Comparison of different antibody–conjugate derivatives for the development of a sensitive and specific progesterone assay

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Antibodies raised against progesterone with hormone-carrier protein bridges placed at four different carbon positions were used to compare the sensitivity and specificity of homologous and heterologous enzyme–hormone conjugates. All heterologous assays were at least twice as sensitive as the corresponding homologous assays. The best results were obtained by using antibodies against 7 α -carboxyethyl-thioether-progesterone with 6 β -hemisuccinate–progesterone conjugate (or hemimaleate). The sensitivity with human sera was 0.25 ng ml⁻¹ and, the highest crossreaction 10% with 5 β -pregnane-3,20-dione, and reproducibility, recovery and accuracy were satisfactory. The correlation coefficient with radioimmunoassay in 103 human sera tested was $r = 0.915$. The assay was successfully applied for the diagnosis of pregnancy in dairy cattle.

Introduction

Early methods for measuring progesterone concentration in the plasma or milk used radioimmunoassay (RIA) techniques (Robertson and Sarda, 1971; Heap *et al.*, 1973; Hoffmann and Hamburger, 1973). Enzymeimmunoassays (EIA) are advantageous compared with RIA once problems inherent to EIA could be solved. Homologous EIA systems developed originally using 11 α -hydroxyprogesterone 11-hemisuccinate-base (Joyce *et al.*, 1977; Sauer *et al.*, 1981; Munro and Stabenfeldt, 1984) were unsatisfactory. Considerable improvements in sensitivity and specificity were reported with heterologous systems using antibodies to the above antigen in combination with conjugates 11 α -hydroxyprogesterone 11-glucuronide (Sauer *et al.*, 1986) or progesterone-3-*O*-carboxymethyl oxime (Munro and Stabenfeldt, 1984; Marcus and Hackett, 1986), and antibodies to progesterone-7 α -carboxyethylthioether with 6 β -hydroxyprogesterone-6-hemisuccinate as conjugate (van de Wiel and Koops, 1986). The above assays evaluated only one antibody–conjugate system without a clear indication of which system was preferable. In the study reported here, polyclonal antibodies were produced to all of the above-mentioned progesterone–BSA antigens and were used to compare combinations of homologous and heterologous EIA systems for measuring progesterone. An assay was developed based on the best antibody–conjugate combination and its characteristics were assessed.

Materials and Methods

Reagents

All steroids used in the study were purchased from Steraloids (Wilton), unless otherwise stated. Organic solvents and *N*-bromosuccinimide, sym-collidine, mercaptopropionic acid, methyl-

morpholine, isobutyl chloroformate, maleic anhydride, succinic anhydride and triethalonamine were purchased from Merck (Darmstadt), BSA and Freund's adjuvants from Sigma (St Louis, MO), horseradish peroxidase (HRP) from Boehringer Mannheim (Mannheim) and microtitre plates from Nunc (Kamstrup). The immunogens 11 α -hydroxyhemisuccinate–progesterone–BSA and 6 β -hydroxy-hemisuccinate–progesterone–BSA were obtained from Steraloids, 3-*O*-carboxymethyl oxime–progesterone–BSA from Sigma and 7 α -carboxyethyl thioether–progesterone–BSA was synthesized (Grover and Odell, 1977; van de Wiel and Koops, 1986).

Antibodies

Antiserum was raised in rabbits against the four immunogens and the immunoglobulin fractions were purified with caprylic acid (Hatzidakis *et al.*, 1990).

Conjugates

3-*O*-carboxymethyl oxime–progesterone, 11 α -hydroxyhemisuccinate–progesterone, 11 α -hydroxyhemimaleate–progesterone, 6 β -hydroxyhemimaleate–progesterone, 6 β -hydroxyhemisuccinate–progesterone and 7 α -carboxyethyl thioether–progesterone were conjugated to HRP using the mixed anhydride method (Liebermann *et al.*, 1959). The hormone : HRP ratios were 1:1.

Enzymeimmunoassay procedure

Briefly, flat-bottomed microtitration plates (Maxisorb, Nunc) were coated 100 μ l per well (1 μ g ml⁻¹) with the appropriate anti-progesterone purified immunoglobulin in carbonate–bicarbonate buffer (50 mmol l⁻¹; pH 9.6), overnight at 4°C. Excess antibody

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Table 1. Assay sensitivities

Antibody	Conjugate	Sensitivity (pg) ^a
3-O-carboxymethyl oxime–progesterone–BSA	3-O-carboxymethyloxime–progesterone	400
	6β-hemisuccinate–progesterone	200
11α-hemisuccinate–progesterone–BSA	11α-hemisuccinate–progesterone	400
	11α-hemimaleate–progesterone	150
	3-O-carboxymethyl oxime–progesterone	150
6β-hemisuccinate–progesterone–BSA	6β-hemisuccinate–progesterone	120
	6β-hemimaleate–progesterone	50
	3-O-carboxymethyl oxime–progesterone	50
	7α-carboxyethyl thioether–progesterone	50
7α-carboxyethyl thioether–progesterone–BSA	7α-carboxyethyl thioether–progesterone	30
	6β-hemimaleate–progesterone	12
	6β-hemisuccinate–progesterone	12

^aDose for 50% inhibition of tracer binding.

was washed off with distilled water containing 0.05% Tween 20 (Merck, Darmstadt) and the coated wells were blocked with 200 µl per well of 1% w/v BSA in saline for 1 h at room temperature. Test samples (serum or milk), 10 µl, or solutions with standard concentrations of progesterone were added to wells in duplicate, plus 200 µl of hapten–peroxidase conjugate at 1/50 000. The mixture was incubated at room temperature for 1 h. After three washes with distilled water, the antibody–antigen reaction was revealed by adding 100 µl of substrate (0.25 mol 3,3',5,5' tetramethylbenzidine l⁻¹, 0.03% v/v hydrogen peroxidase in sodium acetate buffer (50 mmol l⁻¹; pH 5.2) to each well. The reaction was stopped after 15 min by the addition of 50 µl sulfuric acid (2 mol l⁻¹) per well. The absorbance was measured at 450 nm (Multiskan, Flow, Finland).

Results

Comparison of different antibody–conjugate assay systems

All homologous assays gave lower sensitivities (Abraham, 1969) than the corresponding heterologous systems (Table 1). Two of the most sensitive antibody–conjugate systems were assessed for specificity by measuring crossreactivity with a number of relevant commercially available steroids (Steraloids, Wilton) (Table 2). The best sensitivity and specificity were obtained with the combination of 7α-carboxyethyl thioether–progesterone antibodies with 6β-hemisuccinate–progesterone (or the hemimaleate version) as conjugate. This combination was further assessed as an assay for measuring progesterone in human sera and bovine milk.

Assay characteristics in human sera and bovine milk

Progesterone standards in human serum were prepared by adding appropriate amounts of hormone from a stock solution (45 µg ml⁻¹ in ethanol, $E_{240} = 171$ mmol l⁻¹) to a selected

serum with no detectable progesterone. Eight standards were made which covered the intended range (0–100 ng progesterone ml⁻¹ serum). Reproducibility of the calibration curve was tested by repeat measurements of these standards (Table 3). Similar results were observed with four standards (0, 5, 10 and 20 ng progesterone ml⁻¹) prepared in bovine whole milk which was taken from cows 8–10 days after giving birth and which contained no detectable progesterone. The range of the milk standards was selected to be sufficient in the discrimination between pregnant and nonpregnant animals.

The sensitivity of the serum assay, calculated as the concentration of progesterone detected at three standard deviations from the zero standard, was 0.25 ng ml⁻¹. The sensitivity of the milk assay was not determined as concentrations below 1 ng ml⁻¹ were considered not to be of diagnostic use.

Precision was tested by replicate measurement of two human serum samples, one with low and one with high progesterone concentrations. The intra-assay mean value for the first sample was 1.30 ng ml⁻¹ ($n = 64$, coefficient of variation, CV = 3.0%) and for the second sample 15 ng ml⁻¹ ($n = 56$, CV = 5.9%), whereas the interassay values (different batches of reagents) were 1.27 ng ml⁻¹ ($n = 4$, CV = 3.4%) and 14 ng ml⁻¹ ($n = 4$, CV = 9.8%), respectively. Similar tests with two bovine whole milk samples gave intra-assay values of 2.4 ng ml⁻¹ ($n = 50$, CV = 5.3%) and 50 ng ml⁻¹ ($n = 34$, CV = 4.0%), and inter-assay values of 2.4 ng ml⁻¹ ($n = 6$, CV = 5.0%) and 4.9 ng ml⁻¹ ($n = 4$, CV = 9.1%), respectively.

Accuracy of the readings was tested by comparing values obtained from the assay with those determined by a commercially available RIA (Amerlex, Amersham) in 103 samples of human sera. The correlation coefficient between the two assays was $r = 0.915$. Accuracy of measurement was further tested by determining recovery by adding 1.0, 5.0 and 15 ng progesterone ml⁻¹ to two human sera with native progesterone concentrations of 1.8 and 28 ng ml⁻¹ respectively, and a bovine milk sample with 2.4 ng ml⁻¹ (Table 4).

Table 2. Assay specificity

Steroid	% Crossreactivity ^a	
	6 β -hs-prog and 6 β -hm-prog ^b	7 α -ct-prog- 6 β -hs-prog ^c
Progesterone	100.0	100.0
11 α -Hydroxyprogesterone	3.3	1.0
5 α -Pregnane-3,20-dione	33.0	2.8
5 β -Pregnane-3,20-dione	1.2	10.0
3 α -Hydroxy-5 β -pregnane-20-one	Not tested	1.8
3 β -Hydroxy-5 β -pregnane-20-one	4.2	4.0
21-Hydroxy-4-pregnene-3,20-dione	<0.1	1.2
21-Hydroxy-4-pregnene-3,20-dione acetate	0.3	<0.1
17-Hydroxy-4-pregnene-3,20-dione	1.5	<0.1
Oestriol	<0.1	<0.1
Oestrone	0.2	<0.1
Oestradiol	<0.1	<0.1
Corticosterone	0.3	<0.1
Cortisol	<0.1	<0.1
4-Androstene-3,17-dione	<0.1	<0.1
3 β -Hydroxy-5-androsten-17one	<0.1	<0.1
Testosterone	<0.1	<0.1

^aCrossreactivity was defined as the amount of steroid (pg), relative to progesterone, required to reduce label binding by 50%.

^bAntibody, 6 β -hemisuccinate-progesterone and conjugate, 6 β -hemialeate-progesterone.

^cAntibody, 7 α -carboxyethyl thioether-progesterone and conjugate, 6 β -hemisuccinate-progesterone.

Table 3. Reproducibility of the calibration curve for progesterone in human serum

Reference standard	Progesterone (ng ml ⁻¹)	Intra-assay values ^a		Interassay values ^b	
		Mean absorbance (450 nm)	CV%	Mean absorbance (450 nm)	CV%
1	0.0	1.090	4.6	1.063	2.5
2	0.4	0.900	3.1	0.890	3.5
3	1.0	0.650	4.0	0.645	3.2
4	2.5	0.500	3.1	0.490	3.9
5	5.0	0.390	3.2	0.390	3.9
6	10.0	0.290	4.7	0.280	4.0
7	30.0	0.170	5.1	0.165	5.5
8	100.0	0.110	7.9	0.100	5.2

^aThe results were obtained from 12 replicates.

^bThe results were obtained from four different batches of reagents, tested on four different occasions.

CV: Coefficient of variation.

Application of the assay for early pregnancy diagnosis in dairy cattle

The assay was evaluated as a cattle pregnancy diagnostic tool. Pregnancy testing was performed on 49 cows by taking milk samples on the day of insemination and 19 days later. Accuracy of positive and negative diagnoses was assessed with data from oestrous returns or subsequent calving (Table 5).

Discussion

This is the first report on the influence of different chemically modified progesterone conjugates and antibodies to an EIA, thus allowing a more rational design of a progesterone assay. Antibodies directed to 7 α -carboxyethyl thioether-progesterone gave the greatest competitive inhibition in contrast to antibodies against 3-*O*-carboxymethyl-oxime-progesterone and

Table 4. Recovery of progesterone-added samples

Type of sample	Basal progesterone (ng ml ⁻¹)	Progesterone added (ng ml ⁻¹)	Observed value (ng ml ⁻¹)	% Recovery I	% Recovery II
Human serum	1.8	1.0	3.0	120	107
		5.0	7.0	104	103
		15.0	17.0	101	101
Human serum	28.0	1.0	30.0	—	103
		5.0	34.0	120	103
		15.0	42.0	93	98
Bovine milk	2.4	1.0	3.5	110	103
		5.0	7.0	92	95
		15.0	17.0	97	98

% recovery I: (increment found/increment added) × 100; % recovery II: {(observed value)/(basal value + increment added)} × 100.

Table 5. Pregnancy diagnosis with bovine milk

	Inseminated	Positive test ^a on day 19	Calved	Negative test on day 19	Return to oestrus
Number of animals	49	39	33 (84.6%) ^b	10	10 (100%)
Mean progesterone concentration (ng ml ⁻¹) (range)	1.45 (1 < to 5)	22.8 (10–58)		1.92 (1 < to 5)	

^aPositive: Progesterone ≥ 10 ng ml⁻¹; ^baccuracy based on calving rate.

11 α -hemisuccinate–progesterone which gave poor inhibition. In all situations, assays with heterologous conjugates were at least twice as sensitive as with the corresponding homologous conjugates. Previous studies regarding homologous EIAs reported just adequate sensitivity (Joyce *et al.*, 1977; Sauer *et al.*, 1981; Foulkes *et al.*, 1982).

The use of heterologous progesterone conjugates with antibodies increased sensitivity considerably. The improvement was approximately the same for any given antibody, irrespective of the type or position of the bridge in the conjugate. Our results suggest that the overall sensitivity of a heterologous system is governed by the antibody rather than by the conjugate. This appears to be the case here and in other studies that describe heterologous progesterone EIAs (Munro and Stabenfeldt, 1984; Marcus and Hackett, 1986; Sauer *et al.*, 1986; van de Wiel and Koops, 1986). In our study the best combination was the 7 α -carboxyethyl-thioether–progesterone/6 β -hemisuccinate–progesterone system, which is similar to the system described by van de Wiel and Koops (1986).

Specificity is a well-recognized problem with progesterone assays (Grover and Odell, 1977). The heterologous EIA tests crossreact strongly with three related steroids: with 11 α -hydroxyprogesterone, 95% (Marcus and Hackett, 1986), 75.8% (Sauer *et al.*, 1986), 21.4% (Munro and Stabenfeldt, 1984) and 5.6% (van de Wiel and Koops, 1986); with 5 α -pregnane-3,20-dione, 50% (Marcus and Hackett, 1986), 29.5% (Munro and

Stabenfeldt, 1984) and 25.0% (van de Wiel and Koops, 1986); and with 5 β -pregnane-3,20-dione, 48% (Marcus and Hackett, 1986) and 6.8% (Sauer *et al.*, 1986). The corresponding cross-reactivity of our 7 α -carboxyethyl-thioether–progesterone and 6 β -hemisuccinate–progesterone assay was 1.0%, 2.8% and 10.0%, respectively, which represents a considerable improvement in specificity. Although our assay and that described by van de Wiel and Koops (1986) are essentially similar, the differences in sensitivity may be attributed to the different starting materials.

The assay is simple and convenient to use, as it permits direct addition of undiluted sample and conjugate, and requires a short incubation period. The good sensitivity of the assay is reflected by the volume of the sample used (10 μ l per test) and by the displacement of the conjugate at low concentrations (17.5% displacement by 0.4 ng progesterone ml⁻¹), with at least comparable performance to commercially available reagents.

The diagnostic results with the bovine milk agree with results obtained in previous studies using RIA (Robertson *et al.*, 1971) and EIA (Sauer *et al.*, 1986; van de Wiel *et al.*, 1986). As the test requires less than 90 min to complete, a major application in cattle could be centralized testing of large numbers of samples, thus allowing close supervision by trained veterinarians for the correct interpretation of the results which could improve the overall management of cows.

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