concentration would allow one to determine if the structure was a PAF or LAF and subsequently determine whether it would respond to prostaglandins. Administration of prostaglandins to LAF's resulted in a rapid decline of plasma progesterone levels to < 1.0 ng/ml within 48 hours (31). The ability to assess the concentration of progesterone in a mare would inherently allow one to determine luteal function which would also answer the questionable ovulation as well as other situation such as whether a mare has a mature CL that will respond to prostaglandins and allow her to be short-cycled (40). When diagnosing a granulosa cell tumor in a mare an endocrine panel of inhibin, testosterone and progesterone is most commonly performed. Progesterone is a helpful indicator as concentrations are almost always low (< 1 ng/ml), consistent with the absence of luteal tissue. If progesterone is greater than 1 ng/ml this is suggestive that a mare does not have a GCT as the tumor does not produce progesterone and affected mares do not develop follicles and ovulate (30).

Elevated levels of progesterone during gestation are important as progesterone is required for the maintenance of pregnancy. Mares that had pregnancy loss between days 11 and 15 showed significantly lower progesterone concentrations than mares that were able to maintain pregnancy. A drop in progesterone was a rare cause of pregnancy loss between days 17 to 42 (26). Whether a mare has low progesterone due to original formation of a small CL or has a CL that seems to be regressing due to failure of maternal

recognition of pregnancy or for other reasons, the resulting inadequate levels of progesterone will most likely cause a loss of pregnancy. Assessment of the concentration of progesterone would allow one to determine if levels were sufficient to maintain pregnancy. If levels are considered to be low, exogenous progesterone supplementation may be required. The minimal level of progesterone considered to be adequate to maintain pregnancy in the first trimester is 4 ng/ml (22,47,49). If a mare has endogenous progesterone levels below 4 ng/ml, supplementation is suggested and if levels are above 4 ng/ml the mare theoretically has adequate progesterone to maintain pregnancy.

Clinical applications for a progesterone test include assessment of progesterone levels during pregnancy, determination of the presence or absence of a corpora luteum in mares that are acyclic or during the spring transition period, confirmation of ovulation (23), confirmation of progesterone levels in mares that do not display behavioral signs of estrus ("silent heat"), and determination of progesterone patterns in infertile mares (11). Clinical applications will be discussed further in Chapter 3.

Every year, thousands of mares throughout the world receive exogenous progestagen therapy during part or all of their pregnancy as insurance against embryonic or fetal death and abortion due to insufficient endogenous progesterone (2). Pregnant mares that are ovariectomized during the first three months of pregnancy or mares that have received a luteolytic dose of prostaglandin $F_{2\alpha}$ remained pregnant if supplemented with progesterone (9). Several of the progestagens commonly administered to horses in an attempt to prevent abortion were proven to be unable to maintain pregnancy during 18 to 30 days of gestation in the absence of endogenous progesterone secretion by the CL (33). Altrenogest is a synthetic progestagen and is the only compound confirmed to have

bioactivity in the mare. This progestin is capable of maintaining pregnancy in the mare when endogenous progesterone levels are inadequate. The two main options for progesterone supplementation of pregnant mares include natural progesterone and altrenogest. Natural progesterone is available in a short- and long-acting formulations for intramuscular injection. Altrenogest, commercially available as ReguMate® [Intervet/Schering-Plough Animal Health, Millsboro, DE] is available in an oral formulation that is administered daily or as an injectable compounded formulation. The compounded formulation consists of 500 mg altrenogest suspended in microparticles and lasts for 30 days [BET Pharmacy, Lexington, KY] (9,54). The latter regimen has not been proven effective for the maintenance of pregnancy in mares, only the suppression of estrus.

Testing for Progesterone

Radioimmunoassay (RIA) has been the most widely used method to assess the concentration of progesterone in serum. The technique was first developed by Dr. Rosalyn Yalow in 1960 who was awarded a Nobel Prize for her work. Radioimmunoassay is used to quantify small amounts of substance present in biological fluids or tissues. Quantification is achieved by measuring the ability of the ligand (ie. progesterone) to compete with a radioactive form of the ligand for binding sites on a specific antibody. (Figure 1.5). Radioimmunoassay development requires three important components: an antibody specific for the ligand, a radioactive form of the ligand and a method for separating the ligand bound to antibody from that remaining free

in solution (38). Currently, the most common radioisotope used to label progesterone is ^{125}iodine.

There are several cumbersome steps when performing a radioimmunoassay. The first is iodination. This is the process of attaching the radioactive iodine to the ligand (ie. progesterone). As with the entire assay, radioactive materials are being handled and the appropriate precautions must be taken to ensure the safety of all individuals in the laboratory. Extraction is another tedious step involving the use of petroleum ether to extract progesterone from serum. The test tube containing the serum/petroleum ether mixture is immersed in a dry ice/methanol bath. The serum layer is frozen in the dry ice/methanol bath and the petroleum ether, which now contains the progesterone, is decanted and evaporated in a heating block under a stream of nitrogen gas. Extraction is a somewhat hazardous and time consuming process that is performed under a fume hood. There are other methods used to avoid extraction which will be discussed later.

Separation of the antibody-ligand complexes from unbound ligand is another step that requires considerable effort. This has been done in the past in a number of ways including solid-phase primary antibody, solid-phase adsorption of ligands, and chemical precipitation. More recently immunoprecipitation has been used as there are no limitations and it can be applied to almost any radioimmunoassay. This "double-antibody" procedure involves the addition of a second antibody raised against immunoglobulin's from the species in which the primary antibody was produced (38). For example, if the primary antibody was produced in rabbits, then immunoglobulins from rabbits would be used to immunize goats and the antiserum would then be collected and processed to achieve the secondary antibody. After the reaction between the primary

antibody and ligand is complete, the secondary antibody is added to the mixture. The reaction between the secondary antibody and primary antibody-ligand complex yields an insoluble immune complex leaving the unbound or "free" ligand in solution to then be decanted. Radioimmunoassay is still considered the gold standard (outside of mass spectrometry) when determining the concentrations of progesterone.

An enzyme linked immunosorbent assay, or ELISA, is another technique used to determine the concentration of an unknown in tissue or blood. There are many different types of ELISA's performed though our design of interest involves a competitive ELISA assay. This requires coating polystyrene plates with antibody and incubating for a set period of time, usually overnight at 4°C. This is followed by a wash procedure and addition of both standard or unknown sample and an enzyme conjugate. The mixture is then incubated for a set period of time at which point it will be washed and a substrate added to cause fluorescence or a color change (37). Depending on the particular protocol, a stop agent may be added and the plate read by a microplate reader at a wavelength that maximizes sensitivity. The ELISA technique can be performed using a single antibody or by use of two antibodies (Figure 1.6). In the single antibody technique, the plate is coated with a polyclonal rabbit anti-progesterone antibody. In the double antibody technique, the plate is coated with a goat anti-rabbit IgG. When performing the assay with the double antibody technique, the anti-progesterone antibody is added prior to addition of the standards or samples and conjugate.

Another component of an ELISA assay is the type of antibody being used. Either a polyclonal or monoclonal antibody can be used in the assay. Polyclonal antibodies are the product of an immunized animal and polyclonal serum contains many different

antibodies against the various antigens injected. In the case of progesterone, since it has a low molecular weight and would be insufficient in creating an immunogenic response on its own, it is attached to a carrier protein, usually bovine serum albumin (BSA). Once the hapten is attached to a carrier protein in an adjuvant and injected into the organism for immunization, an antibody response is generated to both the hapten (progesterone) and carrier protein (BSA). Therefore, the polyclonal antiserum will contain anti-progesterone and anti-BSA antibodies at the very least. The antiserum is usually heterogeneous at all levels including the antibodies' specificity, classes and subclasses, titers and affinities. The response to individual epitopes may be diverse in nature and antibodies of different affinities may compete for the same epitope (8). Monoclonal antibodies can provide advantages over polyclonal antisera. Polyclonal antibodies may bind several antigenic sites on the hormone of interest, possibly reducing sensitivity; however, a monoclonal antibody will only bind to a single site and leave other epitopes free (45). The monoclonal antibody is monospecific in nature and therefore has a single affinity for a distinct epitope (8). The disadvantage arises with the fact that the immune response will generate a wide range of specificities and antibodies and therefore must undergo a selection process. The production of monoclonal antibodies is complex, difficult and time consuming as it may take a great length of time to produce the antibody of interest. To obtain monoclonal antibodies it is necessary to isolate and propagate single antibody-secreting B cell clones, this is possible by the combination of methods of in-vitro cell fusion, selection and cloning. The single antibody-producing cells are immortalized by fusion of B-lymphocytes to myeloma cells. This forms a hybridoma clone which lives forever and secretes antibody of the desired specificity. A crucial part in the production

of monoclonal antibodies is the screening process. A stringent selection procedure is necessary for the hybridoma cells as to eliminate hybrids that are either producing no antibody or, importantly, antibody that is of either the wrong specificity or of too low an affinity to be useful (45). Due to the great efforts to produce monoclonal antibodies and the expensive end product, they are often not used in hormone immunoassays.

The conjugate utilized in an ELISA can be either a homologous or heterologous combination with the antibody of choice. For instance, the anti-progesterone antibody used in most ELISA assays is raised against progesterone 11α-hemisuccinate-BSA. A homologous combination would include the use of the enzyme conjugated to progesterone 11α-hemisuccinate-HRP whereas a heterologous assay might consist of progesterone-3(O-carboxymethyloxime)-HRP. There are several studies that have assessed the performance of ELISA's with use of numerous combinations of antibody and conjugate preparations, both heterologous and homologous (18,37,56). It has been shown that heterogeneity between the conjugate and antibody greatly increases the sensitivity of an assay (37,56). The increase in sensitivity is most likely attributable to the antibody (18). A study was performed by Dr. Niswender (1973) showing the influence of the site of conjugation on specificity of antibodies to progesterone. Conjugation of BSA to the 11 position of progesterone provided the greatest specificity and is the reason the anti-progesterone antibody is raised against progesterone 11α-hemisuccinate-BSA (39). Figure 1.7 illustrates the difference in the sensitivity and performance for both combinations as reported by Yoon et al (1995).

As mentioned previously, there are several techniques that may be used to avoid extraction when performing an RIA or EIA. Conventional assays involve extraction of

progesterone from serum by organic solvents and are laborious and hazardous. However, in the establishment of direct assays it is important to establish the accuracy of the new protocol by comparing the results with those of existing methods involving extraction (42). One technique used in lieu of extraction are displacing agents that can be employed for the direct assay of unextracted serum using danazol, cortisol, dexamethaxone, salicylate or 8-anilino-1-naphthalene sulphonic acid (ANS) (32,43,51). A potential disadvantage of ANS is that at its effective blocking concentration it may partially reduce specific binding in an assay (42). When performing an assay with unextracted serum it is necessary to remove progesterone from its carrier proteins or displace the hormone from the carrier protein. In the case of progesterone, the carrier proteins of concern are CBG and albumin. Danazol is a synthetic steroid that has been used to displace progesterone. Danazol, cortisol, and dexamethasone all have high affinities for CBG and have been used in the direct assay of progesterone (42,51). It has been shown that when used in an RIA, danazol did not alter the standard curve and results for the progesterone assayed in the direct RIA (unextracted) had a very high correlation, ($r = 0.99$), with those obtained using an extraction RIA. In a study by Carrière and Lee (1994), 200 ng of danazol diluted in assay buffer was added to 25 µl of plasma sample in duplicate (5). The amount of danazol added to the samples was equivalent to a 1000-fold excess of the maximum levels of progesterone likely to be measured though it did not cross-react with the progesterone anti-serum. They concluded that danazol effectively displaces all of the progesterone bound to plasma proteins and inhibits the recombination with freed hormone (5). A similar study by McGinley et al. (1979) reported comparable results

using 400 ng of danazol and had a correlation of 0.91 between the unextracted and extracted assay (32).

Cortisol has also been used with success in the radioimmunoassay for progesterone in unextracted serum (19). Adding cortisol released progesterone from its binding protein, CBG. Cortisol binds to CBG with a greater affinity than progesterone and competes for the one binding site. Cortisol was diluted in assay buffer to a concentration of 200 ng/100 µl and 100 µl was added to 25µl of serum. This amount of cortisol did not cross-react with the progesterone antiserum. The results correlated very well (r = 0.97) between the unextracted method and traditional method involving solvent extraction (19). A disadvantage of these displacers is that at their blocking concentrations, some of them reduce the specific binding of the antigen with the antibody and in some cases they cross-react with the antibody though that was not the case in the above studies (51). These displacing agents save considerable time and money in the lab by bypassing the extraction step and do not compromise precision.

Other direct methods used to avoid extraction involve lowering the pH, heat treatment and use of proteolytic enzymes (42,51). Low pH quantitatively displaces steroids from serum binding proteins and denatures the proteins. Lowering the pH to approximately 4.0 has been used to effectively displace progesterone from serum proteins (43). The optimal pH for progesterone binding with cortisol binding globulin is 8.5. The binding of cortisol and progesterone is sensitive to pH. Cortisol binding globulin is irreversibly denatured at a pH of 4.0. In regards to temperature, the binding of cortisol and progesterone is highly temperature dependant and polymerization has been shown to occur when heated for 15 minutes at 60°C (6). Various other temperatures and times

have been shown to denature the high-affinity binding proteins and effectively allow the assay of progesterone (17). Cortisol binding globulin is also a member of the serine protease inhibitors and substrates superfamily (SERPINS). CBG can be attacked by elastase, a serine protease (46). Interactions between CBG and specific proteinases destroy its ability to functionally bind steroid (17).

Progesterone Kits

There are many instances in equine reproductive management in which a rapid and precise progesterone assay would be helpful in determining the presence or absence of a functional corpus luteum. Confirmation of ovulation, confirmation of progesterone levels in mares that do not display behavioral estrus, and determining luteal function in acyclic or transitional mares are probably the most classic examples (14). There are several stall-side tests and kits that are available for use today and some have been used in the equine. It should be noted that immunoassays in general are subject to inter-species variability and it is not recommended that an assay optimized for a component in one species be used to measure the same component in another species, unless the assay has been validated for that particular species (10). Direct assay kits used to measure progesterone in human samples may not be suitable for use in other species. Bovine serum has been shown to interfere with the assay probably due to species differences in serum proteins (5). The stall-side tests are generally regarded as being qualitative or semi-quantitative. Several companies market rapid EIA progesterone kits such as Norden Laboratories (ClinEase®-EP), American Diagnostics (Equicheck®), AgriTech Systems (CITE® Semi-Quant™), and BioVet (Ovucheck® Premate 10) (12,41). Total time

required for these rapid EIA's is approximately 10 minutes. Each kit comes with either one or two standards, typically 1.0 ng/ml and 3.0 to 4.0 ng/ml. The protocol involves simply adding either standards or unknown samples and reagents into cups or wells, allowing them to incubate at room temperature, and visually comparing the color changes in the unknown samples to that of standards. Pinto et al. (2006) utilized the Ovucheck® Premate 10 kit in the mare (41). This kit was originally designed to determine serum or plasma progesterone concentrations in dogs and cats. However, in this study 255 blood samples were analyzed for progesterone by both the semi-quantitative ELISA kit and by RIA in a blind experimental design. Overall, agreement between the two assays was 92.15%. It was determined that the assay predicted values below 3 ng/ml and above 10 ng/ml the best (41). Another problem with this semi-quantitative test is that one cannot easily differentiate values between 0.0 and 1.0 ng/ml. The non stall-side kits are considered to be quantitative and require the use of a microplate reader and micropipettes. Some of these kits include those made by Cambridge Life Sciences (Ovusure® Plasma/Serum Progesterone 96 well Kit) and Hoechst-Roussel Agri-Vet (Enzygnost® Serum Progesterone Test Kit) (10,12). These quantitative kits are generally regarded as having more validity when performing progesterone concentrations though are more time consuming and require the proper equipment. Eckersall and Harvey used the Ovucheck® Bovine Plasma kit produced by Cambridge Life Sciences (similar to Ovusure®) to determine the efficacy of use in equine, ovine and canine. They assayed progesterone by ELISA and radioimmunoassay involving solvent extraction. They found that this ELISA kit proved to be acceptable for the analysis of progesterone in plasma of

each of the species, and in particular for the detection of the low concentrations found during estrus and of the subsequent rise during the luteal phase of the estrous cycle.

Parameters for assessing the validity of an immunoassay

Specificity is an important parameter in a hormone assay. The accuracy of an assay is only as good as the specificity. This refers to the ability of the immunoassay, whether it is an RIA or EIA, to demonstrate that it quantifies only the ligand that it was intended to measure. Specificity is defined as the lack of interference from substances other than the parameter being measured. With a polyclonal antibody the specificity is complicated due to the heterogeneity of the antibodies, which have different affinities even against the same epitope. Monoclonal antibodies are better though even minor variations in the same epitope can affect the specificity. The measurement of specific activity is complicated by the existence of cross-reactivities and the variation in specific response against a required single antigenic site. Factors include the existence of endogenous molecules that are structurally similar to the principle analyte, the in vivo production of metabolites of the principal analyte with common cross-reactive epitopes, and the possible administration of similar analytes as vaccines or medications. In a radioimmunoassay it is essential that the radioactive ligand is pure to insure that nonspecificity of the assay is minimized. If the immunoassay is used to measure concentrations of a ligand in serum or plasma, then it is necessary to demonstrate that other components in the blood do not interfere with the measurement of the ligand of interest (7,38).

Sensitivity is the second most important criteria of an immunoassay. This is the assay's capacity to measure the smallest amount of ligand under standard conditions. If the assay does not have sufficient sensitivity to quantify the ligand of interest, its application is not relevant. The limiting factor in reducing the sensitivity seems to be the form of the detector (28) though some state it is a function of the affinity of the antibody and the specific activity of the radioactive ligand (in the case of RIA), or enzyme conjugate (in the case if EIA) (38). Typically, radioimmunoassays are regarded as being the most sensitive methods for quantifying ligands though ELISA's have become increasingly more sensitive over the years. The sensitivity is determined by measuring the variation associated with binding of labeled ligand in the absence of unlabeled ligand (7,38).

Precision is another parameter measured and is regarded as the reproducibility of a given set of measurements for the same sample. This is a statistical measure of the variation in samples by taking repeated measurements of the same sample either within the same assay or between assays. Precision is usually expressed as the coefficient of variation and calculated by the standard deviation divided by the mean. Precision will vary depending on the given point of the standard curve; therefore, three measurements should be taken, a high, medium and low coefficient of variation (CV). This will provide the estimation of error at three different concentrations (38). One should measure both the intra- and inter-assay coefficients of variation. The intra-assay CV is regarded as the precision within runs on the same day in the same assay. The inter-assay CV is the precision between runs from day to day or between assays, this CV typically contains more variation. An acceptable precision is approximately a maximum value of 10%

(7,55) however others stated that a CV of less than 20% is acceptable though the degree of variation is left to the judgment of the investigators (10,38).

Accuracy is the last parameter measured and is the extent of agreement between the average of an infinite number of measurements of a particular ligand and the true amount of ligand present in the sample. Assessment of accuracy requires that the ligand of interest be measured using a procedure other than an individuals immunoassay technique. If there is good agreement between the two techniques, comparison of data should result in a line with a slope of 1.0 and a high correlation approaching 1.0 (38). All of the above parameters should be assessed when examining the validity of an immunoassay.

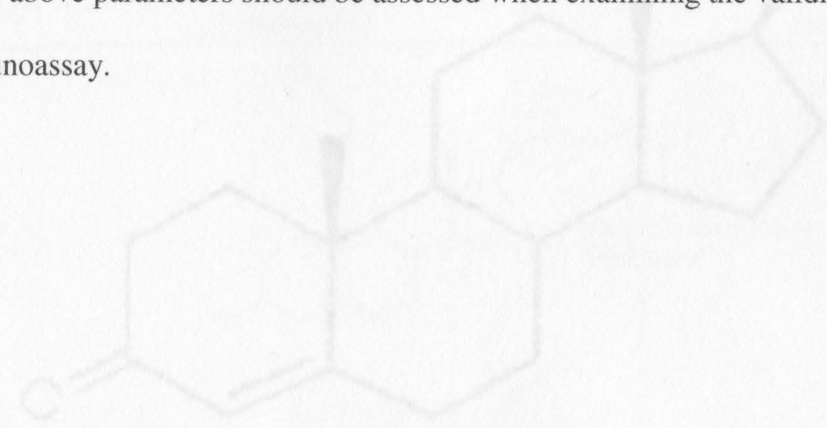


Figure 1.1. Chemical structure of progesterone, (29)

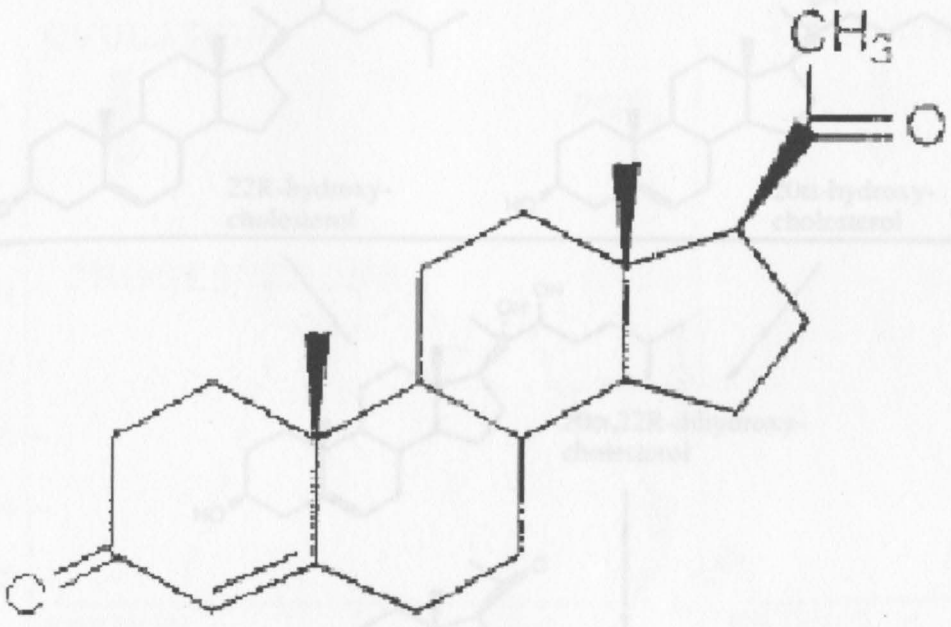


Figure 1.1. Chemical structure of progesterone. (29)

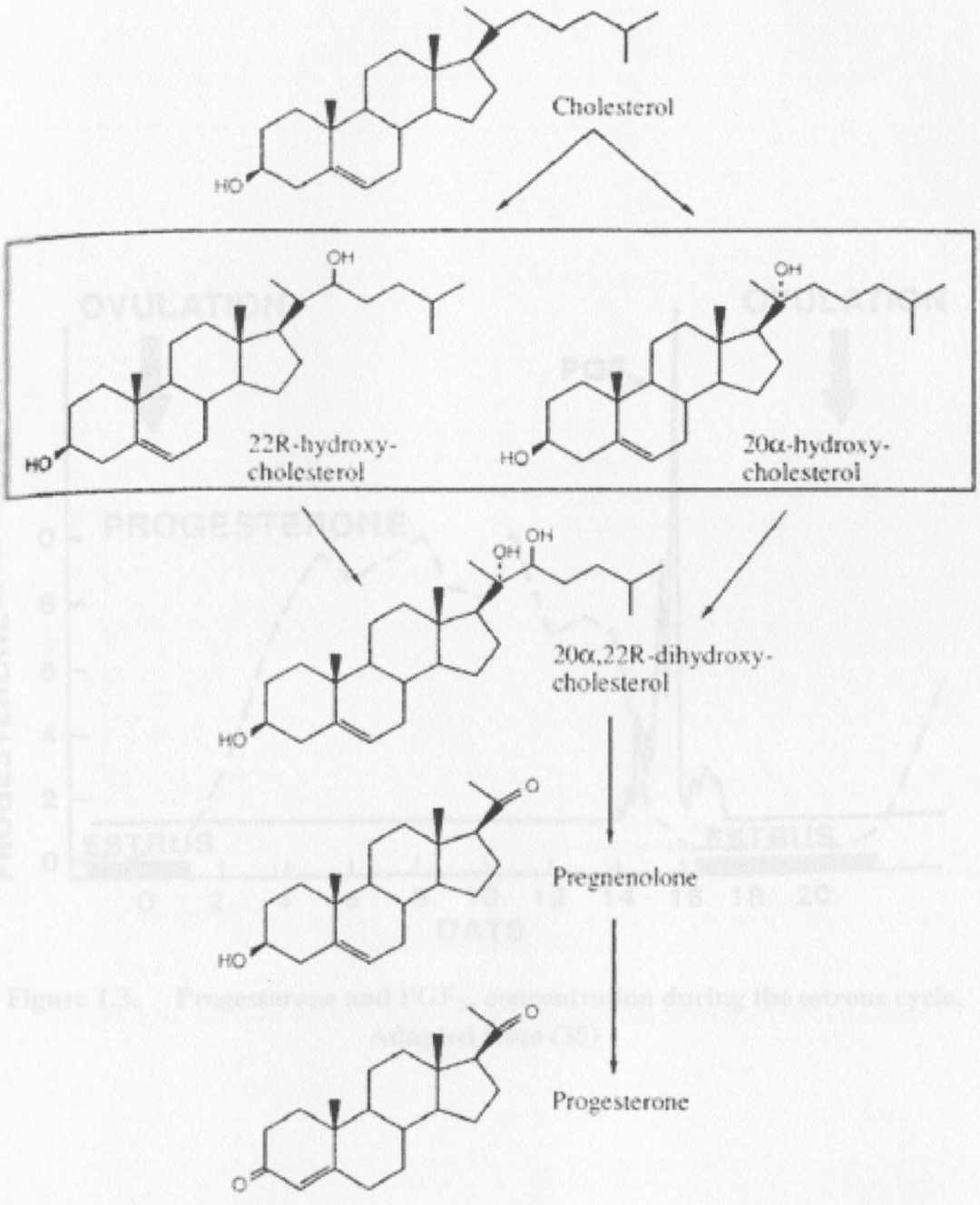


Figure 1.2. Progesterone biosynthesis. Adapted from (29)

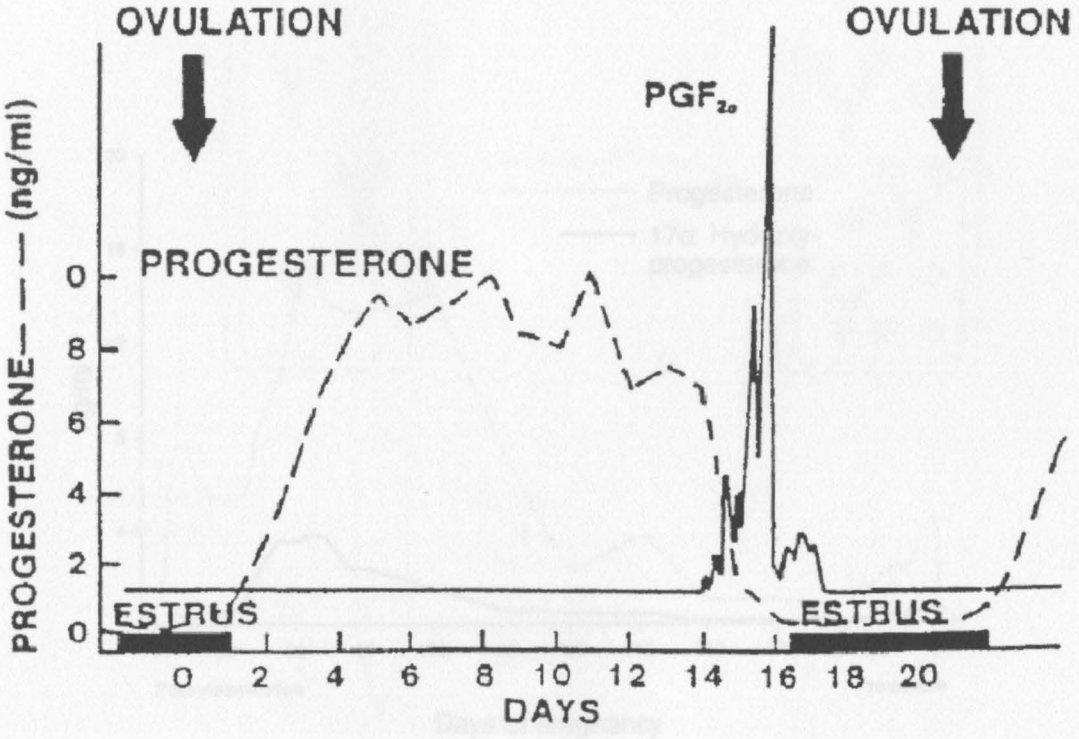


Figure 1.3. Progesterone and $PGF_{2\alpha}$ concentration during the estrous cycle. Adapted from (35)

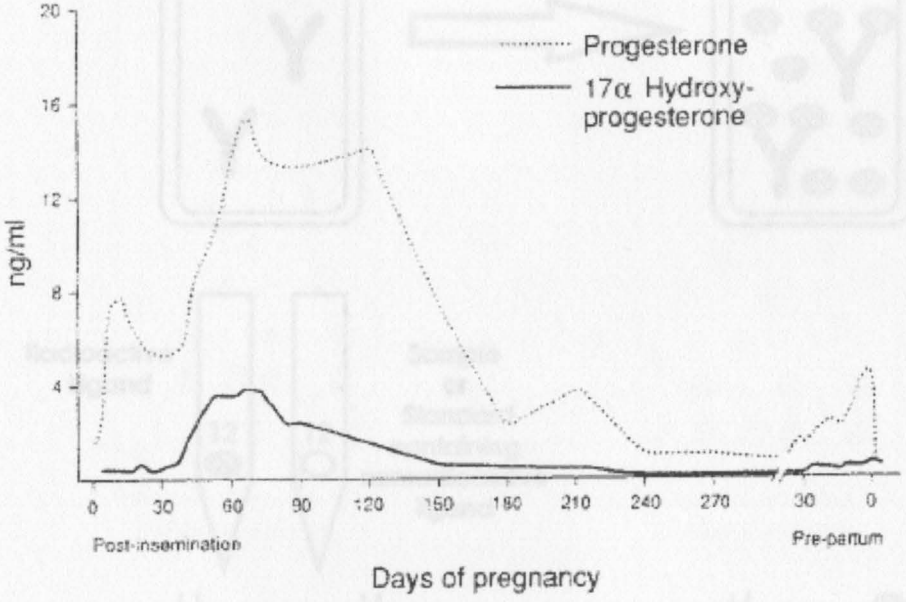


Figure 1.4. Progesterone concentrations during gestation in the mare. Adapted from (35)

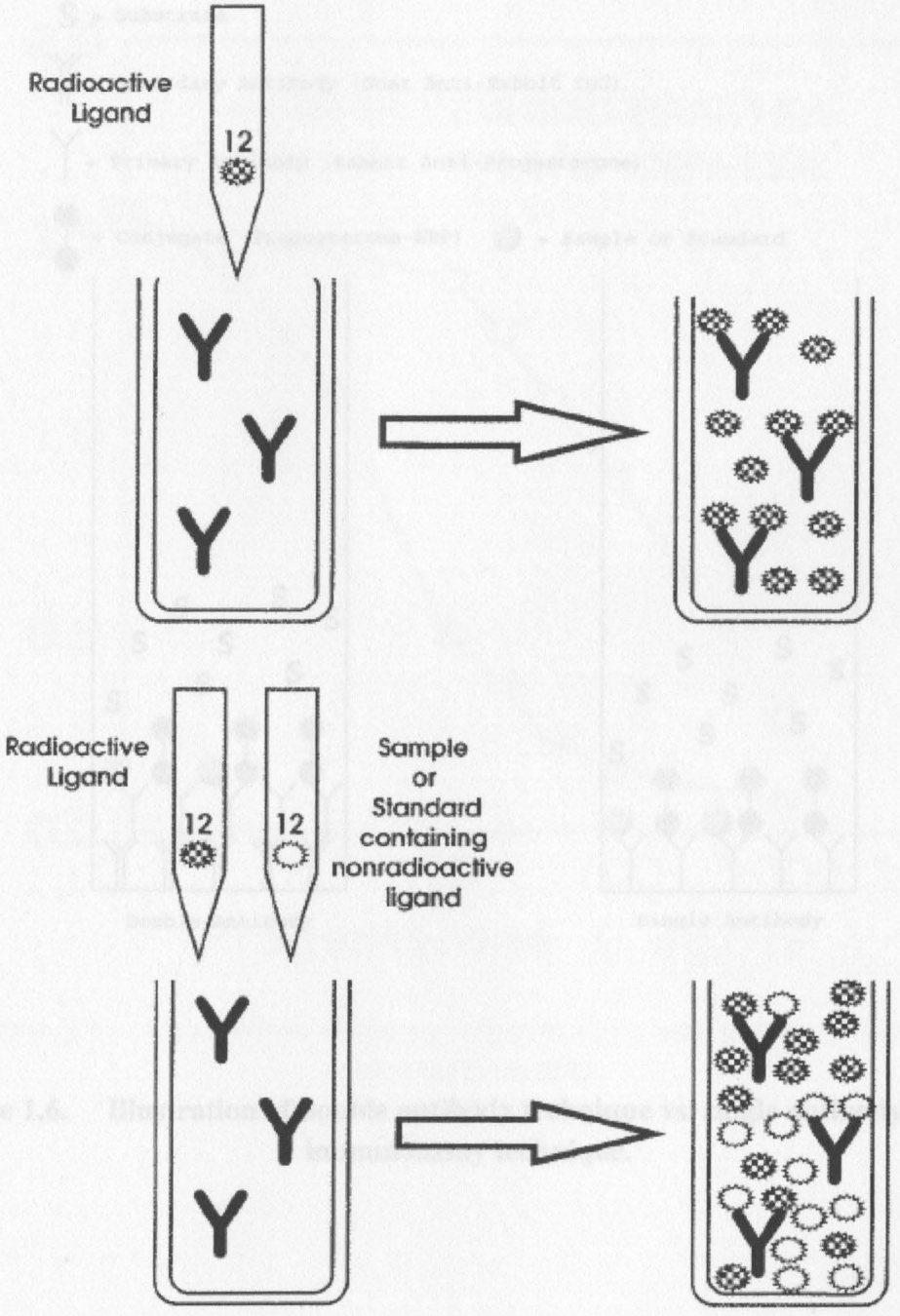


Figure 1.5. Illustration of competition in RIA between radioactive ligand and nonradioactive ligand. Adapted from (38)

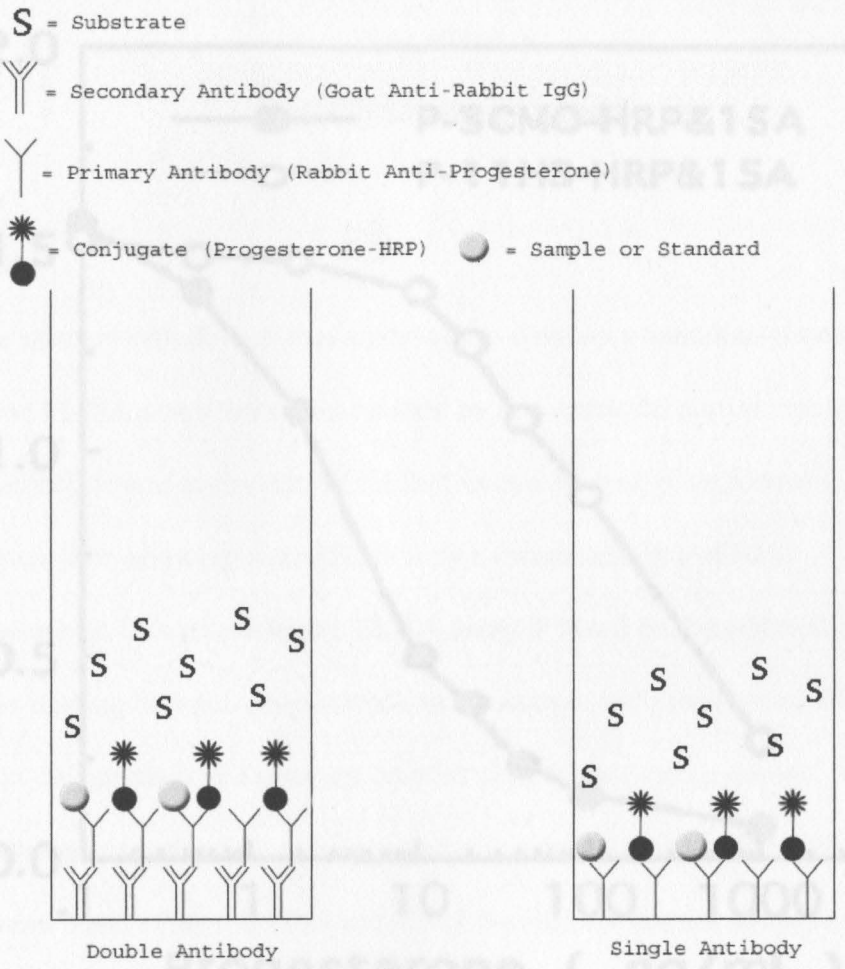


Figure 1.6. Illustration of double antibody technique vs. single antibody enzyme immunoassay technique.

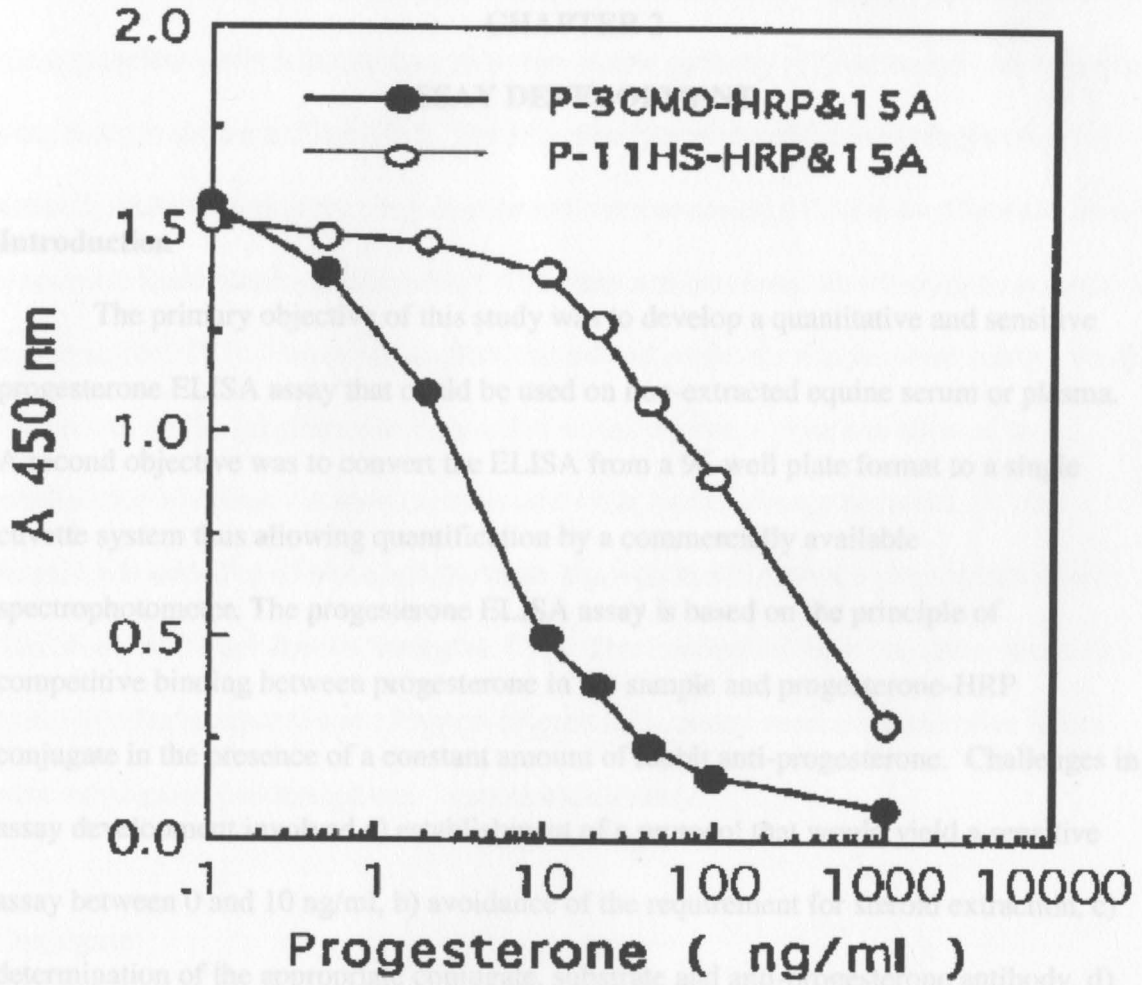


Figure 1.7. Comparison of response between two conjugate combinations. Adapted from (56)

CHAPTER 2

ASSAY DEVELOPMENT

Introduction

The primary objective of this study was to develop a quantitative and sensitive progesterone ELISA assay that could be used on non-extracted equine serum or plasma. A second objective was to convert the ELISA from a 96-well plate format to a single cuvette system thus allowing quantification by a commercially available spectrophotometer. The progesterone ELISA assay is based on the principle of competitive binding between progesterone in the sample and progesterone-HRP conjugate in the presence of a constant amount of rabbit anti-progesterone. Challenges in assay development involved a) establishment of a protocol that would yield a sensitive assay between 0 and 10 ng/ml, b) avoidance of the requirement for steroid extraction, c) determination of the appropriate conjugate, substrate and anti-progesterone antibody, d) adaptation of the assay to a cuvette system, and e) employment of immunoassay stabilizers.

Double Antibody ELISA

Development of the progesterone assay began with the use of a double antibody system that was initially designed using a protocol similar to that of a human progesterone ELISA assay in regards to the components, incubation times and volumes.

Originally, 96-well plates were coated with a goat anti-rabbit IgG (2° Antibody) and then incubated overnight at 4°C. After incubation, the assay was set up and serum samples were extracted using petroleum ether. Samples, standards and conjugate were added to the appropriate wells followed by anti-progesterone antibody (1° Antibody). An example plate setup is shown in Figure 2.1. The 1° antibody was the rabbit anti-progesterone antibody (R348) used in the progesterone radioimmunoassay (RIA) at the Colorado State University Endocrinology Laboratory. The plate was incubated for 90 minutes at room temperature (22°C). Unbound sample/standard and conjugate was removed using a wash buffer. An ABTS substrate was then added across the entire plate and allowed to incubate for 30 minutes at room temperature while a color change occurred. A stop reagent was added to all wells and the plate was read at 415 nm on a plate reader [Bio-Rad Model 680 Plate Reader, Hercules, CA]. The standard curve of this assay was noted to be very flat between 0 and 10 ng/ml. (Figure 2.2). Assay sensitivity and other issues were subsequently addressed one component at a time.

Conjugate

The sensitivity of the ELISA assay was the first area of concern. The slope of the curve was not sufficient to allow for differentiation of progesterone concentration between 0 and 10 ng/ml. The anti-progesterone antibody used in the original assay was raised against the 11α-hemisuccinate. This was achieved by immunizing New Zealand white rabbits with progesterone 11α-hemisuccinate-BSA and collecting the antiserum. The conjugate was produced by coupling horseradish peroxidase (HRP) to an 11α-hemisuccinate derivative of progesterone. This homologous combination of antibody and

conjugate both being raised against the 11α-hemisuccinate derivative was found to be the cause of the lack of sensitivity within the assay. According to Munro and Stabenfeldt (1984) and Yoon et al. (1995), a heterologous combination of the antibody and conjugate greatly increases the sensitivity of an assay (37,56). The conjugate was subsequently changed to one in which HRP was coupled to a 3-O-carboxymethyl-oxime (3-CMO) derivative of progesterone. The use of a progesterone 3-CMO HRP and anti-progesterone antibody raised against the 11α-hemisuccinate position greatly increased the sensitivity of the assay. (Figure 2.3).

Antibody

A second major change in the assay was employment of a different antibody, and movement to a single-antibody system. Plates were coated with anti-progesterone antibody instead of the goat anti-rabbit IgG used in the double-antibody system. This eliminated a step in the assay and made the assay easier to perform. The anti-progesterone antibody was acquired from the UC Davis Endocrinology Laboratory (R4859). The antibody change was made because the original antibody obtained from the CSU Endocrinology Laboratory was not purified and contained anti-BSA from the immunization of progesterone 11α-hemisuccinate-BSA. Progesterone is conjugated to bovine serum albumin (BSA) because immunization with progesterone alone does not produce an immunogenic response due to its low molecular weight. A large proportion of the original antiserum consisted of anti-BSA antibodies which may bind with BSA in the EIA buffer, thus decreasing the sensitivity of the assay and causing non-specific hindrance. In contrast, the new anti-progesterone antibody was purified to remove the

anti-BSA fraction. The change in antibody and conjugate likely both contributed to the increased sensitivity seen in Figure 2.3.

Extraction

After the antibody and conjugate changes were completed, the next focus was on the extraction procedure. In order to obtain accurate progesterone values, samples were originally extracted to dissociate progesterone from carrier proteins. As mentioned earlier, progesterone is naturally bound to CBG and albumin. Progesterone must be freed from the binding proteins. Extraction was performed by adding 2.0 mls of petroleum ether to 100 µl of serum sample in a 13x100 mm glass test tube. The tubes were then vortexed for 30-45 seconds and placed in a dry ice and methanol bath. Progesterone would be dissolved into the petroleum ether. Insertion into the methanol/dry ice bath resulted in freezing of the bottom serum component, with the petroleum ether supernatant remaining unfrozen. The petroleum ether containing the progesterone was decanted into a 12x75 mm glass test tube. The petroleum ether was then evaporated in a heating block under a steady stream of nitrogen gas. Standards were prepared with progesterone values of 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0, and 20.0 ng/ml. These standards consisted of ethanol spiked with progesterone. The standards were also evaporated under a stream of nitrogen gas. The samples and standards were both reconstituted with buffer containing conjugate according to the procedures initially described by Munro and Stabenfeldt (1984) (37). Samples and standards were then pipetted into the appropriate wells of a 96-well plate (Figure 2.1). Standards were pipetted in quadruplicate and samples in duplicate. Columns 1, 2, 11 and 12 contained only conjugate and served as B_0 wells and were used

in the calculation of "percent bound." The B_0 wells represent the maximum amount of HRP binding as there was no competition with progesterone occurring and wells contain solely HRP which in turn binds all sites on the antibody. Percent bound was calculated by dividing the average optical density of the samples/standards by the average optical density of all B_0 wells. Percent bound was then plotted on the Y-axis and progesterone concentration on the X-axis. A standard curve was established and unknown values were then interpolated using a computer program (GraphPad Prism 5 [La Jolla, CA]). The extracted ELISA values were compared to values obtained by radioimmunoassay (RIA) at the CSU Endocrinology Laboratory. It was determined that the ELISA assay (using extraction) was highly correlated with the RIA which also used extraction.

Cortisol

Unfortunately, the process of extraction is laborious, involves hazardous materials, and requires specialized equipment and facilities. In order for an assay to be used in the field or outside of diagnostic laboratories, a technique to avoid extraction is necessary. The first attempt to avoid extraction involved the use of cortisol. Endogenous cortisol binds to cortisol binding globulin (CBG), which is the same binding protein as that for progesterone. Cortisol has a higher affinity for CBG than progesterone. Our hypothesis was that an excess of cortisol in the assay may result in the release of progesterone from CBG. Several reports from the late 1970's used this method with apparent success. According to Haynes et al. (1980), cortisol was used in a progesterone RIA and a high correlation ($r = 0.97$) was achieved between progesterone values in non-extracted serum and progesterone values following extraction (19). A concentration of

200 ng cortisol/100 µl assay buffer was determined to be optimal in the experiment by Haynes et al. In our research, 10 mg cortisol was added to 10 ml 100% ethanol. To achieve a final concentration of 200 ng/100 µl, 10 µl cortisol in ethanol was added to 5 ml EIA buffer. The first experiment with cortisol compared extracted samples, samples with cortisol at 200 ng/100 µl and blocking buffer, and samples with no cortisol and blocking buffer. The blocking buffer used was Superblock® (Thermo Scientific, Rockford, IL) and was used to block the binding of non-specific free proteins in the assay and help eliminate background noise. It was later determined that use of a blocking buffer was not needed and it was consequently eliminated. In this experiment, all wells containing serum (25 µl), with or without cortisol, did not exhibit any color change, showing that no P4-HRP was bound to the coating antibody. The addition of cortisol was later tried with varying concentrations ranging between 50 ng/100 µl and 400 ng/100 µl. Again, all wells containing serum had no color change. This seemed to show that the serum was either interfering with the binding sites of the antibody or cortisol was cross-reacting with the antibody at the high concentrations used.

In a preliminary test with cuvettes when the double antibody system was used, a color change did occur and though the trial involved a low sample size, the addition of cortisol seemed to have a positive effect as samples containing cortisol showed a linear increase that followed with an increase in the true progesterone concentration whereas values with no cortisol remained scattered. Consequently, the double antibody system was tried again. Several 96-well plates were run to determine the optimal dilution of goat anti-rabbit IgG and the best dilution was noted to be 1:500. Plates were coated at 1:500 with goat anti-rabbit IgG and incubated 24 hours at 4°C. The assay was run with anti-

progesterone antibody followed by standards/samples and progesterone-HRP conjugate. Cortisol was added to the plates at a concentration of 200 ng/100 µl. All standards and extracted sample values came out as expected, but any well containing serum had no color change. This trial showed that a) the use of the double antibody system had no distinct advantage over the single antibody system and b) cortisol was not effective as a method to avoid extraction.

In other efforts to develop a direct (non-extracted) assay, a progesterone ELISA kit was obtained from MP Biomedicals [Solon, OH]. This kit was specified for use with human serum or plasma and did not involve extraction. Our goal was to determine if the kit worked with equine serum. If so, we would determine the reagent or method used in that assay to avoid extraction and adapt it to our ELISA assay. Standards, controls, extracted and unextracted samples were all run on the plate. The standard curve was appropriate, although not as sensitive as anticipated. The internal controls were within the expected ranges and the values from extracted and unextracted samples were not significantly different from each other; however, none of the values were near the progesterone concentrations previously determined by RIA. This was later determined to be due to differences between human and equine serum.

A commercial product labeled as "Direct ELISA Assay Diluent" (BioFX, Owings Mills, MD) was also evaluated and none of the wells containing the diluent exhibited a color change (extracted or unextracted). It was theorized that the diluent inhibited the binding of progesterone-HRP to the anti-progesterone antibody.

The original serum samples from mares had been completely used and pooled gelding serum was subsequently spiked with progesterone. Serial dilutions were made to

contain samples that consisted of 0, 0.625, 1.25, 2.5, 5.0, 10, and 20 ng/ml of progesterone. Exact progesterone concentrations were determined by RIA. The new standards were used in subsequent experiments. Eventually, additional standards were produced in a similar manner with ovariectomized mare serum.

Heat Treatment

Heat treatment was the next approach evaluated as a method to avoid extraction. The hypothesis was that heat treatment would denature CBG as well as other non-specific serum proteins, and therefore releasing bound progesterone (6,42). Samples were heated in a water bath for 10 and 15 minutes at 65°C as well as 10 and 15 minutes at 75°C. All serum samples heat treated to 75°C turned to a gel and were therefore not run in the assay. Although there was a very slight color change noted in the samples heated to 65°C, it was relatively the same color change for all samples. Consequently, it was determined that heat treatment was not a successful method of releasing progesterone as accurate results were not obtained.

pH – Round 1

Next, lowering the pH of serum was tried as a means of bypassing extraction. The hypothesis was that lowering the pH of serum would cause denaturation of proteins, including CBG. Progesterone would therefore be dissociated from CBG and be available in a "free" form for subsequent assay. Chan et al. (1977) reported that lowering the pH of serum below 4.0 irreversibly denatures CBG (6). Original pH of serum assayed was approximately 8.4 and the pH was lowered to just below 4.0. The volume of serum used

in the assay was 40 µl. A larger total volume of serum was used to see if lowering pH alone would allow for accurate results. The original thought was that if the pH and volume were lowered at the same time, one would not be able to distinguish which factor was causing the resulting effect. Results of the original trial using 40 µl of serum at a low pH were variable, with a slight trend toward true progesterone concentrations in samples that contained serum with a lower pH compared to serum samples with no pH change.

Volume

In the next experiment, standards and extracted samples were run along with varying volumes of serum including 40 µl, 20 µl, and 10 µl. The corresponding binding curves are shown in Figures 2.4 – 2.6 respectively. As the volume of serum decreased the standard curve became similar to the standard curve for traditional extracted serum. The next experiment reduced the volume even more by making a 1:1 dilution by adding 20 µl serum to 20 µl EIA buffer and analyzing either the whole 40 µl, or 20 µl, or 10 µl of the diluted sample. The corresponding binding curves are shown in Figures 2.7 – 2.9 respectively. After evaluation of the binding curves of the three volumes, it was theorized that lowering the volume could be a successful method to avoid extraction. The graph of binding when 10 µl of 1:1 dilution of serum:EIA buffer (Figure 2.9) showed a large change in percent bound as progesterone concentration increased. As a follow-up, the spiked gelding serum samples were run with the same low volume (10 µl of a 1:1 dilution) and various mare samples were also run using the same protocol. It was expected that one should be able to interpolate the true progesterone concentrations of the

mare samples from the curve established by serial progesterone concentrations in gelding serum. However, progesterone concentrations of the mare samples did not match the expected values as determined by RIA. In fact, all values were approximately 1.5 to 2.0 ng/ml and did not correspond to RIA values. It was theorized that the progesterone added to gelding serum or ovariectomized mare serum remained free and did not re-associate or bind to CBG. In contrast, intact mare samples had endogenous progesterone that was still bound to CBG. Consequently, it was determined that a volume had been identified that eliminated interference from non-specific serum proteins, but progesterone would still need to be released from its binding protein.

pH – Round 2

The lowering of serum pH was re-evaluated and used in conjunction with the low volume (10 µl of a 1:1 dilution). It was determined that 60 µl of 1 N HCl was required to lower the pH of 1.0 ml of serum to 4.0. When planning the next experiment, it was proposed that since CBG is supposedly irreversibly denatured at a pH of 4.0, we would lower the pH to approximately 4.0, allow the sample to incubate 10 minutes and then neutralize the pH by adding the same volume of 1 N NaOH. The goal of pH neutralization was to prevent denaturation of the anti-progesterone antibody coating the plate. The assay was performed after adding either 60 µl or 120 µl 1 N HCl to 1.0 ml of serum, allowing it to incubate for 10 minutes and then adding an equivalent amount of 1 N NaOH. This was followed by adding either 880 µl or 760 µl of EIA buffer to achieve a 1:1 dilution. From this, 10 µl of the 1:1 dilution was assayed using the standard protocol. Progesterone values still did not correspond to those obtained by RIA.

Success was finally achieved when a) the incubation time of 10 minutes was eliminated, b) various volumes of 1 N HCl were added to 1.0 ml of serum followed by the appropriate proportion of EIA buffer to achieve a 1:1 dilution and c) the use of NaOH to neutralize the pH was eliminated. Volumes of HCl used included 90 µl, 240 µl and 500 µl 1 N HCl. When interpolated back to the standard curve, progesterone concentrations of mare samples were very close to those obtained by RIA when using the protocol involving 500 µl 1 N HCl. Standard curve of new direct ELISA with 500 µl HCl is shown in Figure 2.10. Examples of progesterone measured by RIA and ELISA using this protocol are found in Table 2.1.

To be sure that a higher volume of hydrochloric acid would not provide more accurate results, 750 µl and 1000 µl of 1 N HCl were tried while taking into account the 1:1 dilution and taking 10 µl to the assay. The higher volumes of hydrochloric acid both made the standard curve flatter and resulted in a lower correlation with RIA data. Figure 2.11 depicts the standard curve involving 1000 µl 1 N HCl.

The assay using 500 µl of 1 N HCl and then diluting the mixture 1:1 with EIA buffer was performed multiple times to insure repeatability. The new assay proved to be repeatable and results were highly correlated to RIA values. A direct protocol was now established combining the addition of hydrochloric acid and drastically lowering the volume of serum brought to the assay. The issue of extraction was now resolved.

Substrate

Now that a method to avoid extraction had been discovered, the substrate used in the assay was addressed. Originally an ABTS (2,2′-Azino-bis(3-ethylbenzthiazoline-6-

sulfonic acid)) substrate was used. It was deemed desirable to move away from this substrate as it must be prepared immediately prior to use to avoid auto-oxidation and involved the use of three separate components including 0.05 M Citric acid, 40 mM ABTS, and 0.5 M H_2O_2 (2.0 %). In addition, the ABTS substrate yielded a green color when reacted with HRP and the ARS densimeter works best at a higher wavelength (ie. blue color). A TMB (3,3′,5,5′-Tetramethylbenzidine) substrate was evaluated for use in the new ELISA assay. This product was considered ideal because a) it consists of one stable component that does not require mixing, b) has a shelf life of approximately three years, c) has a shorter incubation time than ABTS, and d) yields a blue color change if not stopped. If stopped with sulfuric acid, the blue color changes to yellow. The major absorbance peaks of ABTS are 410 and 650 nm, whereas the major absorbance peaks of TMB are 370 and 652 if not stopped (blue) and 450 nm if stopped (yellow). The TMB substrate was tested and produced a standard curve similar to that of the ABTS substrate, figure 2.12. It was decided that TMB would be the substrate of choice for the ELISA assay.

Serum vs. Plasma

Another characteristic assessed was the use of serum vs. plasma in the ELISA assay. It was shown that either serum or plasma could be used in the direct ELISA assay developed. The correlations (r) were determined between red (no anticoagulant), green (sodium heparin) and purple (EDTA) blood collection tubes. The correlations between red:green, red:purple, and green:purple were 0.9986, 0.9998, and 0.9976 respectively. (Table 2.2).

Reading results

Optical densities are reported by the plate reader and thus entered into an Excel spreadsheet. The percent bound is then calculated for each standard and sample by taking the average optical densities for a given standard or sample and dividing it by the average of all B_0 wells (columns 1,2,11,12 except 1A and 1B). GraphPad Prism 5 [La Jolla, CA] is then used to further analyze the data. Based on known concentrations of standards and the corresponding percent bound, a standard curve is established by a non-linear regression curve. For unknown samples the percent bound is entered and the progesterone values are interpolated from the standard curve. Correlation coefficients of the standard curve are also checked to insure a good fit.

Assay Characteristics

The *specificity* is the lack of interference from substances other than the parameter being measured, in this case progesterone. The % cross reactivities of the anti-progesterone antibody used were tested by the UC Davis Endocrinology Laboratory and are shown in Table 2.3.

Sensitivity is the assay's capacity to measure the smallest amount of ligand under normal conditions. Sensitivity was calculated to be two standard deviations from the 0 ng/ml concentration on the standard curve. In the direct ELISA assay it was determined to be 0.018 ng/ml or 18 pg/ml.

The *precision* is known as the reproducibility of a given set of measurements for the same sample within the same run or between runs. The precision of the direct ELISA assay is reported as the coefficients of variation. The intra-assay coefficients of variation

were categorized using high (8 ng/ml), medium (4 ng/ml) and low progesterone (2.0 ng/ml) serum samples since the sensitivity varies depending on the concentration of progesterone measured. The values were 8.7%, 8.4%, and 11.2% respectively. The inter-assay coefficients of variation were also categorized using high, medium and low progesterone serum samples. They were 10.2%, 11.7%, and 3.6% respectively

Accuracy is the extent of agreement between the measurement of a particular ligand and the true amount of ligand present in the sample. This requires the ligand to be measured by a procedure other than the test immunoassay technique. Therefore direct ELISA values were compared to RIA (extracted) as this is often considered a gold standard, however the true gold standard is mass spectrometry. Accuracy of an assay is characterized by a high correlation and a line with a slope near 1.0. Figures 2.13 and 2.14 show the correlation and slope that correspond to the direct ELISA assay. Figure 2.13 shows the correlation ($r = 0.92$) between a large number of samples compared between the direct ELISA and RIA extracted values. Figure 2.14 shows the high correlation ($r = 0.95$) between the ELISA (unextracted) assay and RIA extracted values for RIA values less than 5 ng/ml. This is the concentration of greatest interest in clinical equine endocrinology. One would like to be able to assess the function of a corpus luteum or tell if a pregnant mare has a progesterone concentration greater than or less than 4 ng/ml. Progesterone levels greater than 5 ng/ml are of somewhat less importance as this simply tells that a CL is present, functional and that concentrations are adequate for the maintenance of pregnancy.

Summary

Numerous parameters were evaluated and subsequently changed in development of the direct ELISA assay. The antibody was changed to a single rabbit anti-progesterone antibody raised against the 11α-hemisuccinate position. The conjugate employed was a progesterone 3-CMO HRP providing a heterologous combination between antibody and conjugate and thus increasing sensitivity. The substrate was changed from an ABTS to a TMB for convenience and practicality of the commercial end result. Sensitivity of the assay was not adversely affected by implementation of the new substrate. Through comprehensive research to avoid organic solvent extraction, it was determined that the combination of a low volume of serum used in the assay (10 µl of a 1:1 dilution between serum and EIA buffer) and low serum pH (500 µl 1 N HCl added to 1.0 ml serum) would allow for direct (non-extracted) assay of progesterone and provide accurate and repeatable results. The assay was further validated and showed acceptable assay characteristics as well as the ability to be performed on serum or plasma.

Mare #	Date	Day of Cycle	Progesterone (ng/ml) ELISA	RIA
16048	3/4/2010	0	0.25	0.26
16048	3/5/2010	1	0.83	0.74
16048	3/6/2010	2	2.33	2.13
16048	3/7/2010	3	4.14	5.76
16048	3/8/2010	4	7.52	7.82
16048	3/9/2010	5	7.72	9.11
8104	3/4/2010	0	0.00	0.09
8104	3/7/2010	3	6.01	6.17
8104	3/8/2010	4	11.00	9.09
8104	3/9/2010	5	6.98	9.63
8104	3/10/2010	6	9.95	10.71
2105	3/6/2010	0	0.50	0.27
2105	3/7/2010	1	0.83	0.94
2105	3/8/2010	2	1.90	2.53
2105	3/9/2010	3	5.39	4.59
2105	3/10/2010	4	10.11	6.77
2105	3/11/2010	5	9.76	10.12
2105	3/12/2010	6	12.07	8.44
957	3/9/2010	0	0.52	0.39
957	3/10/2010	1	1.73	1.69
957	3/11/2010	2	4.68	6.85
957	3/12/2010	3	5.66	9.21
957	3/13/2010	4	8.81	13.74
957	3/14/2010	5	20.89	15.08
957	3/15/2010	6	23.68	14.52

Correlation and slope for Progesterone Concentrations < 15 ng/ml:
r = 0.9221
Slope = 0.9605 ± 0.0576

Mare #	Date	Day of Cycle	Progesterone (ng/ml) ELISA	RIA
0305	3/7/2010	1	1.36	1.27
0305	3/8/2010	2	2.89	5.16
0305	3/9/2010	3	4.93	6.33
0305	3/11/2010	5	11.68	9.96
0305	3/12/2010	6	9.45	10.28
0250	3/4/2010	0	0.00	0.16
0250	3/6/2010	2	3.27	3.05
0250	3/7/2010	3	5.60	6.59
0250	3/8/2010	4	7.45	9.66
0250	3/9/2010	5	9.35	10.03
0250	3/10/2010	6	8.36	11.34
0137	3/6/2010	0	0.63	0.79
0137	3/7/2010	1	1.77	2.06
0137	3/8/2010	2	5.28	4.73
0137	3/9/2010	3	3.73	4.44
0137	3/10/2010	4	3.98	5.61
0137	3/11/2010	5	7.47	8.08
0137	3/12/2010	6	7.65	6.85
0480	3/5/2010	0	0.75	0.04
0480	3/6/2010	1	2.03	1.31
0480	3/8/2010	3	7.14	5.59
0480	3/9/2010	4	9.30	10.41
0480	3/11/2010	6	9.03	10.71
383	3/10/2010	0	0.00	0.06
383	3/11/2010	1	1.33	1.95
383	3/12/2010	2	2.68	3.65
383	3/13/2010	3	6.52	5.93
383	3/15/2010	5	13.54	12.03

Table 2.1 Displays progesterone values for mares tested by RIA (extracted) and direct ELISA

	Progesterone (ng/ml)		
	Serum	Plasma	
Mare ID	Red Top (No Anticoagulant)	Green Top (Sodium Heparin)	Purple Top (EDTA)
1374	2.80	2.73	2.55
096	3.01	2.84	2.76
1348	4.05	3.23	3.93

	Correlation (r)
Red:Green	0.9986
Red:Purple	0.9998
Green:Purple	0.9976

Table 2.2. Progesterone concentrations and correlations for serum vs. plasma performed by direct ELISA.

Anti-Progesterone (R4859) Antibody raised against 11α-hemisuccinate progesterone

Cross-Reacting Steroids	% Cross Reaction
Progesterone	100.00
11α-OH-Progesterone	40.00
5α-Pregnane-3,20-dione	12.19
17α-OH-Progesterone	0.38
20α-OH-Progesterone	0.13
20β-OH-Progesterone	0.13
Pregnanediol	< 0.01
Pregnenolone	0.12
Estradiol 17β	< 0.01
Estrone	< 0.01
Testosterone	< 0.01
Cortisol	< 0.04

* Tested by UC Davis Endocrinology Laboratory

Table 2.3. Specificity of anti-progesterone antibody

Date 5/7/2010 Experiment Standards and Samples with Low pH protocol – 500 µl 1 N HCl

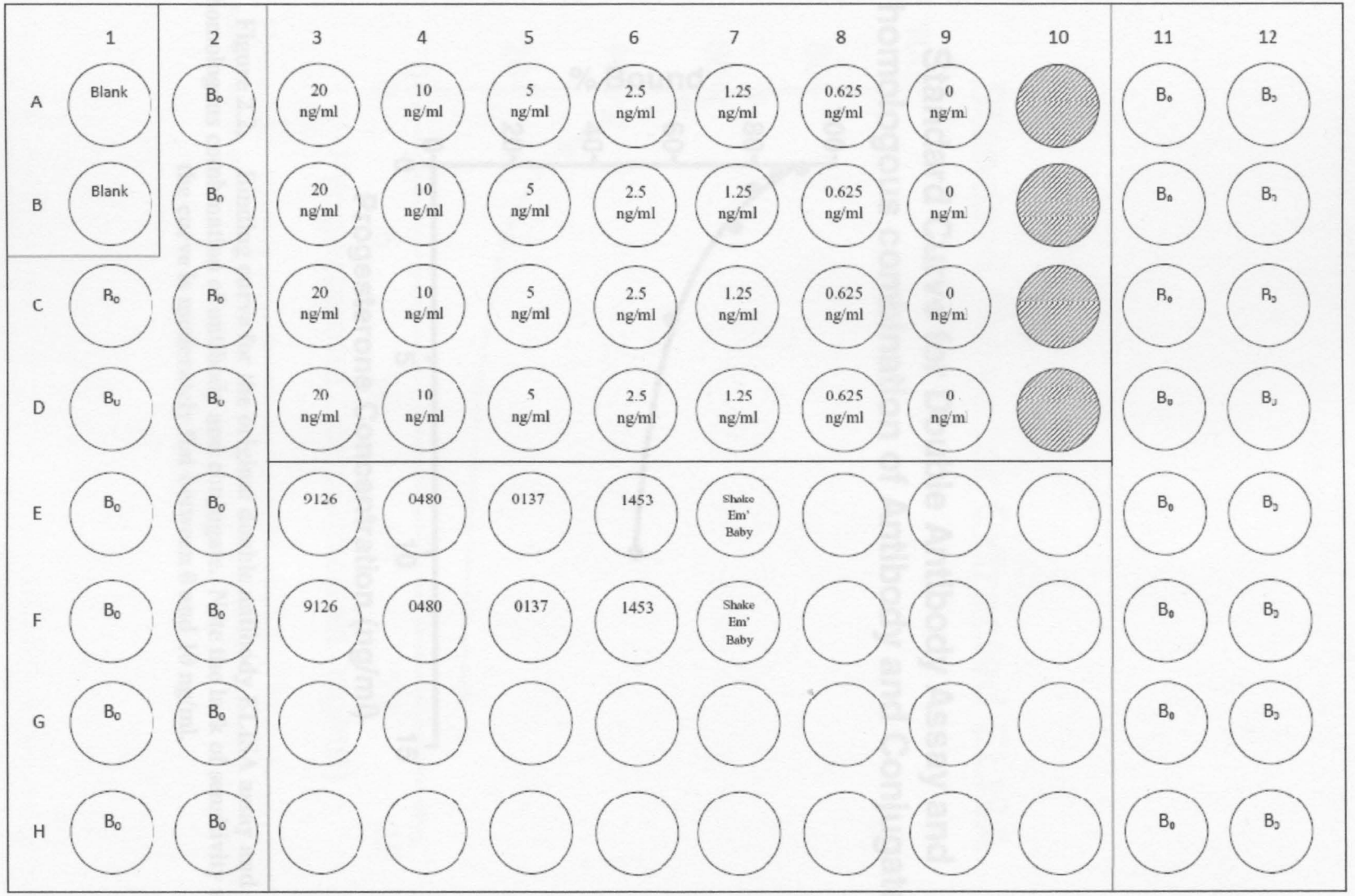


Figure 2.1. Example of assay setup on a 96-well plate

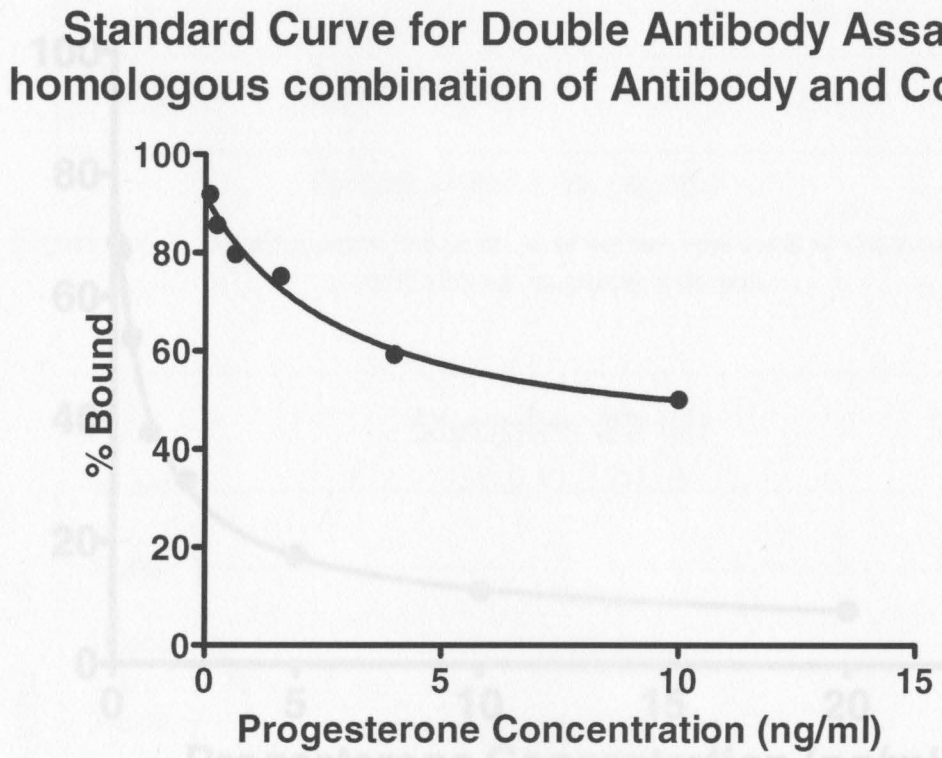


Figure 2.2. Binding curve for the original double antibody ELISA assay and homologous combination of antibody and conjugate. Note the lack of sensitivity as the curve is moderately flat between 0 and 10 ng/ml.

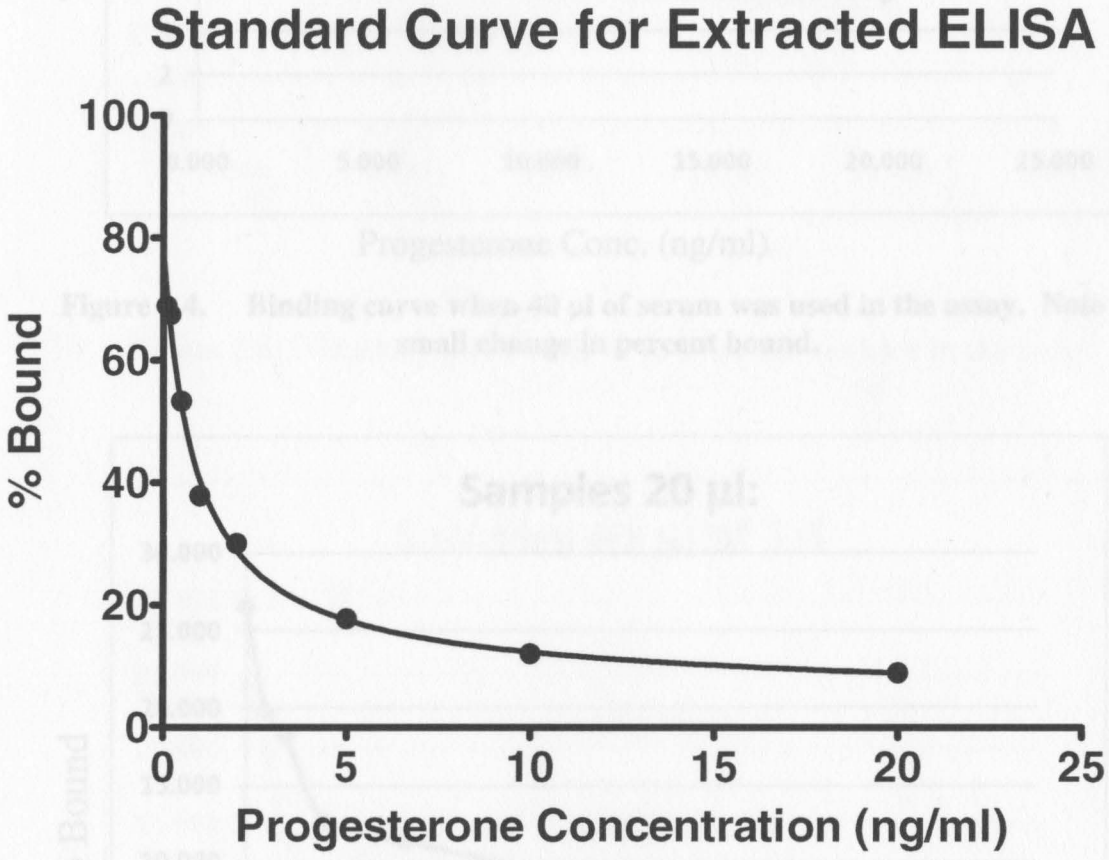


Figure 2.3. Binding curve for the extracted ELISA assay. Note the increased sensitivity in extracted ELISA employing a heterologous combination of antibody and conjugate.

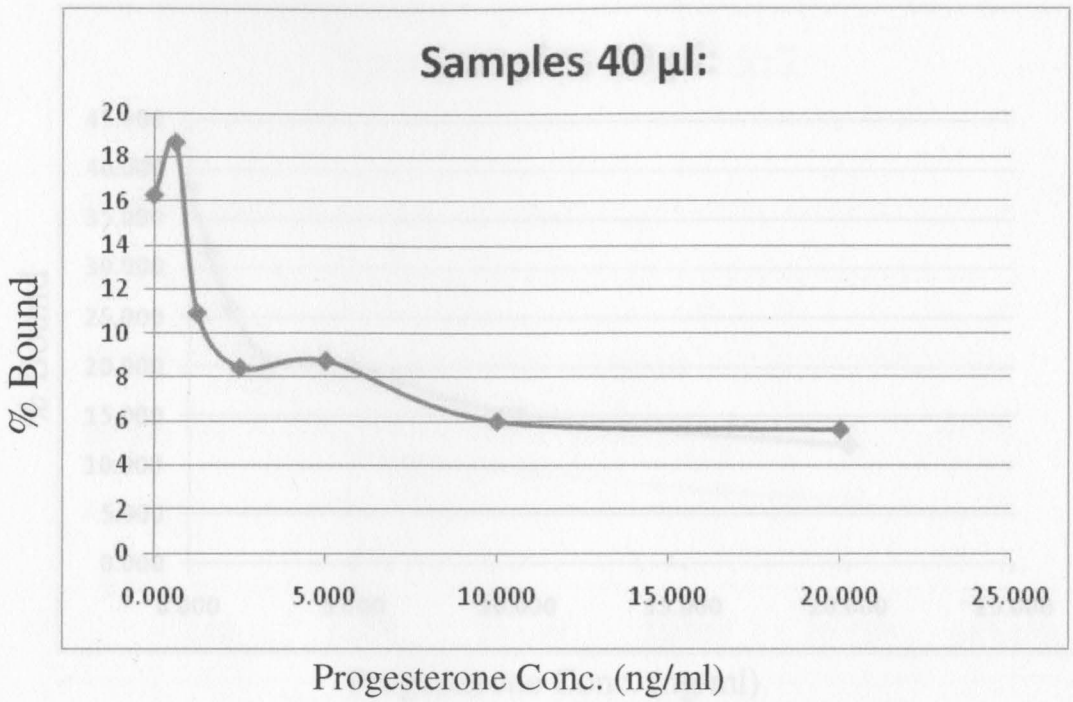


Figure 2.4. Binding curve when 40 µl of serum was used in the assay. Note the small change in percent bound.

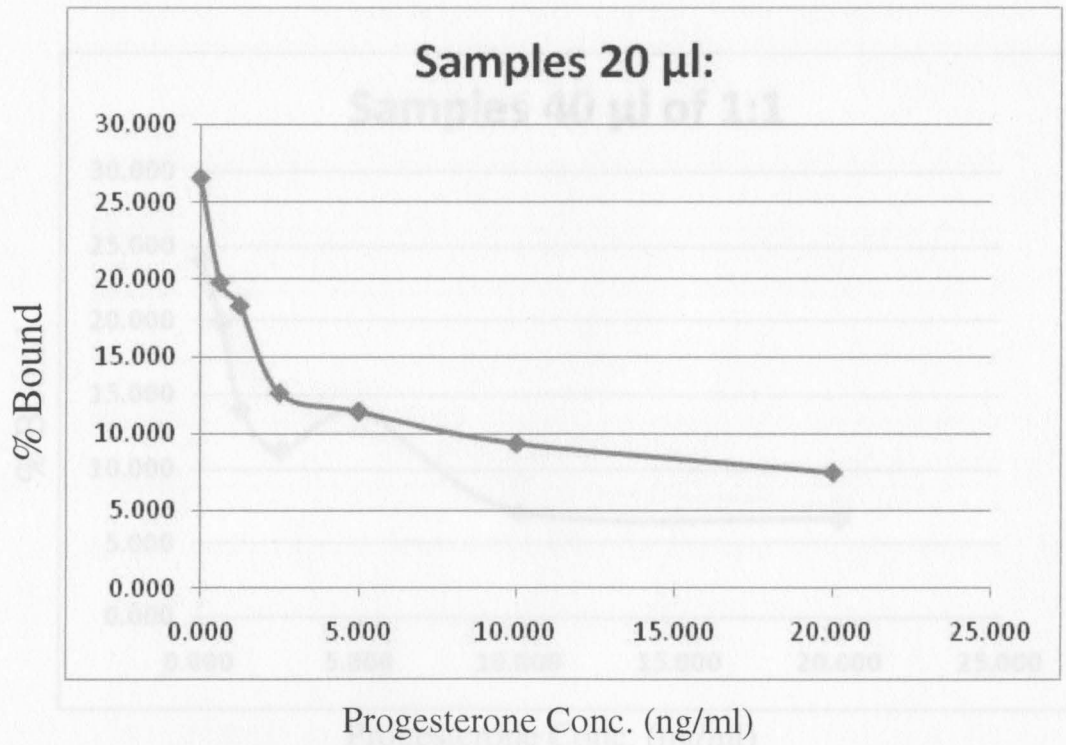


Figure 2.5. Binding curve when 20 µl of serum was used in the assay. Note the small change in percent bound.

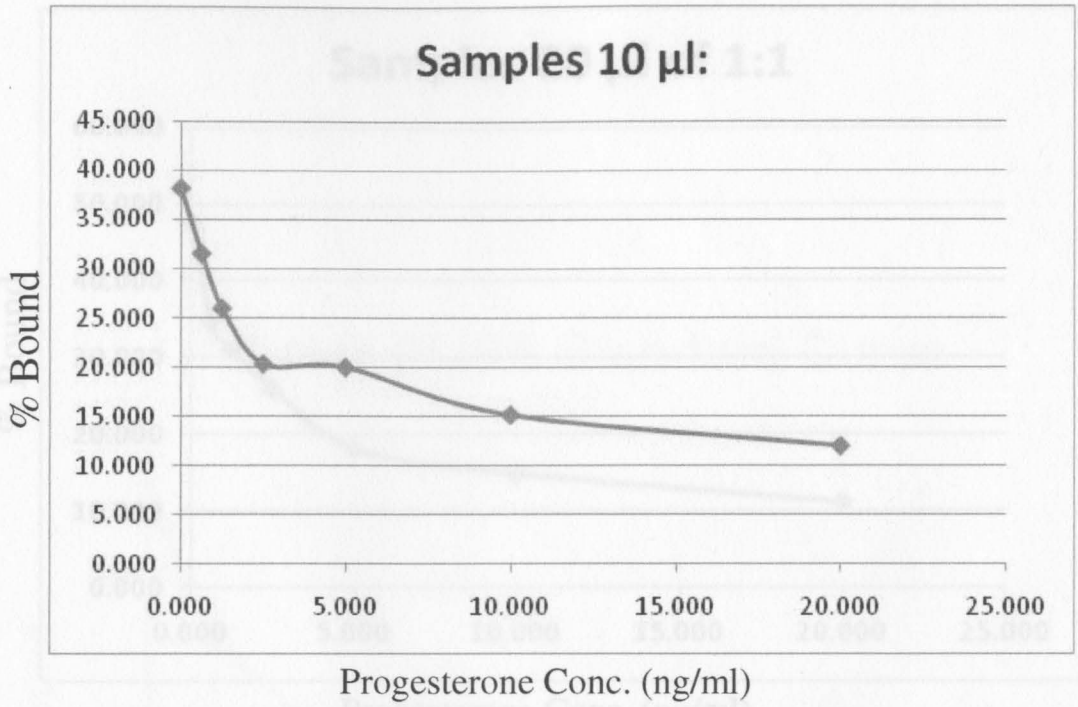


Figure 2.6. Binding curve when 10 µl of serum was used in the assay

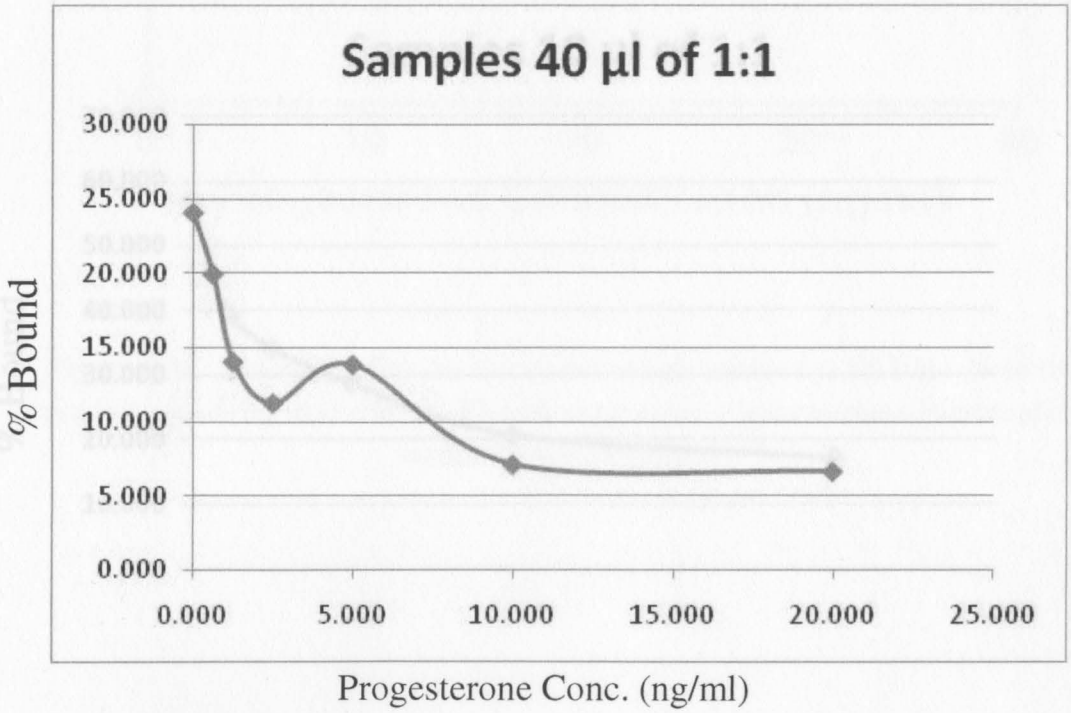


Figure 2.7. Binding curve when 40 µl of 1:1 (20 µl serum + 20 µl EIA buffer) was used in the assay.

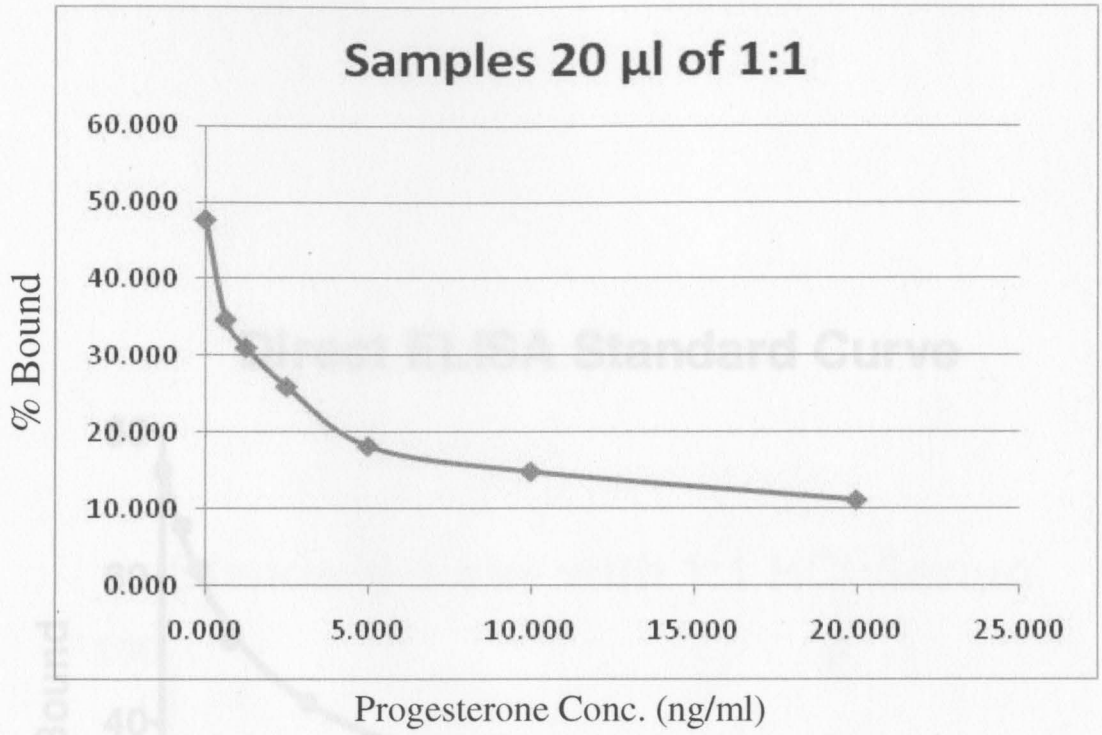


Figure 2.8. Binding curve when 20 µl of 1:1 (20 µl serum + 20 µl EIA buffer) was used in the assay

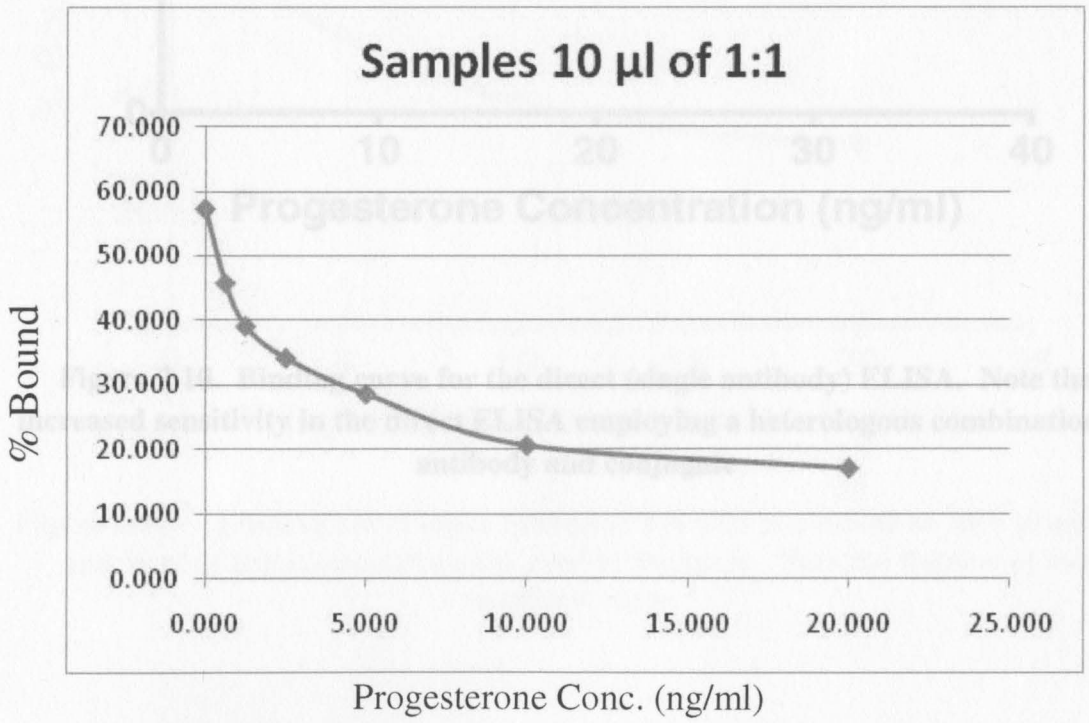


Figure 2.9. Binding curve when 10 µl of 1:1 (20 µl serum + 20 µl EIA buffer) was used in the assay. Note the large change in percent bound.

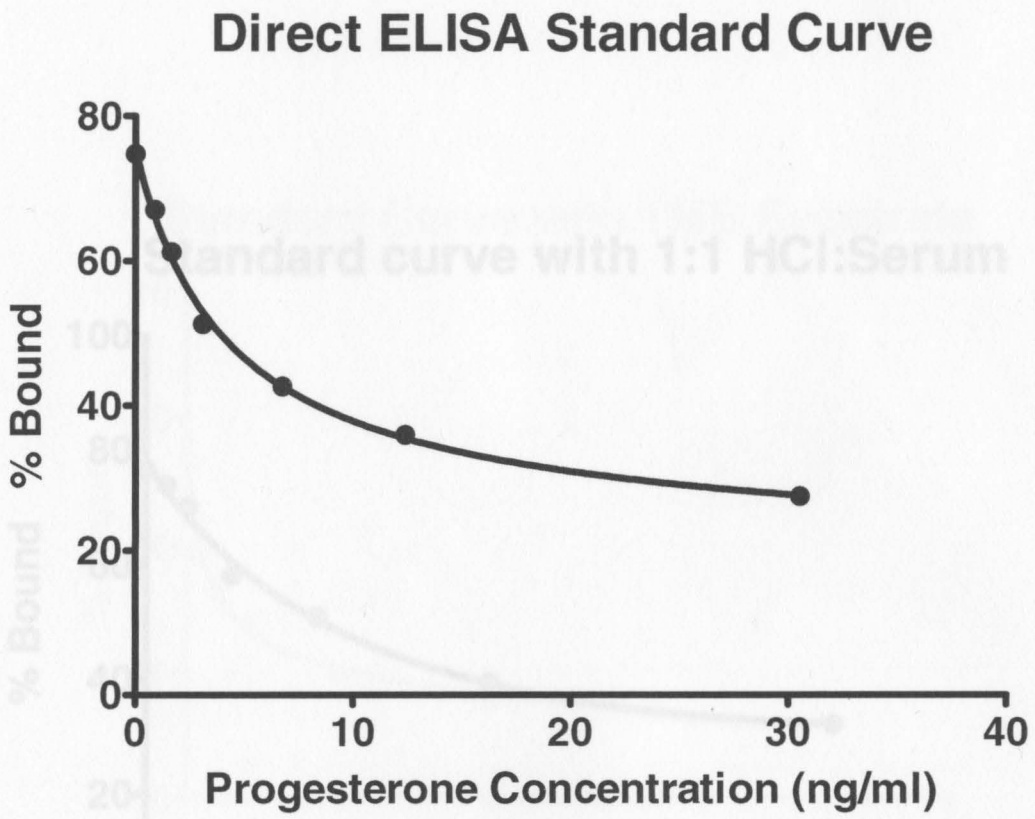


Figure 2.10. Binding curve for the direct (single antibody) ELISA. Note the increased sensitivity in the direct ELISA employing a heterologous combination of antibody and conjugate

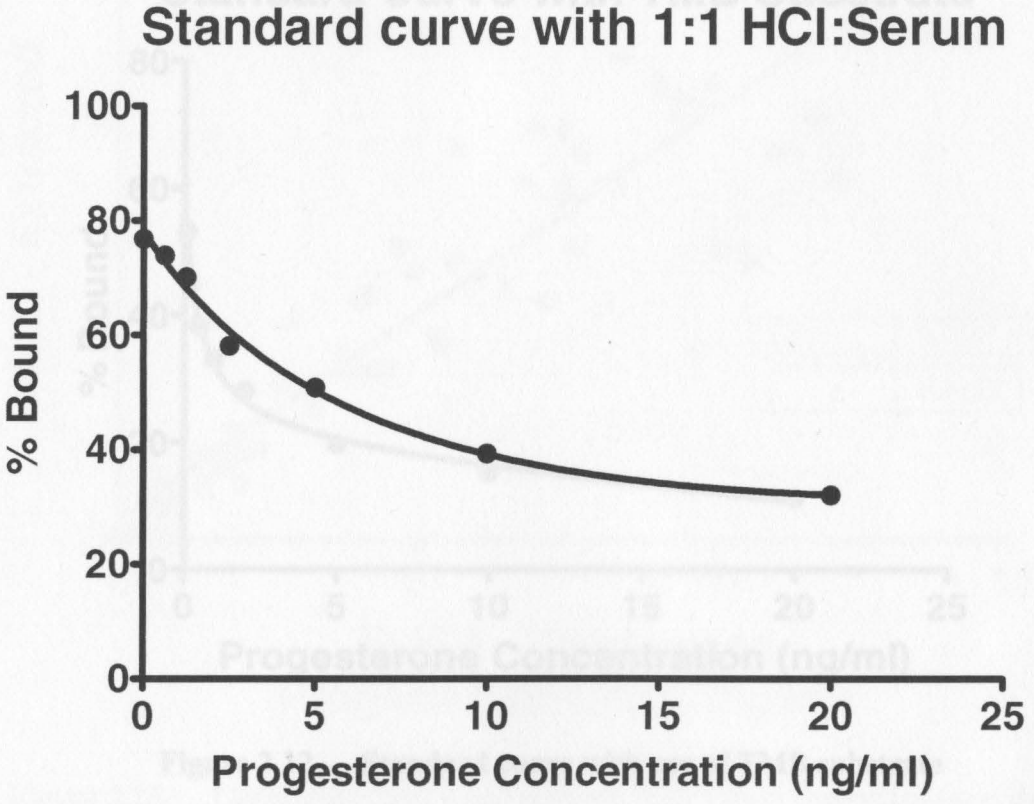


Figure 2.11. Binding curve when 1000 µl of 1 N HCl was added to 1000 µl serum and 10 µl of this combination was used in the assay. Note the flatness of the standard curve.

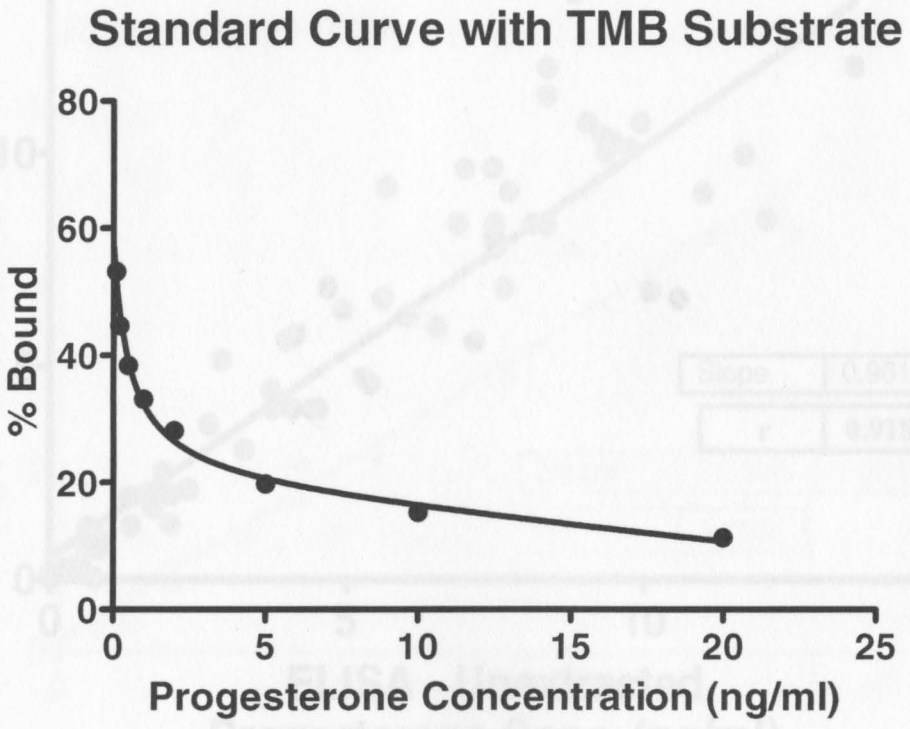


Figure 2.12. Standard curve with use of TMB substrate

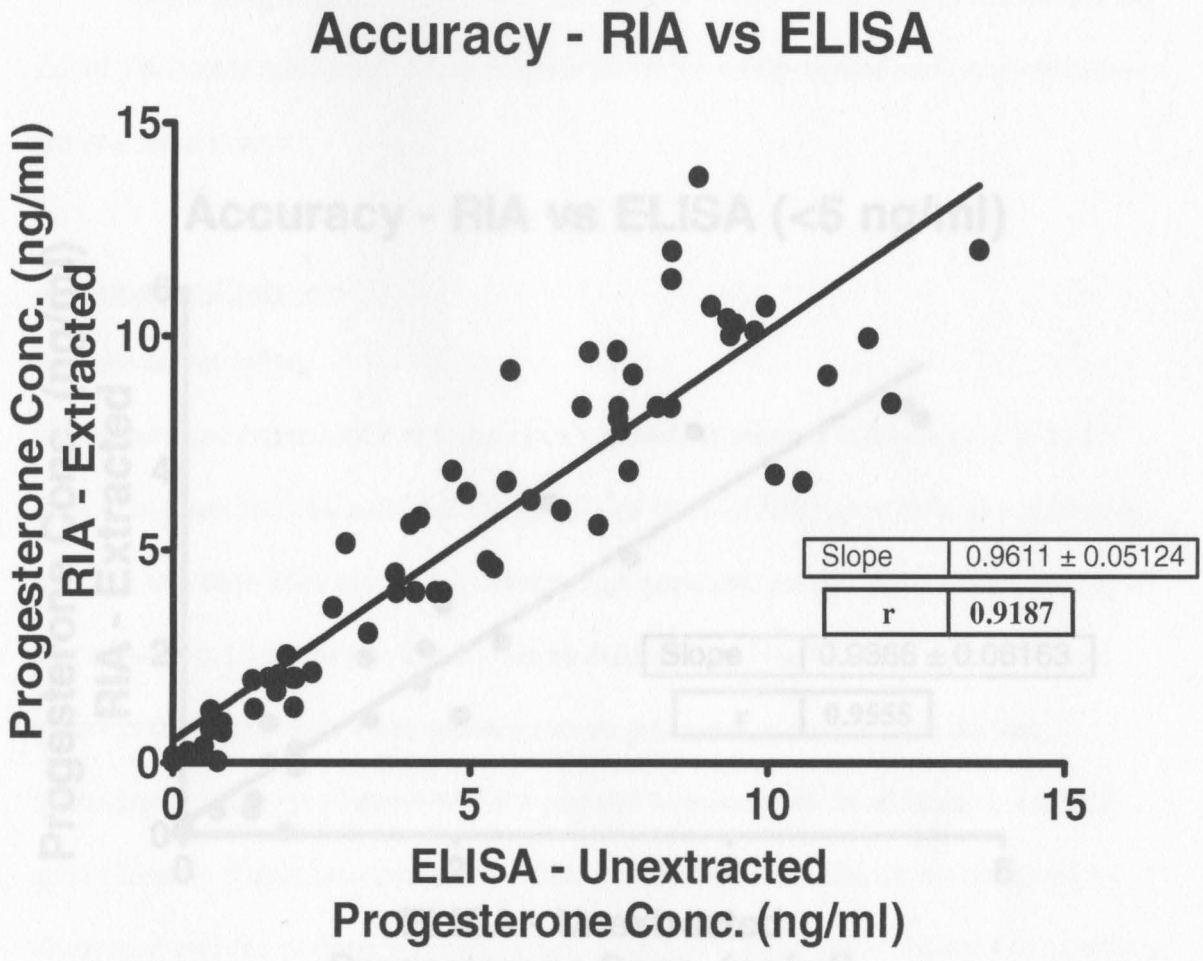


Figure 2.13. **Correlation and slope between RIA values (extracted) and direct ELISA values**

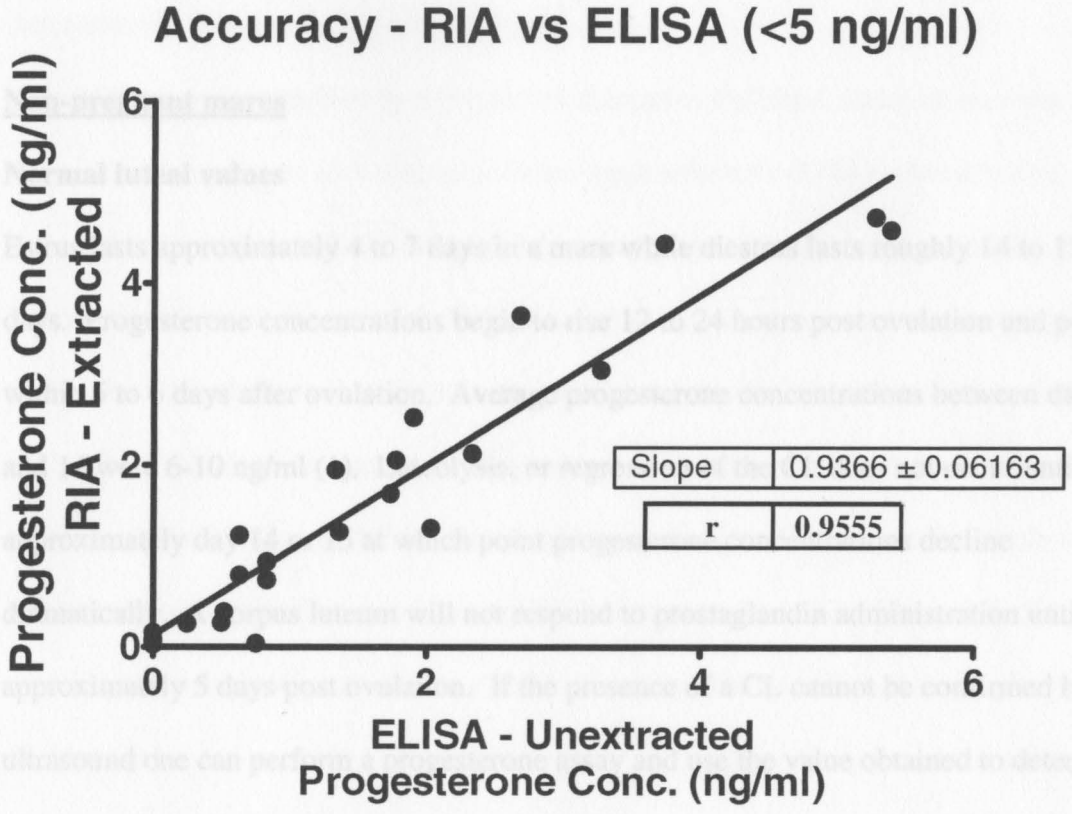


Figure 2.14. Correlation and slope between RIA values (extracted) and direct ELISA values for samples less than 5 ng/ml

CHAPTER 3

CLINICAL APPLICATIONS

In breeding management of the mare there are many clinical applications for the use of a progesterone assay. Several applications for non-pregnant and pregnant mares are discussed below:

Non-pregnant mares

Normal luteal values

Estrus lasts approximately 4 to 7 days in a mare while diestrus lasts roughly 14 to 15 days. Progesterone concentrations begin to rise 12 to 24 hours post ovulation and peak within 5 to 6 days after ovulation. Average progesterone concentrations between days 5 and 14 were 6-10 ng/ml (1). Luteolysis, or regression of the CL does not occur until approximately day 14 or 15 at which point progesterone concentrations decline dramatically. A corpus luteum will not respond to prostaglandin administration until approximately 5 days post ovulation. If the presence of a CL cannot be confirmed by ultrasound one can perform a progesterone assay and use the value obtained to determine if a CL is present.

Confirming ovulation in cycling mare

In a small percentage of mares throughout each breeding season we are unable to confirm ovulation by ultrasonography. A large follicle will have been noted during estrus, the

mare will have been bred and upon examination 1 to 2 days later no large follicle is present but also no collapsed follicle or corpus hemorrhagicum and subsequently no corpus luteum. A blood sample may be taken 2 to 3 days after disappearance of a large follicle for a progesterone assay. If concentrations are above 1 ng/ml, luteal tissue is present and ovulation most likely occurred. Another sample 1 to 2 days later should show an increase in progesterone concentrations and verify the diagnosis.

Anovulatory follicles

Progesterone concentrations may be used to determine the luteal status of anovulatory follicles. A majority of anovulatory follicles (approximately 85%) eventually luteinize and produce moderate to large amounts of progesterone (31). Prostaglandins, administered 9 to 10 days after the first observation of echogenic particles or strands within the follicular lumen, will induce regression of the luteinized anovulatory follicle (31). However, if we can assess the progesterone concentration and determine if the anovulatory follicle is an LAF we can administer prostaglandins at an earlier time as it has been shown by McCue and Squires (2002) that luteinized anovulatory follicles will respond to prostaglandins and show a decline in progesterone to less than 1 ng/ml within 48 hours (31).

Mares that cannot be palpated per rectum

There is great advantage for using a progesterone assay in reproductive management of the estrous cycle of a mare that cannot be palpated per rectum due to a previous rectal tear, small physical size, or adverse behaviors that do not permit palpation per rectum.

Teasing may be used in conjunction with daily progesterone samples to determine when to breed and estimate the day of ovulation. Daily blood samples should be collected beginning shortly after the onset of behavioral estrus and continue until approximately one day after behavioral estrus subsides. Knowing the day of ovulation is very important in potential management of twins in a mare that carries her own pregnancy and the day of a flush in an embryo transfer mare. Progesterone concentrations will be less than 1 ng/ml during estrus and begin to rise significantly 12 to 24 hours after ovulation. Evaluation of serial progesterone concentrations will allow for the determination of the day of ovulation and the subsequent 14 day pregnancy exam or embryo flush procedure.

Transitional mare

Management of the transitional mare can be enhanced by sampling of progesterone concentration. To determine when a mare has ended the spring transition period and ovulated for the first time in a given season, a progesterone sample may be taken once weekly (or more if desired) instead of constantly palpating per rectum. Progesterone concentrations above 1 ng/ml indicates that luteal tissue is present and that the mare has ovulated. Once a mare has ovulated in the spring, she will generally continue to ovulate at 21-day intervals throughout the physiologic breeding season.

Evaluate therapy of native P4

Native progesterone therapy may be evaluated by performing a progesterone assay. Administration of a short- or long-acting form of natural progesterone should cause a transient (ie. 24 hours) or prolonged (ie. 10 to 14 days) rise in serum progesterone levels.

In contrast, Regu-mate® or altrenogest will not be detected in a conventional progesterone assay. This synthetic progestin does not cross-react with the antibody used in progesterone assays. This can be advantageous in a pregnant mare receiving supplementation as one can continue to supplement with altrenogest and also assay for endogenous progesterone at various time points (ie. after formation of secondary corpora lutea) and determine if therapy can be discontinued.

Embryo transfer recipients

The level of serum progesterone may be used as another criterion when evaluating recipients for embryo transfer. Currently, most facilities assess the normality of the recipients cycle, tone of the uterus and cervix, presence of corpora lutea, and absence of uterine edema or free fluid within the lumen of the uterus. Progesterone samples may be another parameter used as low progesterone often correlates with poor tone or an inadequate CL. This could become a routine assessment on 5-day checks of recipient mares.

Pregnant mares

Confirm P4 levels

Progesterone is most frequently assayed in pregnant mares to determine if the mare is producing adequate progesterone to maintain pregnancy. Clinically, a mare may arrive for an ultrasound pregnancy exam and upon examination has a small CL or no CL, slight uterine edema and/or poor tone in the uterus. At this time we would recommend submission of a blood sample for progesterone analysis. The initial pregnancy

examination is most commonly performed 14 to 16 days after ovulation during the period of maternal recognition of pregnancy. Beyond day 40 of gestation progesterone values may increase due to formation of secondary/accessory corpora lutea. At around 70 – 90 days of gestation the placenta takes over the role of progesterone production and by day 100 supplementation is no longer needed. Some owners may keep high-risk mares on exogenous progesterone treatment for the entire duration of pregnancy. Typically, if the serum progesterone level is ≥ 4 ng/ml a mare is considered able to maintain pregnancy. If endogenous progesterone levels are less than 4 ng/ml during the first trimester when ovarian corpora lutea are responsible for maintenance of pregnancy, exogenous progestin supplementation would then be advised. Regu-Mate® and compounded long-acting natural progesterone are the two most common formulations used to supplement pregnant mares.

Activity of 2° CL's

One can assess the activity of secondary corpora lutea in pregnant mares by performing a progesterone assay. Endometrial cups form around day 35 of pregnancy and produce the luteotropin eCG which subsequently causes the formation of secondary/accessory corpora lutea in most mares. After approximately day 40 of gestation, a further rise is serum progesterone levels should be seen indicating the presence of 2° CL's.

Prior to foaling

Progesterone concentrations may possibly be used as an indicator of impending parturition. Progesterone levels begin to rise about 310 days of gestation and

dramatically decline just prior to parturition. In one mare sampled in the current study, progesterone concentrations were high (> 25 ng/ml) until one day prior to foaling at which time levels dropped to around 16 ng/ml and continued to decline until the mare foaled. (Figure 3.1) A potential clinical use of the progesterone assay may be in maiden mares that fail to "bag up" and therefore do not have sufficient "milk" to allow tests such as milk calcium to be performed. Detection of a sharp decline in progesterone levels could a valuable tool to predict the time of foaling in these pregnant mares.

Failure of maternal recognition of pregnancy

Maternal recognition of pregnancy fails to occur in a number of mares. However, if this is determined early enough we are able to save the pregnancy with exogenous progesterone supplementation. If a mare is examined at 14 days after ovulation and a small CL is observed along with an embryonic vesicle, by 16 days the CL is often no longer visible. Regression of the corpus luteum results in decline in the production of progesterone and subsequent loss of the pregnancy. Affected mares are supplemented with exogenous progesterone if the CL is thought to be of poor quality. However, a blood sample could be collected to determine if endogenous progesterone is truly deficient and whether supplementation is truly needed.

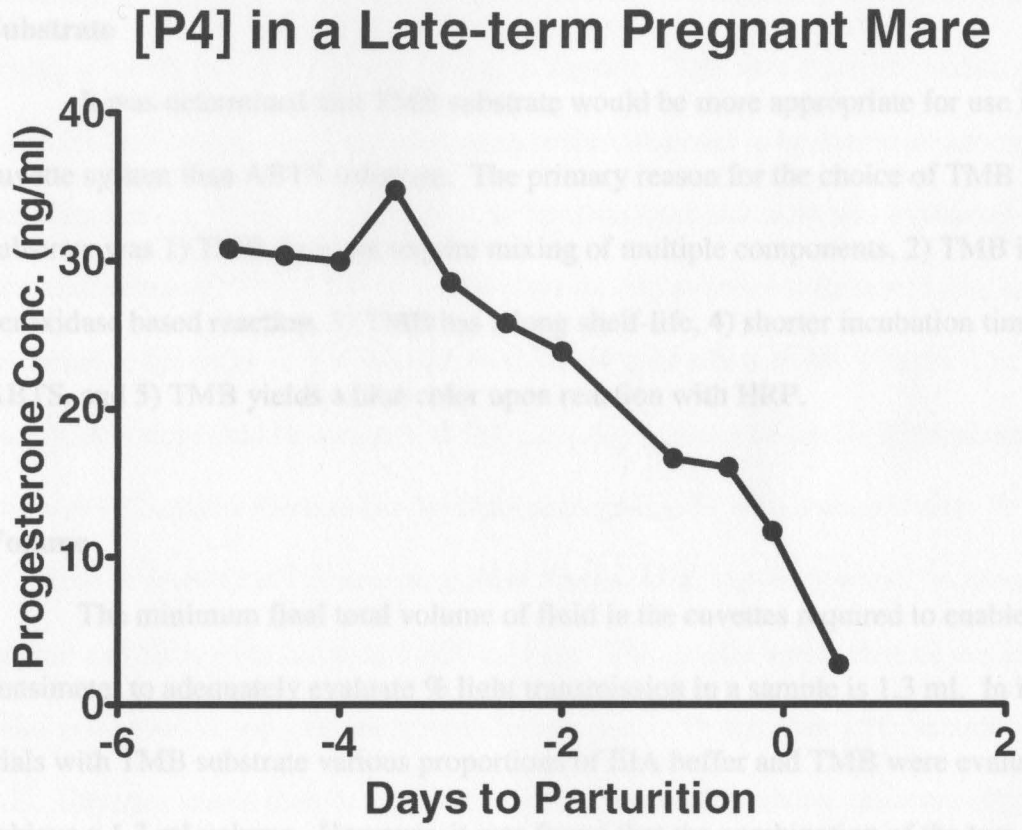


Figure 3.1. Concentration of progesterone in days prior to foaling. (Day 0 = Day of Parturition)

CHAPTER 4

CUVETTE CONVERSION

Substrate

It was determined that TMB substrate would be more appropriate for use in the cuvette system than ABTS substrate. The primary reason for the choice of TMB substrate was 1) TMB does not require mixing of multiple components, 2) TMB is a non-peroxidase based reaction, 3) TMB has a long shelf-life, 4) shorter incubation time than ABTS, and 5) TMB yields a blue color upon reaction with HRP.

Volume

The minimum final total volume of fluid in the cuvettes required to enable the densimeter to adequately evaluate % light transmission in a sample is 1.3 ml. In initial trials with TMB substrate various proportions of EIA buffer and TMB were evaluated to achieve a 1.3 ml volume. However, it was found that the combination of the two caused a precipitate which adhered to the polystyrene wall of the cuvettes and thus interfered with light transmission. It was subsequently determined that the addition of 1.4 ml TMB substrate alone in the absence of EIA buffer was successful. It is currently recommended that the solution be mixed at the end of the incubation period to make the color change homogeneous throughout the cuvette before the % light transmission is measured.

Antibody Coating

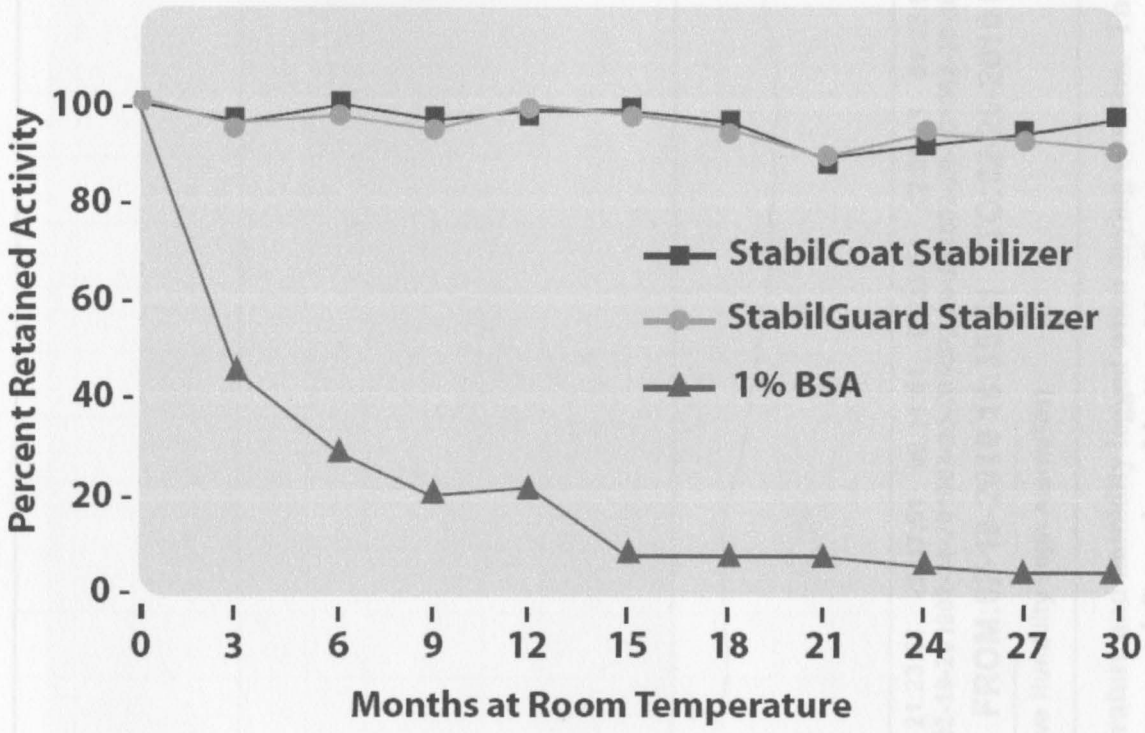
Coating of cuvettes with anti-progesterone antibody was evaluated. Cuvettes were coated at the same 1:5,000 dilution of anti-progesterone antibody used in the 96-well plates. However, cuvettes were coated with 200 µl instead of the 50 µl used in the 96-well plate. Once plates or cuvettes are coated, they were incubated at 4°C for a minimum of 24 hours. The coated plates or cuvettes were used for a maximum of 3 to 4 weeks at which time the antibody begins to degrade. This would not be conducive to batch production and shipment of cuvettes to the consumer to be used over an entire breeding season. Therefore, the use of an immunoassay stabilizer was evaluated as a potential solution. The stabilizer would allow coated cuvettes to be stored dry, at room temperature for up to 30 months with near 100 % antibody activity. (Figure 4.1) In theory, cuvettes could be coated with 200 µl anti-progesterone antibody, incubated for 48 hours at 4°C, inverted to remove the fluid, and washed 5x with a wash buffer. A volume of 200 µl of StabilCoat® [Surmodics, Eden Prairie, MN] stabilizer would be added to the cuvette and allowed to incubate for 30 minutes. The cuvette would then be emptied and dried in an oven at approximately room temperature with less than 15% humidity (Figure 4.2). Cuvettes would then be capped and packaged in a bag with a desiccant (Figure 4.3).

Complete Assay Kit

A progesterone ELISA kit was designed that could be used in the ARS Densimeter. The kit contained all solutions and materials needed to run the direct (non-extracted) progesterone ELISA assay (Figure 4.4). The progesterone-HRP conjugate was stabilized at a working concentration of 1:200,000 with the product StabilZyme®

[Surmodics, Eden Prairie, MN]. This would eliminate user error when making the working dilution of conjugate as well as make the conjugate stable for approximately two years (Figure 4.5). Several trials were run to insure that all components of the kit had a good efficacy and yielded progesterone concentrations in equine serum samples that were correlated with RIA values. Standards were run in the cuvettes and read in the ARS densimeter to show that the antibody would bind, produce a color change and appropriate a standard curve (Figure 4.6).

Figure 4.1. Percent activity when StabilCoat® is used as an immunoassay stabilizer. Compare the percent retained activity for StabilCoat® vs. 1% BSA which is the absence of a stabilizer. (Adapted from Surmodics, Inc.)



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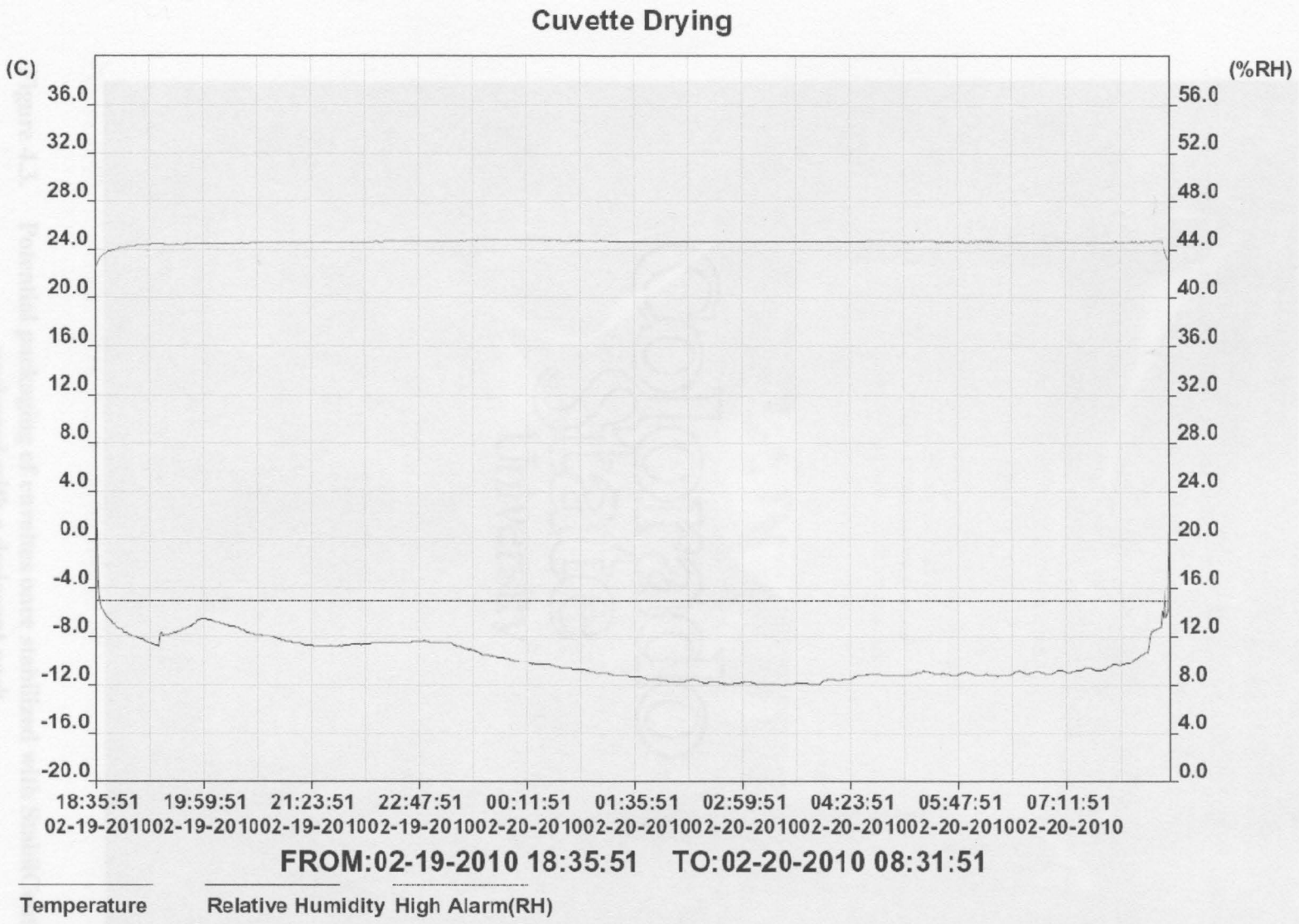

Figure 4.2. Temperature and humidity logged when drying cuvettes. The top red line represents temperature and is shown on left y-axis and bottom blue line represents humidity and is shown on the right y-axis

Figure 4.3. Potential packaging of cuvettes once stabilized with StabilCoat® and packaged with a desiccant pack.

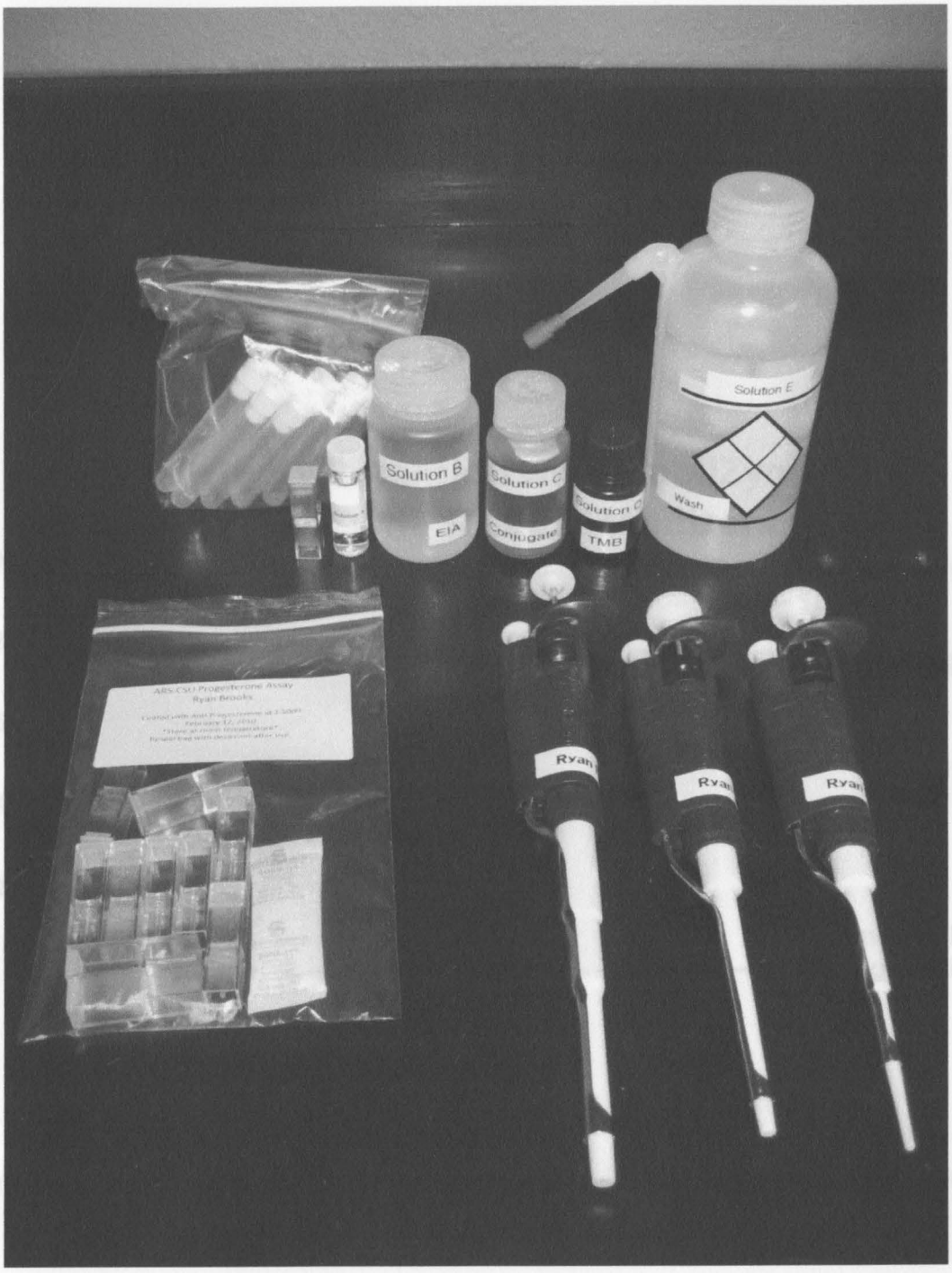

Figure 4.4. Potential kit to run progesterone ELISA assay in the ARS Densimeter.

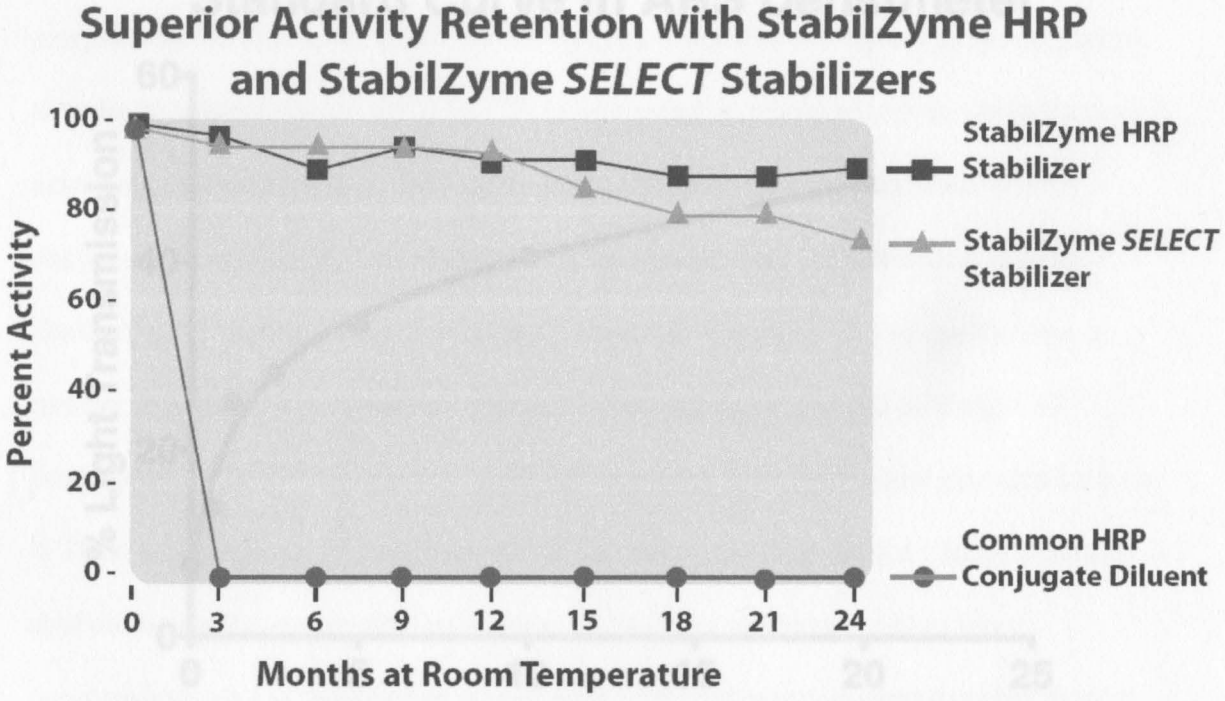

Figure 4.5. Percent activity of HRP Conjugate when stabilized with StabilZyme®. (Adapted from Surmodics, Inc.)

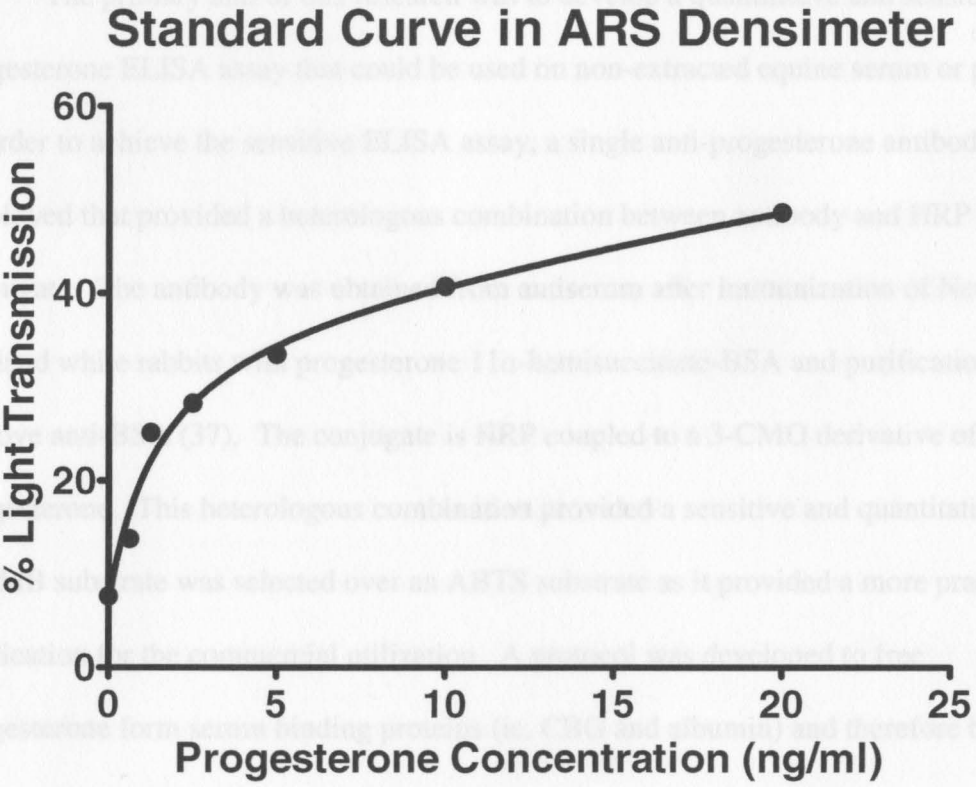

Figure 4.6. Standard curve for the direct progesterone ELISA comparing progesterone concentration (ng/ml) with % light transmission.

CHAPTER 5

CONCLUSIONS

The primary aim of this research was to develop a quantitative and sensitive progesterone ELISA assay that could be used on non-extracted equine serum or plasma. In order to achieve the sensitive ELISA assay, a single anti-progesterone antibody was employed that provided a heterologous combination between antibody and HRP conjugate. The antibody was obtained from antiserum after immunization of New Zealand white rabbits with progesterone 11α-hemisuccinate-BSA and purification to remove anti-BSA (37). The conjugate is HRP coupled to a 3-CMO derivative of progesterone. This heterologous combination provided a sensitive and quantitative assay. A TMB substrate was selected over an ABTS substrate as it provided a more practical application for the commercial utilization. A protocol was developed to free progesterone form serum binding proteins (ie. CBG and albumin) and therefore bypass the need for organic solvent extraction. Hydrochloric acid was used to lower serum pH and denature albumin and CBG thus freeing progesterone from binding proteins for subsequent assay. A low volume of serum was used in the assay as this eliminated interference caused by non-specific serum proteins binding to the antibody. The end volume analyzed was 10 µl of a mixture of 1.0 ml serum, 500 µl 1 N HCl, and 500 µl EIA buffer. This volume did not influence the sensitivity of the assay. The new direct

(non-extracted) ELISA had a high correlation ($r = 0.9555$ at P4 concentrations < 5 ng/ml) with a traditional RIA that requires organic solvent extraction, a sensitivity of 0.018 ng/ml, and intra- and inter-assay coefficients of variation of 11.2% and 3.6%, 8.4% and 11.7%, and 8.7% and 10.2% for progesterone concentrations of 2.0, 4.0, and 8.0 ng/ml, respectively.

A second objective was to convert the ELISA from a 96-well plate format to a single cuvette system thus allowing quantification by a commercially available spectrophotometer. This was achieved by coating cuvettes and performing the assay as initially developed for the 96-well plate with slight increases in volumes of components. Cuvettes and conjugate were used in conjunction with immunoassay stabilizers to provide a longer shelf life, ease of use and ease of shipment of the commercial product. An assay kit and protocol were developed that contained all components needed to perform the direct progesterone ELISA and quantify results with use of the ARS Densimeter. A standard curve was tested to verify sensitivity and appropriate binding in the cuvette system. The main clinical applications of the progesterone ELISA are 1) determination of the presence or absence of active luteal tissue in non-pregnant mares and 2) determination of progesterone levels in pregnant mares. Other potential applications include diagnosis of specific types of ovarian pathology (ie. persistent corpus luteum and luteinized anovulatory follicles) and prediction of impending parturition.

APPENDIX I

Assay Components

1. **Coating Buffer**: Sodium Bicarbonate Buffer 0.05 M, pH 9.6

Na_2CO_3	1.59 g
$NaHCO_3$	2.93 g
Distilled Water	1000 mls

Stored at 4°C

2. **EIA Buffer**: Phosphate Buffered Saline containing 0.1% BSA 0.1 M, pH 7.0

$NaH_2PO_4.H_2O$	5.42 g
Na_2HPO_4	8.66 g
NaCl	8.70 g
BSA	1.00 g
Distilled Water	1000 mls

Check pH before adding BSA, raise pH to 7.0

Stored at 4°C

3. **Wash Solution**: 0.15 M NaCl, 0.05% Tween 20

1.5 M NaCl	8.77 g
0.5% Tween 20	0.50 mls
Distilled Water	1000 mls

Store at room temperature

4. **ABTS Substrate Solutions**:

A. **0.05 M Citric Acid** pH 4.0

Citric Acid	9.605 g
Distilled Water	1000 mls

Adjust pH to 4.0, pH is critical or substrate will not work

Store at 4°C

B. **40 mM ABTS**

0.439 g in 20.0 mls distilled water

Store at 4°C

C. **0.5 M H_2O_2 (2.0%)**

H_2O_2 8.0 M (30%)	0.5 ml
Distilled Water	7.5 ml

To make 25 mls ABTS substrate:

24.6 mls 0.05 M Citric Acid, 250 µl 40 mM ABTS, and 80 µl 0.5 M H_2O_2

5. **TMB Substrate Solution**

Commercial product used – 1-Step Ultra TMB-ELISA from Thermo Scientific

6. **Stop Solutions**:

A. **0.15 M Hydrofluoric Acid with 0.006 M NaOH**

Hydrofluoric Acid (48%)	6.24 g 48% liquid HF
NaOH	1.2 mls 5.0 M NaOH
Distilled Water	1000 mls

Place empty 1 liter bottle on scale and add HF dropwise to weigh 6.24 g

Store at room temperature

B. **1.0 M Edetic Acid (EDTA)**

EDTA	19.0 g
Distilled Water	50.0 mls

To make 25 mls working Stop Solution:

25 mls 0.15 M HF with 0.006 M NaOH and 25 µl 1.0 M EDTA

APPENDIX II

Assay Protocols

Preparation of antiserum (Anti-P_4) and conjugate (P_4-HRP)

Anti-progesterone comes as 400 µl of undiluted stock

1. Take 200 µl undiluted stock, add 1.8 ml coating buffer (1:10 dilution)
   Refreeze undiluted stock immediately
2. Aliquot 100 µl of 1:10 into micro-centrifuge tubes and freeze

Conjugate comes as 250 µl of undiluted stock

1. Take 50 µl undiluted stock, add to 4.95 ml EIA buffer to give 1:100 dilution
   Refreeze undiluted stock immediately
   Store the 1:100 conjugate solution at 4°C
2. For use in the assay, dilute to working conjugate (1:200,000 for lot used)

Coating Microtitre (96-well) plates

1. Thaw one aliquot of 1:10 antiserum
2. Dilute in coating buffer to required concentration (1:5,000 for lot used)
3. Coat all wells with 50 µl except 1A and 1B as they will serve as blanks
4. Tilt plate and tap edges to disperse antibody and cover entire well evenly
5. Cover and seal tightly with acetate plate sealers, place plate in resealable bag
6. Store at 4°C for a minimum of 24 hours and maximum of 3 to 4 weeks

Standards and Extracted Samples:

Make up four standard stock solutions

a. Weigh 1.0 mg progesterone and add 10.0 ml ethanol (EtOH) = 100,000 pg/µl

b. Take 400 µl 100,000 pg/µl, add 3.6 ml EtOH = 10,000 pg/µl [A]

c. Take 400 µl 10,000 pg/µl [A], add 3.6 ml EtOH = 1,000 pg/µl [B]

d. Take 400 µl 1,000 pg/µl [B], add 3.6 ml EtOH = 100 pg/µl [C]

e. Take 400 µl 100 pg/µl [C], add 3.6 ml EtOH = 10 pg/µl [D]

Make up 8 working standards in 12x75 mm glass test tubes

a. 0.1 ng/ml = 25 µl [D] added to 2.5 ml EtOH

b. 0.2 ng/ml = 50 µl [D] added to 2.5 ml EtOH

c. 0.5 ng/ml = 125 µl [D] added to 2.5 ml EtOH

d. 1.0 ng/ml = 25 µl [C] added to 2.5 ml EtOH

e. 2.0 ng/ml = 50 µl [C] added to 2.5 ml EtOH

f. 5.0 ng/ml = 125 µl [C] added to 2.5 ml EtOH

g. 10 ng/ml = 25 µl [B] added to 2.5 ml EtOH

h. 20 ng/ml = 50 µl [B] added to 2.5 ml EtOH

Dry the standards down under a steady stream of nitrogen gas

Reconstitute by adding 2.5 ml working conjugate (1:200,000) to each tube, vortex 15 sec.

Extraction of serum samples

1. Pipette 100 µl serum sample into 13x100 mm glass test tube
2. Add 2.0 ml petroleum ether
3. Vortex 30-45 seconds
4. Place tubes in dry ice/methanol bath
5. Serum layer will freeze immediately
6. Decant off petroleum ether layer into similarly labeled 12x75 mm glass test tube

(Be sure serum layer does not thaw before pouring off)

7. Discard 13x100 mm test tube and serum layer
8. Dry down petroleum ether under a steady stream of nitrogen gas
9. Reconstitute by adding 150 µl working conjugate to evaporated samples
10. Vortex 8-10 seconds

Running the Assay

1. Reconstitute standards and samples as mentioned above and have ready for use
2. Invert coated plate and wash 5x with wash solution
3. Dry plate by firmly tapping upside down on paper towel
4. Pipette 50 µl EIA buffer across entire plate
5. Pipette 50 µl reconstituted standards in quadruplicate in columns 3-10
6. Pipette 50 µl reconstituted samples in duplicate in columns 3-10
7. Pipette 50 µl working conjugate across entire plate
8. Cover plate and seal tightly with acetate plate sealer
9. Incubate 1 hour and 45 minutes at room temperature
10. Invert plate and wash 5x with wash solution
11. Dry plate by firmly tapping upside down on paper towel
12. Pipette 100 µl ABTS substrate across entire plate
13. Cover plate and allow to incubate for 45 minutes at room temperature
14. Pipette 100 µl stop solution across entire plate
15. Read plate at 415 nm on plate reader

Standards and Samples per direct (non-extracted) method:

Preparation of standards

1. Collect and pool approximately 200 ml gelding or ovariectomized mare serum
2. Add 1.0 mg of progesterone to 10 ml EtOH [A]
3. Add 100 µl [A] to 900 µl pooled serum [B]
4. Add 200 µl [B] to 1800 µl pooled serum [C]
5. Add 1.0 ml [C] to 49 ml pooled serum = 20 ng/ml [D]
6. Add 25 ml [D] to 25 ml pooled serum = 10 ng/ml [E]
7. Add 25 ml [E] to 25 ml pooled serum = 5.0 ng/ml [F]
8. Add 25 ml [F] to 25 ml pooled serum = 2.5 ng/ml [G]
9. Add 25 ml [G] to 25 ml pooled serum = 1.25 ng/ml [H]
10. Add 25 ml [H] to 25 ml pooled serum = 0.625 ng/ml [I]
11. Add 25 ml pooled serum = 0 ng/ml [J]
12. Aliquot standards [D-J] into 1250 µl in micro-centrifuge tubes, freeze

Running the Assay

1. Pipette 1.0 ml of standards [D-J] and samples into 12x75 mm glass test tubes
2. Pipette 500 µl 1 N HCl into each test tube
3. Vortex for 20 seconds
4. Pipette 500 µl EIA buffer into each test tube
5. Vortex for 20 seconds
6. Invert coated plate and wash 5x with wash solution
7. Dry plate by firmly tapping upside down on paper towel
8. Pipette 50 µl EIA buffer across entire plate

9. Pipette 10 µl of standards in quadruplicate in columns 3-10
10. Pipette 10 µl of samples in duplicate in columns 3-10
11. Pipette 50 µl of working conjugate across entire plate
12. Cover plate and seal tightly with acetate plate sealer
13. Incubate 1 hour and 45 minutes at room temperature
14. Invert plate and wash 5x with wash solution
15. Pipette 100 µl ABTS substrate across entire plate
16. Incubate 45 minutes at room temperature
17. Pipette 100 µl stop solution across entire plate
18. Read plate at 415 nm on plate reader

APPENDIX III

Contents and protocol for cuvette progesterone assay for use in the ARS Densimeter:

Contents:

- 2 mL cuvettes coated with antibody and stabilized
- Plastic test tubes with caps
- Solution A (Contains acidic solution – CAUTION)
- Solution B (EIA buffer)
- Solution C (Conjugate)
- Solution D (TMB Substrate)
- Solution E (Wash)
- Zero Calibration Cuvette

Protocol:

1. Collect blood sample
    a. If collected in blood collection tube without anticoagulant, allow sufficient time to clot before proceeding to Step 2
    b. If collected in blood collection tube containing anticoagulant, proceed immediately to Step 2
2. Centrifuge blood sample and remove serum; discard remainder of sample appropriately

3. Add 1.0 mL serum to a plastic test tube
4. Add 500 µl of "Solution A", (1 N HCl), to the plastic test tube containing serum sample, mix well (gently invert 5 to 7 times)
5. Add 500 µl of "Solution B", (EIA buffer), to plastic test tube, mix well (gently invert 5 to 7 times)
6. Wash coated Cuvette 5x with "Solution E", (Wash Solution)
7. Add 100 µl of "Solution B", (EIA buffer), to washed Cuvette
8. Add 20 µl from plastic test tube mixture to Cuvette
9. Add 100 µl of "Solution C", (Conjugate), to Cuvette
10. Cap Cuvette and incubate 1 hour and 45 minutes at room temperature
11. Empty Cuvette and wash 5x with "Solution E", (Wash Solution)
12. Add 1.4 ml of "Solution D", (TMB Substrate), to Cuvette
13. Cap Cuvette and incubate exactly 30 minutes at room temperature
    a. Note: Shorter or longer incubation periods will result in calculation of erroneous values
14. After incubation, gently invert cuvette 5 to 7 times to make color change homogeneous.
15. Place the Zero Calibration Cuvette in Densimeter and Press "Zero"
16. When asked to "Add Sample", place test Cuvette in Densimeter and Press "Count"
17. Concentration of Progesterone will be displayed in ng/ml

Coating of cuvettes and stabilization

1. Thaw one 100 µl aliquot of 1:10 antiserum
2. Dilute in coating buffer to required concentration (1:5,000 for lot used)
3. Coat cuvettes with 200 µl antibody dilution
4. Tap cuvette gently to distribute antiserum evenly over bottom of cuvette
5. Cap cuvettes and incubate for 48 hours at 4°C
6. Uncap cuvette, invert and wash 5x with wash solution
7. Pipette 200 µl StabilCoat® into cuvettes
8. Incubate 30 minutes at room temperature
9. Empty cuvettes by inversion and tap firmly on paper towel
10. Dry completely in oven at room temperature and less than 15% humidity

    Place in oven with pan of dessicant rocks on bottom to absorb moisture

    (Temperature and humidity were logged and are shown in Figure 4.2)

11. Cap cuvettes and package with dessicant pack

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