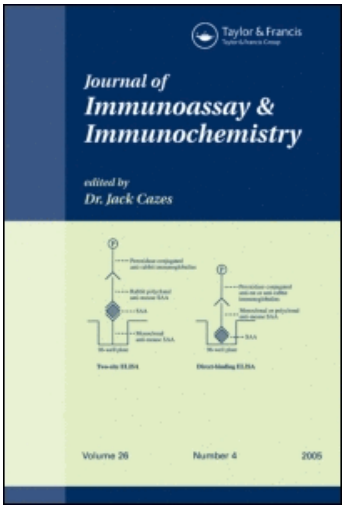

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New RIA Kit for the Determination of Progesterone in Cows' Milk

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Abstract: A new, single-step, specific, simple, economical, and ready to use RIA test for the determination of progesterone in cow's milk was developed. Tubes coated with the specific polyclonal anti-progesterone antibody and progesterone-CMO-^{125}I-histamine as the tracer were used. Progesterone in buffer and fat-free cow's milk, without progesterone were the matrix for the standard curve preparation. Fifty μL of milk and 500 μL of tracer were incubated in the tube for 2 h at room temperature, decanted, and coated. Assay range was 0–270 nmol of progesterone $\cdot l^{-1}$; sensitivity, <1 nmol $\cdot l^{-1}$; recovery, 94–104%; precision as CV%, 2.44–6%.

Keywords: Radioimmunoassay, Steroid radioiodination, Progesterone-RIA, Milk, Progesterone, Cows' milk, RIA kit

INTRODUCTION

Progesterone, the major hormone secreted from the corpus luteum, has two main biological functions. First, it transforms the estrogen stimulated endometrium into the secretory phase, which allows implantation of the fertilized ovum. Secondly, it protects pregnancy by decreasing uterine contractility. In cows, the presence of a corpus luteum is necessary for the maintenance of pregnancy, as it is in the vast majority of mammals.

Progesterone has been found to be of significant clinical value in most domestic species. The cyclic behavior of progesterone levels during the oestrous cycle and the relatively high levels during pregnancy result in this hormone being widely used as a pregnancy test. Also, plasma progesterone,

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progesterone in whole milk, milk fat, and fat-free milk have been estimated and a high correlation has been found between the values.

The development of radioimmunoassay (RIA) techniques for hormone determination in domestic species created laboratory procedures that are relatively simple to perform, inexpensive, specific, and sensitive, and which have potential usefulness as diagnostic aids in reproductive studies.

One of the most important veterinary applications of RIA, in terms of economy, is the determination of progesterone in cow's milk as a fertilization test for cattle. By determination of progesterone concentration in milk, insemination failures can be detected as early as 21–23 days after mating or attention can be drawn to the cows inseminated when not in estrus and a second insemination attempt should follow. The economic advantage of this approach is obvious. Fertilization checks should be considered as a routine procedure, allowing a better utilization of the cow's reproductive potential in which an insemination failure would be have been detected much later without determination of progesterone in milk.

To make the processing of such large numbers of samples economical, in terms of cost and time, progesterone determination should be attempted directly in whole milk, without prior extraction. In the laboratory, the best results have been obtained when fat-free milk was used. The different fat concentration in milk sampled before, during, or after milking affects the progesterone concentration and separation of the fat, thus, substantially diminishes this effect.[1]

The aim of this work was to prepare a new, ready-to-use RIA kit for the determination of progesterone in cow's milk. In the developed kit, a solid phase coated with specific antibodies is used. The assay is simple in operation, fast (one-step), and acceptably accurate.

EXPERIMENTAL

As the solid phase, tubes coated with polyclonal anti-progesterone antibody were used (Orion Diagnostica, Finland). The cross reactivity of the progesterone antiserum coated onto the tubes was as follows: progesterone, 100%; pregnenolone, 3.9%; corticosterone, 0.9%; other progesterone derivatives, $<0.7\%$; other steroids, $<0.01\%$.

As the raw material for the preparation of the tracer, the 3-(O-carboxymethyloxime-progesterone) derivative (Sigma Chemical Co.) was used. Tri-n-butylamine, dioxane, iso-butylchloroformate, chloramine T, cortisol, danasol, and acetonitrile were from Merck (Germany); histamine dihydrochloride and ursodeoxycholic acid were from Sigma Chemical Co.; Na ^{125}I was from Izotop (Hungary). As the raw material for preparation of standards, progesterone (99%) from Sigma Chemical Co. was used.

A high performance liquid chromatography (HPLC) system with a Lichrospher RP-18 column (250 × 4 mm, 5 μm), was obtained from Merck

(Germany), UV detectors (at 240 nm for progesterone and 220 nm for histamine) and an isotopic detector were used for purification. All milk samples (reference samples from non-pregnant cows and unknown samples from pregnant cows) were received from the Institute of Animal Breeding, Polish Academy of Science. Milk, without progesterone (reference), was obtained from cows 8–10 days after calving, when the function of the corpus luteum was absent. All samples contained preservative and were defatted using centrifugation for 30 min at 1,200 g at 4°C, and lyophilized.

An indirect, modified, three-step procedure of 3-CMO-progesterone iodination[2–5] was used: step 1, activation with tri-n-butylamine and isobutylchlorformate in dry dioxane at 10°C, 45 min; step 2, histamine iodination with Na-^{125}I using the chloramine T method, for 90 s at room temp; step 3, conjugation 38.7 μg (100 nmol) of activated progesterone derivative in dioxane with iodinated histamine (3.65 μg) in an ice-bath for 60 min.

The reaction mixture, after conjugation, was purified by HPLC, using 65% acetonitrile and 35% water (v/v) as eluent.[6–10] The radioactive peaks were identified. The collected peaks were diluted with tracer diluent buffer (PBS with 0.05% BSA and blockers) and their immunoreactivities were obtained.

RESULTS AND DISCUSSION

Tracer

The HPLC elution profile of the reaction mixture, after conjugation, is presented in Fig. 1. The first peak (1) was identified as ^{125}I-histamine (RT = 1.88 min, 10% of radioactivity). The second (2) (RT = 11.17 min, 41% of radioactivity) and the third (3) (RT = 13.19 min, 49% of radioactivity) were identified as iodinated progesterone derivatives. The first peak was not immunoreactive, as it did not show any binding with the antibody coated tubes. Immunoreactivity (B_0/T) of the second peak was 8% and of the third – 37%. The third radioactive peak was used for preparing the tracer (^{125}I–progesterone) by dilution in tracer diluent. Specific activity of the tracer, determined by displacement analysis,[11] was 4.1 $MBq \cdot \mu g^{-1}$ (112 $\mu Ci/\mu g$). Tracer in the kit had an activity about 130 kBq in 11 mL of tracer dilution buffer (quantity for 100 test tubes). Ready-to-use tracer was prepared by dilution of 11 mL of tracer in 45 mL of tracer dilution buffer.

Standards

The optimum buffer for preparation of standards was PBS buffer, pH 7.4, containing 0.5% BSA and 0.1% sodium azide. The range of the assay of

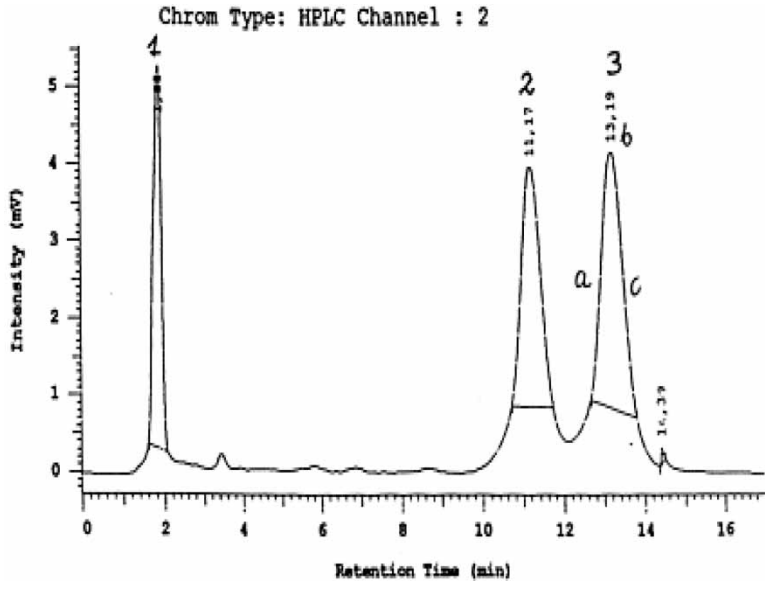

Figure 1. Radiochromatogram of the reaction mixture of progesterone-3-CMO with ^{125}I-histamine on HPLC after iodination.

progesterone in milk should be different than in serum. The optimum range of our assay was 0–300 nmol progesterone $\cdot$ L^{-1}. Six levels of standards were prepared: 0, 3, 15, 45, 135, and 270 nmol of progesterone $\cdot$ L^{-1}. Each standard of 0.5 mL volume was pipetted into a vial and kept in solution at 4–8°C.

$$\text{Progesterone (ng} \cdot \text{mL}^{-1}) = \text{progesterone (nmol} \cdot \text{L}^{-1}) \times 0.314$$

Milk Zero Matrix

The optimal matrix for preparing the standard curve was fat-free milk with zero progesterone. It was lyophilized in 1 mL portions and stored at −20°C.

Control Samples

Control samples were prepared from pregnant cow's milk samples after dilution with fat-free milk zero and was lyophilized in 0.5 mL portions.

Optimization of the Conditions for Assay

To develop a simple, fast, and reproducible assay with adequate sensitivity, the conditions of the assay were optimized.

Because of the height of coating in the tube, the total volume of all reagents pipetted into the tubes was 550 μL. To 45 mL of tracer diluent buffer, 11 mL of the tracer was added, and 500 μL of the prepared tracer solution was pipetted into the coated tubes. The optimum standard zero binding (B_0/T) and shape of standard curve were obtained when the activity of the pipetted tracer was 35–50,000 cpm per tube. For obtaining comparable results of progesterone concentration in unknown milk samples with the commercial kit, it was necessary to use fat-free milk zero matrix for preparation of the standard curve. The optimal proportion of milk zero matrix to the standard solution pipetted into the test tubes was 1 : 1 and the optimal quantities of these components was 25 μL. The volumes of pipetted control and unknown milk samples were 50 μL. The optimal incubation conditions were 2 hours at room temperature without rotation. Under these conditions, the assay system reached equilibrium.

Similarly as in the test for determination of progesterone in human serum, the solution, after incubation, was decanted (or aspirated) and the tubes were not washed.

The final optimized assay protocol of the Progesterone RIA Veterinary test is as follows: Add 25 μL of the standard sample, along with 25 μL fat-free milk zero matrix to the coated tubes for the standard curve and 50 μL of milk controls and unknown samples aliquot, then 500 μL diluted tracer; mix and incubate at room temperature for 2 h, decant the tubes, and measure for radioactivity. The typical calibration curve of the Progesterone Veterinary RIA assay (OBRI POLATOM) is presented in Fig. 2.

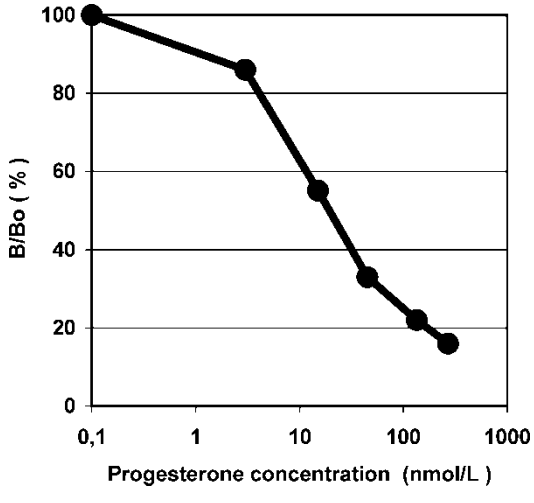

Figure 2. Typical calibration curve of Progesterone Veterinary RIA assay.

Analytical Characteristics of the Progesterone Veterinary RIA

Assay Sensitivity

The detection limit of the assay, defined as the concentration corresponding to a signal with 2 SD under the mean of 20 replicates of zero calibrator, was 0.77 nmol of progesterone $\cdot L^{-1}$.

Recovery

Different concentrations of progesterone in fat-free milk zero were added to three fat-free milk samples (with the progesterone concentration in the range 7–26 nmol $\cdot L^{-1}$), analyzed for progesterone content, and the recovery of added analyte was calculated (observed to expected values). The recoveries ranged from 94 to 104% (Table 1).

Parallelism Testing

Parallelism was tested by dilution of four fat-free milk samples (with high progesterone concentrations) with standard zero matrix and estimating the progesterone values in these samples using the developed kit. Measured values were between 88–104% of the expected values. The values of two serially diluted milk samples, on plotting, gave a response curves parallel to the standard curve, as depicted in Fig. 3. This suggests the identity of behavior between milk samples and the standard matrix in the Progesterone Veterinary RIA developed test. As is shown in Fig. 4, the values determined (measured) for the diluted samples correlated well with the expected values.

$$Y = 0.9362x + 0.7813; \quad R^2 = 0.9956$$

Table 1. Recovery test for Progesterone Veterinary RIA kit

Progesterone in sample (nmol $\cdot L^{-1}$)	Progesterone added (nmol $\cdot L^{-1}$)	Expected progesterone (nmol $\cdot L^{-1}$)	Observed progesterone (nmol $\cdot L^{-1}$)	Recovery (%)
7.4	6.8	14.2	13.8	97
7.4	16.0	22.4	21.0	94
7.4	41.0	48.4	49.0	101
14.0	6.8	20.8	20.0	96
14.0	16.0	30.0	29.0	97
14.0	41.0	55.0	53.0	104
26.3	6.8	33.1	33.0	100
26.3	16.0	42.3	40.0	95
26.3	41.0	67.3	65.0	97

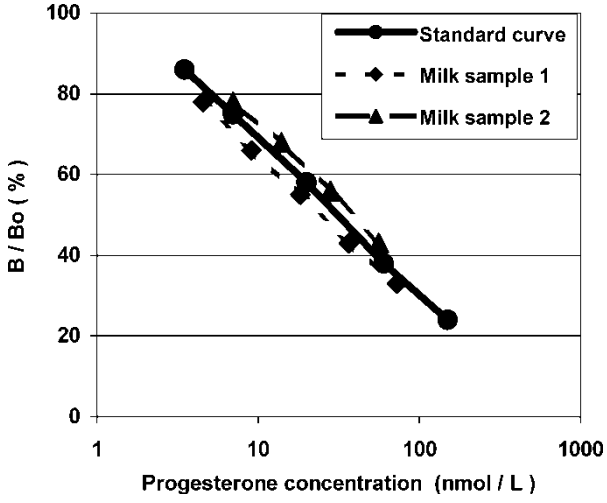

Figure 3. Parallelism between milk samples (73 and 56 nmol of progesterone $\cdot L^{-1}$) and standards in Progesterone Veterinary RIA assay.

Precision

Intra-assay and inter-assay variations were estimated for the developed kit using six milk samples. These results are shown in Table 2. The variation coefficients (CV%) were between 2.44 and 6.0%.

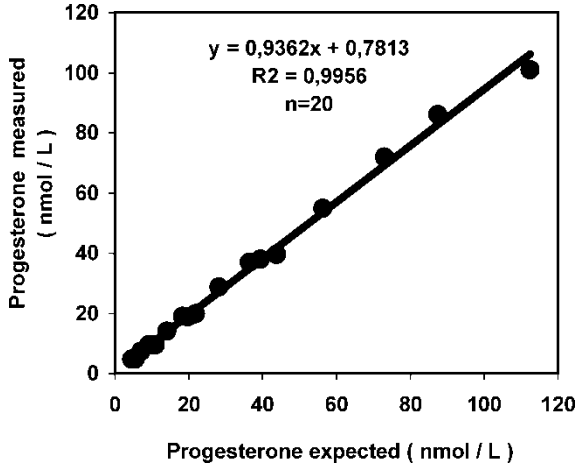

Figure 4. Comparison between measured and expected values of four milk samples with high progestrone levels, diluted with standard zero milk matrix.

Table 2. Precision of Progesterone Veterinary RIA assay

Sample number		Mean value ($nmol \cdot L^{-1}$)	SD ($nmol \cdot L^{-1}$)	CV (%)
Intra-assay precision	Number of replicates			
1	20	6.46	0.23	3.6
2	20	22.3	1.06	4.8
3	20	33.0	1.80	5.8
4	20	42.6	1.67	3.9
5	20	104.5	5.18	4.9
6	20	181.6	9.96	5.5
Inter-assay precision	Number of duplicates			
1	5	6.7	0.34	5.2
2	5	20.0	1.0	5.0
3	5	35.13	1.06	2.9
4	5	61.5	1.5	2.4
5	5	101.3	2.89	2.9
6	5	220	13.2	6.0

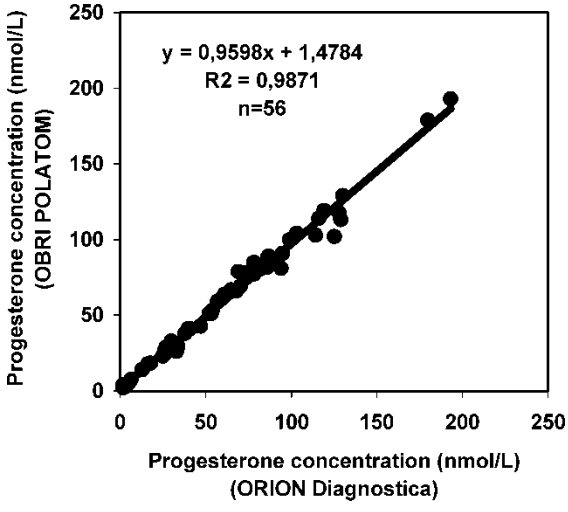

Figure 5. Correlation between progesterone values in milk samples using the developed Progesterone Veterinary RIA (OBRI POLATOM) and Spectria Veterinary Progesterone RIA assay (Orion Diagnostica).

Comparison with the Commercial Kit

Progesterone levels in milk samples from pregnant and non-pregnant cows were determined using Spectria Veterinary Progesterone RIA (Orion Diagnostica) kit and the new Progesterone Veterinary RIA (OBRI POLATOM) kit. Results were compared by regression analysis. The correlation coefficient reflects good agreement between these two kits ($R^2 = 0.9871$) (see Fig. 5).

CONCLUSIONS

From the work carried out for the development of an assay for progesterone in human serum, animal serum, or animal milk, it was observed that the main problem was to prepare a tracer suitable for antibody coating onto the tubes. It is necessery to select a suitable progesterone derivative for labelling[2–4] as the progesterone derivative used for preparing antibodies that were, in turn, used for coating, was unknown in the case of commercial coated tubes.The second main problem was the choice of the right matrix for preparing of the standards which, in dilution, would be parallel to diluted cow's milk samples.[1] In the commercial kit from Orion (Progesterone Veterinary SPECTRIA RIA), human serum is used as the matrix for preparing standards. We observed the best results using, for standards preparation, a 1 : 1 mixture of buffer containing bovine serum albumin and fat-free milk without progesterone (milk zero matrix).

Finally, optimization of the assay should be done, taking into account that the assay should be simple, fast, and possible to use with normal fat containing milk.

The best method for preparing the tracer was indirect labelling (three steps) using conjugation of activated progesterone derivative with iodinated histamine (^{125}I-histamine)[2–4] and the best method of purification was HPLC using a reverse phase (RP-18) column.[5–10] Iodinated progesterone-CMO derivative (progesterone-CMO-^{125}I-histamine) showed a binding of 36% to coated tubes from Orion. Despite low maximal binding in Progesterone Veterinary RIA assay, the standard curve in this assay is steep and B/B_o, of the first standard was 85%, and last standard was 16%. Incubation in this kit is short and washing of the tubes was unnecessary. The sensitivity was very good, i.e., 0.77 $nmol \cdot L^{-1}$. The quality of the obtained Progesterone Veterinary RIA kit was good in terms of quality control aspects; for example, recovery was 94–104%, recovery in the parallelism test was 88–104%, precision as CV% was 2.44–6.0%.

The only commercial kit for progesterone assay in cow's milk is manufactured by Orion Diagnostica (Finland). The results of progesterone concentration in control and pregnant cow's milk samples, obtained using RIA kit OBRI POLATOM and the commercial Orion kit were very similar. In general, the results of progesterone concentration in fat-free milk, compared with normal milk samples were more repeatable.

Stability of the optimized kit was quite good (up to 9 weeks at 4–8°C). During this time, maximal binding (B_0/T) and progesterone concentration in the control milk samples were constant.

The work carried out has resulted in a good quality Progesterone Veterinary RIA test which is simple, quick, and specific for the assay of progesterone in cow's milk.

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