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Immunochromatographic Assay for Quantitation of Milk Progesterone

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We describe a rapid immunochromatographic method for the quantitation of progesterone in bovine milk. The method is based on a 'competitive' assay format using the monoclonal antibody to progesterone and a progesterone–protein conjugate labelled with colloidal gold particles. The monoclonal antibody to progesterone is immobilized as a narrow detection zone on a porous membrane. The sample is mixed with colloidal gold particles coated with progesterone–protein conjugate, and the mixture is allowed to migrate past the detection zone. Migration is facilitated by capillary forces. The amount of labelled progesterone–protein conjugate bound to the detection zone, as detected by photometric scanning, is inversely proportional to the amount of progesterone present in the sample. Analysis is complete in less that 10 min. The method has a practical detection limit of 5 ng of progesterone per ml of bovine milk.

A thin layer immunochromatographic detection system for proteins has been recently described.[1] This method was based on the 'sandwich' assay format using monoclonal antibodies (Mabs) of two distinct specificities. The Mab of one specificity was immobilized as a definite, narrow detection zone on a porous membrane, while the Mab of the other specificity was coupled to colored latex microparticles. The sample was mixed with latex-labelled Mab and allowed to migrate past the detection zone. The intensity of the color within the detection zone was proportional to the concentration of protein antigen in the sample.[1] In another recent publication,[2] a competitive immunochromatographic assay for plasma lipoprotein A was described. In this assay, lipoprotein A labelled with colloidal selenium was mixed with sample and allowed to migrate through a porous membrane, containing several consequent narrow detection zones prepared by immobilizing monoclonal antibodies to lipoprotein to the membrane. Increasing amounts of lipoprotein A in the sample caused an increasing number of detection zones to become visible, owing to partial inhibition of binding of selenium-labelled lipoprotein A to antibody at the early detection zones of the migration path.[2] Here, we report a competitive immunochromatographic assay using only one detection zone for the steroid hormone progesterone. This novel assay significantly increases the speed and reduces the complexity of progesterone analysis. We foresee it to have application in the field of pregnancy testing in cattle.

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Experimental

Materials. Samples of bovine milk containing pretitrated amounts of progesterone and monoclonal antibody to progesterone were obtained from Cambridge Veterinary Sciences Ltd. (Littleport, UK). Samples of bovine milk were also obtained from local farmhouses. The monoclonal antibody was purified by protein A affinity chromatography as described.[3] Nitrocellulose membrane having a nominal pore size of 12 μm was obtained from Sleicher and Schull (Darmstadt, Germany). Progesterone 11α-hemisuccinate, estrone, β-estradiol, corticosterone, 5α- and 5β-dihydroprogesterone, 11α, 17α- and 20β-hydroxyprogesterone, pregnenolone, Ultrawet 60 L, 4-androstene-3,17-dione, dehydroisoandrosterone, hydrocortisone, hexanesulfonic acid, ovalbumin, 1,1′-carbonyldiimidazole, [tris(hydroxymethyl)amino]methane (Tris) and bovine serum albumin (type V) were obtained from Sigma (St. Louis, Mo., USA). Citric acid (trisodium salt dihydrate), sucrose, NaCl, $NaHCO_3$, gold chloride [tetrachloroauric(III) acid, $HAuCl_4 \cdot 3H_2O$] and NaN_3 were obtained from Merck (Darmstadt, Germany). Testosterone was obtained from MC (Jerusalem, Israel). Progesterone was obtained from Serva (Heidelberg, Germany).

Preparation of progesterone–ovalbumin conjugate. Progesterone 11α-hemisuccinate was coupled to ovalbumin by using carbonyldiimidazole.[4] Progesterone hemisuccinate (100 mg) was dissolved in 4 ml of *N,N*-dimethylformamide and 100 mg of carbonyldiimidazole were added with continuous stirring. The solution was incubated at

80 °C for 30 min and then allowed to cool to room temperature. This solution of activated progesterone hemisuccinate was added dropwise to 20 ml of 0.1 M sodium bicarbonate containing 500 mg of ovalbumin. The solution was incubated overnight at 4 °C with continuous stirring and dialysed first against 0.1 M sodium bicarbonate at 4 °C for 4 h and then twice against distilled water at the same conditions. The conjugate was stored at 4 °C with 0.1% NaN_3 until use. As judged from spectrophotometry at 249 nm (log ε of progesterone in water is 4.23),[5] the obtained progesterone-ovalbumin conjugates contained 16 mol of progesterone per mole of ovalbumin.

Preparation of colloidal gold particles. Colloidal gold particles with an average diameter of 40 nm were prepared as described.[6] Briefly, 1 ml of a 1% (w/v) stock solution of gold chloride was added to 200 ml of distilled water and heated to the boiling point. A freshly made 1% solution of sodium citrate (1 ml) was added to the gold solution with constant stirring and the mixture was boiled for approximately 10 min until it changed its color to red. After an additional 5 min of boiling, the solution was cooled to 4 °C for further processing. The particle size was confirmed by electron microscopy (data not shown).

Coating of colloidal gold particles with progesterone–ovalbumin conjugate. Coating of colloidal gold particles with progesterone–ovalbumin conjugates was carried out as described.[6] Colloidal gold (10 ml) was quickly added to 1 ml of progesterone–ovalbumin conjugate solution with vigorous stirring and the mixture was incubated at room temperature for 5 min. The protein concentration of progesterone–ovalbumin conjugate used was 31.4 mg l^{-1}. After incubation the coated colloidal gold was stabilized by addition of 10% (w/v) of bovine serum albumin (113 µl) to a concentration 0.1% (w/v). The labelled conjugate was centrifuged at 10000*g* for 20 min and washed five times with 1 ml of 10 mM Tris pH 8.5 buffer containing 0.1% (w/v) of bovine serum albumin and 0.9% NaCl (TBS with BSA). The final volume of label was 1 ml. The labelled conjugate was stored at 4 °C with 0.1% NaN_3.

Preparation of immunostrips. The detection zone was set up on the nitrocellulose membrane essentially as described.[1] One side of the membrane was coated with a plastic film. The solution of antibody was pencilled on a plastic stamp and stamped on the naked side of the membrane giving a zone width of approximately 1 mm. The amount of antibody used was 0.8 µl cm^{-1}. The detection zone was allowed to dry in air. The dried membrane was soaked for 5 min in blocking solution (3% BSA, 5% sucrose, 0.1% Ultrawet L60 or hexanesulfonic acid and 0.1% NaN_3 in water), washed with 10 mM TBS pH 8.5, and air dried.

The detection zone was in 10 mm from the lower end of the immunostrip (50 × 4 mm, Fig. 2). The detection zone was overlaid with plastic film (10 × 4 mm) and the lower part of membrane was divided into two separated zones (Fig. 2) also with plastic film (3 × 4 mm). The membrane was cut into strips like those in Fig. 2. The strips were stored at 4 °C.

Assay procedure. Aliquots of milk (10 µl) were pipetted onto the sample loading area (Fig. 2) of the test strip positioned horizontally on a flat surface. Immediately 10 µl of gold-labelled progesterone–ovalbumin conjugate were pipetted onto the label area (Fig. 2) of the strip. When both sample and label were completely absorbed by the test strips, the strips were scanned by photometry. In some experiments, milk samples (500 µl) were sonicated for 10 s at a power of 4 W cm^{-2} with Vibra-Cell 375 High Intensity Ultrasonic Processor sonicator (Sonics & Materials, Danbury, USA) equipped with a microtip.

Detection. The developed strips were scanned with a chromatography scanner at 565 nm. The light source of the scanner was a green light-emitting diode, having an emission maximum at 565 nm. Transmitted green light was detected through a 0.5 mm wide slit with phototransistor at the opposite side of the strip. The signal was amplified and recorded.

Cross-reactivity. Steroid standards were prepared in ethanol, diluted in 10 mM TBS with BSA and the assay performed as described. The cross-reactivities were determined as described[7] (Table 1).

Correlation with RIA. Radioimmunoassay of progesterone was carried out by use of a commercial RIA kit (Veterinary progesterone RIA, Orion Diagnostica, Turku, Finland) according to the instructions of the manufacturer.

Table 1. Cross-reactivity of various steroids with the anti-progesterone antibody in thin-layer immunochromatographic assay system.

Steroid	Cross reaction (%) [b]
Pregnenolone[a]	18.7
Corticosterone	0.9
Estrone	<0.1
5α-Dihydroprogesterone[a]	13.0
17α-Hydroxyprogesterone [a]	3.3
5β-Dihydroprogesterone[a]	5.9
β-Estradiol	<0.1
20β-Hydroxyprogesterone[a]	<0.1
4-Androstene-3,17-dione	0.1
Dehydroisoandrosterone	0.4
Hydrocortisone	<0.1
Testosterone	<0.1
Progesterone	100.0
11α-Hydroxyprogesterone[a]	39.1

[a] Metabolites of progesterone detected in bovine milk.[9]
[b] Cross-reactivity is defined as the ng quantity of steroid relative to progesterone (ng progesterone/ng steroid) required to reduce label binding by 50%.

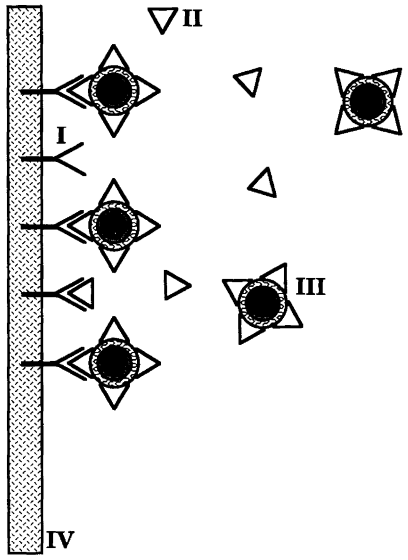

Fig. 1. The principle of the competitive thin-layer immunoaffinity chromatography: I, monoclonal antibody to progesterone immobilized to membrane; II, progesterone from milk sample; III, colloidal gold labelled progesterone–ovalbumin conjugate; IV, nitrocellulose membrane strip.

Results and discussion

Monitoring of milk progesterone is important for the assessment of reproductive status of cattle.[8] A variety of radioimmunoassay and enzyme immunoassay methods have been developed for this purpose.[9] While these methods offer high specificity and sensitivity, they are time-consuming and critically dependent on expensive laboratory instrumentation. The immunochromatographic test system[1] holds the potential that important analytes could be quantitated without expensive instruments. However, this system has so far been used only with protein analytes.[1,2] The recent work was initiated in order to apply the immunochromatographic principle in the assay of small-sized analytes.

A schematic description of the competitive immunochromatographic assay procedure used in the present study is shown in Fig. 1. The Mab to progesterone was immobilized on a thin porous nitrocellulose membrane by physical adsorption. The sample and the labelled progesterone–ovalbumin conjugate were allowed to migrate past the Mab zone driven by capillary forces. At the detection zone the analyte competitively inhibited the binding of the label into the detection zone. The colloidal gold label particles were bound to the detection zone and gave a red color within the detection zone (Fig. 2). The presence of progesterone in the sample could then be qualitatively estimated by visual examination of the intensity of the red color in this defined region of the strip. Alternatively, the intensity of the color could be quantitated by scanning photometry.

A photograph of the developed strips is shown in Fig. 3. The concentration of progesterone in the samples ranged from 1 to 30 ng ml^{-1}. As shown in Fig. 3, the intensity of the color at the detection zone decreased as the antigen concentration increased. As judged by visual inspection, binding of colored articles to the detection zone was almost completely absent at 30 ng ml^{-1}. A significant reduction in the intensity of the color at the detection zone could be visually detected at 5 ng ml^{-1}, as compared with samples containing 1 ng ml (Fig. 3). By using visual inspection, the practical detection limit of the present method was 5 ng ml^{-1}. However, when the results were read by photometric analysis, the detection limit was about 2 ng ml^{-1}. The signal was reduced to about 40% of the original signal at a progesterone concentration of 30 ng ml^{-1}.

The developed strips were scanned with a chromatography scanner as described above. A typical series of these scans is shown in Fig. 4. The peak corresponding to the detection zone was symmetrical, and the peak

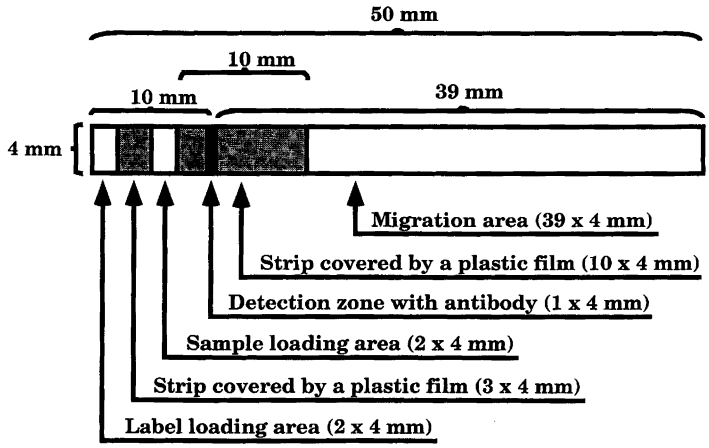

Fig. 2. Schematic of assay strip design. The assay strip consists of a label loading area, sample loading area, detection zone and migration area. The detection zone consists of one capture bar containing 6.4 ng of anti-progesterone antibody.

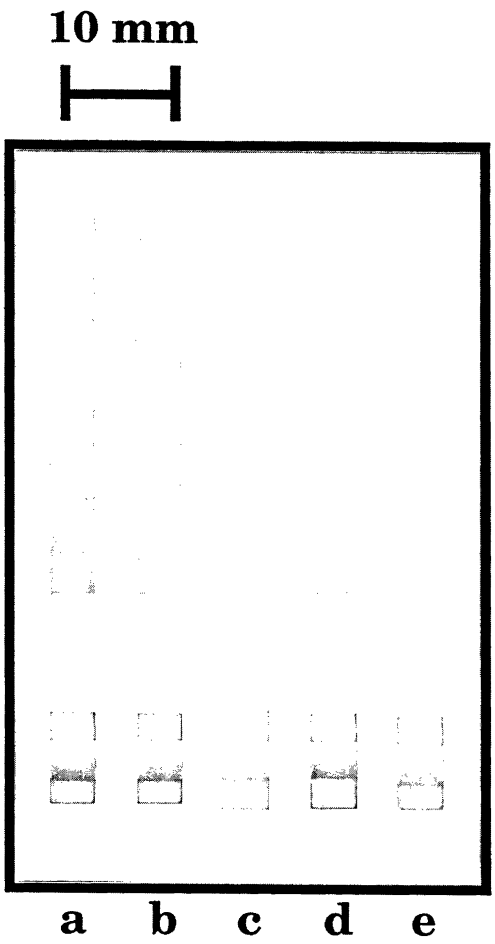

Fig. 3. Photograph of strips developed with samples of bovine milk having various progesterone concentrations. The concentrations of progesterone were (a) 1 ng ml^{-1}; (b) 5 ng ml^{-1}; (c) 10 ng ml^{-1}; (d) 15 ng ml^{-1}; (e) 30 ng ml^{-1}.

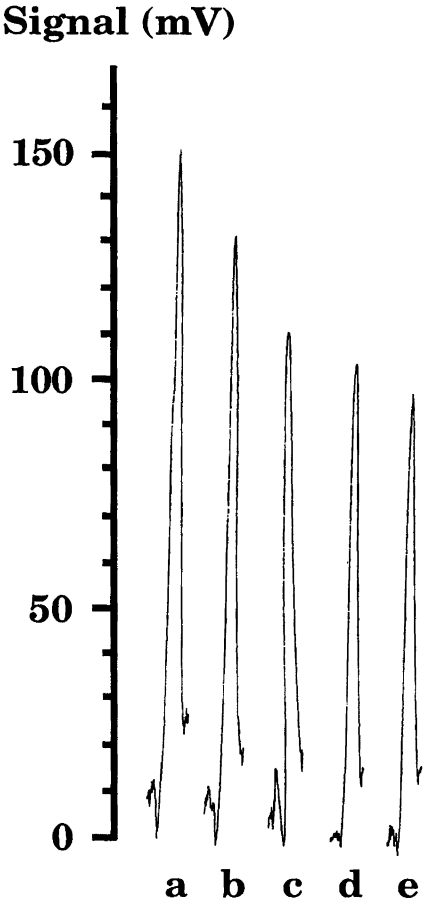

Fig. 4. Series of photometric scanning for the developed strips. Progesterone concentrations were (a) 1 ng ml^{-1}; (b) 5 ng ml^{-1}; (c) 10 ng ml^{-1}; (d) 15 ng ml^{-1}; (e) 30 ng ml^{-1}.

height decreased as the antigen concentration increased. Also, the peak width remained relatively constant for all the antigen concentrations tested. Therefore, the peak heights, rather than peak areas, were used to construct the standard curve. The heights of the peaks obtained from the photometric scans were plotted against antigen concentration to give a standard curve (Fig. 5). As shown in Fig. 5, peak heights were related to the logarithm of antigen concentration in the concentration range 1–30 ng ml^{-1}. The weighted linear regression coefficient was 0.99. The intra-assay variation coefficient was 14.4% ($n = 26$) and interassay variation coefficient was 6.6% ($n = 15$).

In the assay scheme presented here, we used the Mab immobilized to the detection zone and progesterone conjugated with ovalbumin and labelled with colloidal gold particles for each assay in the ratio 1:5 (ng of Mab/ng of progesterone). This ratio of Mab to labelled conjugate was adapted as a result of a series of experiments carried out to optimize the performance of the test (data not shown). In this system, we have been able visually to detect approximately 50 pg (160 fmol) of progesterone in 10 µl of milk sample. This sensitivity appears sufficient for the present assay to be used in pregnancy testing in

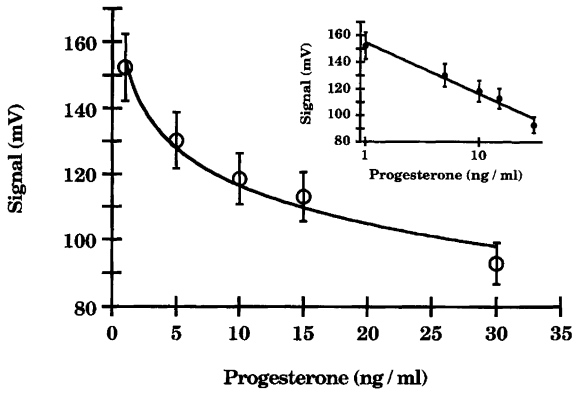

Fig. 5. Progesterone standard curve ($r = 0.99$). Vertical axis represents the signal from photometric scans. Values are the mean of three days±SD. Insert: standard curve for progesterone. Progesterone concentrations are presented on a logarithmic scale.

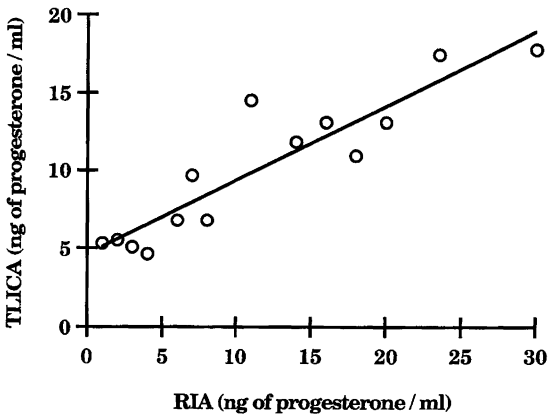

Fig. 6. Comparison of progesterone concentration determined by thin-layer immunochromatographic assay (TLICA) and RIA in milk samples (r=0.92). The samples were obtained from local farmhouses.

cattle, as a milk progesterone concentration exceeding 10 ng ml^{-1} (30 nmol l^{-1}) signifies pregnancy.[10]

The present assay system appeared to be specific for progesterone, although three metabolites of progesterone (pregnenolone, 5α-dihydroprogesterone and 11α-hydroxyprogesterone) did show significant cross-reaction (Table 1). The results obtained with the recent assay showed correlation with those obtained with RIA (Fig. 6), the weighted linear regression coefficient being 0.92.

To our knowledge, this is the first study on the use of an immunochromatographic system for assay of analytes of small molecular size. The sandwich format of the immunochromatographic test system[1] is not feasible with small analytes that cannot bind to two different Mabs at a time. The competitive system with multiple detection zones[2] might possibly be applied for the detection of small analytes, but it is a rather complex method as compared with the test system presented here.

It is significant in the present assay that the sample was first allowed to pass through the detection zone, followed by the colloidal gold-labelled progesterone–ovalbumin conjugate. In our hands, this increased the sensitivity of the assay as compared with the situation where the sample and the labelled conjugate were mixed prior to their passing through the detection zone (data not shown). It is possible to pipette both the sample and the label at the same time onto the membrane because the label does not mix with the milk in the thin membrane and cannot be passed under the milk drop separated by the plastic film (Fig. 2). The gold-label starts migrate through the slide only when all of the milk sample has soaked into nitrocellulose membrane. The reason for this effect is not well understood.

The relatively high variation observed in the present assay could probably be improved by preparing the detection zone with an ink jet. However, we feel that even in its present form our assay system demonstrates the feasibility of the competitive immunochromatographic test in the quantitation of small-sized analytes such as progesterone. Also, the assay does not require extraction, dilution, centrifugation or any other preparation of samples. For semiquantitative results, the test strips can be read visually.

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