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Development of an enzyme immunoassay for determination of steroid hormones to improve the reproductive efficiency of domestic animals

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家畜의 繁殖效率增進을 위한 steroid hormones의 酵素免疫分析法 開發

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초록 : 젖소에서 progesterone과 testosterone의 측정을 위하여 solid phase 酵素免疫分析法를 개발하였다. 1차 항체로서 11 α-hemisuccinate-progesterone bovine serum albumin과 4-androsten-17 β-ol-3-one-carboxymethyloxime bovine serum albumin에 대한 토끼 血淸을 각각 抗血淸으로 사용하였고, 2차 항체로서 면약 IgG를 사용하였다.

Conjugates로서는 각각 progesterone-11 α-hydroxy-hemisuccinate-horseradish peroxidase와 4-androsten-17 β-ol-3-hemisuccinate-horseradish peroxidase를 사용하였다.

Progesterone과 testosterone에 대한 最低測定値는 각각 6.7 pg/well과 1.0 pg/well이었다.

Key words : enzyme immunoassay, progesterone, testosterone.

Introduction

Radioimunoassay(RIA) has been the most predominant analytical technique in endocrinology during the last 20 years. However, RIA has several limitations which are inherent to the use of radioactive isotopes. Atempts have been made during the last decade to circumvent the use of radioactive isotopes by applying other labels, such as enzymes.[1~3] Use of enzymeimmunoassay(EIA) in endocrinology, pioneered by Engvall and Perlmann[4,5] and Van Weemen and Schuurs[6,7], has offered an attractive alternative to RIA to detect antibodies and antigens from body fluids.

This report describes the development of a sensitive and rapid EIA for progesterone and testosterone using solid-phase microtitre plates.

Meterials and Methods

Reagents : Horseradish peroxidase(EC 1.11.1.7., R.Z. of approximately 3.0), 5-Pregnen-3 β-ol-20-one, 4-Pregnen-3,20-dione, 4-Pregnen-17 α-ol-3,20-dione, 4-Androsten-3.17-dione, 4-Androsten-17 β-ol-3-one, 4-Androsten-17 α-ol-3-one, 5-Androsten-3 β-ol-17-one, 5-Androsten-3 α, 17 β-diol, 5-Androsten-3 β, 17 β-diol, Estrone, Estradiol-17 α, Estradiol-17 β and Estriol were purchased from Sigma, München, Germany ; 11 α-hemisuccinate-progesterone, 4-androsten-17 β-ol-3-one carboxymethyloxime and bovine serum albunim(BSA) were obtained from Steraloids, New Hampshire, USA ; cyanogen bromide, isobutylchloroformate and sodium chloride were obtained from

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Fluka, Buchs, Switzerland ; N, N-dimethylformamide, sodium azide, 2,2'-azino-di-(3-ethylbenzthiazoline sulphonic acid) diammonium salt(ABTS), Tween 20 and citric acid were obtained from Sigma, München, Germany. All other reagents were purchased from Merck, Darmstadt, Germany. Organic solvents were distilled before use. Flat-bottomed polystyrene 96-well micro-ELISA plates were purchased form Dynatec Lab., Virginia, USA.

Antiserum : Antiserum against 4-pregnen-11 α -ol-3,20 dione-hemisuccinate and 4-androsten-17 β -ol-3-one-caboxymethyloxime conjugated to BSA were raised in rabbit. Characterization of these antisera for RIA was described previously by Choi.[8] Antirabbit Ig G antiserum was raised in sheep and was purified by protein A affinity chromatography.

Enzyme conjugate : Horseradish peroxide(HRP) was coupled to the 4-pregnen-11 α -ol-3,20-dione-hemisuccinate and 4-androsten-17 β -ol-3-one-carboxymethyloxime by the mixed anhydride method of Erlanger et al[9] as modified by Dawson et al.[10] Separation of the conjugate from any remaining low molecular weight material was achieved by column chromatography with Sephadex G 25. The charge number of the conjugate(progesterone/enzyme ratio) approached the theoretical ideal value of 1.0 as estimated by RIA. The conjugate have been stable for 1 year at 4℃ with no loss of enzymic and immunological activity.

Plasma samples : Heparinized blood samples were taken daily from 3 dairy cows during the estrous cycle and centrifuged immediately(10 min, 3000g) and the plasma samples were strored at -20℃ until use.

Progesterone and testosterone standards : Progesterone and testosterone were stored in ethanol at 4℃ at a concentration of 1.0 mM. When required, dilutions were prepared in assay buffer.

Stock solutions : 1) coating buffer : 0.05 M sodium bicarbonate, pH 9.6 ; 2) washing solution : 0.15 M NaCl containing 0.05% Tween 20 ; 3) assay buffer : 0.1 M sodium phosphate, pH 7.0, containing 0.87% NaCl and 0.1% BSA(this phosphate buffer was used for the dilution of standards and conjugate) ; 4) substrate : 6.0 mM hydrogen peroxide and 3.7 mM o-phenylenediamine ; 5) stopping reagent : 25mℓ stopping solution contained 25$\mu\ell$ 1.0M EDTA and 25mℓ 0.15M hydrofluoric acid(HF) containing 0.6mM NaOH.

EIA procedure : Flat-bottomed polystyrene 96-well microtitre plates were coated with 50$\mu\ell$ per well of the antirabbit Ig G antiserum in coating buffer at a dilution of : 2,000 except for the first column for the blank. The plate was sealed tightly with a waterproof plate-sealer cover and incubated overnight at 4℃, although plates could be used in as little as 4 hours or could be stored at 4℃ for several weeks in unwashed or washed state. To remove antiserum that was not bound to polystyrene, wells of the plate were emptied, washed four times with washing solution and dried inverted on paper towels.

The steroids were extracted from the 100$\mu\ell$ plasma with 2mℓ petroleum ether(40~60℃ bp). Before the assay. 12$\mu\ell$ assay buffer was added to each tube containing plasma extract, and 50$\mu\ell$ aliquots(equivalent to 40$\mu\ell$ plasma) were added to the wells in duplicate. In order to facilitate the procedure of aliquoting, assay buffer was added to all 40 samples and were mixed by vortex befor duplicate pipetting of samples. For the standard curve, 50$\mu\ell$ of progesterone and testosterone standards were used(eight wells/standard, 1.6~240 pg and ten wells/standard, 0.55~300 pg, respecitively). This was followed immediately by 50$\mu\ell$ of diluted progesterone-and testosterone-HRP conjugate in assay buffer of initiate a competition reaction.

Immediately after the addition of conjugate, plates were covered and incubated for exactly 3 hours at 20℃ which was then washed 5 times. The amount of conjugate bound was determined by the addition of the 100$\mu\ell$ ABTS to each well with care being taken to keep plates and the unused substrate solution sealed to prevent any non-specific auto-oxidation. The substrate reaction was terminated after 1 hour at 20℃ by adding 100$\mu\ell$ 0.15M HF stopping solution to each well. Absorbance was then measured at 492 nm in the automatic micro-ELISA plate reader(Titertek Multiskan MCC/340MK II, Flow Lab.).

Results and Discussion

Specificity was determined by measuring cross-reactivity with a number of steroids and by determining parallelism between the standard curve and serial dilutions of samples(Table 1).

Cross-reactivity was defined as the amount of progesterone or testosterone causing a 50% reduction of the

Table 1. Percentage cross-reaction of various steroids with anti-4-pregnen-11 α -ol-3,20-dione-hemisuccinate bovine serum albumin and anti-4-androsten-17 β -ol-3-one-carboxymethyloxime bovine serum albumin

Steroids	Antibody	
	Progesterone	Testosterone
5-Pregnen-3 β -ol-20-one	–	–
4-Pregnen-3,20-dione	100.0	<0.1
4-Pregnen-17 α -ol-3,20-dione	<0.1	–
4-Androsten-3,17-dione	<0.1	<0.1
4-Androsten-17 β -ol-3-one	<0.1	100.0
4-Androsten-17 α -ol-3-one	<0.1	–
5-Androsten-3 β -ol-17-one	<0.1	–
5-Androsten-3 α ,17 β -diol	<0.1	–
5-Androsten-3 β ,17 β -diol	<0.1	1.4
Estrone	<0.1	–
Estradiol-17 α	–	<0.1
Estradiol-17 β	1.1	0.5
Estriol	<0.1	<0.1

Table 2. Titration of serial dilutions of anti-11 α -hemisuccinate-progesterone bovine serum albumin against serial dilution of 11 α -hemisuccinate-progesterone-horseradish peroxidase

Enzyme levels	Progesterone added(pg)	Antibody dilution(OD 492)			
		1 : 4,000	1 : 8,000	1 : 16,000	NSB*
1 : 500	0	>	>	2.36	1.27
	5	>	>	2.23	1.41
1 : 1,500	0	>	>	1.81	0.66
	5	>	>	1.80	0.61
1 : 4,500	0	>	1.92	0.97	0.22
	5	>	1.72	0.94	0.22
1 : 13,000	0	1.96	0.96	0.44	0.07
	5	1.99	0.88	0.42	0.07

>=Absorbance displacement, more than 2.36

* NSB=Non-specific binding

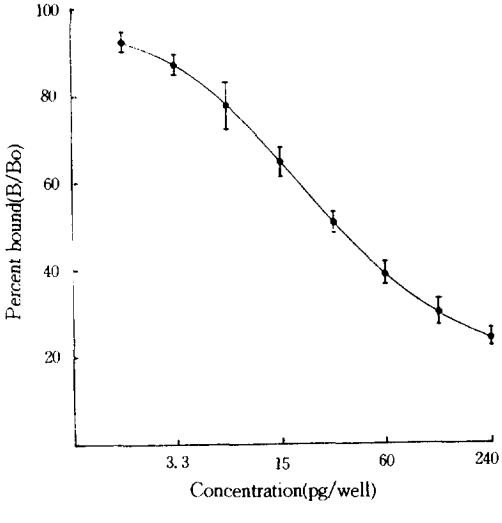

Fig 1. Standard curve for progesterone(mean ± S.D).

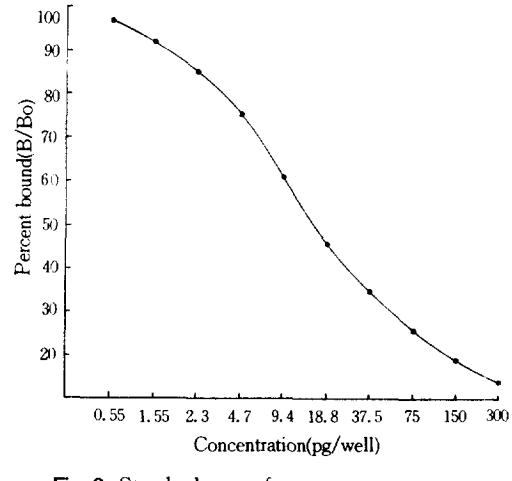

Fig 2. Standard curve for testosterone.

initial binding in the standard curve, divided by the amount of a corss-reacing steroid which caused same the reduction.

Sheep antirabbit gamma globulin serum was used in the solid phase microtitre plate EIA, which was similar to the method of Nakao et al.[11] Concentration of 0.1 mg/mℓ(1:2000) sheep Ig G appeared to be adequate compared to 0.4 mg/mℓ Ig G for coating on each plate done by Nakao et al.[11]

Diluted antiserum in assay buffer and labeled progesterone were incubated with or without added progesterone, and dilution factor of antiserum was determined by displacement of absorbance(Table 2).

Two dilution combinations indicated high displacement, and less antibody and labeled progesterone combination was adopted to improve the detection limit.

Sensitivity calculated from initial binding(Bo) values minus 2 SD was 6.7 pg per well for progesterone and 1.0 pg per well for testosterone. Sensitivity, defined as the amount of progesterone of testosterone which causes a 50% reduction of the Bo in the standard curve, varied between 1.6 and 240 pg per well for progesterone(Fig 1) and between 0.55 and 300 pg per well for testosterone(Fig 2). The result indicated the preference in high reactivity of progesterone for horseradish peroxidase and for bovine serum albumin conjugation(Fig 1) which was supported by Arnstadt and Cleer.[12] It shows that o-phenylendiamine was successfully used as a substrate for horseradish peroxidase in the competitive microtitre plate EIA. The sensitivity in the EIA of testosterone was 7 times greater than that of progesterone. The use of the heterologous system involving two different chemical bridges(hemisuccinate for immunogen and carboxymethyloxime for conjugate) conferred considerably greater sensitivity to the testosterone EIA.[13~15]

Summary

A rapid, solid-phase microtitre plate enzyme immunoassay(EIA) to determine the concentration of progesterone and testosterone in dairy cow is described. Both steroid hormones were analysed employing antibodies against 11 α -hemisuccinate-progesterone bovine serum albumin and 4-androsten-17 β -ol-3-one-carboxymethyloxime bovine serum albumin, respectively, as primary antibodies and sheep Ig G as secondary antibody.

The conjugated used as labels for progesterone and testosterone was progesterone-11 α -hydroxy-hemisuccinate-horseradish peroxidase and 4 α -androsten-17 β -ol-3-hemisuccinate-horseradish peroxidase, respectively.

Detection limit of microtitre plate EIA was 6.7 pg/well for progesteone and 1.0 pg/well for testosterone.

Reference

1. Voller A, Bidwell DE, Bartlett A. Enzyme immunoassy in diagnostic medicine. Theory and practice. *Bull WHO* 1976 ; 53 : 55~65.
2. Wisdom GB. Enzyme-immunoassay. *Clini Chemis* 1976 ; 22 : 1243~1255.
3. Schuurs AHWM, Van Weemen BK. Enzyme-immunoassay : a powerful analytical tool. *J Immunoassay* 1980 ; 1 : 229~249.
4. Engvall E, Perlmann P. Enzyme-linked immunosorbent assay(ELISA). Quantitative assay of immunoglobin G. *Immunochemistry* 1971 ; 8 : 871~874.
5. Engvall E, Perlamenn P. Enzyme-linked immunosor bent assay, ELISA. Quantitation of specific antibodies by enzyme-labelled anti-immunoglobulin in antigen-coated tubes. *J Immunol* 1972 ; 109 : 129~135.
6. Van Weemen BK, Schuurs AHWM. Immunoassay using antigen-enzyme conjugates, *FEBS Letters* 1971 ; 15 : 232~236.
7. Van Weemen BK, Schuurs AHWM. Immunoassay using hapten-enzyme conjugates, *FEBS Letters* 1971 ; 24 : 77~81.
8. Choi HS. Immunologische Bestimmung von Sexualsteroiden zur Fertilitästkon-trolle bei Rind, Schwein und Pferd. *Wein tierärztl Mschr* 1987 ; 74 : 14~22.
9. Erlanger B, Borek F, Beiser SM, et al. Steroid-protein conjugates. I. Preparation and characterization of conjugates of bovine serum albumin with testosterone and with cortisol. *J Biol Chem* 1957 ; 228 : 713~727.
10. Dawson EC, Dennison AEM, Van Weemen BK. A simple and efficient method for raising steroid antibodies in rabbit. *Steroids* 1980 ; 31 : 357~366.
11. Nakao T, Sugihashi A, Saga N, et al. An improved enzyme immunoassay of progesterone applied to bovine milk. *Br Vet J* 1983 ; 139 : 109~118.
12. Arnstadt KI, Cleere WF. Enzyme-immunoassay for determination of progesterone in milk from cows. *J*

Reprod Fert 1981 ; 62 : 173~180.

13. Joyce BG, Wilson DW, Read GF, et al. An improved enzyme immunoassay for progesterone in human plasma. *Clini Chemis* 1978 ; 24 : 2099~2102.
14. Bosch AMG, Dijkhuizen DM, Schuurs AHWM, et al. Enzyme immunoassay for total oestrogens in pregnancy plasma or serum. *Clinica Chimica Acta* 1978 ; 89 : 59~70.
15. Gros C, Flecheux O, Dray F. Enzymatic tracers for steroid EIA. In : Malvano Red. *In Immunoenzymatic assay techniques*. 1st ed. Netherlands ; Nijhoff Co. 1980 ; 45~58.