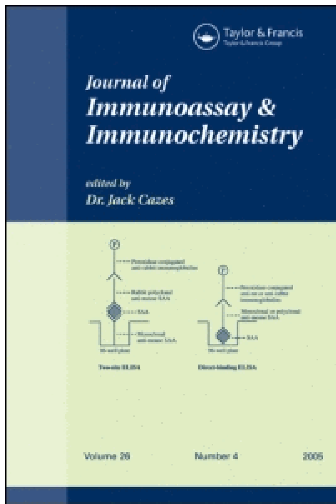
This article was downloaded by:
On: *16 January 2011*
Access details: *Access Details: Free Access*
Publisher *Taylor & Francis*
Informa Ltd Registered in England and Wales Registered Number: 1072954 Registered office: Mortimer House, 37-41 Mortimer Street, London W1T 3JH, UK



Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

A Sensitive Microtitre Plate Enzyme Immunoassay of Oestradiol-17β in the Cow and Mare

Ilona Jones[a]; A. Madej[a]
[a] Department of Clinical Chemistry, College of Veterinary Medicine, Swedish University of Agricultural Sciences, Uppsala, Sweden

To cite this Article Jones, Ilona and Madej, A.(1988) 'A Sensitive Microtitre Plate Enzyme Immunoassay of Oestradiol-17β in the Cow and Mare', Journal of Immunoassay and Immunochemistry, 9: 3, 349 — 365
To link to this Article: DOI: 10.1080/01971528808053221
URL: http://dx.doi.org/10.1080/01971528808053221

PLEASE SCROLL DOWN FOR ARTICLE

Full terms and conditions of use: http://www.informaworld.com/terms-and-conditions-of-access.pdf

This article may be used for research, teaching and private study purposes. Any substantial or systematic reproduction, re-distribution, re-selling, loan or sub-licensing, systematic supply or distribution in any form to anyone is expressly forbidden.

The publisher does not give any warranty express or implied or make any representation that the contents will be complete or accurate or up to date. The accuracy of any instructions, formulae and drug doses should be independently verified with primary sources. The publisher shall not be liable for any loss, actions, claims, proceedings, demand or costs or damages whatsoever or howsoever caused arising directly or indirectly in connection with or arising out of the use of this material.

JOURNAL OF IMMUNOASSAY, 9(3&4), 349-365 (1988)

A SENSITIVE MICROTITRE PLATE ENZYME IMMUNOASSAY OF OESTRADIOL-17β IN THE COW AND MARE

Ilona Jones and A. Madej

Department of Clinical Chemistry,
College of Veterinary Medicine,
Swedish University of Agricultural Sciences,
Box 7038, S-750 07 Uppsala, Sweden

Downloaded At: 11:56 16 January 2011

ABSTRACT

Microtitre plates were coated with antiserum against oestradiol-17β-6-(O-carboxymethyl)-oxime bovine serum albumin raised in sheep. The plasma samples (0.2-1.0 ml) were extracted with peroxide-free diethyl ether prepared daily by treatment with Al_2O_3. The enzyme conjugate was prepared by coupling oestradiol-17β-6-(O-carboxymethyl)-oxime to horse-radish peroxidase. The conjugate was chromatographed on a Sephadex G-25 column. The standard curve ranged from 0.37 to 18.40 fmol/well of oestradiol-17β. The amount of oestradiol-17β causing a 50% reduction of maximum binding was 4.4 fmol/well.

Standards and samples were incubated overnight at 4^oC. The conjugate solution was added followed by further incubation for 2 h at 4^oC. Tetramethylbenzidine was used as a chromogen, and the optical density was measured at 450 nm. The patterns of oestradiol-17β during a normal oestrus cycle in the cow and mare are presented.

INTRODUCTION

In radioimmunoassays of steroids the specific activity of the tritium-labelled tracer is a limiting factor for the sensitivity of the test. An increase of assay sensitivity is possible

Copyright © 1988 by Marcel Dekker, Inc.

through introduction of either radioiodine-labelled tracers or enzyme-labelled tracers. Although several studies dealing with enzyme immunoassay of oestrogens have been published (4-8), none of them is sensitive enough to measure oestradiol-17β in the non-pregnant cow or mare. In this paper we describe a highly sensitive enzyme immunoassay for oestradiol-17β in blood plasma, which is suitable for use in these species.

MATERIALS AND METHODS

Preparation of Antiserum

Antiserum, obtained from Boilert et al. (9), was raised in sheep against oestradiol-17β-6-(O-carboxymethyl)-oxime-bovine serum albumin conjugate. The antiserum was purified by us on a Sephacryl-S-300 (Pharmacia, Sweden) column, 2.6x90 cm, and eluted with 0.05 M Tris-HCl buffer, pH 7.4. The immunoglobulin fractions were pooled and concentrated to the initial antiserum volume and transferred to a new buffer solution (0.04 M phosphate-buffered saline, pH 7.0) using Amicon filter equipment (Danvers, MA, USA). Finally, the antiserum solution was lyophilized in small aliquots from which the stock solution, diluted 1:1000 in assay buffer, was prepared and kept at 4°C.

Preparation of the Enzyme Conjugate

Oestradiol-17β-6-(O-carboxymethyl)-oxime (E_2-6-CMO) (Steraloids, Witton, N.H., USA) was conjugated to horse-radish peroxi-

Downloaded At: 11:56 16 January 2011

dase (HRP, RZ-3, Sigma, St. Louis, MO, USA) using the mixed anhydride reaction modified by Dawson et al. (10). E_2-6-CMO, 18.6 mg, was dissolved in 1 ml of dimethylformamide and 7.5 µl of methylmorpholine. The mixture was cooled to -15°C, and 6.5 µl of sec-butylchloroformate added, and the content then stirred for 3 min. Thereafter, the HRP solution (100 mg HRP in 1 ml distilled water and 0.75 ml dimethylsulphoxide) was added dropwise and stirring continued at -15°C for 60 min and at 0°C for 120 min. Then 10 mg of $NaHCO_3$ was added. The conjugate mixture was dialyzed against distilled water and filtered through a Sephadex G-25 column (1.0x10.0 cm) to separate enzyme-labelled from non-enzyme-labelled oestradiol. The enzyme conjugate fractions were pooled and stored in small aliquots at -70°C. A stock solution of the HRP-oestradiol conjugate diluted 1:10 in assay buffer (0.04 M Na_2HPO_4/0.15 M NaCl, pH 7.2, containing 0.1% BSA and 0.02% Thimerosal), was kept at 4°C and diluted further in the same buffer to 1:40,000 just prior to use.

Procedures for Purification of Diethyl Ether and Extraction

Peroxide-free diethyl ether was prepared daily by purification on an Al_2O_3 90 active basic (technical information, Merck, Darmstadt, FRG) column (1.5x10.0 cm). About 30 g of Al_2O_3 is required for purification of 250 ml of diethyl ether (analytical grade, 0.01% water, Merck). All blood plasma extractions were performed twice with 2 ml of ether and shaken by hand for 1 min

Downloaded At: 11:56 16 January 2011

each time. Then the tubes were placed in a methanol bath (-20°C). The organic phases were decanted, pooled and evaporated under a stream of air. The extraction recovery was estimated by adding tritium-labelled oestradiol-17β (7000 dpm) to samples of calf plasma.

Assay Procedure

All glassware used was thoroughly rinsed in 96% ethanol. Flat-bottomed microtitre plates (M 129B, Dynatech Lab. Ltd., Sussex, UK, and Cat. No. 3590, Costar Co, Cambridge, MA, USA) were coated with 100 µl of purified oestradiol antiserum diluted 1:500 000 in coating buffer (0.05 M Na_2CO_3/$NaHCO_3$, pH 9.6) The plates were incubated overnight at 4°C. Just before assay, the coated plates were washed 3 times with 400 µl per well of washing liquid (0.05% Tween 80 in saline). Standards and samples were analyzed in quadruplicates, and aliquots of 100 µl were transferred to each well. The standards prepared in fresh assay buffer contained 0, 0.37, 0.92, 1.84, 3.68, 9.19 and 18.4 fmol/100 µl. The evaporated extract from 1 ml plasma was dissolved in 500 µl of fresh assay buffer, and 100 µl per well of this solution was analyzed. For the quality control pooled samples of low, medium and high levels of oestradiol-17β were analyzed on every plate. The plasma with the low concentration of oestradiol-17β (approx. 9 pmol/l) was prepared by charcoal treatment (50 mg/ml plasma). The plates were covered with self-adhesive plastic sheet (x-film,

Downloaded At: 11:56 16 January 2011

Overath, FRG) and incubated overnight at 4°C. A cold (4°C) solution of the oestradiol-HRP conjugate, 50 µl per well, was then added and the incubation continued at 4°C for 2 h. After the plates had been washed (3x400 µl/well) 150 µl substrate solution (25 ml 0.1 M acetate/citrate buffer, pH 5.5; 100 µl 1% H_2O_2; 300 µl of 6% tetramethylbenzidine solution in dimethylsulfoxide) was added and the plates were incubated for 40 min in the dark at room temperature. The enzyme reaction was stopped by adding 50 µl of 2 M H_2SO_4 (3). The optical density was then measured at 450 nm (Multiscan, Dynatech).

Downloaded At: 11:56 16 January 2011

Blood Samples

Daily blood samples (7-10 ml) were collected in heparinized tubes from the jugular vein of a postpartum cow and a mare with regular oestrous cycle. The blood was immediately centrifuged and the plasma stored at -20°C until assayed.

Radioimmunoassays

Procedures for radioimmunoassay of progesterone (11) and LH (12) in bovine plasma and radioimmunoassay of progesterone and oestrone sulphate in equine plasma (13) have been described earlier.

RESULTS

Validation of Assay

A mean standard curve for oestradiol-17β, calculated from 18 assays, is shown in Fig. 1. The sensitivity of the assay, calcu-

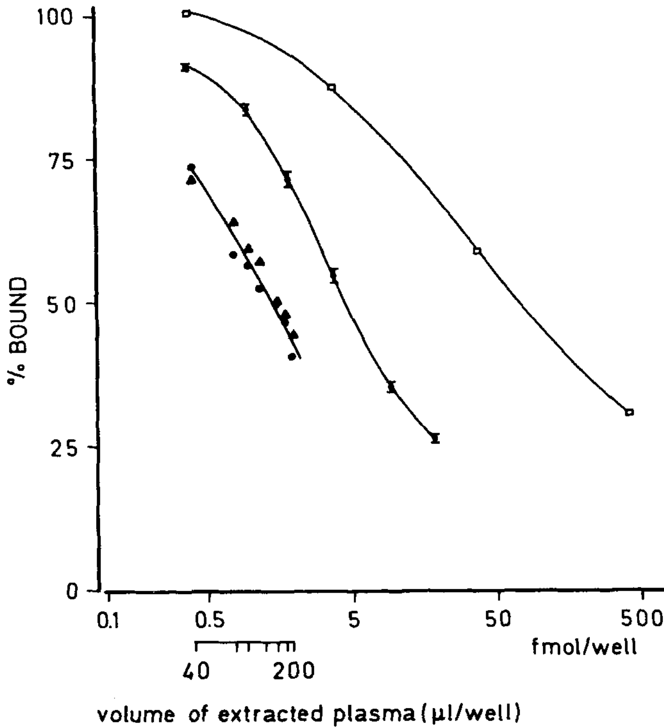


FIGURE 1. Standard curve for oestradiol-17β (●, mean±S.E.M., n=18), the cross reaction of oestrone (□) and the dilution curve of extracted plasma (●, plasma no. 1; ▲, plasma no. 2).

lated from the precision profile, was 0.22 fmol/well (1.1 pmol/l). The amount of oestradiol-17β causing a 50% reduction of maximum binding in the standard curve was 4.4 fmol/well. The relative cross-reactions of the antibody were 7% with oestrone (Fig. 1), 1% with oestradiol-17α, 0.3% with oestriol, 0.1% with

Downloaded At: 11:56 16 January 2011

oestrone sulphate and less than 0.01% with cortisol, progesterone and testosterone (data not shown). A dilution curve of extracted plasma (from 200 µl to 40 µl) from a heifer in oestrus was parallel to the standard curve (Fig. 1). The same parallelism was obtained with equine and mink plasma (data not shown).

Known amounts of oestradiol-17β were added to plasma containing 1063 fg/well (19 pmol/l) of oestradiol-17β and the results are given in Table 1. The recovery of oestradiol-17β ranged from 82.0% to 103.0% with an average of 94.0±7.0%.

The average extraction recovery of tritium-labelled oestradiol-17β added to 1 ml of bovine plasma was 92.0±1.0%. The same extraction recovery was obtained when the shaking was done with a Multi-tube vortexer SMI (Dade, CA, USA). Less than 0.2% of tritium-labelled oestrone sulphate was extracted using diethyl ether.

No measurable oestradiol could be found in extracted water.

The intra-assay (intra-plate) coefficients of variation of the optical density at 0, 22.5 and 90 pmol/l varied between 6.2 and 7.3% (Table 2). Precision profile of our assay indicated that the coefficient of variation decreased from 20% at 5 pmol/l to 17% at 10 pmol/l and remained below 15% for concentrations exceeding 20 pmol/l.

The inter-assay coefficients of variation for the quality control samples containing low, medium and high levels of oestradiol-17β were 27.1%, 12.9% and 5.9%, respectively (Table 3).

Downloaded At: 11:56 16 January 2011

TABLE 1

Recovery of Oestradiol-17β Added to Bovine Plasma

	Added E_2-17β fg/well	Found E_2-17β fg/well	Recovery %
Plasma	-	1063	100
	100	955	82
	200	1078	85
	500	1532	98
	1000	1909	93
	2000	3142	103
	3000	4018	99
	4000	4916	97
	5000	5796	96
			$\bar{x}$=94±7%

Downloaded At: 11:56 16 January 2011

Plasma Oestradiol-17β Concentration

To evaluate the assay, oestradiol-17β levels during the first normal luteal phase in a postpartum cow were determined (Fig. 2). The preovulatory LH peaks followed by the normal progesterone increase were recorded on days 22 (data not shown) and 44 post partum. Two oestradiol-17β peaks (26 and 33 pmol/l,

TABLE 2

The Intra-assay Variation in the Enzyme Immunoassay of Oestradiol-17β

absorbance	1.434	0.512	0.264
pmol/l	0	22.5	90
n	96	96	96
SD	0.089	0.037	0.018
CV%	6.2	7.2	6.8

Downloaded At: 11:56 16 January 2011

TABLE 3

The Inter-assay Variation in the Enzyme Immunoassay of Oestradiol-17β

pmol/l	8.5	14.0	28.9
n	8	8	8
SD	2.3	1.8	1.7
CV%	27.1	12.9	5.9

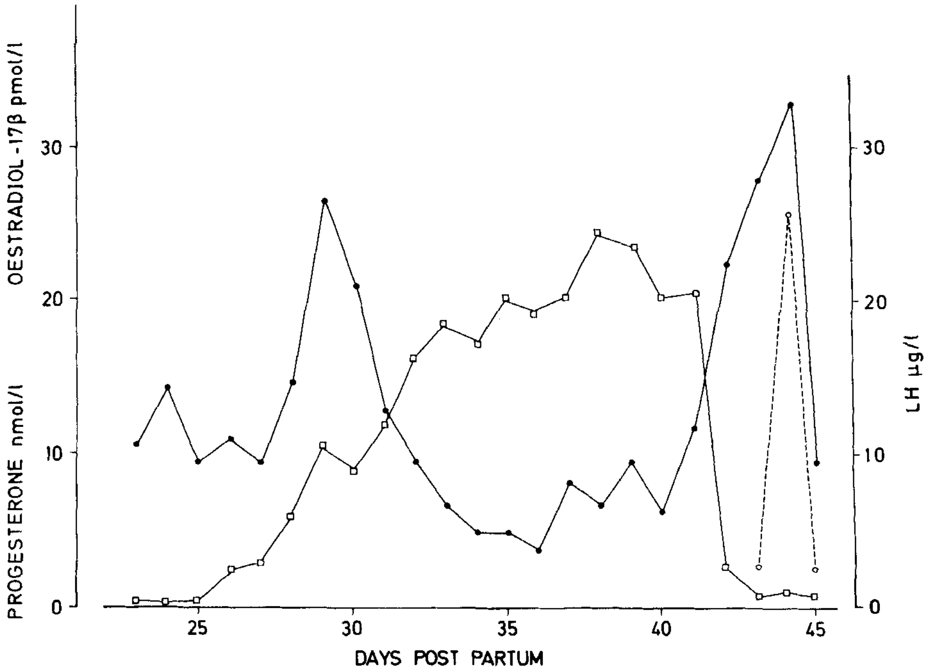


FIGURE 2. Plasma oestradiol-17β (●), progesterone (□) and LH (o) levels during the first normal luteal phase in a postpartum cow.

Downloaded At: 11:56 16 January 2011

respectively) were recorded: the first occurred 7 days after the LH peak and the second occurred simultaneously with the next preovulatory LH surge on day 44. The basal level of oestradiol-17β fluctuated between 3 and 14 pmol/l.

The maximum plasma oestradiol-17β observed on day 4 of the oestrous cycle in the mare was 120 pmol/l (Fig. 3). The mid-luteal levels of oestradiol-17β varied between 20 and 40 pmol/l.

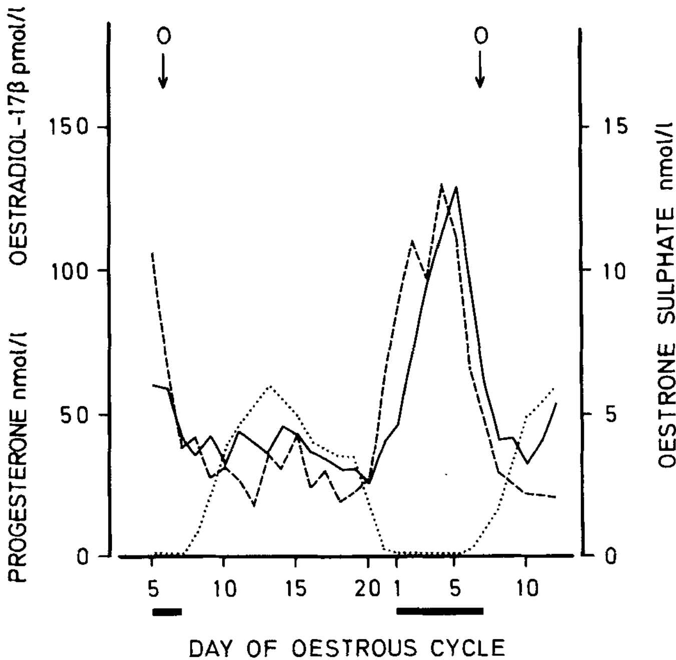


FIGURE 3. Plasma oestradiol-17β (---), oestrone sulphate (——) and progesterone (....) levels during the oestrous cycle in one mare. The horizontal black bars indicate oestrus; arrows denote the time of ovulation.

Fig. 3 also depicts the plasma levels of oestrone sulphate in the same mare. The oestrone sulphate level started to increase one day before the onset of oestrus and reached its maximum on day 5 of the oestrous cycle (12 nmol/l). The basal level of oestrone sulphate varied between 2 and 4 nmol/l.

Downloaded At: 11:56 16 January 2011

DISCUSSION

This enzyme immunoassay for determination of oestradiol-17β is very sensitive, allowing a substantial reduction of the plasma volume required for extraction. The sensitivity, 0.22 fmol (1.1 pmol/l) per assay unit, is better than that reported by Lindberg et al. (1) using a radioiodine-labelled tracer and the same oestradiol-17β antiserum. When the antiserum was used in radioimmunoassay with tritium-labelled tracer the practical detection limit was 25 pmol/l (14). Indeed, a 20-fold increase of the sensitivity was achieved by introducing the enzyme-labelled tracer. This finding agrees with those described for progesterone by van de Wiel and Koops (3). Recently Roda et al. (15) developed a direct chemiluminescent assay and Maurel et al. (16) a fluorescent enzyme immunoassay of oestradiol-17β with almost the same detection limit as the present assay (0.55 fmol/ tube and 0.37 fmol/well, respectively).

The findings of no measurable oestradiol-17β when assaying water is in agreement with Peters (17) and Saumande and Batra (18), who also reported that the water samples did not decrease the maximum binding.

Van de Wiel and Koops (3) reported that a second coating of the wells with bovine serum albumin solution reduced non-specific binding of the conjugate to the surface of the plate. In contrast, we found that a second coating of the wells with bovine serum albumin solution had no influence on either the intra-assay variation or the standard curve.

Downloaded At: 11:56 16 January 2011

The accuracy and precision of the test is strongly affected by the quality of the microtitre plate. Hence, a batch of microtitre plates was only accepted for assay when one or two randomly selected plates showed an intra-assay coefficient of variation at maximal binding (absorbance at least 0.9) lower than 7%. Only the microtitre plates from Dynatech and Costar fulfilled our requirements, so far.

The concentrations of oestradiol-17β during a normal luteal phase in the cow are very similar to those published earlier (e.g. 19,20,21).

The maximum concentrations of oestradiol-17β found here in the mare at oestrus are somewhat lower than those reported by Pattisson et al. (22), but almost the same as those reported by Nelson et al. (23). However, the basal level of oestradiol-17β during the luteal phase of the oestrous cycle was about half that reported by Nelson et al.(23), who used the same oestradiol-17β antiserum. It is interesting to note the good agreement between the oestradiol-17β and oestrone sulphate pattern during the oestrous cycle in the mare. The concentration of oestrone sulphate is 100 times higher than that of oestradiol-17β. Still this high level of oestrone sulphate will not affect the oestradiol determination because only 0.2% is extracted using diethyl-ether and the cross-reactivity of the present antiserum to oestrone sulphate is 0.1%.

Downloaded At: 11:56 16 January 2011

It is well known that in steroid assays, the sensitivity of the assay is improved by the use of a heterologous "bridge system" (24-27). It can also be improved by a homologous bridge system but with different configurations at the site of attachment (28,29). However, in some instances (i.e. testosterone assay: 29) the use of a homologous bridge system with the same configurations resulted in a very sensitive assay. The homologous system for oestradiol-17β employed here support these findings of Webb et al. (29).

In conclusion, the microtitre plate enzyme immunoassay described here is suitable for measuring oestradiol-17β in a small volume of bovine or equine plasma. Consequently, it is also possible to apply this technique to study the pattern of oestradiol in relatively small animals, e.g. mink. Moreover, the determination of steroids by enzyme immunoassay is relatively inexpensive and rapid.

Downloaded At: 11:56 16 January 2011

ACKNOWLEDGEMENTS

This study was supported by grants from the Swedish Council for Forestry and Agricultural Research.

REFERENCES

1. Lindberg P. and Edqvist L.-E. The use of 17β-oestradiol-6-(O-carboxymethyl)oxime- ^{125}I tyramine as tracer for the radioimmunoassay of 17β-oestradiol. Clin. Chim. Acta 1974; 53:169-174.

2. Chearskul S., Rincon-Rodriguez I., Sufi S.B., Donaldson A. and Jeffcoate S.L. Simple direct assays for measuring oestr-

adiol and progesterone in saliva. In: Proceedings of an International Symposium on Radioimmunoassay and Related Procedures in Medicine, Vienna 1982, Proceedings Series, IAEA, Vienna, 1982:265-274.

3. Van de Wiel D.F.M. and Koops W. Development and validation of an enzyme immunoassay for progesterone in bovine milk or blood plasma. Anim. Reprod. Sci. 1986;10:201-213.

4. Numazawa M., Haryu A., Kurosaka K. and Nambara T. Picogram order enzyme immunoassay of oestradiol. FEBS Lett. 1977;79:396-398.

5. Exley D. and Abuknesha R. A highly sensitive and specific enzyme-immunoassay method for oestradiol-17β. FEBS Lett. 1978;91:162-165.

6. Tamamura F., Nakao T., Tsunoda N. and Kawata K. An enzyme immunoassay of estrone in swine serum. Steroids 1982;39:657-666.

7. Bosch A.M.G., Dijkhuizen D.M., Schuurs A.H.W.M. and van Weemen B.K. Enzyme immunoassay for total oestrogens in pregnancy plasma or serum. Clin. Chim. Acta 1978;89:59-70.

8. Sugiyama S., Nakao T., Tsunoda N. and Kawata K. An enzymeimmunoassay of serum oestrone sulphate and its application to early pregnancy diagnosis in pigs. Br. vet. J. 1985;141:60-68.

9. Boilert B., Edqvist L.-E., Johansson E.D.B., Lindberg P. and Martinsson K. The influence of conjugated estrogens in radioimmunoassays using different antibodies against estradiol-17β. Steroids 1973;22:891-894.

10. Dawson E.C., Denissen A.E.H.C. and van Weemen B.K. A simple and efficient method for raising steroid antibodies in rabbits. Steroids 1978;31:357-366.

11. Kindahl H., Edqvist L.-E., Granström E. and Bane A. The release of prostaglandin $F_{2\alpha}$ as reflected by 15-keto-13,14-dihydroprostaglandin $F_{2\alpha}$ in the peripheral circulation during normal luteolysis in heifers. Prostaglandins 1976;11:871-878.

12. Stupnicki R. and Madej A. Radioimmunoassay of LH in blood plasma of farm animals. Endokrinologie 1976;68:6-13.

Downloaded At: 11:56 16 January 2011

13. Kindahl H., Knudsen O., Madej A. and Edqvist L.-E. Progesterone, prostaglandin $F_{2\alpha}$, PMSG and oestrone sulphate during early pregnancy in the mare. J. Reprod. Fert. 1982; Suppl. 32:353-359.

14. Kunavongkrit A. Kindahl H. and Madej A. Clinical and endocrinological studies in primiparous zero-weaned sows: 2. Hormonal patterns of normal cycling sows after zero-weaning. Zbl. Vet. Med. A. 1983;30:616-624.

15. Roda A., Girotti S., Piacentini A.L., Preti S. and Lodi S. Development of a sensitive, direct luminescent enzyme immunoassay for plasma estradiol-17β. Anal. Biochem. 1986; 156:267-273.

16. Maurel M.C., Labrousse H., Terqui M. and Ayrameas S. Microtitre plate enzyme immunoassay of oestradiol-17β. J. steroid Biochem. 1986;25:Suppl., 48S.

17. Peters A.R. Effect of exogenous oestradiol-17β on gonadotrophin secretion in post-partum beef cows. J. Reprod. Fert. 1984;72:473-478.

18. Saumande J. and Batra S. K. Superovulation in the cow: Comparison of oestradiol-17β and progesterone patterns in plasma and milk of cows induced to superovulate; relationships with ovarian responses. J. Endocr. 1985;107:259-264.

19. Dobson H. and Dean P.D.G. Radioimmunoassay of oestrone, oestradiol-17α and 17β in bovine plasma during the oestrous cycle and last stages of pregnancy. J. Endocr. 1974;61:479-486.

20. Abeyawardene S.A., Hathorn D.J. and Glencross R.G. Concentrations of oestradiol-17β and progesterone in bovine plasma and defatted milk during the post-partum anovulatory period, during oestrous cycles and following ovariectomy. Br. vet. J. 1984;140:458-467.

21. Dieleman S.J., Bevers M.M., van Tol H.T.M. and Willemse A.H. Peripheral plasma concentrations of oestradiol, progesterone, cortisol, LH and prolactin during the oestrous cycle in the cow, with emphasis on the peri-oestrous period. Anim. Reprod. Sci. 1986;10:275-292.

22. Pattison M.L., Chen C.L., Kelley S.T. and Brandt G.W. Luteinizing hormone and estradiol in peripheral blood of mares during estrous cycle. Biol. Reprod. 1974;11:245-250.

Downloaded At: 11:56 16 January 2011

23. Nelson E.M., Kiefer B.L., Roser J.F. and Evans J.W. Serum estradiol-17β concentrations during spontaneous silent estrus and after prostaglandin treatment in the mare. Theriogenology 1985;23:241-262.

24. Van Weemen B.K. and Schuurs A.H.W.M. The influence of heterologous combinations of antiserum and enzyme-labeled estrogen on the characteristics of estrogen enzyme-immunoassays. Immunochemistry 1975;12:667-670.

25. Van Weemen B.K., Bosch A.M.G., Dawson E.C., Van Hell H. and Schuurs A.H.W.M. Enzyme immunoassay of hormones. Scand. J. Immunol. 1978; 8 (Suppl. 7): 73-82.

26. Corrie J.E.T. Immunoassays for steroid hormones using radioiodinated tracers. Br. vet. J. 1982;138:439-442.

27. Mitsuma M., Kambegawa A., Okinaga S. and Arai K. A sensitive bridge heterologous enzyme immunoassay of progesterone using geometrical isomers. J. steroid Biochem. 1987; 28:83-88.

28. England B.G., Niswender G.D. and Midgley A.R.Jr. Radioimmunoassay of estradiol-17β without chromatography. J. clin. Endocr. Metab. 1974;38:42-50.

29. Webb R., Baxter G., McBride D., Nordblom G.D. and Shaw M.P.K. The measurement of testosterone and oestradiol-17β using iodinated tracers and incorporating an affinity chromatography extraction procedure. J. steroid Biochem. 1985;23:1043-1051.

Downloaded At: 11:56 16 January 2011