Onderstepoort J. vet. Res., 47, 179–185 (1980)

PLASMA PROGESTERONE IN CATTLE. I. DEVELOPMENT AND VALIDITY OF THE ASSAY

H. M. TERBLANCHE and J. M. LABUSCHAGNE, Department of Genesiology, Faculty of Veterinary Science, P.O. Box 12580, Onderstepoort 0110

ABSTRACT

TERBLANCHE, H. M. & LABUSCHAGNE, J. M., 1980. Plasma progesterone in cattle. I. Development and validity of the assay. *Onderstepoort Journal of Veterinary Research*, 47, 179–185 (1980).

The development of a practical competitive protein-binding assay for plasma progesterone in cattle is described. With an intra-assay coefficient of variation of 5,46% and an interassay coefficient of variation of 14,25%, the method is sufficiently accurate and sensitive for practical purposes, and for use in routines and surveys. The statistical level of sensitivity was found to be in the region of 0,25 ng/mℓ based on the confidence limits of zero dose and 0,25 ng/mℓ, with the practical sensitivity level at 0,50 ng/mℓ. Method and reagent blanks were found to be negligible. The specificity of the assay is based entirely on the partial specificity of the petroleum ether used for the extraction of progesterone (87,5% extraction, n=141).

Résumé

PROGESTERONE DU PLASMA BOVIN. I. DÉVELOPPEMENT ET VALIDITÉ DE L'ESSAI

Le développement d'un essai compétitif pratique de conjugation de protéine pour le progesterone du plasma bovin est décrit. Avec un coéfficient de variation intra-essai de 5,46% *et un coéfficient de variation inter-essai de* 14,25%, *la méthode est suffisamment précise et suffisamment sensible pour des fins pratiques ainsi que pour un usage dans des travaux de routine et d'examen. Le niveau statistique de sensibilité a été trouvé se situer dans la région de* 0,25 *ng/ml basé sur la confiance limitée de la dose zéro et de* 0,25 *ng/ml, avec le niveau pratique de sensibilité à* 0,50 *ng/ml. La méthode et les réactifs ont rarement faites faillite. La spécificité de l'essai est entièrement basée sur la spécificité partiale du pétrole ether utilisé pour l'extraction du progesterone* (87,5% *d'extraction, n*=141).

INTRODUCTION

The development and application of routine assays for plasma progesterone have developed very rapidly during the past 15–20 years. A major breakthrough occurred in 1963 when the first competitive protein-binding assay for steroid hormones was published by Murphy, Engelberg & Pattee (1963). This assay served as a basis for the development of the first competitive protein-binding assay for bovine plasma progesterone in 1968 (Surve, Randel, Erb & Callahan, 1968). During 1971 yet another important event occurred with the publication of the first radioimmunoassay for progesterone by Abraham, Swerdloff, Tulchinsky & Odell (1971) based on the pioneer work of Yalow & Berson (1959).

The gynaecologist and clinician can nowadays obtain much information from progesterone assays of a great variety of clinical cases. It is therefore the aim of this study to describe the development of a competitive protein-binding assay which can be used for the routine determination of plasma progesterone in cattle under various conditions and to provide proof of its validity in terms of several reliability criteria in such a way as to conform to the requirements recently forwarded by the editors of a scientific journal (Anon., 1978).

MATERIALS AND METHODS

Chemical reagents

Unless otherwise indicated, all the chemical reagents used in this study were of special purity (GR reagents) and were used without further purification.

Isotopes

Tritium-labelled isotopes were obtained from the Radiochemical Centre, Amersham, UK* and were stored at 4 °C in diluted concentrations in a 9:1= benzene : ethanol solution.

* Weil Organisation (Pty) Ltd, Johannesburg

Received 27 May 1980—Editor

Petroleum ether 40–60 °C

Sufficient petroleum ether* was freshly distilled immediately before the extraction of the samples and the distillate collected between 40 and 50 °C. The efficiency of this process was checked in the assay by determination of reagent blank values of purified and non-purified petroleum ether. This was done by assaying the progesterone content of 1 mℓ of petroleum ether samples. In addition, the percentage extraction of progesterone from a pool of low steroid plasma was determined with purified and non-purified petroleum ether. These results were statistically analysed in a Hewlett-Packard HP-97 calculator with a pre-recorded programme by testing the null hypothesis in a t-test for 2 means.

Florisil 60–100 *ASTM*

Florisil* was washed 4 times in deionized water before use and all fines were decanted between separate washings. The wet Florisil powder was dried overnight at 80–100 °C in a laboratory oven fitted with an extraction fan. Dry powder was kept in a desiccator with silica gel containing a moisture indicator. The powder was routinely dried in the same way once a week.

Reference plasma

Blood was collected in heparin from a heifer which had been oophorectomized 6 months earlier, centrifuged, and the plasma used as a source of low steroid plasma. Batches were stored at −15 °C.

Sample extraction

Extraction of 1 mℓ of plasma samples was performed in 142×17 mm glass tubes with 5 mℓ of freshly distilled petroleum ether by being shaken in a horizontal shaking machine for 5 minutes. The tubes were then centrifuged for 10 minutes at 2 000 rpm at 10 °C and immersed for approximately 1 minute in a

* Merck Chemicals (Pty) Ltd, Johannesburg

methylated spirits bath containing dry ice. The unfrozen petroleum ether fraction was decanted into 12×80 mm glass tubes and evaporated under nitrogen* in a water-bath at 40 °C. The dried extract was reconstituted with 1 mℓ of petroleum ether, shaken for 30 seconds on a vortex shaker and again evaporated in the same way.

Scintillation counting

Counting was performed in a Packard Scintillation Spectrometer Model 3385. Ten mℓ of Aquagel** was used as scintillation fluid and all the samples were counted for 10 minutes.

Procedural losses

Procedural losses were assessed by determining the percentage recovery of tritiated progesterone in the extraction procedure described above. Approximately 4000 cpm tritiated progesterone was evaporated in extraction tubes, 1 mℓ of reference plasma added, the tube shaken for 30 seconds on a vortex shaker, incubated for 5 minutes in a water-bath at 40 °C and again shaken for 30 seconds on a vortex shaker. The plasma was then extracted as described and the recovery percentage determined after scintillation counting.

Binding protein

Adult male dogs*** were used as donors of corticosteroid-binding globulin (CBG). The dogs were bled every fortnight and the plasma stored at −15 °C until required. Tritiated corticosterone (1,5 μCi) was evaporated in a 100 mℓ volumetric flask to which 1,5 mℓ of dog plasma was added, and the flask was gently shaken. Deionized water was then added (98,5 mℓ) and the flask gently shaken. The solution was left for at least 1 hour before use, used for 4 days and stored at 4 °C between whiles.

Protein binding

The dried extract obtained after extraction was dissolved in 200 μℓ of Tris buffer (pH 8) by shaking on the vortex shaker for 30 seconds. One mℓ of the binding protein solution was then added, the tubes shaken for 30 seconds on the vortex shaker and transferred to a water-bath for 5 minutes at 40 °C. The tubes were then again shaken on the vortex shaker for 30 seconds and left in an ice-bath for 10 minutes at 4 °C.

Separation of bound and free hormone

Separation of bound and free hormone was achieved by the addition of approximately 90 mg of Florisil powder. The tubes were subsequently shaken for 30 seconds on the vortex shaker and 0,5 mℓ of the supernatant removed. This was then transferred to counting vials containing 10 mℓ of scintillation fluid.

Batch procedure

The whole process of protein binding and separation of bound and free hormones was done in batches of 16 tubes. The procedure was controlled by a stop-watch which was started when the first tube was removed from the ice-bath. Florisil was added at 10 seconds, shaking was commenced at 15 seconds and stopped at 45 seconds and the tube was returned to the ice-bath at 50 seconds. The second tube was removed at 1 minute and the whole process repeated until all 16 tubes had been treated in this way. The first tube was then again removed at the minute, 0,5 mℓ supernatant removed at 10 seconds, the tube put aside and the second tube again removed at the minute and the process repeated. This is a unique aspect of this assay and was used to improve the reproducibility of the assay.

* Afrox (Pty) Ltd, Pretoria

** Chemlab (Pty) Ltd, Johannesburg

*** Departments of Genesiology and Medicine, Faculty of Veterinary Science, University of Pretoria, P.O. Box 12580, Onderstepoort 0110

Standard curve

Two mg of crystalline progesterone* was dissolved in 1 ℓ of ethanol and used as a stock solution. Five mℓ of this solution was then diluted and made up to 500 mℓ as a working standard containing 20 ng per mℓ. A range was then established from 0; 1; 2; 4; 5; 6; 8 to 10 ng per 0,5 mℓ in ethanol and these solutions were then used for construction of a standard curve. Ethanol was used for the zero point.

Duplicate 12×80 mm tubes were set up and 0,5 mℓ of the appropriate standard solution pipetted into the tubes. The tubes were then placed in a water-bath at 40 °C and the contents evaporated under a stream of nitrogen. Buffer and binding protein solutions were then added as described and the binding and separation processes as well as the radioactivity counting performed as described above. Aliquots (0,5 mℓ) of the binding solution were also counted and these represented total activity or 100% binding counts.

The binding percentages at the various standard points were then assessed and a standard curve constructed. Progesterone concentrations were read directly from the curve and, if desired, the values corrected for procedural losses. Alternatively, log/logit transformation of the curvilinear dose response curve has been achieved with a Hewlett-Packard HP-97 calculator with a pre-recorded programme of this nature. The correlation coefficient, the slope and the intercept of the least-squares regression line are provided so that, if required, the curve can be constructed. Progesterone values can be read from the curve or can be obtained from the calculator. Comparisons between the 2 methods have been made at the various progesterone concentrations mentioned below These results were statistically evaluated with the aid of a programme provided for the calculator by testing the null hypothesis in a t-test for 2 means.

Accuracy

The accuracy of the assay was determined by doing a series of determinations during the same and subsequent weeks of known amounts of progesterone added to a reference plasma of low progesterone content. Tubes containing 0; 0,25; 0,5; 1,0; 2,0; 4,0; 5,0 and 8,0 ng per mℓ of progesterone were set up in duplicate and 1 mℓ of reference plasma added to the tubes. The progesterone content and the progesterone content of the reference plasma were determined as described above. The results were statistically evaluated in a Hewlett-Packard HP-97 calculator programmed for basic statistics providing the mean, standard error, standard deviation and coefficient of variation. Confidence limits were also calculated at the various concentrations according to the formula P (A ≤ real unknown concentration ≤ B) = 0,95 where A = measured response minus $1{,}96\sqrt{\frac{SD}{n}}$ and B = measured response plus $1{,}96\sqrt{\frac{SD}{n}}$.

* Steraloids, Taeuber & Corssen (Pty) Ltd, Johannesburg

Precision

The precision of the assay was determined from replicate assays of a specific sample obtained from a cow during the luteal phase of the cycle. From these results the intra-assay and interassay coefficients of variation were calculated according to the formula

$$CV = \frac{\sqrt{\frac{d^2}{2n}}}{\bar{x}} \times 100$$

where d=the difference between duplicate pairs, n=the number of duplicate pairs and $\bar{x}$=the mean, for the intra-assay coefficient of variation, and $CV = \frac{SD}{\bar{x}} \times 100$ for the interassay coefficient of variation. The precision is also expressed as the standard deviation (SD) of a large number (68) of replicate assays of the same sample.

Sensitivity

The sensitivity was determined from the 95% confidence limits of the measured response to progesterone at zero dose by replicate assays of the progesterone content at zero dose and at 0,25 ng per mℓ. Confidence limits were established as mentioned above. The sensitivity was further examined by determination of the method blank, reagent blank and plasma blank. Tubes containing 1 mℓ of deionized water were assayed as described to establish the method blank while tubes containing 1 mℓ of petroleum ether were assayed as described to establish the reagent blank. The plasma blank was determined by assaying 1 mℓ samples of plasma obtained from the oophorectomized heifer.

Specificity

The specificity of the assay was examined by the extraction of various steroids with the petroleum ether used for progesterone extractions. Approximately 2500–3500 cpm of tritiated cortisol, corticosterone, oestradiol, oestrone, testosterone and 17α-hydroxyprogesterone was separately added to extraction tubes, evaporated and dissolved in 1 mℓ of oophorectomized heifer plasma, and extracted as previously described. The petroleum ether extract was decanted after freezing into empty counting vials, evaporated, filled with 10 mℓ of scintillation fluid and counted. The activity thus extracted was converted into a percentage of the activity initially added before extraction.

Laboratory glassware

All laboratory glassware was rinsed under running tap water after use and soaked overnight in a 2% solution of Decon 75* in deionized water. The glassware was then rinsed under running tap water for approximately 12 hours and again soaked overnight in deionized water. It was finally autoclaved before use.

RESULTS

Reagent blank values

The reagent blank value of purified petroleum ether was 0,05 ng±0,05 (n=24) and of non-purified petroleum ether 0,15 ng±0,21 (n=24). Because of this negligible amount, consideration of the reagent blank value in the final determination of the progesterone content of the samples was omitted.

* Protea Laboratory Services (Pty) Ltd, Johannesburg

Procedural losses

The percentage progesterone extraction with non-purified petroleum ether was 78,52±9,25 (n=60), and with purified petroleum ether 87,52±8,53 (n=140). The coefficient of variation was 11,74% for the non-purified petroleum ether and 9,75% for the purified petroleum ether. The t-value obtained in a t-test comparing the reagent blanks of purified and non-purified petroleum ether was 2,31 (46 df) which represents a significant difference ($P<0,05$).

Procedural losses from extraction amounted to 12,48% (n=140). This figure should therefore be used to determine the correction factor (1,14) in the rapid technique described, if so desired.

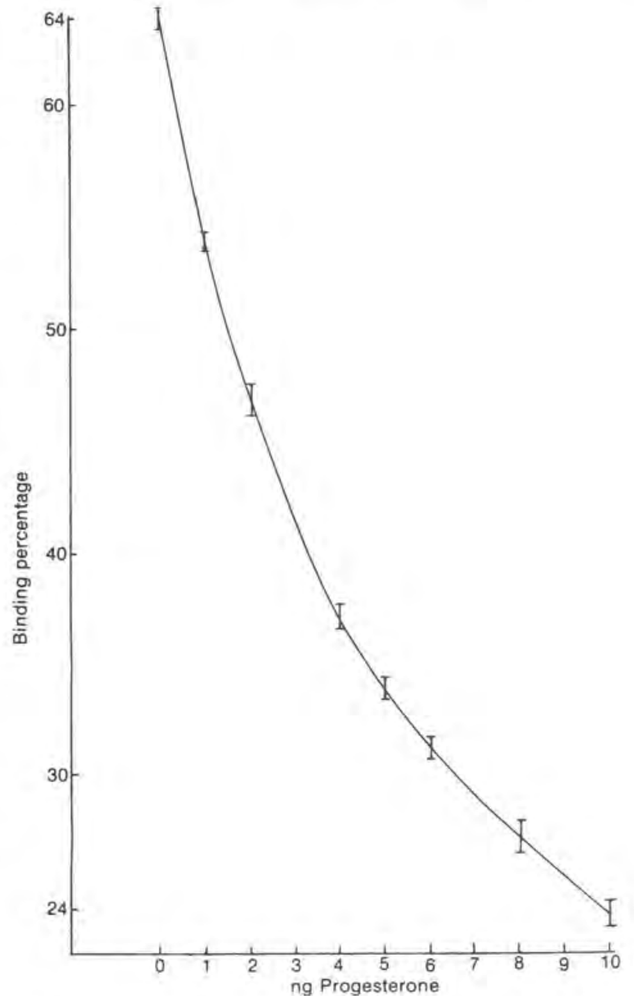

FIG. 1 Typical curvilinear standard curve with 1,5 μCi ^{3}H corticosterone and 1,5% canine CBG

Standard curve

A typical curvilinear standard curve obtained in the assay is represented in Fig. 1. The deviation between duplicate pairs is shown as vertical bars resulting in a progesterone variation of approximately 0 ng at the 0 ng level, 0,1 ng at the 1 ng level, 0,06 to 0,1 ng at the 2 ng level, 0,06 to 0,2 at the 4 ng level, 0,1 to 0,2 ng at the 5 ng level, 0,2 ng at the 6 ng level, 0,3 ng at the 8 ng level and 0,3 ng at the 10 ng level.

Accuracy

The results of the replicate assays of known progesterone concentrations between zero and 8 ng

after the final correction of values for the progesterone content of the reference plasma and procedural losses are given in Table 1. The progesterone content of the oophorectomized heifer plasma was 0,07 ng± 0,08 when read from the curve and 0,02 ng±0,04 when calculated from the log/logit plot. The corrected values were 0,08 ng and 0,02 ng respectively.

The confidence limits at the various progesterone concentrations for the final corrected progesterone values are given in Table 2. Analysis of the null hypothesis for 2 means—curve reading against log/logit calculation with t-statistics have shown non-significant differences at 0 ng; 0,25 ng; 1,0 ng; 5,0 ng and 8,0 ng progesterone ($P>0,05$) and significant differences at 2,0 ng and 4,0 ng progesterone ($P<0,05$).

Precision

The replicate assay of duplicate pairs of a reference plasma during the same week yielded a progesterone concentration of 4,84±0,46 ng when determined by direct reading from the standard curve and 5,24± 0,58 ng when calculated from a log/logit plot. The coefficient of variation was calculated to be 4,67% and 5,46% respectively for 15 duplicate pairs. Replicate assays of the same reference pool over several weeks and months yielded a progesterone concentration of 4,19±0,74 ng when read directly from the curve, and 4,56±0,65 ng when calculated from the log/logit plot. The coefficient of variation in these cases was calculated to be 17,66% and 14,25% respectively for 19 duplicate determinations. These results are also presented in tabular form in Table 3.

TABLE 1 Final recovery of progesterone at various known concentrations after deduction of reference plasma and correction for procedural losses

Amount of steroid added in ng	Progesterone recovered in ng		Standard deviation		Standard error		CV*		Number of replicate assays
	Curve	Log/Logit	Curve	Log/Logit	Curve	Log/Logit	Curve	Log/Logit	
0	0,11	0,12	0,06	0,05	0,01	0,01	54,80	40,68	31
0,25	0,34	0,33	0,09	0,09	0,02	0,02	25,55	26,44	32
0,50	0,48	—	0,16	—	0,02	—	34,03	—	58
1,00	0,91	—	0,30	—	0,03	—	33,01	—	95
	0,89	0,87	0,22	0,25	0,05	0,05	24,56	28,82	23
2,00	1,86	—	0,33	—	0,03	—	17,78	—	91
	1,78	1,95	0,20	0,25	0,04	0,05	11,36	12,85	23
4,00	4,03	—	0,45	—	0,05	—	11,07	—	85
	3,92	4,17	0,47	0,39	0,09	0,08	11,93	9,44	25
5,00	5,09	—	0,56	—	0,06	—	11,01	—	82
	5,10	5,11	0,66	0,57	0,13	0,12	12,95	11,19	24
8,00	7,53	—	0,69	—	0,08	—	9,22	—	70
	7,65	7,54	0,71	0,64	0,10	0,09	9,32	8,54	51

* CV=Coefficient of variation

TABLE 2 Confidence limits for the final measured progesterone values at the various known concentrations ($P=0,05$)

Known progesterone concentration in ng	Confidence limits	
	Curve	Log/Logit
0	0,02–0,20 (0,11)	0,04–0,20 (0,12)
0,25	0,24–0,44 (0,34)	0,23–0,43 (0,33)
0,50	0,38–0,58 (0,48)	
1,00	0,80–1,02 (0,91)	
	0,70–1,14 (0,89)	0,67–1,07 (0,87)
2,00	1,74–1,98 (1,86)	
	1,60–1,96 (1,78)	1,75–2,15 (1,95)
4,00	3,89–4,17 (4,03)	
	3,65–4,19 (3,92)	3,93–4,41 (4,17)
5,00	4,93–5,25 (5,09)	
	4,78–5,42 (5,10)	4,81–5,41 (5,11)
8,00	7,34–7,72 (7,53)	
	7,42–7,88 (7,65)	7,32–7,76 (7,54)

TABLE 3 Replicate assays of a plasma pool during the same and subsequent weeks

	Progesterone in ng (SD)		CV(%)		Number of duplicate pairs
	Curve	Log/Logit	Curve	Log/Logit	
Same week (intra-assay)	4,84±0,46	5,24±0,58	4,67	5,46	15
Subsequent weeks (interassay)	4,19±0,74	4,56±0,65	17,66	14,25	38

The standard deviation of 68 replicate assays of the same sample performed in the same and subsequent weeks was 0,71 (average concentration 4,47 ng/mℓ and the coefficient of variation 15,78%) when read directly from the curve, and 0,70 (average concentration 4,88 ng/mℓ and coefficient of variation 14,29%) when calculated from a log/logit plot.

Sensitivity

The sensitivity of the assay was calculated from the confidence limits presented in Table 2. These limits were regarded as a non-sensitive statistical test and, as no overlap occurred in these limits, 0,25 ng was regarded as the statistical level of sensitivity of the assay being significantly different from zero at the 95% confidence limit.

The remaining parameters of sensitivity, viz. method blank, reagent blank and plasma blank, are given in Table 4. These parameters were not regarded as significant in the determination of the sensitivity of the assay.

TABLE 4 Parameters of sensitivity: method blank, reagent blank and plasma blank

Parameter of sensitivity	Concentration in ng		Number of replicate assays
	Curve	Log/Logit	
Method blank....	0,0	0,0	24
Reagent blank....	0,05	0,04	24
Plasma blank.....	0,07	0,02	18

Specificity

The results of the percentage extraction of various steroids with petroleum ether are given in Table 5. These results confirm the partial specificity of the method for progesterone when using petroleum ether. Attention must, however, be drawn to the fact that 141 replicate recoveries were performed for progesterone whereas only 48 replicate recoveries were performed for the 6 steroids examined in this study.

TABLE 5 Percentage extraction of various steroids with petroleum ether

Steroid	% Extraction	SD	$S\bar{x}$	CV	n
Progesterone......	87,52	8,53	—	—	141
Cortisol..........	0,85	0,32	0,05	37,43	48
17α-hydroxyprogesterone.......	45,97	2,90	0,42	6,3	48
Testosterone.......	40,40	2,42	0,35	5,98	48
Corticosterone.....	0,66	0,07	0,01	10,81	48
Oestradiol........	3,91	0,29	0,04	7,49	48
Oestrone..........	22,61	1,29	0,19	5,71	48

DISCUSSION

The significant difference in the reagent blank of purified and non-purified petroleum ether seems to indicate the necessity for the purification of petroleum ether. This is in accordance with Fassora & Luisi (1971), Hoffmann & Hamburger (1973), Luisi, Levanti & Franchi (1971), Moore, Barrett & Brown (1972), Pichon & Milgrom (1973), Reeves, De Souza, Thompson & Diczfalusy (1970) and Wishart, Head & Horth (1975), who regard such purification as a necessary precaution.

Some workers, however, do not include such a step but use solvents of a very high degree of purity (Edqvist, Ekman, Gustafsson & Åström, 1970; Johansson, 1969a; Neill, Johansson, Datta & Knobil, 1967; Pizarro, 1971). This principle is supported by the present finding that the reagent blank values of purified and non-purified petroleum ether both fell within the confidence limits calculated for zero ng progesterone.

The assay, based on the results of Johansson & Gemzell (1969), Johansson & Wide (1969), Pichon & Milgrom (1973) and Shemesh, Lindner & Ayalon (1971), who have reported similar progesterone concentrations with assays including and excluding such steps, was developed without extract purification steps. This is in accordance with the assays of Attal & Engels (1971), Edqvist (1972), Edqvist *et al.* (1970), Johansson (1969a; 1969b; 1970), Moore *et al.* (1972), Pichon & Milgrom (1973), Robertson (1972), Robertson & Sarda (1971), Shemesh, Ayalon & Lindner (1973), Shemesh *et al.* (1971), Smith, Edgerton, Hafs & Convey (1973), Smith, Fairclough, Payne & Peterson (1975) and Sprague, Hopwood, Niswender & Wiltbank (1971).

The exclusion of internal standards in the present assay is in accordance with the technique of other rapid assays (Attal & Engels, 1971; Bassett & Hinks, 1969; Donaldson, Bassett & Thornburn, 1970; Edqvist, 1972; Edqvist *et al.*, 1970; Johansson, 1969a; 1969b; 1970; Luisi *et al.*, 1971; Moore *et al.*, 1972; Robertson, 1972; Robertson & Sarda, 1971; Shemesh *et al.*, 1973; Smith *et al.*, 1975).

The use of canine CBG as a binding protein is in accordance with many other workers (Abilay, Mitra & Johnson, 1975; Bassett & Hinks, 1969; Booth, Newcomb, Strange, Rowson & Sacher, 1975; Fassora & Luisi, 1971; Hoffmann & Karg, 1970; Luisi *et al.*, 1971; Randel, Brown, Erb, Niswender & Callahan, 1971; Robertson, 1972; Robertson & Sarda, 1971; Smith *et al.*, 1973). The use of Florisil for the separation of bound and free hormone is likewise in accordance with the technique of others (Edqvist *et al.*, 1970; Fassora & Luisi, 1971; Hoffmann & Karg, 1970; Johansson, 1969a; 1969b; 1970; Lipsett, Doerr & Bermudez, 1970; Luisi *et al.*, 1971; Neill *et al.*, 1967; Pizarro, 1971; Robertson & Sarda, 1971; Smith *et al.*, 1973; 1975). The strict adherence to a time schedule is in accordance with the suggestions of Batra (1976) and Hoffmann & Karg (1970) to improve the reproducibility, sensitivity and precision of the assay.

The addition of 200 $\mu\ell$ of Tris buffer to all tubes immediately prior to the protein-binding step has greatly improved the reproducibility of the assay and has also resulted in much reduced variation between duplicate pairs. Because of the very subjective way used in the construction of the standard curve as well as in the reading of binding percentages on the curve to obtain the actual progesterone content, the need for a more reliable method has led to the transformation of the curvilinear dose response curve into a straight line by log/logit plotting on a programmed calculator. This procedure is in accordance with Lipsett *et al.* (1970). The final progesterone concentrations are then calculated by a calculator to 2 decimal points which provide a more reliable and unbiased answer.

The definition of accuracy as explained by Cekan (1975), Reeves & Calhoun (1970) and Strott (1975) has been applied to the present assay along the lines of suggestions forwarded by Strott (1975) to measure the recovery of added known amounts of progesterone. The results presented in Table 1 compare very favourably with those published by other workers in this field (Attal & Engels, 1971; Edqvist *et al.*, 1970; Fassora & Luisi, 1971; Johansson, 1969a; Luisi *et al.*, 1971; Martin, Cooke & Black 1970; Robertson, 1972; Smith *et al.*, 1975). The recovery of added progesterone at the various concentration levels differed non-significantly at the 95% limits of confidence from the known added amounts, since all the known concentrations fell within the calculated confidence limits (Table 2). The only exception was at 8 ng, which indicates the relative inaccuracy of the assay at this concentration and implies the necessity of diluting or halving of the aliquot to be extracted when values in excess of 6 to 8 ng/mℓ are expected.

A comparison of the results obtained from curvilinear reading and log/logit transformation indicates good agreement between the 2 methods, as significant differences were only observed at the 2 ng and 4 ng levels. The reason for this is not known but could possibly be related to the curvilinear dose response curve which normally undergoes alterations in the slope at about the 4 to 5 ng level. Once again it would seem that the flatness of the curve at the 8 ng level need not necessarily pose a problem because of the non-significant differences found between the 2 methods at this concentration.

The precision of the assay was determined from the coefficient of variation of replicates within and between assays as suggested by Cekan (1975) and, Strott (1975). The present results indicate that the assay is well within the limits of acceptability proposed by Strott (1975). He considers an intra-assay (within assay) coefficient of variation below 15% and an interassay (between assay) coefficient of variation below 30% as acceptable. The present results of 4,67–5,46% within assay variation and 14,25–17,66% between assay variation compare indeed very favourably. This is also in agreement with the results of Attal & Engels, 1971; Edqvist *et al.*, 1970; Fassora & Luisi, 1971; Johansson, 1969a; 1970; Martin *et al.*, 1970; Pizarro, 1971; Reeves *et al.*, 1970; Smith *et al.*, 1975; Yoshimi & Lipsett, 1968).

The standard deviation of replicate assays also used as a measure of precision (Murphy & Pattee, 1964; Reeves *et al.*, 1970; Smith *et al.*, 1975) is in good agreement with figures reported by a number of other workers (Attal & Engels, 1971; Johansson, 1969a; Martin *et al.*, 1970; Pizarro, 1971; Reeves *et al.*, 1970).

The sensitivity of the assay is not only by definition the smallest concentration of steroid significantly distinguishable from zero (Reeves & Calhoun, 1970; Strott, 1975) but also the ability of the assay to distinguish between zero and any other concentration and between one concentration and any other concentration (Cekan, 1975). The confidence limits given in Table 2 clearly indicate that the sensitivity of this assay is above 0,2 ng/mℓ because the limits of zero dose extent to 0,2 ng/mℓ. The next concentration above 0,2 ng to be tested was 0,25 ng/mℓ and, because of the absence of overlap between 0 and 0,25 ng/mℓ, the latter concentration is regarded as the statistical limit of sensitivity for this assay at the 95% level of confidence. The use of these confidence limits constitutes a non-sensitive statistical test (Groeneveld—personal communication).

The low reagent blank and method blank values found (Table 3) seem to be of no importance in the assay since they all fall within the confidence limits of zero dose. This is in agreement with the results of Attal & Engels (1971), Clarke & Gurpide (1972), Fassora & Luisi (1971), Luisi *et al.* (1971), Martin, Cooke & Black (1969), Martin *et al.* (1970) and Yoshimi & Lipsett (1968).

The statistically significant ($P<0,05$) sensitivity limit of this assay of 0,25 ng progesterone and the accepted practical sensitivity limit of 0,5 ng progesterone agree very favourably with the results of many other workers (Attal & Engels, 1971; Edqvist *et al.*, 1970; Fassora & Luisi, 1971; Johansson, 1969a; 1970; Luisi *et al.*, 1971; Martin *et al.*, 1970; Moore *et al.*, 1972; Neill *et al.*, 1967; Pichon & Milgrom, 1973; Pizarro, 1971; Reeves *et al.*, 1970; Smith *et al.*, 1975).

The petroleum ether used for extraction in this study showed a high affinity for progesterone, moderate affinity for 17α-hydroxyprogesterone and testosterone, relatively poor affinity for oestrone and a very poor affinity for cortisol, corticosterone and oestradiol-17β. This finding agrees favourably with the requirements forwarded by Johansson (1969a; 1970) and Morgan & Cooke (1972) as far as progesterone extraction is concerned, fairly favourably as far as the corticosteroids are concerned but poorly as far as 17α-hydroxyprogesterone is concerned. The 2 steroids of importance in terms of interference in the assay, which are also extracted to some degree by the petroleum ether used, are therefore 17α-hydroxyprogesterone and testosterone. Both these steroids, however, seem to have limited competitive displacement affinities for progesterone in the CBG-binding system employed (Fassora & Luisi, 1971; Luisi *et al.*, 1971). Edqvist *et al.* (1970) have reported that the only possible interfering steriod in the bovine is 20 β-hydroxyprogesterone. They reported a 48–55% extraction of this steroid with petroleum ether, but fortunately very low levels in the cow. For this reason the influence of this steroid was not evaluated in the present assay.

In the light of these discussions and for practical purposes, the assay is sufficienty specific for progesterone. This is further strengthened by the reports of Robertson (1972) and Robertson & Sarda (1971) who have reported negligible amounts of interfering substances in bovine plasma during various reproductive stages. In addition, Schiavo, Matuszczak, Oltenacu & Foote (1975) have stated that "most of the substance measured by the competitive protein binding assay is true progesterone". It is therefore our contention that the described rapid assay is of such sufficient reliability and specificity so as not to warrant the use of time-consuming extract purification steps nor the protective measures employed by Leyendecker, Wardlaw & Nocke (1972).

ACKNOWLEDGEMENTS

Acknowledgement is hereby given to the Atomic Energy Board and the University of Pretoria whose financial aid has made this research possible. The senior author also wishes to express his sincere appreciation to Prof. J. S. van Heerden and the staff members of the Department of Genesiology for their support during the time it took to complete this study.

REFERENCES

ABILAY, T. A., MITRA, R. & JOHNSON, H. D., 1975. Plasma cortisol and total progestin levels in Holstein steers during acute exposure to high environmental temperature (42 °C) conditions. *Journal of Animal Science*, 41, 113–117.

ABRAHAM, G. E., SWERDLOFF, R., TULCHINSKY, D. & ODELL, W. D., 1971. Radioimmunoassay of plasma progesterone. *Journal of Clinical Endocrinology and Metabolism*, 32, 619–624

ANON., 1978. The validation of assays, the statistical treatment of results and steriod nomenclature. *Journal of Endocrinology*, 76, 1–8.

ATTAL, J. & ENGELS, J. A., 1971. A simple technique for the assay of progesterone in pregnancy. *Journal of Endocrinology*, 50, 703–704.

BASSET, J. M. & HINKS, N. T., 1969. Micro-determination of corticosteroids in ovine peripheral plasma: effects of venipuncture, corticotrophin, insulin and glucose. *Journal of Endocrinology*, 44, 387–403.

BATRA, S., 1976. New simplified procedures for the determination of progesterone by competitive protein binding and radioimmunoassay. *Journal of Steriod Biochemistry*, 7, 131–134.

BOOTH, W. D., NEWCOMB, R., STRANGE, H., ROWSON, L.E.A. & SACHER, H. B., 1975. Plasma oestrogen and progesterone in relation to superovulation and egg recovery in the cow. *Veterinary Record*, 97, 366–369.

CEKAN, Z., 1975. Assessment of reliability of steroid radioimmunoassays. *Journal of Steroid Biochemistry*, 6, 271–275.

CLARK, H., & GURPIDE, E., 1972. Measurement of the solubility of progesterone under competitive protein binding assay conditions. *Journal of Clinical Endocrinology and Metabolism*, 34, 1085–1086.

DONALDSON, L. E., BASSET, J. M. & THORBURN, G. D., 1970. Peripheral plasma progesterone concentration of cows during puberty, oestrous cycles, pregnancy and lactation and the effects of undernutrition or exogenous oxytocin on progesterone concentration. *Journal of Endocrinology*, 48, 599–614.

EDQVIST, L. E., 1972. Peripheral plasma levels of oestrogens and progesterone with special reference to bovine pregnancy. Stockholm: Tryckeri Balder AB.

EDQVIST, L. E., EKMAN, L., GUSTAFSSON, B. & ÅSTRÖM, G., 1970. Progesterone levels in the bovine peripheral plasma measured by the competitive protein binding technique. *Zentrablat für Veterinär Medizin*, A 17, 899–908.

FASSORA, C. & LUISI, M., 1971. The measurement of progesterone in human plasma by competitive protein-binding radioassay. *Hormones*, 2, 153–163.

HOFFMANN, B. & HAMBURGER, R., 1973. Progesterone in der Milch: Radioimmunologische Bestimmung, Beziehungen zur Gelbkörperfunktion und Milchfetkonzentration. *Zuchthygiene*, 8, 154–162.

HOFFMANN, B. & KARG, H., 1970. Determination of progesterone in bovine peripheral plasma by competitive protein binding. *Annales Endocrinologica*, 31, 823–827.

JOHANSSON, E. D. B., 1969a. Progesterone levels in peripheral plasma during the luteal phase of the normal human menstrual cycle measured by a rapid competitive protein binding technique. *Acta Endocrinologica*, 61, 592–606.

JOHANSSON, E. D. B., 1969b. Plasma levels of progesterone in pregnancy measured by a rapid competitive protein binding technique. *Acta Endocrinologica*, 61, 607–617.

JOHANSSON, E. D. B., 1970. A simplified procedure for the assay of progesterone. *Acta Endocrinologica Supplementum*, 147, 188–203.

JOHANSSON, E. D. B. & GEMZELL, C., 1969. The relation between plasma progesterone and total urinary oestrogens following induction of ovulation in women. *Acta Endocrinologica*, 62, 89–97.

JOHANSSON, E. D. B. & WIDE, L., 1969. Periovulatory levels of plasma progesterone and luteinizing hormone in women. *Acta Endocrinologica*, 62, 82–88.

LEYENDECKER, G., WARDLAW, S. & NOCKE, W., 1972. Gamma globulin protection of radioimmunoassay and competitive protein binding saturation analysis of steroids. *Journal of Clinical Endocrinology and Metabolism*, 34, 430–433.

LIPSETT, M. B., DOERR, R., & BERMUDEZ, J. A., 1970. Saturation assays for plasma progesterone and 17-hydroxyprogesterone. *Acta Endocrinologica Supplementum*, 147, 155–170.

LUISI, M., LEVANTI, S. & FRANCHI, F., 1971. The description and evaluation of a competitive protein-binding radioassay of plasma progesterone in pregnancy. *Fertility and Sterility*, 22, 435–445.

MARTIN, B. T., COOKE, B. A. & BLACK, W. P., 1969. Evaluation of a competitive protein-binding method for determining progesterone in peripheral plasma. *Journal of Endocrinology*, 45, iv-v.

MARTIN, B. T., COOKE, B. A. & BLACK, W. P., 1970. Evaluation of a rapid method for the measurement of plasma progesterone by competitive protein binding. *Journal of Endocrinology*, 46, 369–377.

MOORE, N. W., BARRETT, S. & BROWN, J. B., 1972. Progesterone concentrations in maternal and foetal blood plasma of ewes. *Journal of Endocrinology*, 53, 187–194.

MORGAN, C. A. & COOKE, I. D., 1972. A comparison of the competitive protein-binding assay and radioimmunoassay for plasma progesterone during the normal menstrual cycle. *Journal of Endocrinology*, 54, 445–456.

MURPHY, B. E. P., ENGELBERG, W. & PATTEE, C. J., 1963. Simple method for the determination of plasma corticoids. *Journal of Clinical Endocrinology and Metabolism*, 23, 293–300.

MURPHY, B. E. P. & PATTEE, C. J., 1964. Determination of plasma corticoids by competitive protein-binding analysis using gel filtration. *Journal of Clinical Endocrinology and Metabolism*, 24, 919–923.

NEILL, J. D., JOHANSSON, E. D. B., DATTA, J. K. & KNOBIL, E., 1967. Relationship between the plasma levels of luteinizing hormone and progesterone during the normal menstrual cycle. *Journal of Clinical Endocrinology and Metabolism*, 27, 1167–1173.

PICHON, M. F. & MILGROM, E., 1973. Competitive protein-binding assay of progesterone without chromatography. *Steriods*, 21, 335–346.

PIZARRO, M. A., 1971. A modified procedure for the measurement of plasma progesterone by reliance on protein binding. *Journal of Steroid Biochemistry*, 2, 321–333.

RANDEL, R. D., BROWN, B. L., ERB, R. E., NISWENDER, G. D. & CALLAHAN, C. J., 1971. Reproductive steroids in the bovine. II. Comparison of freemartins to fertile heifers. *Journal of Animal Science*, 32, 318–326.

REEVES, B. D. & CALHOUN, D. W., 1970. Reliability criteria for saturation analysis of steroids by competitive protein binding. *Acta Endocrinologica Supplementum*, 147, 61–78.

REVES, B. D., DE SOUZA, M. L. A., THOMPSON, I. E. & DICZFALUSY, E., 1970. An improved method for the assay of progesterone by competitive protein binding. *Acta Endocrinologica*, 63, 225–241.

ROBERTSON, H. A., 1972. Sequential changes in plasma progesterone in the cow during estrous cycle, pregnancy, at parturition and post-partum. *Canadian Journal of Animal Science*, 52, 645–658.

ROBERTSON, H. A. & SARDA, I. R., 1971. A very early pregnancy test for mammals: its application to the cow, ewe and sow. *Journal of Endocrinology*, 49, 407–419.

SCHIAVO, J. J., MATUSZCZAK, R. L., OLTENACU, E. B. & FOOTE, R. H., 1975. Milk progesterone in post-partum and pregnant cows as a monitor of reproductive status. *Journal of Dairy Science*, 58, 1713–1716.

SHEMESH, M., AYALON, N. & LINDNER, H. R., 1973. Early pregnancy diagnosis based upon plasma progesterone levels in the cow and ewe. *Journal of Animal Science*, 36, 726–729.

SHEMESH, M., LINDNER, H. R. & AYALON, N., 1971. Competitive protein-binding assay of progesterone in bovine jugular venous plasma during the oestrous cycle. *Journal of Reproduction and Fertility*, 26, 167–174.

SMITH, J. F., FAIRCLOUGH, R. J., PAYNE, E. & PETERSON, A. J., 1975. Plasma hormone levels in the cow. I. Changes in progesterone and oestrogen during the normal oestrous cycle. *New Zealand Journal of Agricultural Research*, 18, 123–129.

SMITH, V. G., EDGERTON, L. A., HAFS, H. D. & CONVEY, E. M., 1973. Bovine serum estrogens, progestins and glucocorticoids during late pregnancy, parturition and early lactation. *Journal of Animal Science*, 36, 391–396.

SPRAGUE, E. A., HOPWOOD, M. L., NISWENDER, G. D. & WILTBANK, J. N., 1971. Progesterone and luteinizing hormone levels in peripheral blood of cycling beef cows. *Journal of Animal Science*, 33, 99–103.

STROTT, C. A., 1975. Use of plasma binding-proteins for steroid hormone assays. *Methods in Enzymology*, 36, 34–48.

SURVE, A. H., RANDEL, R. D., ERB, R. E. & CALLAHAN, C. J., 1968. A modified CPB method for the quantitation of blood plasma progesterone in cows. *Journal of Animal Science*, 27, 1789.

WISHART, D. F., HEAD, V. A. & HORTH, C. E., 1975. Early pregnancy diagnosis in cattle. *Veterinary Record*, 96, 34–38.

YALOW, R. S. & BERSON, S. A., 1959. Assay of plasma insulin in human subjects by immunological methods. *Nature*, 184, 1648–1649.

YOSHIMI, T. & LIPSETT, M. B., 1968. The measurement of plasma progesterone. *Steroids*, 11, 527–540.