AN ABSTRACT OF THE THESIS OF

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Title: Development of an Enzyme Immunoassay Using Whole Plasma to Determine Progesterone Concentrations During Early Pregnancy in the Mare.

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A modified solid phase competitive enzyme labelled immunoassay (ELISA) for progesterone using whole plasma is described. Microtitre plates were coated with a 1:5000 dilution of rabbit anti-progesterone-11-BSA and stored at 4 C for approximately 12 hours. After the plates were washed, progesterone standards diluted in whole plasma and whole plasma samples were added, followed by the addition of a 1:20,000 dilution of progesterone-3-O-carboxymethyloxime horseradish peroxidase as the steroid enzyme conjugate. Plates were covered and incubated for 2 h at room temperature. Following incubation and washing, the substrate, 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulphonic acid) was added and allowed to react for approximately 45 min to 1 h. Plate absorbance was read at 405 nm. The sensitivity of the assay was 19.5 pg/well or .391 ng/ml, with the standard curve usable to 25 ng/ml. Progesterone of 5, 10 and 15 ng/ml were added to whole plasma to determine

recovery, which was linear (y = -.062 + .984x). Intra- and interassay coefficients of variation for low and high (5 and 15 ng/ml) progesterone were 4.4, 3.5 and 9.1, 15.5, respectively. Of nine structurally related steroids tested for cross reactivity, 5α-pregnane-3,20-dione (5αDHP) reacted at 23.9 % and pregnenolone at .5 %. All other steroids did not significantly cross react (<.1%). A commercially available radioimmunoassay (RIA) was used to test standards diluted in human serum, charcoal extracted or equine whole plasma. A one-way analysis of variance followed by least significant difference (LSD) indicated that standards diluted in charcoal extracted plasma measured progesterone concentrations lower ($P<.05$) than human serum or whole plasma.

Normal pregnant mare samples were assayed using standards diluted in charcoal extracted or whole plasma. Plasma progesterone measured using standards diluted in charcoal extracted plasma were low and inconsistent with other reports. A paired t-test indicated that there was a difference in progesterone concentrations when using standards diluted in charcoal extracted or whole plasma ($P<.05$). Results by analysis of variance indicated a difference in plasma progesterone over time, using standards diluted in charcoal extracted or whole plasma ($P<.05$). These results indicated that treatment of standard and sample plasma must be the same. Overall, plasma progesterone concentrations using standards diluted in whole plasma were similar to values reported using RIA and competitive binding radioassay methods.

Plasma progesterone was measured from four normal pregnant mares from

Day 0 to 200 using standards diluted in whole plasma. The levels and pattern were similar to those previously reported. Plasma progesterone was also determined from two mares treated with 5αDHP and one mare with prostaglandin-$F_2\alpha$ ($PGF_2\alpha$). Progesterone decreased to 0 ng/ml within 48 h following luteolysis with $PGF_2\alpha$. Plasma progesterone decreased one and two days following $PGF_2\alpha$ in both mares treated with 5αDHP but never reached 0 ng/ml. This was presumably due to the cross reactivity of 5αDHP.

Development of an Enzyme Immunoassay Using Whole Plasma to Determine Progesterone Concentrations During Early Pregnancy in the Mare

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Most of all, a special thank you to Mom and Bob. The road was long and bumpy, but you were there every step of the way supporting me, believing in me and encouraging me. I would never have made it this far without you!

To those of you starting graduate school or struggling to finish, I would like to leave you with these thoughts:

" When things go wrong as they sometimes will,
When the road you're trudging seems all up hill,
When the funds are low, and the debts are high,
And you want to smile, but you have to sigh,
When care is pressing you down a bit,
Rest if you must, **but don't you quit.**
Success is failure turned inside out,
The silver tint of the clouds of doubt.
And you never can tell how close you are,
It may be near when it seems afar.

So, stick to the fight when you're hardest hit-

It's when things go wrong

that **you mustn't quit"**.

author unknown

Table of Contents

List of Figures

Figure | Page

List of Appendix Figures

List of Tables

Development of an Enzyme Immunoassay Using Whole Plasma to Determine Progesterone Concentrations During Early Pregnancy in the Mare

INTRODUCTION

Plasma progesterone during the estrous cycle and pregnancy was first measured by Short (1959) using ultraviolet absorption technique. Because of the limited detection of this technique, other methods for measuring progesterone such as radioimmunoassay (RIA) and enzyme labelled immunosorbent assay (ELISA) were developed. Due to the concerns associated with the use of RIA such as handling and disposal of radioactive material, the development of an alternate method that would be equal to or superior to RIA was attempted.

The ELISA was first introduced in the field of endocrinology in the early 1970's. Since then, it has become increasingly popular in many disciplines. Enzyme immunoassay systems have been developed for measuring progesterone in plasma and milk of many domestic species. Some of the ELISA's utilize whole plasma, but many still require ether extraction of samples. Enzyme immunoassay systems specifically developed (and validated for use in horses) to measure plasma progesterone concentrations in the horse appear to be few. A commercially available enzyme immunoassay kit (Enzygnost Serum Progesterone test kit, distributed by Hoechst-Roussel Agri-Vet company, Somerville, NJ) which has been validated for measuring plasma progesterone in the horse, is not always easily obtained and more expensive when assaying

large quantities of samples for research purposes. Seeger et al. (1979) developed an ELISA for progesterone in horse plasma. The assay appeared to measure progesterone concentrations similar to those measured by RIA, but was less sensitive and involved extraction of samples. Bosch et al. (1978) developed an ELISA for measuring total estrogens in serum or plasma during pregnancy. The results were similar to those obtained by RIA, but also involved sample hydrolysis and dilutions to avoid interference of plasma factors.

The goal of this thesis work was; 1) To adapt a workable ELISA for our laboratory in which whole plasma is used, eliminating the cumbersome process of ether extraction; and, 2) To measure progesterone concentrations of whole plasma samples from mares during different physiological states such as early pregnancy (ovulation to Day 200) and following treatment of 5αDHP and $PGF_2\alpha$. This would demonstrate that the assay is measuring plasma progesterone concentrations consistent with those reported in the literature.

LITERATURE REVIEW

Introduction

The mare is known as a long-day polyestrous seasonal breeder with fertile estrous cycles in the spring and summer (Hines, 1987). The gestational length of the mare is approximately 11 mo or 335 to 345 d. Due to this long gestational period and the arbitrary assignment of January 1 as the birth date for foals born within a year, a demand has been stimulated by the equine industry for advancing the onset of the natural breeding season (Hart et al, 1984). In northern latitudes, reproductive cyclicity occurs from early May to October (Ginther, 1974). Research in the last two decades has led to the acceptance of photoperiod as the primary stimulus for the regulation of seasonal reproduction in birds and mammals (Hart et al., 1984). Photoperiod is the main factor that influences the mare's reproductive cycle. Maximum and minimum ovulatory activity coincides with the time of long and short daily photoperiods, respectively (Freedman et al., 1979). Sharp and Ginther (1975), and Kooistra and Ginther (1975), showed that the onset of the ovulatory season in pony mares can be hastened by approximately two months by exposing mares during the anovulatory season to spring environmental conditions of artificial long daily photoperiod. Oxender et al. (1977), demonstrated that a 16 h photoperiod exposure to anestrous mares in December caused normal cycles to begin in two months.

Tissue Control of Reproductive Activity

The four tissues that play a vital role in controlling reproductive processes are the pineal gland, hypothalamus, pituitary, and gonads.

The pineal gland, located above the thalamus and attached to the roof of the third ventricle, is largely responsible for controlling reproductive seasonality (Hines, 1987). The pineal acts as a mediator between the light receptors and the hypothalamic-pituitary axis, by converting a neural input to a hormonal output (Ginther, 1979). Light enters the retina of the eye and travels to the pineal via a neural pathway. As the mare perceives increasing photoperiod, secretion of the antigonadal factor melatonin, produced by the pineal, is inhibited, which allows an increase in gonadotropic releasing hormone (GnRH) from the hypothalamus. This releasing hormone (GnRH) travels to the anterior pituitary via the portal system to activate an increase in gonadotropic hormone release from the anterior pituitary. The gonadotropic hormones released from the anterior pituitary travel through the general circulation to the ovaries and stimulate their function.

The hypothalamus through the action of GnRH, which is released in a pulsatile manner, controls the release of the gonadotropins follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Hines, 1987). The hypothalamic content and secretion of GnRH is decreased during anestrus compared to the breeding season (Johnson, 1987). Although the secretion of GnRH varies with

season, the total GnRH receptor number in the pituitary remains unchanged (Johnson, 1987). This would indicate that seasonal anestrus is the result of decreasing amounts of GnRH released from the hypothalamus and not due to pituitary responsiveness to GnRH.

Two important hormones released from the anterior pituitary are LH and FSH. These are glycoprotein hormones each containing two subunits, α and ß, in which the α subunits are similar within species but with differing ß subunits. It is the ß subunits that regulate the binding of the hormone to various target cells and define the biological function of each hormone. In comparison to other species, a pulsatile release of LH and FSH are not usually found to occur in the mare. However, reports by Irvine (1984) and Fitzgerald et al. (1985) have indicated that LH and FSH may be released in a pulsatile manner. Equine LH differs from the LH of other species in that it contains a large amount of sialic acid (6-7%), which renders unusual properties to the LH molecule (Irvine, 1984). This high amount of sialic acid allows the LH molecule to interact with or locate target molecules in the gonadal (ovarian) cells and contributes to the high biological potency and long circulatory halflife of 5 h. This is in comparison to human LH halflife of 2 h (sialic acid 2%) and ovine LH halflife of 20-30 min (no sialic acid). The FSH molecule from all species has 5-7% sialic acid and about the same halflife as each corresponding LH halflife (Irvine, 1984).

There are several unique properties concerning LH in the mare as reported

by Irvine (1984). First, the preovulatory phase of the LH surge is prolonged encompassing 6 d, in comparison to 1 d in other species. Secondly, LH levels continue to rise 1-2 d postovulation instead of declining up to 24 h before ovulation, as in most species. Thirdly, LH levels decline slowly to baseline levels. Therefore, in the mare, the function of LH is similar to other species except that there is a very slow rise which extends follicular maturation and the length of estrus. The rate of FSH and LH release are regulated by the amount of GnRH that is released from the hypothalamus, which varies with season. Alexander and Irvine (1986) found that the amount of LH released in response to a similar dose of GnRH was similar in anestrus compared to cycling mares. Thus, the pituitary seems capable of releasing LH and/or FSH, but the decreased release of GnRH depresses pituitary release of FSH and LH.

Estrous Cycle and Ovulation

The length of the estrous cycle in the mare is approximately 21 d with estrus lasting 6 d and diestrus 15 d. On the average, the estrous cycle is two days longer in ponies than in horses (Ginther, 1979). In one published study by Ginther (1979), the length of the interovulatory interval was 22.7 ± 0.7 d (mean ± SEM) for horses and 25.0± 0.6 d for ponies.

Ovulation usually occurs 24 h before the end of estrus. A distinctive feature of ovulation in the mare, compared to that in other species, is the

occurrence of ovulation from a specialized area, the ovulation fossa. The cortical and noncortical areas of the adult mare's ovary is unusual in comparison to other species. One would normally expect the medullary region of the ovary to be the soft region in the center of the ovary and the cortical region to be the outer portion. However, this is not true of the equine ovary. The ovary of the adult mare is structured so that the medullary region is superficial and the cortical region, which contains the follicles (parenchyma), is in the interior of the organ. The parenchyma reaches the surface only at the depression (ovulation fossa) from which normal ovulation occurs. Because ovulation occurs inward, it is more difficult to detect the corpus luteum by rectal palpation. Ovulation occurs due to a reduction in the strength of the rupturing point (ovulation fossa) by proteolytic enzymes (Ginther, 1979). The hormonal stimulant that most likely allows ovulation to occur is LH. Right after ovulation takes place, a structure known as the corpus hemorrhagicum, forms. The corpus hemorrhagicum is a fluid filled (primarily blood) structure that precedes the formation of the corpus luteum or CL also known as a "yellow body". The CL is the primary source of progesterone secretion and very necessary for the maintenance of early pregnancy in the mare.

Endocrinology of Pregnancy

After fertilization has occurred, the conceptus takes approximately 4 to 5 d to travel down the oviduct into the uterus. At this time the conceptus is completely mobile and is free to migrate between the right and left uterine horns. Maternal recognition of pregnancy occurs between Day 14 and 16, as reported by Hershmann and Douglas (1979), but may be as early as Day 11 to 13 (Betteridge et al., 1985; Goff et al., 1987). According to Hershmann and Douglas (1979), blastocyst removal on Day 16 after ovulation resulted in pseudopregnancy characterized by a prolonged interestrous interval, prolonged interovulatory interval and the maintenance of uterine tonicity and continued progesterone production. Kooistra and Ginther (1976) found that removing equine embryos surgically on Day 24 resulted in pseudopregnancy and the continued secretion of progesterone. These results indicated that the cyclic life-span of the CL is not affected by the presence of the blastocyst within the mare's uterus until after Day 14 postovulation.

Fixation of the equine blastocyst usually occurs at approximately Day 15 of gestation (Kastelic et al., 1987). The fetal placenta is composed of three membranes (amnion, chorion and allantois) and is described as being diffuse and of the nondeciduate type with superficial villous attachments. True microvillous attachment of the placenta to the uterine endometrium begins at approximately Day 35 and is completed by Day 150 of gestation.

One specialized feature of the mare is the formation of endometrial cups. The cups are of maternal and fetal origin, that appear as raised areas above the surface of the endometrium. Formation of the cups are apparent by Day 35 and begin to secrete a hormone, pregnant mares' serum gonadotropin (PMSG), also known as equine chorionic gonadotropin (eCG), unique to the mare. Pregnant mares serum gonadotropin has primarily FSH activity, peaking in secretion by Day 60 and virtually undetectable by Day 120 to 150 (Evans et al., 1990).

The two steroid hormones estrogen and progesterone are present at various concentrations in the plasma of mares during the estrous cycle and pregnancy. Allen and Hadley (1973) reported plasma progesterone concentrations during the estrous cycle to increase on Day 2 postovulation and to be maintained on a plateau from Day 5 to 14. Diestrus progesterone concentrations varied from 6.4 ng/ml to 13.4 ng/ml which occurred between Days 5 and 14. Within 2 to 3 days thereafter, levels dropped to approximately 1 ng/ml. If conception occurred, maximal plasma progesterone concentrations were found to range from 9.4 ng/ml to 17.4 ng/ml from Day 7 to 17 postovulation. Sato et al. (1977) reported progesterone concentrations in cycling mares to rapidly increase between Day 0 to Day 3 postovulation (4.5 ng/ml), reach maximal levels between Day 7 and 15 (7.8 ng/ml), and then fall sharply. Progesterone concentrations remained elevated after Day 15 if the mares were pregnant.

Holtan et al. (1975) measured progesterone concentrations during

Holtan et al. (1975) measured progesterone concentrations during pregnancy by competitive protein binding radioassay. Progesterone concentrations were found to increase from 1.1 ± .4 ng/ml on Day 0 to 7.5 ± 1.2 ng/ml on Day 8, declining to 4.8 ± .4 ng/ml on Day 28. From Day 28 to 44 progesterone increased, reaching a maximal concentration of 15.2 ± 1.4 on Day 64, then decreased and remained between 1 and 2 ng/ml from Days 180 to 300. During the last 30 days of gestation, progesterone increased to 4.4 ± 1.1 ng/ml 5 days prepartum, and then dropped to .5 ng/ml 1 to 3 days postpartum. Ginther (1979) summarized the following changes: Days 0-8, progesterone concentrations increase similar to the estrous cycle; Days 9 to 27, slight decrease; Days 28 to 44, increasing; Days 56 to 120, the greatest concentration; Days 120 to 180, decreasing; Days 180 to 300, very low concentrations; and the last thirty days, slight increase. After parturition, progesterone concentrations decrease only after delivery of the placenta as described by Holtan et al. (1975).

Nett et al. (1973) have shown that plasma estrogens start to increase approximately by Day 45 of gestation and increase at a rapid rate beginning by Day 90. Estrogen concentrations continue to rise until Day 210 with maximal concentrations found at Day 240. This pattern coincides with urinary estrogen concentrations as described by Cole and Saunder (1935). The concentrations of estrone (E1), equilin and equilenin remained below 20 pg/ml from breeding to Day 80 of gestation and then increased to 828 ± 151 pg/ml at Day 210 and

declined until parturition. The concentration of estradiol-17ß (E2) remained below 15 pg/ml until Day 90, increased to 71 ± 18 pg/ml at Day 240, declined until Day 300 and remained unchanged until parturition (Nett et al., 1973).

Progesterone Sources

Progesterone derived either from the CL, placenta or both, have been shown to be essential for the maintenance of pregnancy in every species (Sitteri et al., 1977). As reviewed by Gomes and Erb (1965), maternal ovaries with functional CL are necessary throughout gestation in the rabbit, sow, goat, cow and bitch, but for a much smaller proportion of pregnancy in the ewe, primate, guinea pig, and mare, with the latter species deriving their progesterone from the placenta. The essential role of the CL was first discovered by Frankel in 1903 as reported by Ginther (1979). The CL is formed at the site where ovulation occurred. Burns and Fleeger (1975) reported plasma concentrations of progesterone, produced by the primary CL to reach 13 ng/ml by Day 5 postovulation and persist until Day 30. Sato et al. (1977) reported mean progesterone concentrations in cycling mares to increase rapidly between Day 0 to 3 postovulation (4.5 ng/ml), were maximal (7.8 ng/ml) between Day 7 and 15, and then fell sharply. In pregnant mares, levels remain elevated after Day 15. According to Ginther (1979) the primary CL is maintained until approximately Day 160 to 180 of pregnancy, at which time it regresses. The

secondary CL, whether or not from an ovulatory or lutenized follicle, begin to form by Day 40, develop into functional luteal tissue and also regress with the primary CL by Day 180 (Ginther, 1979).

The placenta takes over progesterone production at some stage during the first half of gestation (Squires and Ginther, 1975). It is not known exactly when the ovarian source of progesterone is no longer primarily responsible for the maintenance of pregnancy, but early reports by Hart and Cole (1934) and Amoroso (1955) demonstrated that mares who were ovariectomized at Day 150 or 200 of gestation do not abort. More recently, Holtan et al. (1979) reported that the ovaries of the mare are dispensable between Day 50 and 70 of gestation. This was demonstrated by ovariectomizing mares between Days 50 and 70, of which 11 out of 20 mares maintained pregnancy, in comparison to all of the mares who were ovariectomized before Day 50 of gestation who either aborted or resorbed the conceptus. This time period is considerably earlier than reported by Hart and Cole (1934) and Amoroso (1955). It appears that the ovarian source of progesterone is critical up to Day 50 of gestation but that a nonovarian progesterone source is responsible for maintaining pregnancy after this time (Holtan et al., 1979). It was suggested by Short (1957) that the placenta or conceptus may be the nonovarian source of progesterone which is not established until after Day 45, as ovariectomized mares maintained with exogenous progesterone daily from Day 25 to 45 aborted following treatment.

Progesterone Metabolites

Three progestagen-like compounds have been detected during pregnancy and have been identified as 5α-pregnane-3,20-dione (5αDHP), 3ß-hydroxy-5α-pregnan-20-one (3ß-OH5αP), and 20α-hydroxy-5α-pregnan-3-one (20α-OH5αP) by Atkins, et al. (1974), and Holtan et al. (1975). Holtan et al. (1975) identified 5αDHP and 3ß-OH5αP and 20α-OH5αP which appear to be of feto-placental origin. Atkins et al. (1976) confirmed the identification of 5α-pregnane-3,20-dione in the peripheral circulation of the pregnant mare. Atkins et al. (1976) detected 5αDHP in the maternal circulation during early pregnancy (Day 1 to 30) at which time embryo contributions would not be expected to account for the elevated levels that were detected. Atkins et al. (1976) demonstrated that blood levels of progesterone and 5αDHP are similar in that they both decline from Day 10 to Day 40, and then increase. Following the development of the feto-placental unit (80 days), progesterone concentrations decline whereas 5αDHP concentrations either increase or are maintained. Seamans et al. (1979) reported 5αDHP levels to increase to a level of approximately 133 ng/ml 80 h before parturition and decreased to a level of 9.2 ng/ml in the postpartum period. Work done by Moss et al. (1979) demonstrated that progesterone and/or its metabolites are rapidly dispersed into both the maternal and fetal circulations. Tritiated progesterone was injected into mares after which blood samples were taken for 6 h from the uterine artery

and vein and umbilical artery and vein via catheters that were sutured into place. Blood samples were taken during the first 2 h after injection and the level of radioactivity associated with each pregnane determined. Results indicated that umbilical artery levels of 5αDHP are less than umbilical vein which most likely suggests that this compound is not formed in the fetus. Evidence for a placental source of 5αDHP included elevated levels of nonradioactive 5αDHP in plasma samples from the umbilical and uterine vein at 0 h. The inability to detect nonradioactive 20α-OH5αP at 0 h in umbilical plasma but with consistent elevation of radioactive 20α-OH5αP from uterine artery plasma, led to the conclusion that this pregnane is formed primarily from a maternal source. The level of radioactivity associated with 3ß-OH5αP in plasma from the umbilical artery was always greater than in plasma from the uterine artery and vein. Results from this work suggested that radioactive 3ß-OH5αP was formed primarily by the fetus. Holtan et al. (1991) detected 5αDHP and 3ß-OH5αP concentrations in plasma of normal pregnant mares which were similar to progesterone during early pregnancy (Day 10 and on), but remained elevated to term. These results support the suggestion that 5αDHP (Holtan et al., 1975; Atkins et al., 1976) and 3ß-OH5αP (Moss et al., 1979) are of maternal origin, at least during early pregnancy. Other pregnanes were first detected at 60 d and gradually increased throughout pregnancy, with a sharp increase the last 30 to 60 d. The predominate pregnane was 20α-OH5αP which was found to be one of two pregnanes (the other was 5α-

pregnane-3ß-20αdiol) that were higher than all other steroids from Day 170 to term. These progestagens in the latter stages of pregnancy, including 5αDHP and 3ß-OH5αP, appear to involve the feta-placental unit. The function of these progestagen-like compounds still remains unclear and further work needs to be done in identifying their biological activity and function during early and late pregnancy.

Methods for Measuring Progesterone

Progesterone concentration in the blood of mares during the estrous cycle and pregnancy was first measured by Short (1959), using an ultraviolet absorption technique. This technique had a limited detection of approximately 4 ng/ml, which was insufficient for measuring progesterone levels during some periods of estrus and pregnancy. Since then, quicker and more sensitive assay systems have been developed such as competitive protein binding assay (CPB), radioimmunoassay (RIA), and enzyme linked immunosorbent assay (ELISA).

The RIA and ELISA have proven to be accurate, reliable and quick methods for measuring progesterone and estrogens. However, specificity in the antibody is needed to eliminate false positives and cross reactivities (Villahoz et al., 1989). Polyclonal antibodies are currently being used, however, the results may be an overestimation of the steroid being measured (progesterone) due to increased level of cross reactivity. Both RIA and ELISA are sensitive

assays that can detect relatively small amounts of antigen and measure large quantities of samples rather quickly. Although sensitive and accurate, use of the RIA requires careful consideration over the ELISA due to such factors as: a) working with radioactive material; b) short shelf-life of reagents such as ^{125}I ; c) disposal of radioactive material; and, d) expensive equipment to measure radioactivity.

The ELISA was first introduced in the field of endocrinology in the 1970's. Since then, it has become increasingly popular. Enzyme immunoassay systems have been developed for measuring progesterone in plasma and milk of many domestic species. Some of the ELISA's utilize whole plasma, but many still require ether extraction of samples. Enzyme immunoassay systems specifically developed for measuring plasma progesterone in the horse appear to be few. Seeger et al. (1979) developed an ELISA for measuring plasma progesterone in the horse, which required ether extraction of plasma samples. Bosch et al. (1978) developed an ELISA for measuring total estrogens in serum or plasma during pregnancy in the horse. Results were similar to those obtained by RIA, but also involved extraction of samples to avoid interference of plasma factors. More recently, Munro and Stabenfeldt (1984) developed a direct ELISA for measuring plasma progesterone, but still involved ether extraction of samples. Although there is a commercial progesterone kit that has been validated for use in horses, it is not always easily obtained or economically feasible for research purposes.

MATERIAL AND METHODS

Animals

Normal mares. Four pony mares weighing approximately 250 kg and of unknown breeding origin were used. The animals were teased daily with a stallion to determine estrus and the ovaries examined every day by rectal palpation and ultrasound (Aloka 210-DX with a 5-MHz probe, Corometrics Medical Systems, Inc. Wallingford, CT) as described by Ginther (1986). Ovulation was defined as the disappearance of a large follicle (>35mm) and the subsequent appearance of CL tissue as detected by ultrasound. The mares were bred either naturally or by artificial insemination every other day while in estrus. All mares were examined with ultrasound starting on Day 10 for confirmation of pregnancy.

Treated mares. Three pony mares of unknown breeding origin and weighing approximately 250 kg were used. Handling of the animals, blood sampling and hormone analysis were the same as for normal mares. The treatment that these mares received was as follows: a) Mare 1 received 60 mg of 5α-pregnane-3,20-dione (5αDHP) i.m. (dissolved in corn oil) starting on Day 10 of pregnancy with 5 mg of $PGF_2\alpha$ given i.m. on Day 12; b) Mare 2 received the same treatment, but starting on Day 13 of pregnancy with $PGF_2\alpha$ given on Day 15. Treatment of 5αDHP was continued for both mares until loss of

pregnancy, which was confirmed by ultrasound. Mare 3 was used as a positive control, with $PGF_2\alpha$ given on Day 15 of pregnancy.

Blood Handling

Daily blood samples were collected by jugular venipuncture beginning on Day 1 of estrus through Day 2 postovulation, every other day until Day 15 of pregnancy, and then daily to Day 20. From Day 20 to 50 of pregnancy, blood samples were collected every other day, twice weekly from Day 50 to 80, and reduced to a weekly basis until parturition. The blood was withdrawn into evacuated heparinized tubes and immediately centrifuged. The plasma was removed into plastic vials and stored at -20C until hormone analysis.

ELISA

Reagents. The following reagents were purchased from Sigma Chemical Company (St. Louis, MO); bovine serum albumin, polyoxyethylene-sorbitan monolaurate (Tween 20), 2,2'-azino-bis(3-ethylbenz-thiazoline-6 sulfonic acid) (ABTS), citric acid, hydrogen peroxide, progesterone, 5α-pregnane-3,20-dione, 3ß-Hydroxy-5-pregnen-20-one,5-pregene-3ß,20ßdiol,3ß-hydroxy-5α-pregnan-20-one, 20α-hydroxy-5α-pregnan-3-one, 5α-pregnane-3ß,20ß-diol, 5α-pregnane-3ß,20α-diol, and cholesterol.

Disposable Immulon 4 flat-bottomed polystyrene 96-well microelisa plates (12.8 x 8.6 cm) and plate sealers (8.26 x 13.34) were purchased from Dynatech Lab Inc. (Chantilly, VA). A Titertek digital multichannel pipette and Titertek Multiskan Plus II automatic plate reader were both purchased from Flow Laboratories (Covina, CA). A commercially available radioimmunoassay, Coat-A-Count (for progesterone) was purchased from Diagnostic Products Corporation (Los Angeles, CA).

Stock solutions. The following stock solutions were used in the solid phase ELISA : 1) .05 M bicarbonate coating buffer, pH 9.6; 2) .1 M phosphate buffer saline, pH 7.0, contained .1 % bovine serum albumin (PBS-BSA); 3) wash solution contained .05 % polyoxyethylene-sorbitan monolaurate (Tween 20) and .15 M NaCl; 4) citrate buffer, pH 4.0; 5) substrate solution contained 1 ml 2,2-azino-bis(3-ethylbenz-thiazoline-6 sulfonic acid) (ABTS), 98.7 ml citrate buffer and 320 μl of an 8 M hydrogen peroxide solution and was made approximately 10 min before use.

Antibody. A polyclonal antibody, rabbit anti-progesterone conjugated with BSA at the 11 position, was a gift from Dr. G. Stabenfeldt (Univ. Cal, Davis, CA). An initial 1:10 dilution was made in .1M PBS-BSA with 100 μl aliquots stored at -70 C until assay.

Steroid enzyme conjugate. The enzyme-steroid conjugate used was progesterone-3-CMO-horseradish peroxidase, which was also a gracious gift from Dr G. Stabenfeldt. As with the antibody, an initial 1:10 dilution in .1 M PBS-BSA was made with 20 µl aliquots stored at -70 C until assay.

Standards and controls. A 1 mg/ml stock solution of progesterone in ethanol was first made, from which all standards and quality controls were made. The plasma that was used to dilute standards and controls was obtained from a mare in estrus. Dextran coated charcoal was added to plasma to remove free steroid, fat, fibrin and other components in plasma that may cause interference of progesterone binding. Standards were diluted in both charcoal extracted and whole plasma in halving dilutions which ranged from 25 to .391 ng/ml. Quality controls of 5, 10 and 15 ng/ml were made by adding known amounts of progesterone to plasma and assayed (in triplicate) on every plate. This was done to test the assay procedure to measure known added amounts of progesterone and to determine intra- and interassay variability.

Nonspecific binding. Nonspecific binding was determined by adding 50 µl of the following (in duplicate) to each plate during development of the assay; a) PBS-BSA only; b) PBS-BSA without antibody coating; c) PBS-BSA without enzyme conjugate; d) plasma only; e) plasma without antibody coating ; and, f) plasma without enzyme conjugate.

Sample dilution. If progesterone concentrations exceeded the range of the standard curve samples were diluted 1:10 and 1:5 with PBS-BSA or 1:5 with whole plasma.

Steroid cross reactivity. Stock solutions of 1 mg/ml of 9 steroids (listed in the reagent section) were made in methanol and from which log (1 to 10,000 ng/ml) dilutions in whole plasma were made. These steroids were assayed in triplicate to determine which steroids cross reacted with the antibody. Other related steroids such as cortisol, testosterone, estradiol-17ß, and androstenedione were initially tested by Munro and Stabenfeldt (1984).

Hormone analysis. Progesterone concentrations of normal and treated mares were measured by a modified solid phase (direct) competitive ELISA (Munro and Stabenfeldt, 1984) using whole plasma. Various dilutions of antibody and enzyme conjugate were tried during development of the assay. Standards diluted in buffer (PBS-BSA) were first used to determine which dilution of antibody and enzyme conjugate would produce a curve with an optimal slope. Since the purpose of developing this assay system was
to use plasma and avoid ether extraction, further development with buffer standards was eliminated. Standards diluted in charcoal extracted plasma and whole plasma were also tested at various antibody and conjugate dilutions.

Disposable Immulon 4 flat-bottomed polystyrene 96-well microelisa plates

were coated with 50 µl per well of a 1:5,000 dilution of BSA-adsorbed rabbit anti-progesterone in .05 M bicarbonate buffer pH 9.6. The plates were covered with plate sealers and stored at 4 C for approximately 12 h before assay. To remove excess unbound antibody, the plates were individually washed 5x with wash solution, inverted and blotted dry. Fifty µl of .1 M PBS-BSA was added to each well on the plate, followed by the addition of 50 µl whole plasma standards or sample (in triplicate), followed by an additional 50 µl of a 1:20,000 dilution of the steroid enzyme conjugate in .1 M PBS-BSA. Plates were covered and allowed to incubate for 2 h at room temperature. Following incubation, plates were washed 5x, inverted and blotted dry. One hundred µl of substrate solution was added to each well on the plate. After an incubation period of approximately 45 min, plate absorbance was read at 405 nm with a Titertek Multiskan Plus II automatic plate reader. A standard curve was calculated by plotting absorbance against log progesterone concentrations of standards, using a logistic curve-fitting program, Titersoft E.I.A. software version 2.0A (Flow Laboratories, Covina, CA).

Radioimmunoassay. A commercially available RIA was used to compare standards diluted in human serum, charcoal extracted and whole plasma. This was done to determine if the three types of standards were measuring plasma progesterone concentrations of whole plasma samples from mares during early pregnancy (Day 0 to 200) the same.

Statistical Analysis

Sample assay variability and quality controls were analyzed by conventional statistical programs (means ± sem, coefficient of variation, and linear regression). All other statistical analysis was done using SAS program (SAS Institute Inc., Cary, NC) with the exception of a t-test which was hand calculated to determine sensitivity of the assay. GLM-ANOVA with repeated measures was used to compare sample plasma progesterone concentrations of pregnant mares using standards diluted in charcoal extracted or whole plasma. A one-way ANOVA was used to test plasma progesterone concentrations from pregnant mares measured by RIA using standards diluted in human serum, charcoal extracted or whole plasma. Differences were tested with least significant difference (LSD). A paired t-test was done to test plasma progesterone concentrations of individual mares using standards diluted in charcoal extracted and whole plasma. A t-test was done to determine sensitivity of the assay using standards diluted in whole plasma (Zar, 1984).

RESULTS AND DISCUSSION

Assay

Standards initially diluted in buffer (PBS-BSA) were used to determine dilutions of antibody and enzyme conjugate that would produce an optimal standard curve. Results indicated that a 1:5,000 antibody dilution and 1:20,000 enzyme conjugate dilution were appropriate. Further development of the assay was accomplished using standards diluted in charcoal extracted plasma. It was determined early in development that buffer (PBS-BSA) must be added to each well on the plate prior to the addition of standard or sample. Prior to this step, a standard curve comparable to that using standards diluted in buffer (PBS-BSA) was not achieved. The addition of buffer appeared to decrease variability within and between assays.

Plasma in which standards were diluted was charcoal extracted to eliminate existing components in plasma such as free steroids, fat and fibrin that may interfere with progesterone binding to the antibody. An optimal standard curve was achieved using antibody and enzyme conjugate dilutions described for standards diluted in buffer. Figure 1 represents a standard curve utilizing standards diluted in charcoal extracted plasma. The average intra- and interassay coefficient of variation was 4.8 and 6.0, and 12.2 and 12.3, respectively. Quality control (0, 5, 10, and 15 ng/ml of progesterone added to plasma) results appeared to indicate that the assay was measuring known

added amounts of progesterone adequately (y = -.062 + .984x). However, when whole plasma samples were assayed using standards diluted in charcoal extracted plasma, results were low and inconsistent with other reports. Due to the low and inconsistent results using standards diluted in charcoal extracted plasma, whole plasma samples were reassayed using standards diluted in whole plasma. Figure 2 represents a standard curve using standards diluted in whole plasma. The readable portion of the standard curve ranged from .391 ng/ml to approximately 25 ng/ml. The sensitivity of the assay using standards diluted in whole plasma was 19.5 pg/well or .391 ng/ml which was determined by a t-test and defined as the first progesterone standard different from zero; $P < .05$. Munro and Stabenfeldt (1984) reported their assay to have a sensitivity of .25 pg/ml. Plasma progesterone concentrations less than 1 ng/ml are not usually found during early pregnancy. Therefore, a sensitivity of less than 1 ng/ml was not of importance and further attempts to increase sensitivity was not done. Whole plasma quality control (5, 10, and 15 ng/ml of progesterone) results indicated that the assay was measuring known added amounts of progesterone adequately (Figure 3). The intra- and interassay coefficient of variation for low and high progesterone concentrations (5 and 15 ng/ml) were 3.5 and 4.4 and 9.1 and 15.5, respectively.

Nine structurally related steroids listed in Table 1 were tested for cross reactivity. Compared to progesterone at 100 %, 5αDHP reacted at 23.9 % and 3ß-hydroxy-5-pregnen-20-one (pregnenolone) at .5 %. All other steroids listed

did not significantly react (<.1 %). Munro and Stabenfeldt (1984) reported 5αDHP to cross react at 29.5 %, 11α-hydroxyprogesterone at 21.4 % and 20ß-hydroxyprogesterone at 2.38 %. Two other progestins that were tested reacted at <.4 % and the steroids estradiol-17ß, androstenedione, cortisol, and testosterone reacted at <.1%.

Figure 4 compares charcoal extracted and whole plasma standards that were utilized to measure plasma progesterone concentrations of a pregnant mare from Day 0 (ovulation) to Day 200. Plasma progesterone concentrations measured using standards diluted in charcoal extracted plasma remained between 0 and 1 ng/ml from ovulation to approximately Day 40 of pregnancy. This is highly unlikely to occur in a pregnant mare and is inconsistent with other reports of plasma progesterone concentrations during this early pregnancy period. A paired t-test was done with individual normal pregnant mare samples and indicated that there was a difference in plasma progesterone concentrations when using standards diluted in charcoal extracted and whole plasma ($P<.05$). Results from analysis of variance with repeated measures indicated that there was a difference over time in plasma progesterone concentrations of normal pregnant mares when using standards diluted in charcoal extracted or whole plasma ($P<.05$). Overall, plasma progesterone concentrations using standards diluted in whole plasma were higher and consistent with plasma progesterone concentrations measured by radioimmunoassay and competitive binding radioassay as reported by Allen and

Hadley (1973) and Holtan et al. (1975).

The reason as to why charcoal extraction appeared to interfere with the measurement of plasma progesterone concentrations in the ELISA remains unclear. However, it appears that standards and sample must be treated the same for accurate results in the measurement of progesterone. This was also true for samples that exceeded the readable portion of the standard curve and were diluted and reassayed. The diluent used must be of the same matrix as the samples to be assayed. Progesterone concentrations ranged from 50 to 300 ng/ml when diluted with PBS-BSA and 50 to 76 ng/ml when diluted with whole plasma.

To further test and validate the use of whole plasma samples, a commercially available RIA was used to test human serum (provided with the kit), charcoal extracted or equine whole plasma as diluent for standards. From Table 2, it appears that there was no difference between human serum and whole plasma standards when used to measure progesterone concentrations of whole plasma samples. Results from a one-way analysis of variance indicated that there was a difference between sample progesterone concentrations measured using standards diluted in charcoal extracted plasma versus whole plasma or human serum ($P<.05$). Further analysis by least significant difference (LSD) indicated that progesterone measured by charcoal extracted plasma was lower ($P<.05$) than that measured by human serum or whole plasma (see Table 2). Correlation analysis of plasma progesterone

concentrations of fourteen pregnant mare samples assayed by both RIA and ELISA (using whole plasma standards) was highly significant ($P < .05$; $r = .79$).

In conclusion, the modified ELISA using equine whole plasma proved to be a convenient, accurate and quick method for measuring plasma progesterone concentrations during early pregnancy. The results of plasma progesterone concentrations using standards diluted in equine whole plasma were similar and consistent with previous reports. Despite a lowered sensitivity , the assay provides an adequate range of detection for progesterone during early pregnancy of the mare.

Normal mares

Individual plasma progesterone concentrations of four pregnant mares from Day 0 (ovulation) to Day 200 can be seen in Figures 5, 6, 7, and 8. It is apparent from these Figures that there is day to day variation in plasma progesterone concentrations within each mare and between mares. Although progesterone concentrations are different for each mare there appears to be a trend of increasing and decreasing progesterone concentrations at approximately the same time during pregnancy. The overall plasma progesterone concentrations of the mares were consistent with values previously reported.

From Day 80 to approximately 150 plasma progesterone concentrations

From Day 80 to approximately 150 plasma progesterone concentrations increase for each mare, peaking at levels that ranged from 10 to 20 ng/ml. This is normal and would be expected to occur due to accessory ovulations and the formation of secondary CL. However, of the four mares, one mare had exceedingly high plasma progesterone concentrations from Day 70 to 200 (Figure 8). These concentrations are also normal and presumably due to accessory ovulations and the formation of secondary CL.

Figure 9 represents mean plasma progesterone concentrations of these four normal pregnant mares from Day 0 (ovulation) to Day 200. Progesterone concentrations increased following ovulation to approximately 10 ng/ml, decreased and remained at approximately 6 ng/ml from Day 18 to 60. Starting Day 80, levels increased and peaked at approximately 28 ng/ml by Day 100. Standard error of the mean ranged from .175 to 8.0 up to Day 70 due to the one mare with much higher progesterone after Day 70, and from 9.0 to 27.0 from Day 72 to 200.

Since it has been reported by Atkins et al. (1976) that 5αDHP increases (along with progesterone after Day 40) and continues to increase or be maintained until approximately 80 h prior to parturition, plasma progesterone concentrations of all mares during this time may be overestimated due to the cross reactivity of 5αDHP (cross reactivity at 23.9 %).

Treated mares

The control mare (Figure 10a) which was given $PGF_2\alpha$ on Day 15 of pregnancy, appeared to completely resorb the embryonic vesicle within 48 h following luteolysis. Plasma progesterone increased to approximately 9 ng/ml on Day 8 postovulation then slowly declined to 6 ng/ml by Day 13. Within 48 h of $PGF_2\alpha$ injection, progesterone concentrations declined from 6 ng/ml to 0 ng/ml. This is consistent with results reported by Noden et al. (1978), in which progesterone concentrations of mares declined from 7.7 ± 1.1 ng/ml to .6 ± .1 ng/ml within 48 h after injection with $PGF_2\alpha$.

Pregnancy was not maintained after luteolysis with $PGF_2\alpha$ in either of the two mares treated with 5αDHP. The first mare (Figure 10b) which was given 5αDHP starting Day 10 of pregnancy with $PGF_2\alpha$ on Day 12, maintained pregnancy until an abrupt loss on Day 23 as confirmed by ultrasound. The second mare (Figure 10c) which was given 5αDHP starting on Day 13 of pregnancy with $PGF_2\alpha$ on Day 15, maintained pregnancy until Day 21. It should be noted however, that the embryonic vesicle appeared to become irregular in shape starting Day 17 and continued to change shape until complete resorption by Day 21. This irregular shape was not observed to have occurred in mare 1. Plasma progesterone concentrations in the mare which received 5αDHP starting Day 10 of pregnancy decreased to 3 ng/ml one day following $PGF_2\alpha$ injection. Levels were maintained at approximately 3 ng/ml and

appeared to increase at Day 22. Plasma progesterone concentrations of the mare which received 5αDHP starting Day 13 decreased to approximately 1 ng/ml two days following $PGF_2\alpha$ injection. Concentrations remained between 1 and 2 ng/ml until Day 20 and then appeared to increase. In both mares treated with 5αDHP, plasma progesterone levels did not fall to 0 ng/ml following luteolysis with $PGF_2\alpha$. Instead, plasma progesterone concentrations ranged from 1 to 4 ng/ml and appeared to increase after detection of the embryonic vesicle by ultrasound could not be confirmed.

The increase in plasma progesterone concentrations were most likely due to the cross reactivity of 5αDHP (which was determined to be 23.9 %) and/or incomplete luteolysis or subsequent estrus and ovulation. Atkins et al. (1976) found that during early pregnancy both progesterone and 5αDHP decline from Day 10 to 40 in a similar manner, after which time both progestins increase. In one study by Holtan et al. (1991), it was reported that plasma concentrations of 5αDHP during early pregnancy are similar to that of progesterone. It is only toward the end of 150 days gestation that progesterone alone declines and 5αDHP either continues to increase or is maintained.

In summary, it appeared that 5αDHP did not maintain pregnancy. Until the biological role of 5αDHP is determined, the question as to why pregnancy was not maintained remains to be determined. Due to the high levels of 5αDHP at the time of parturition as reported by Atkins et al. (1976) one may question the

biological role of 5αDHP in initiating parturition rather than in maintaining pregnancy. It appeared that 60 mg of exogenous 5αDHP is an inadequate amount for maintaining pregnancy, although an adequate amount of exogenous progesterone. In the future, it seems appropriate to at least test the effects of a higher exogenous dose of 5αDHP and the effects on pregnancy maintenance.

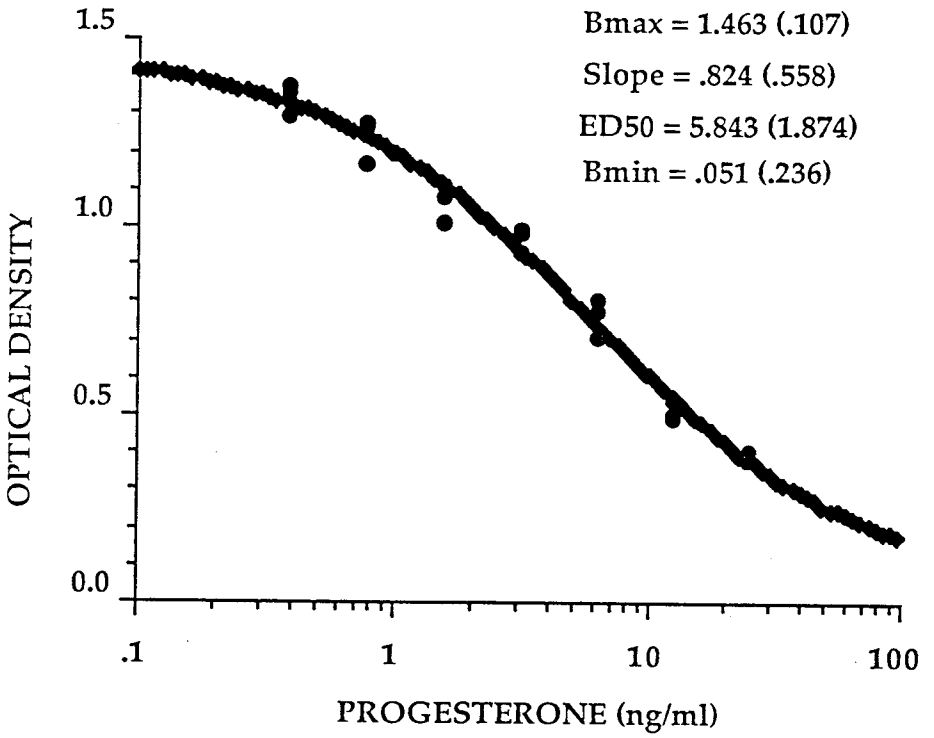

Figure 1. ELISA progesterone standard curve with standards diluted in charcoal extracted plasma, utilizing rabbit anti-progesterone-11-BSA coated plates and progesterone-3-CMO-horseradish peroxidase conjugate.

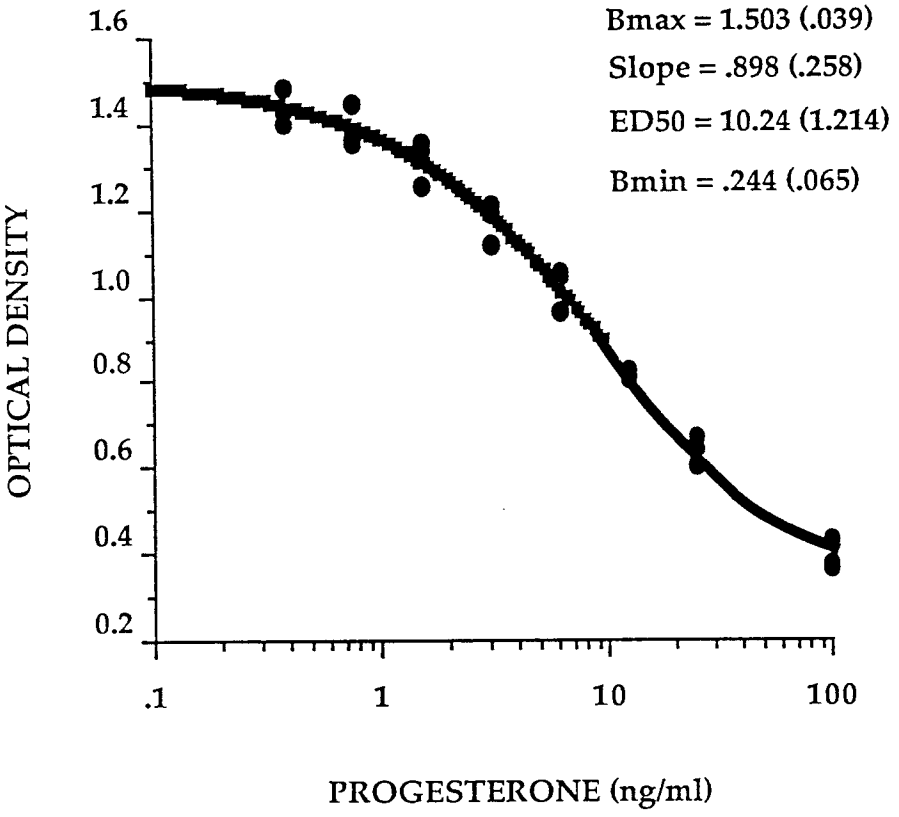

Figure 2. ELISA whole plasma standard curve utilizing rabbit anti-progesterone-11-BSA coated plates and progesterone-3-CMO-horseradish peroxidase conjugate.

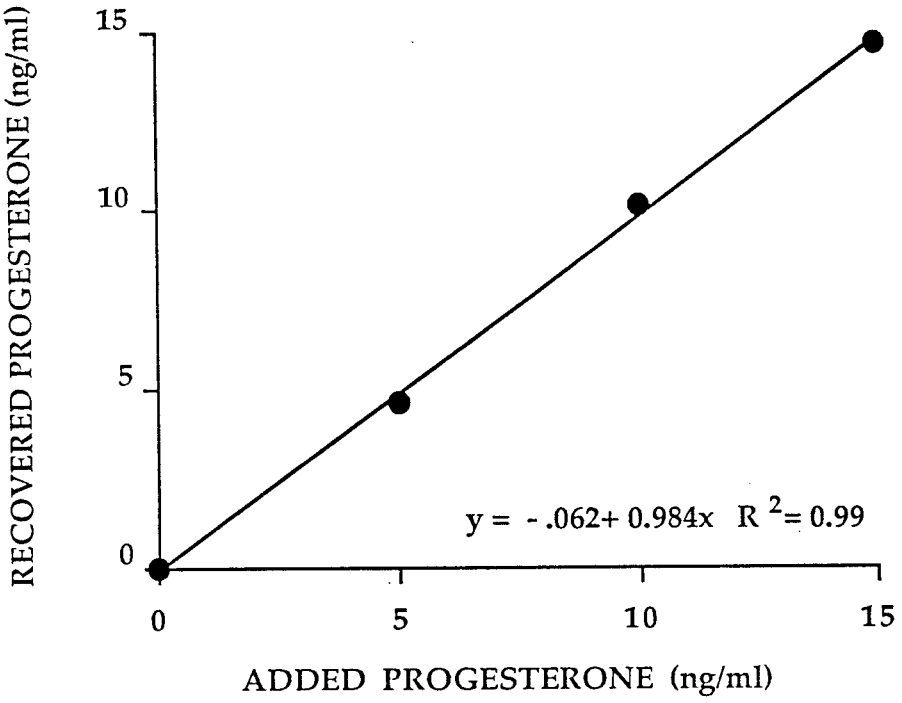

Figure 3. Regression of added and recovered progesterone in plasma quality controls of 5, 10 and 15 ng/ml using standards diluted in whole plasma.

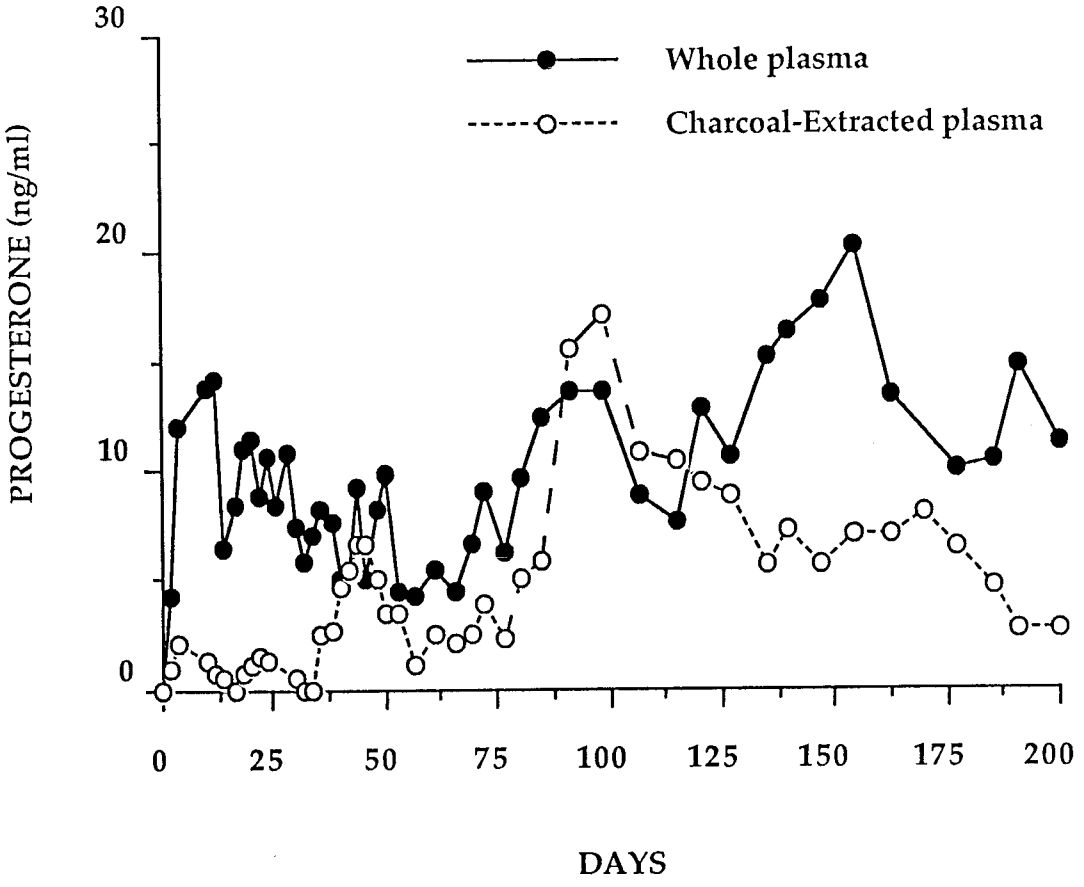

Figure 4. Comparison of progesterone standards diluted in whole plasma vs charcoal-extracted plasma when used to measure progesterone in plasma of a pregnant mare from Day 0 (ovulation) to Day 200.

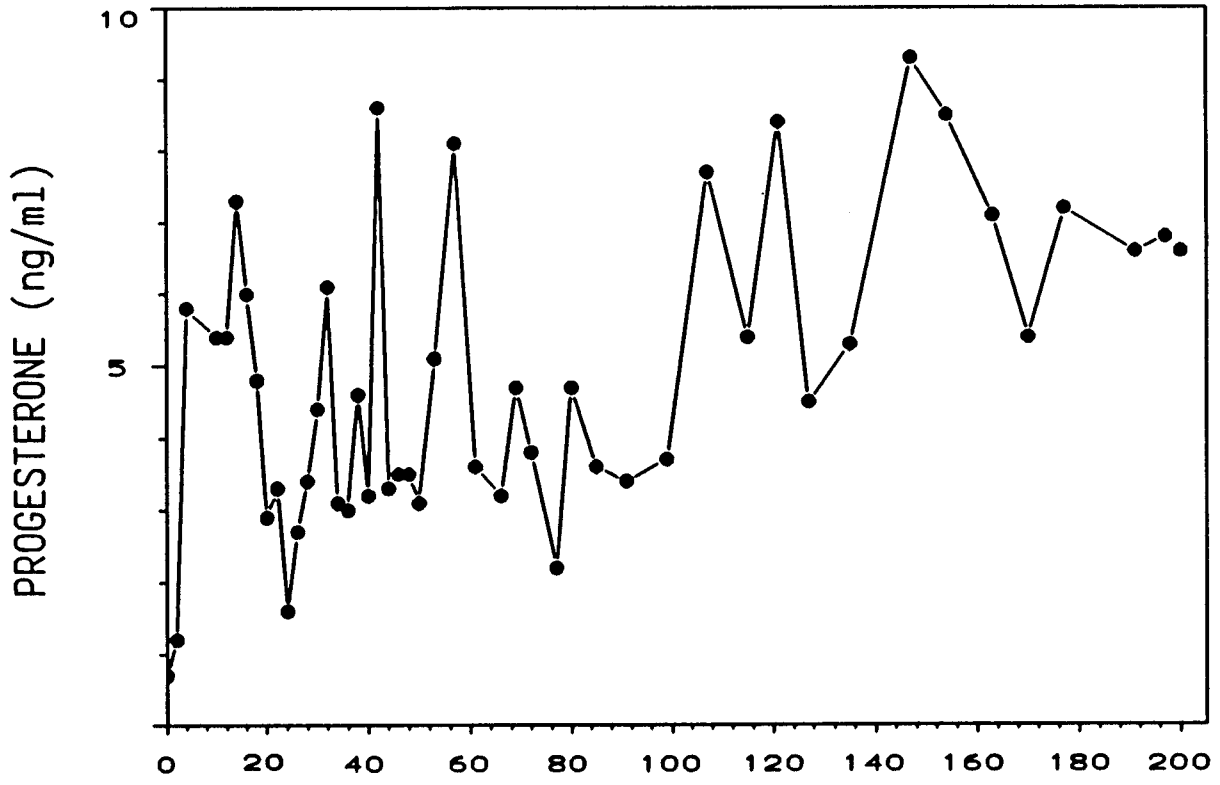

Figure 5. Plasma progesterone concentrations of a pregnant mare from Day 0 (ovulation) to Day 200.

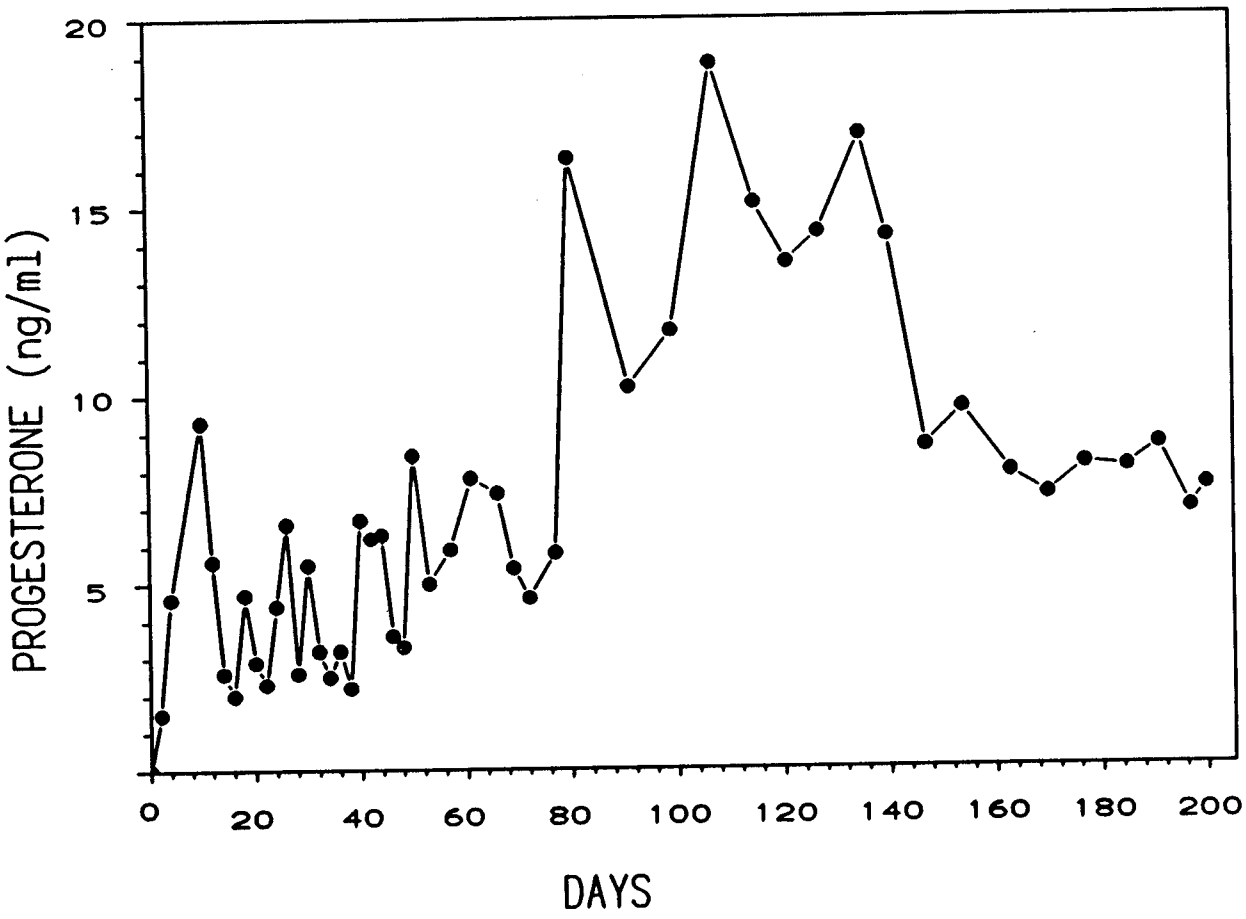

Figure 6. Plasma progesterone concentrations of a pregnant mare from Day 0 (ovulation) to Day 200.

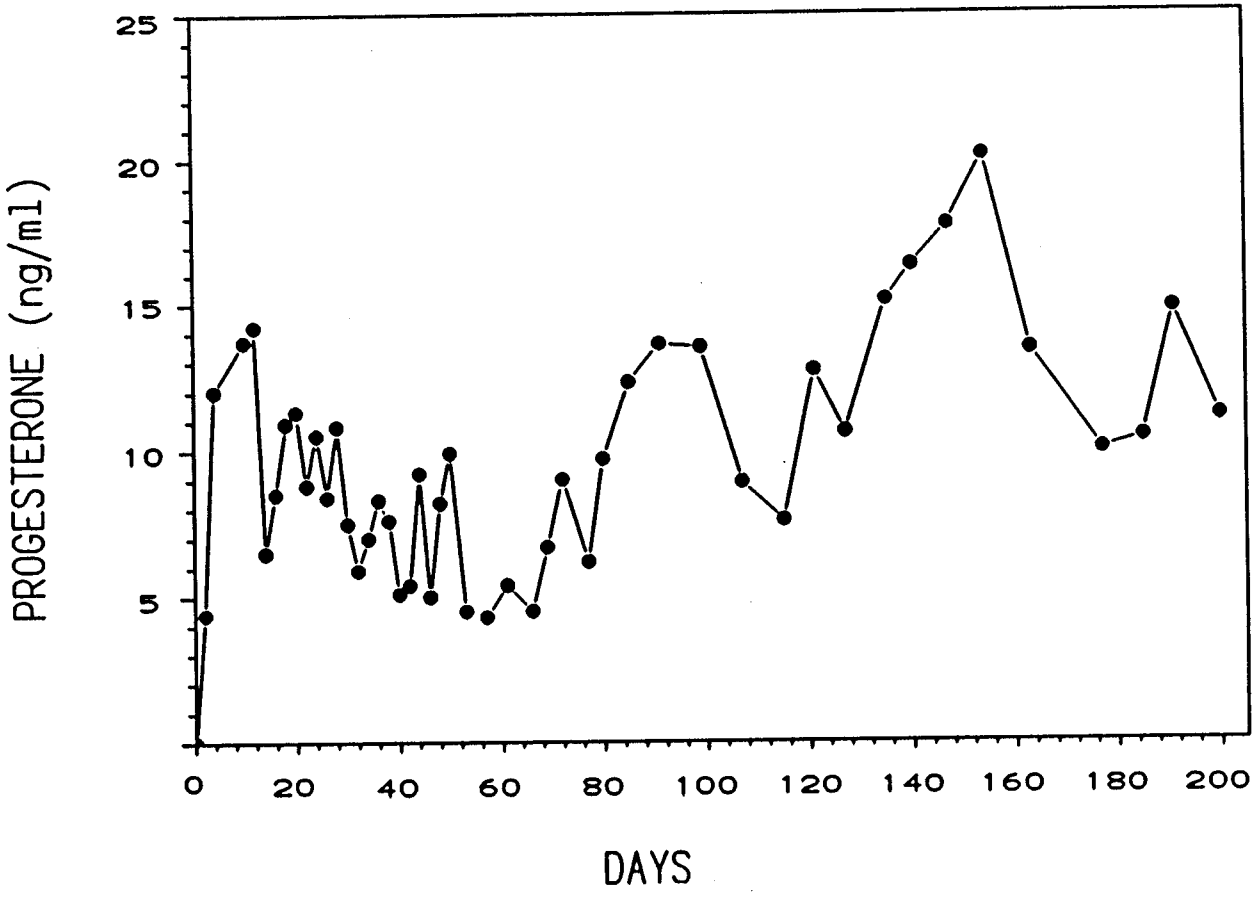

Figure 7. Plasma progesterone concentrations of a pregnant mare from Day 0 (ovulation) to Day 200.

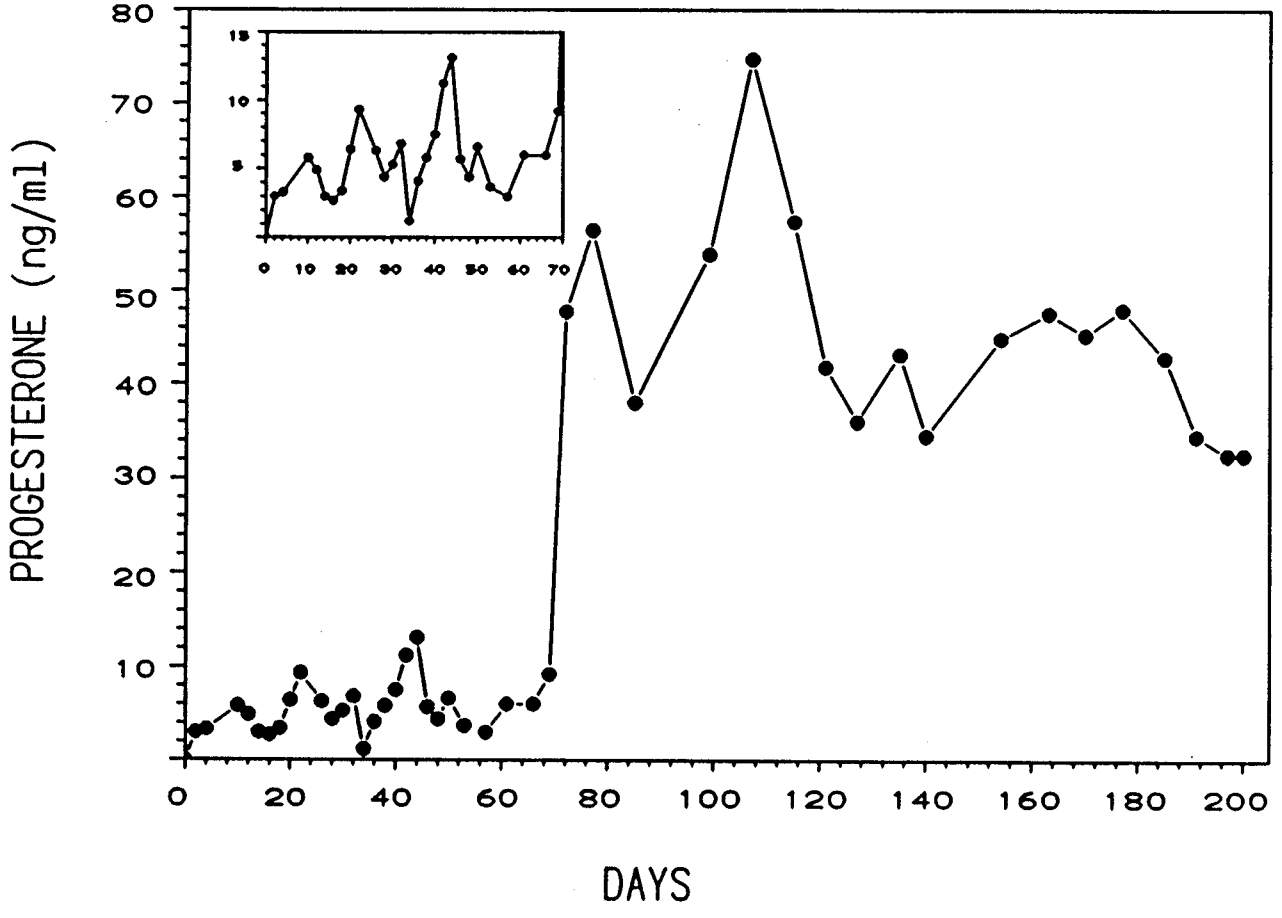

Figure 8. Plasma progesterone concentrations of a pregnant mare from Day 0 (ovulation) to Day 200. Note insert with expanded scale to Day 70.

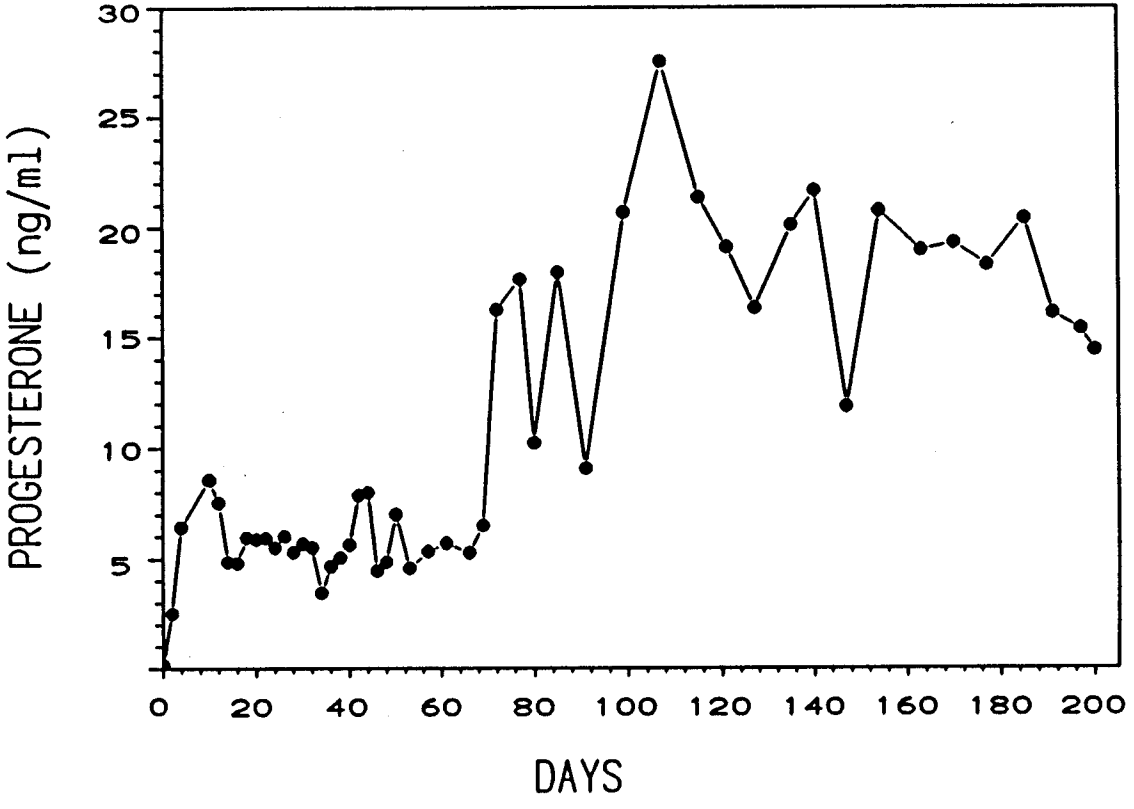

Figure 9. Mean plasma progesterone concentrations of four pregnant mares from Day 0 (ovulation) to Day 200. Standard error of the mean ranged from .175 to 8.0 up to Day 70, and from 9.0 to 27.0 from Day 72 to 200.

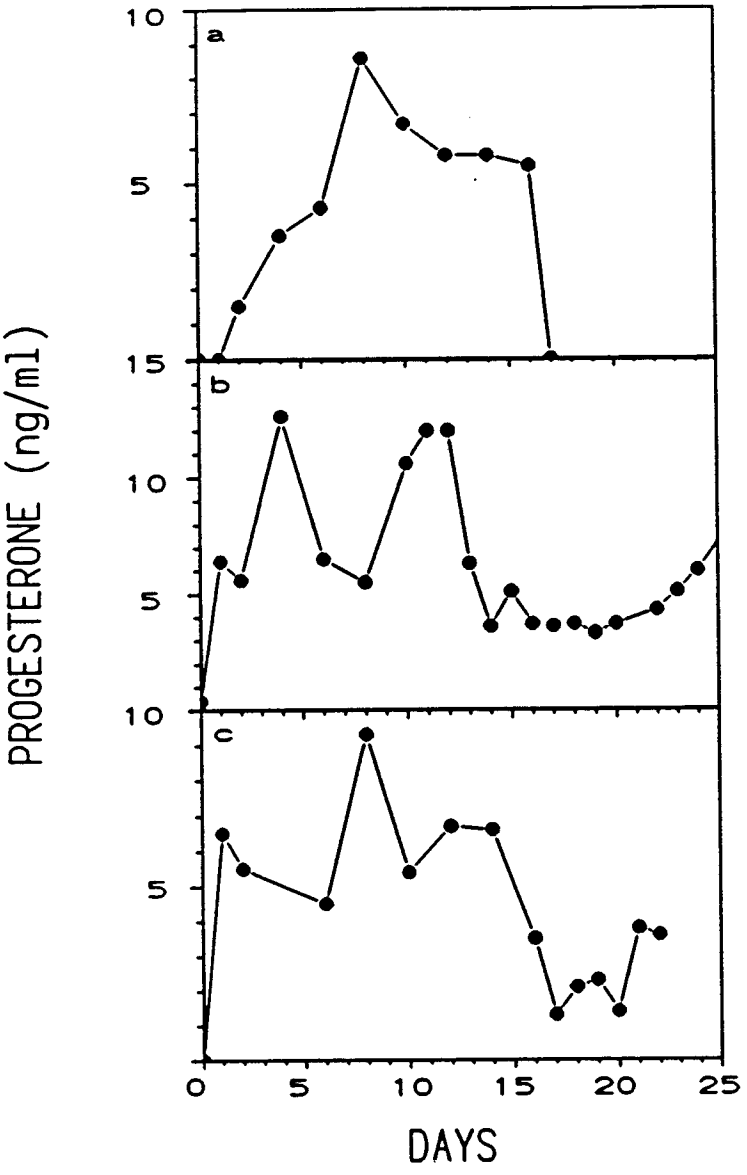

Figure 10. The effect of 5αDHP treatment on plasma progesterone and pregnancy maintanence of mares after luteolysis with $PGF_2\alpha$: a) mare treated with 5 mg $PGF_2\alpha$ Day 15; b) mare treated with daily injections of 60 mg 5αDHP starting Day 10 and 5 mg $PGF_2\alpha$ Day 12; c) mare treated with daily injections of 60 mg 5αDHP starting Day 13 and 5 mg PGF2α Day 15.

TABLE 1. TABLE OF CROSS-REACTIVITIES OF STRUCTURALLY RELATED STEROIDS.

Steroids	Cross-Reactivity (%) at 50% Binding
4-Pregnene-3,20-dione	100
5α-Pregnane-3,20-dione	23.9
3ß-Hydroxy-5α-pregnan-20-one	<.1*
3ß-Hydroxy-5-pregnen-20-one	<.5*
5α-Pregnane-3ß,20ß-diol	<.1*
5α-Pregnane-3ß,20α-diol	<.1*
5-Pregnene-3ß,20ß-diol	<.1*
20α-Hydroxy-5α-pregnan-3-one	<.1*
Cholesterol	<.1*

*Nonsignificant

TABLE 2. COMPARISON OF PROGESTERONE QUANTITATION (ng/ml) BASED ON COMMERCIAL RADIOIMMUNOASSAY UTILIZING DIFFERENT SOURCES OF PLASMA FOR STANDARDS.

	Plasma source		
sample	human	equine whole	equine charcoal extracted
1	.3	0	0
2	5.8	6.9	1.0
3	11.4	13.0	2.5
4	10.6	12.2	2.3
5	3.5	4.4	.4
means	6.3^{a}	7.3^{a}	1.2^{b}

[a,b] Means with different superscripts are different ($P < .05$, least significant difference).

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APPENDIX

Prior to the development of the modified solid phase competitive ELISA using whole plasma, the following sandwich ELISA was attempted due to the availability of commercial antibodies and conjugate. A sandwich ELISA is a competitive assay which utilizes an antigen that is coated onto the plate, followed by a double antibody system. This is in comparison to a direct competitive assay system, which involves the use of only one antibody, which is coated onto the plate.

Sandwich ELISA

Reagents. The following reagents and the company that they were purchased from are as follows: The antigen, 4-pregnen-3,20-dione 3-O-carboxymethyloxime:BSA (P4-3CMO-BSA) was purchased from Steraloids, Inc. (Wilton, NH), the first antibody, sheep anti-progesterone-11α-HS-BSA (#337) was purchased from Colorado State University, and the second antibody, anti-sheep IgG peroxidase conjugate, was purchased from Sigma Chemical Co. (St. Louis, MO). Bovine serum albumin, polyoxyethylene-sorbitan monolaurate (Tween 20), hydrogen peroxide, citric acid, 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS), and progesterone, were all purchased from Sigma Chemical Company (St. Louis, MO). Horse serum (used in the blocking solution) was purchased from Gibco Laboratories (Grand Island, NY). Disposable Immulon 4 flat bottomed polystyrene 96 well microelisa plates and

plate sealers (8.26 x 13.34) were purchased from Dynatech Labs Inc. (Chantilly, VA). A Titerek digital multichannel pipette and Titertek Multiskan Plus II automatic plate reader were purchased from Flow Laboratories (Covina, CA).

Stock solutions. The following stock solutions were prepared: 1) .05 M bicarbonate buffer, pH 9.6; 2) .1 M phosphate buffered saline, pH 7.0, contained .1 % BSA (PBS-BSA); 3) blocking solution that contained 10 % horse serum in .1 M PBS; 4) wash solution contained .05 % Tween 20 and .15 M NaCl; 5) citrate buffer, pH 4.0; and, 6) substrate solution contained 1 ml of 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid), 98.7 ml citrate buffer and 320 μl of a 8 M hydrogen peroxide solution and was made approximately 10 min before use.

Antigen. An initial 1 mg/ml stock solution of P4-3CMO-BSA was made in .05 M bicarbonate buffer pH 9.6, and stored at 4 C until assay.

First antibody. A initial 1:20 dilution of sheep anti-progesterone-11α-HS-BSA in .1 M PBS was made, with 20 μl aliquots stored at -70 C until assay.

Second antibody. The antibody enzyme conjugate which was diluted with .1 M PBS, was diluted each day as needed for assay.

Standards. A 1 mg/ml stock solution of progesterone was made in ethanol, from which subsequent buffer (.1 M PBS-BSA) or plasma standards were made. The plasma was obtained from a mare in estrus, and extracted with dextran coated charcoal. Both buffer and plasma standards ranged from 25 to .195 ng/ml.

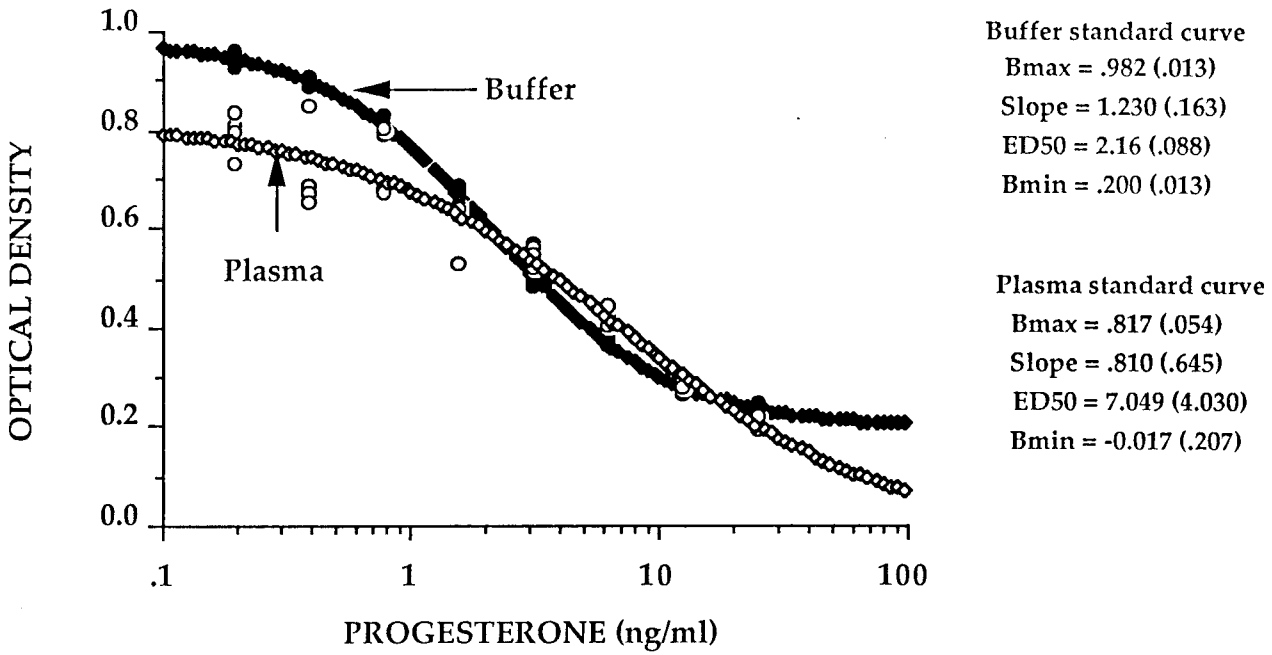
Nonspecific binding. Nonspecific binding was tested by adding 50 μl of the following in duplicate to each plate: a) PBS only; b) PBS with first antibody; c) PBS without first antibody; d) Plasma only; e) Plasma with first antibody; f) plasma without first antibody; g) first antibody only; and, h) antigen only.

Assay. The various dilutions of antigen, first antibody, and second antibody tested are as follows: a) 1:1,000, 1:2,000, 1:4,000; b) 1:10,000, 1:20,000, 1:40,000; c) 1:1,330, 1:2,000 and 1:4,000. Increasing and decreasing amounts of substrate and hydrogen peroxide were added to the citrate buffer to try and determine amounts necessary for optimal color development. Disposable Immulon 4 flat bottomed polystyrene 96 well microelisa plates were coated with 100 μl antigen diluted in .05 M bicarbonate buffer pH 9.6, covered with plate sealers and stored at 4 C approximately 12 h before assay. To remove excess unbound antigen, the plates were individually washed 5x with wash solution, inverted and blotted dry. Fifty μl of blocking solution (10 % horse serum in .1 M PBS-BSA) was then added to each well and allowed to

incubate for approximately 1 h. Following washing and drying of the plates (as described above), 50 μl of standard (s) was added in duplicate, followed by an additional 50 μl of the first antibody (diluted in .1 M PBS-BSA) and allowed to incubate 2 additional hours. The plates were washed and blotted dry, followed by 50 μl of the second antibody (diluted in blocking solution), incubated for 1 h and then washed. One hundred μl of substrate, which was made 10 min before use, was added to each well on the plate and incubated for approximately 45 min. All plates were read at 405 nm with a Titertek Multiskan Plus II automatic plate reader. A standard curve was constructed by plotting absorbance values against standards (ng/ml progesterone) using Graph Pad Inplot version 3.1 (San Diego, CA).

RESULTS AND DISCUSSION

Dilutions of 1:2,000 antigen, 1:20,000 first antibody, and 1:1,330 second antibody produced standard curves for buffer and plasma standards with slopes of 1.23 and .81, respectively (see appendix Figure 1.). On each plate, various reagents listed above were tested for nonspecific binding. It appeared that little or no nonspecific binding was occurring. The assay was tested with and without the use of blocking solution, which did not appear to be necessary. Quality controls were not used since the assay was not ready to use routinely. When comparing the charcoal extracted plasma standard curve to that of the buffer standard curve, there appears to be some interference from the plasma or from components in plasma that cause depression of the curve (see appendix Figure 1). After 3 mo of trying to develop a plasma standard curve in which results were very inconsistent, reagents for the direct ELISA became available and development of a more simplified assay system was undertaken. After development of the modified solid phase competitive ELISA in which results indicated that standard and sample plasma must be treated the same, it seems appropriate to suggest the following modifications that may enhance development using whole plasma: a) Eliminate blocking solution and add PBS-BSA to each well on the plate prior to standard or sample; b) use of whole plasma as diluent for standards.



Appendix Figure 1. Sandwich ELISA comparing buffer and charcoal extracted plasma standard curves utilizing progesterone-3-CMO-BSA as the antigen, sheep anti-progesterone-11-α-HS-BSA as the first antibody, and anti-sheep IgG peroxidase conjugate as the second antibody.