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# TIME-RESOLVED FLUOROIMMUNOASSAY OF PROGESTERONE IN MILK

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**Summary**—A direct, solid-phase, time-resolved fluoroimmunoassay for progesterone in cow's and goat's milk, using europium-chelate-protein A as a label, is described. The coefficients of correlation with the results by RIA were 0.987 and 0.989.

## INTRODUCTION

The progesterone level in cow's and goat's milk is usually measured either for oestrus determination or as an indicator of corpus luteum activity, especially for early diagnosis of pregnancy 20–25 days after artificial insemination [1, 2]. Various radioimmunological [3–6] and immunoenzymatic [7, 8] assay methods have been described. Among procedures not using radioactive isotopes, time-resolved fluoroimmunoassay (TR-FIA) is of interest. This technique employs some europium-chelates for labeling which, because of their excellent emission characteristics, enable the background fluorescence of biological fluids to be eliminated [9]. TR-FIAs for steroids need europium-labeled specific antibodies [10, 11]. Since it would be useful to have a general marker for antibodies to be bound *in situ*, protein A from *Staphylococcus aureus*, which binds rapidly to IgG of various species without influencing its antigen binding capacity [12], was bound covalently to isothiocyanate-EDTA-Eu [13]. This general label had already been used in a TR-FIA for serum progesterone [14]. A direct progesterone TR-FIA to use for cow and goat whole milk is described here. The sensitivity of this procedure enables very small sample volumes to be used, thus reducing the non-specific signal to negligible levels.

## EXPERIMENTAL

### *Materials*

Purified protein A from *Staphylococcus aureus*, progesterone standard, agarose-anti-rabbit IgG raised in goats, 11α-hydroxyprogesterone hemisuccinate, ovalbumin and bovine serum albumin (BSA) were obtained from Sigma (St. Louis, Mo., U.S.A.); [1,2,6,7-$^3$H]progesterone (sp. act. 100 Ci/mmol) was purchased from NEN (Florence, Italy). Rabbit anti-11α-hydroxyprogesterone hemisuccinate-BSA antiserum was obtained through the courtesy of Dr G. Bolelli, Fisiologia della Riproduzione, Università di Bologna, Italy. The antiserum was used at a 1/8000 working dilution. Its cross-reactions were: 11α-hydroxyprogesterone = 26%, 11β-hydroxyprogesterone = 9%, 17α-hydroxyprogesterone = 0.25%, pregnenolone = 0.1%, cortisol = 0.05%, corticosterone < 0.01%, dehydroepiandrosterone < 0.01%, cortisone < 0.01%, oestradiol < 0.01%, testosterone < 0.01%. The scintillation solution Picofluor 40 was purchased from Packard (Groningen, Holland). The enhancement solution (1.0 g/l Triton X-100, 6.8 mM potassium hydrogen phthalate, 0.1 M acetic acid, 0.05 mM tri-n-octyl phosphine oxide, 0.015 mM 2-naphthoyltrifluoroacetone) was purchased from Pharmacia (Uppsala, Sweden). The other chemicals were obtained from Carlo Erba (Milan, Italy).

### *Apparatus*

An Arcus model 1230 fluorometer with time-resolution (Wallac) was used for the europium measurements. Radioactivity was counted with a Packard Tricarb 1500 β-counter.

### *Procedures*

Milk samples were aliquots of a total volume of milk collected manually in the afternoon from normal Friesian cows or from goats and were stored at −20°C without preservatives until time of use. Synthesis of the label, preparation of progesterone-ovalbumin conjugate and europium measurement were as described earlier [15].

### *Coating of polystyrene microtiter strips*

The progesterone-ovalbumin conjugate was adsorbed onto the wells of polystyrene microtiter strips. The wells were coated overnight at 27°C with 0.25 ml of 2 μg/ml conjugate solution in 0.1 M sodium carbonate buffer, pH 9.0. After washing with carbonate buffer, a second coat was made with 0.3 ml of 4% BSA solution in carbonate buffer. After 16 h

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at 27°C, the wells were washed five times with assay buffer by an immunowash NUNC.

*Assay buffer*

0.05 M Tris–HCl, pH 7.9, containing 0.9% NaCl, 0.25% BSA and 0.05% $NaN_3$.

*Immunoassay*

Progesterone standards were prepared by serial dilution in whole milk from a cow or a goat in oestrus. The standards containing 50–10,000 pg of progesterone and the milk samples (10 $\mu$l) were placed in triplicate in coated wells, then specific antiserum diluted in assay buffer was added to each well except those for non-specific binding assay; the incubation was carried out for 1.5 h in a final volume of 200 $\mu$l/well, at room temperature. The liquid phase was discarded and 200 $\mu$l of assay buffer containing an excess (1 pmol/well) of filtered Eu-labeled protein A were added. After 1 h at 20°C, the wells were washed with 0.9% NaCl solution, 250 $\mu$l of enhancement solution were added and the fluorescence read after 15 min. The blank (3700 ± 975 cps) was subtracted from each sample and the results were calculated as the mean of triplicates. RIA was done in duplicate with 20,000 cpm of [$^3$H]progesterone as a tracer, at the same dilution of standard and antiserum as in TR-FIA. The samples were incubated at 4°C overnight and 50 $\mu$l of the agarose-anti-rabbit IgG suspension were added to each well. After 2 h shaking, the bound fraction was separated by centrifugation and the liquid phase transferred into vials containing 10 ml of scintillation liquid and read in the $\beta$-counter.

## RESULTS AND DISCUSSION

The TR-FIA standard curve is shown in Fig. 1. The sensitivity of the method at twice the standard deviation was 50 pg/well. The accuracy was tested by adding different amounts of standard (80, 800 and 8000 pg) to the same pooled milk at known progesterone concentration, equilibrating overnight at 4°C and then assaying. The results corresponded to the expected concentration (Table 1). The coefficient of variation calculated for two samples with low (5.5 ng/ml) and high (31 ng/ml) progesterone content were 8.2 and 9.3% within-assay (average of 10 assays) and 11.4 and 7.4% between-assay (average of 8 assays).

Table 1. Analytical recovery of progesterone (pg/10 $\mu$l) added to pooled milk

| Pooled milk | Added | Observed | Recovery (%) |
|---|---|---|---|
| 65 ± 4 | 80 | 150 ± 2.5 | 103.4 |
| 65 ± 4 | 800 | 908 ± 10.5 | 104.9 |
| 65 ± 4 | 8000 | 8150 ± 63 | 101.0 |

Means of 8 determinations ±SD.

The cow's milk samples assayed by TR-FIA were obtained 19, 21 and 24 days after artificial insemination, to see if we could diagnose pregnancy at these times. Cows with progesterone levels above 5 ng/ml by RIA on these days had diagnoses of pregnancy confirmed by rectal palpation on the 60th day, those with levels below 5 ng/ml by RIA returned to oestrus. The TR-FIA assays confirmed these results, as shown by the regression line in Fig. 2. The coefficient of

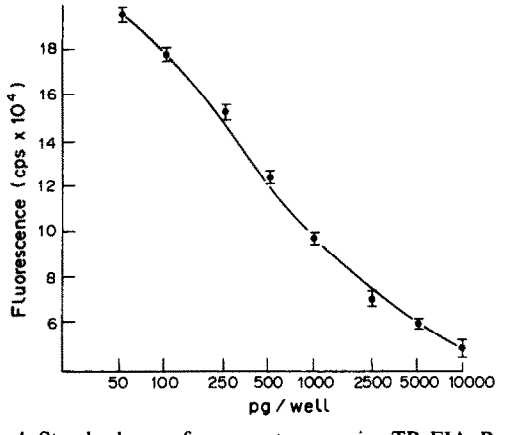


Fig. 1. Standard curve for progesterone using TR-FIA. Bars correspond to ±SD. Average of 10 determinations.

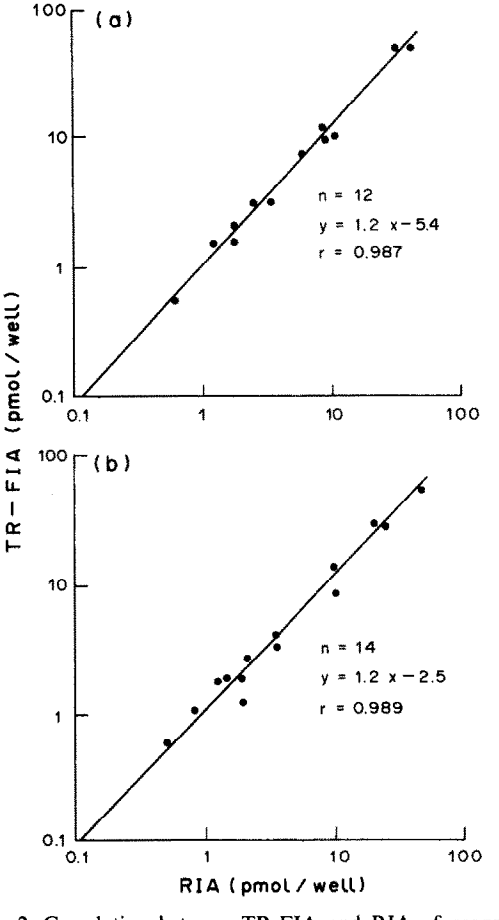


Fig. 2. Correlation between TR-FIA and RIA of progesterone in cow's (a) and goat's (b) milk samples.

correlation between TR-FIA and RIA values was 0.987. For goat's milk, the coefficient of correlation between the two methods was 0.989.

TR-FIA always gave higher levels than the RIA levels, as already reported for an enzyme immunoassay [16], and the difference was greater for cow's milk than for goat's milk. These differences might be the result of slower binding between immobilized antigen and antibody than between free antigen and antibody. In a non-equilibrium system such as ours, less antibody binds to the antigen adsorbed on the microwells than the amount that binds to tritiated progesterone in the RIA method. Nevertheless, because of the good correlation of the results by both methods, the higher values do not invalidate the use of TR-FIA for evaluation of parameters related to progesterone levels, i.e. oestrus cycle behavior and diagnosis of early pregnancy. The range of concentrations that can be measured in a single assay is wider for TR-FIA than for RIA, which means that samples with higher concentrations need not be diluted, and this increases the precision of the assay. In addition, with this method about 100 milk samples can be assayed within 4 h, including the reading, which makes it possible to detect animals that are not pregnant quickly enough to do a second insemination at the next oestrus. Therefore, because of its simplicity, wider concentration range and greater rapidity than RIA, this method is a valid alternative to the use of radioisotopic procedures.

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