Comparison of 2 enzyme immunoassays and a radioimmunoassay for measurement of progesterone concentrations in bovine plasma, skim milk, and whole milk

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Abstract

The objective of this study was to compare 2 enzyme immunoassays (EIAs) with a radioimmunoassay (RIA) as to sensitivity and accuracy in the measurement of the progesterone (P4) concentration in bovine plasma, skim milk, and whole milk. The 72 samples from 24 lactating dairy cows expected to have either a high P4 concentration (cows in diestrus or pregnant) or a low P4 concentration (cows in estrus or anestrus) were analyzed by RIA, solid-phase EIA (SPEIA), which included a solvent extraction step, or direct EIA (DEIA) without solvent extraction. The overall mean concentrations of P4 did not differ ($P < 0.4$) among the assays. However, for the cows that were in diestrus or pregnant, the mean P4 concentrations (and standard error) were higher ($P < 0.03$), regardless of sample type, with RIA than with SPEIA, at 7.3 (0.7) and 6.1 (0.6) ng/mL, respectively. When only the high-P4 samples analyzed by RIA were compared, the mean P4 concentration was higher ($P < 0.001$) in whole milk than in skim milk, at 9.8 (1.0) and 4.1 (0.7) ng/mL, respectively. Although the mean P4 concentrations in the low-P4 samples did not differ ($P < 0.80$) among assays, the proportions of cows with a P4 concentration ≥ 1 ng/mL were 3%, 14%, and 44% for RIA, SPEIA, and DEIA, respectively ($P < 0.01$; DEIA > SPEIA > RIA).

Résumé

L'objectif de cette étude était de comparer deux essais immunoenzymatiques (EIA) à un radio-immunoessai (RIA) quant à leur sensibilité et leur précision pour mesurer la concentration de progestérone (P4) dans le plasma bovin, le lait entier et le lait écrémé. Les 72 échantillons provenant de 24 vaches en lactation, dont on s'attendait à avoir soit une concentration élevée de P4 (vaches en diestrus ou gestantes) ou une concentration faible de P4 (vaches en œstrus ou anestrus), ont été analysés par RIA, EIA en phase-solide (SPEIA) qui comportait une étape d'extraction à l'aide d'un solvant, ou EIA direct (DEIA) sans extraction avec solvant. De manière générale les concentrations moyennes de P4 ne différaient pas (P < 0,4) entre les essais. Toutefois, pour les vaches qui étaient en diestrus ou gestantes, les concentrations moyennes de P4 (et l'écart type) étaient plus élevées (P < 0,03), indépendamment du type d'échantillon, avec le RIA qu'avec le SPEIA, les valeurs étant respectivement de 7,3 (0,7) et 6,1 (0,6) ng/mL. Lorsque seulement les échantillons avec concentration élevée attendue de P4 analysés par RIA ont été comparés, la concentration moyenne de P4 était plus élevée (P < 0,001) dans le lait entier que dans le lait écrémé, étant respectivement de 9,8 (1,0) et 4,1 (0,7) ng/mL. Bien que les concentrations moyennes de P4 dans les échantillons avec concentration faible attendue de P4 ne différaient pas (P < 0,80) entre les essais, les proportions de vaches avec un niveau de P4 ≥ 1 ng/mL étaient de 3 %, 14 % et 44 % respectivement pour le RIA, le SPEIA et le DEIA (P < 0,01; DEIA > SPEIA > RIA).

(Traduit par Docteur Serge Messier)

Introduction

For over 30 y, progesterone (P4) has been used to monitor ovarian activity, estrus detection accuracy, and pregnancy in cattle (1–4). Although the P4 concentration is commonly determined in plasma, it can be readily measured in milk (1–3,5,6), which can be frequently and easily collected from lactating dairy cattle. Radioimmunoassay (RIA) is a well-accepted analytic method to measure the P4 concentration. Although RIA is generally rapid and sensitive, it requires specialized facilities and involves the use of radioactive materials. Conversely, enzyme immunoassay (EIA) for P4 determination uses enzyme or protein conjugates instead of radioactive materials (6–8) and is relatively inexpensive. However, some EIAs require considerable time and may not be as sensitive or accurate as RIA.

The objective of this study was to compare P4 concentrations in bovine plasma, skim milk, and whole milk, as determined by RIA and 2 EIAs.

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Received September 11, 2006. Accepted January 30, 2007.

Materials and methods

Animals and samplings

Lactating dairy cows expected to have either a low P4 concentration (those in anestrus or estrus) or a high P4 concentration (those in diestrus or pregnant) were studied. They were housed and handled in accordance with the guidelines of the Canadian Council on Animal Care (9), and the experimental protocols were approved by the Animal Policy and Welfare Committee, University of Alberta.

Twenty-four samples each of milk and blood were collected. Samples from the 6 cows in estrus and the 6 in diestrus were collected approximately 60 h and 14 d, respectively, after treatment with 25 mg of dinoprost (Lutalyse; Pfizer Animal Health, Montreal, Quebec). Samples were obtained from the 6 anestrous cows between 5 and 8 d after parturition and from the 6 pregnant cows at approximately 90 d of gestation.

Milk samples were collected between milkings by hand-stripping each teat and were pooled into a 30-mL container. The 1st stream of milk from each teat was always discarded. Blood samples were collected by coccygeal venipuncture into an evacuated tube containing sodium heparin (Vacutainer; Becton Dickinson, Franklin Lakes, New Jersey, USA). Blood samples and approximately half of each milk sample were centrifuged at $1500 \times g$ for 20 min at 4°C, and then the plasma and skim milk, respectively, separated. All 3 samples (plasma, skim milk, and whole milk) from each cow were stored at −20°C until assayed for P4. Before assays, all samples were thawed in a water bath at 35°C for approximately 10 min.

Progesterone assays

Radioimmunoassay (RIA) — The P4 concentration was determined, in duplicate, with the use of a commercially available kit (Coat-A-Count Progesterone; Diagnostic Products Corporation, Los Angeles, California, USA). This kit, designed for the direct, quantitative measurement of P4 in serum or plasma, has been validated for bovine milk (10). Polypropylene tubes were coated with the antibody and a set of standards with known concentrations of P4. Then 20 μL of the plasma or milk sample was put into the coated tube, and P4 labeled with radioactive iodine (^{125}I) was added. The tubes were incubated at room temperature for 3 h, decanted, and read in a gamma counter to quantify the radiolabeled P4 against a standard curve. The sensitivity of this assay for plasma is 0.1 ng/mL. The average intra-assay coefficients of variation for the 3 types of sample were 14.4% and 4.9% for mean P4 concentrations of 0.7 and 2.9 ng/mL, respectively.

Solid-phase enzyme EIA (SPEIA) — The P4 concentration was determined, in duplicate, with a SPEIA as previously described (7). Briefly, P4 from plasma and milk samples (250 μL each) was extracted with the use of petroleum ether in a procedure that includes a freezing step. Standards and reconstituted extracts of samples were pipetted into microtiter plate wells previously coated with a solution of mouse monoclonal antibody against rabbit IgG. Progesterone–acetylcholine esterase (Ache) and antiprogesterone were added to each well except for the total activity, blank, and non-specific-binding wells. Buffer solution was used to adjust the wells' volume, and the plates were incubated at room temperature for 18 to 20 h. After incubation, the solutions were aspirated and the wells washed 5 times. Progesterone–ACheE solution was pipetted into the total activity wells, and Ellman's reagent was added to all wells except the blanks. The plates were covered and placed in an orbital shaker in darkness for 2 h, and then optical density was measured at 410 nm. The sensitivity of the SPEIA for plasma is 0.03 ng/mL. The average intra-assay coefficient of variation for the 3 types of sample was 10.3%.

Direct EIA (DEIA) — The P4 concentration was determined, in duplicate, with the use of a commercially available kit (Quanticheck; Faculty of Veterinary Science, Budapest, Hungary). This kit includes a microplate EIA that uses monoclonal antibody against P4 and horseradish peroxidase as the enzyme label. The method was originally developed for P4 determination in equine plasma (8) and has been adapted for quantifying P4 in bovine plasma and milk (11,12), as well as canine serum (13). The sensitivity of the DEIA for plasma is 0.5 ng/mL. The average intra-assay coefficients of variation for the 3 types of sample were 4.5%, 6.5%, and 3.2% for means of 0.97, 2.1, and 10.1 ng/mL, respectively.

Statistical analysis

Throughout this article, data are reported as means and standard error in parenthesis. Probability values ≤ 0.05 were considered significant. A tendency toward significance was indicated if the *P*-value was ≤ 0.1 but > 0.05. The 216 observations (for 24 cows, 3 samples, and 3 assays) were analyzed by the GLM procedure in SAS (version 8.2 for Windows; SAS Institute, Cary, North Carolina, USA). The original model included the main effects as cow reproductive status (estrus, diestrus, anestrus, and pregnant), sample type (plasma, skim milk, and whole milk), assay type (RIA, SPEIA, and DEIA), and their interactions. The P4 concentrations in diestrus and pregnancy and in estrus and anestrus did not differ; hence, the data were combined as expected P4 concentrations (high or low) for further analysis. Means were compared with the protected least-significant-difference test, and equality of variances was compared by Bartlett's test. Pearson correlation coefficients were calculated between RIA, DEIA, and SPEIA; data for cows with high and low P4 concentrations were analyzed separately. The χ^2 test was used for proportional data (proportion of cows with P4 concentrations either $\geq$ or $<$ 1 ng/mL).

Results

As expected, there was an effect of cow status on P4 concentration in all samples. Cows in diestrus or pregnant had higher ($P < 0.01$) mean P4 concentrations (6.0 [0.05] and 7.2 [0.1] ng/mL, respectively) than those in estrus or anestrus (0.8 [0.01] and 0.6 [0.01] ng/mL, respectively). Overall, the P4 concentrations were higher ($P < 0.04$) in plasma (5.0 [0.07] ng/mL) than in skim milk (2.3 [0.06] ng/mL) and intermediate in whole milk (3.6 [0.06] ng/mL). However, there was an interaction between expected P4 concentration and sample type ($P < 0.0001$). The mean P4 concentration in the group expected to have high values (cows in diestrus or pregnant) was higher ($P < 0.001$) in plasma (9.3 [0.7] ng/mL) than in skim milk (3.8 [0.3] ng/mL) or whole milk (6.6 [0.5] ng/mL), regardless of assay technique.

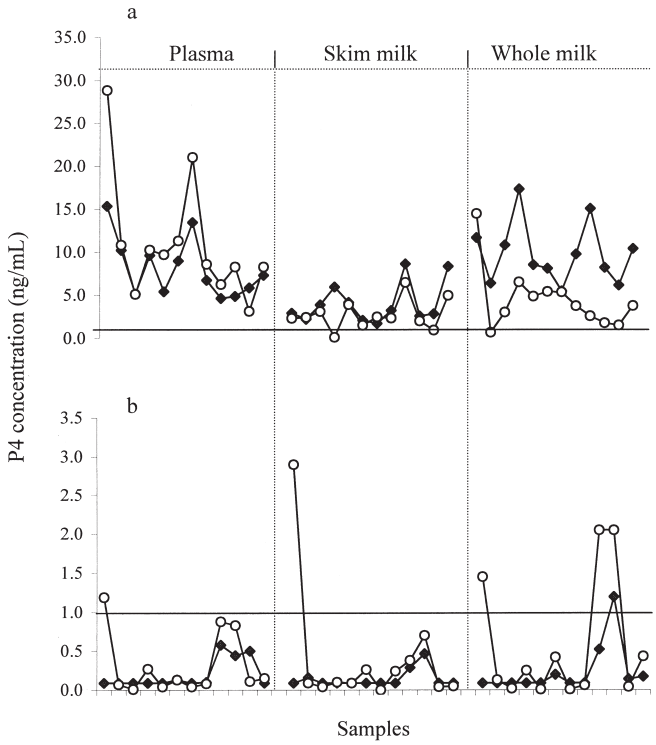

Figure 1. Comparison of progesterone (P4) concentrations in samples of bovine plasma, skim milk, and whole milk from 24 lactating dairy cows as determined with a radioimmunoassay (RIA; black diamonds) or a solid-phase enzyme immunoassay (SPEIA; clear circles); a, data for samples expected to have high values, from diestrous or pregnant cows; b, data for samples expected to have low values, from cows in estrus or anestrus. Correlations between RIA and SPEIA for the data in "a" ($r = 0.60$; $P < 0.01$) and those in "b" ($r = 0.50$; $P < 0.01$) were significant. The 1.0 ng/mL concentration is marked by a solid horizontal reference line in each panel.

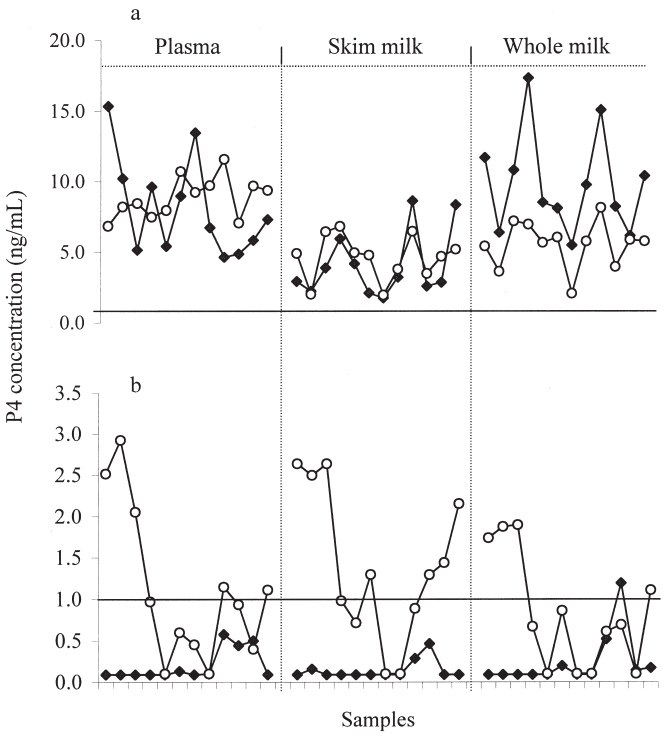

Figure 2. Comparison of P4 concentrations for the same group of cows as determined with RIA (black diamonds) or a direct EIA (DEIA; clear circles). Although the correlation between RIA and DEIA for samples expected to have a high P4 concentration (a) was significant ($r = 0.40$; $P < 0.02$), the correlation was not significant ($r = -0.10$; $P < 0.4$) for samples expected to have a low P4 concentration (b). The 1.0 ng/mL concentration is marked by a solid horizontal reference line in each panel.

Although determination of the P4 concentration did not differ ($P < 0.4$) among assays, an interaction between assay and expected P4 concentration ($P < 0.05$) affected the determination of P4. Regardless of sample type, RIA determined higher P4 concentrations ($P < 0.03$) in diestrus and pregnancy than did SPEIA (7.3 [0.7] versus 6.1 [0.6] ng/mL). The proportions of these cows with P4 concentrations < 1 ng/mL were 0%, 8%, and 0% for RIA, SPEIA, and DEIA, respectively ($P < 0.05$; Figures 1a and 2a). In contrast, DEIA tended to determine higher P4 concentrations ($P < 0.08$) in estrus and anestrus than did RIA (1.1 [0.1] versus 0.2 [0.02] ng/mL). The proportions of these cows with P4 concentrations ≥ 1 ng/mL were 3%, 14%, and 44% for RIA, SPEIA, and DEIA, respectively ($P < 0.01$; DEIA > SPEIA > RIA; Figures 1b and 2b).

There was a significant correlation between RIA and SPEIA for samples expected to have high ($r = 0.60$; $P < 0.01$) and low ($r = 0.50$; $P < 0.01$) P4 concentrations (Figure 1). Although the correlation between RIA and DEIA for samples expected to have high P4 concentrations was significant ($r = 0.40$; $P < 0.02$), the correlation was not significant ($r = -0.10$; $P < 0.4$) for samples expected to have low P4 concentrations (Figure 2).

There was also an interaction between assay, expected P4 concentration, and sample ($P < 0.001$) in the determination of P4 concentration (Table I). When only samples containing high concentrations of P4 and analyzed by RIA were compared, the P4 concentration in whole milk tended to be higher ($P = 0.09$) than that in plasma and was clearly higher ($P < 0.001$) than that in skim milk. However, both EIAs determined greater ($P < 0.001$) P4 concentrations in plasma than in either skim milk or whole milk. When samples containing low concentrations of P4 were analyzed within each immunoassay, the P4 concentrations did not differ ($P < 0.8$) among plasma, skim milk, and whole milk.

Discussion

We chose the Coat-a-Count RIA as the reference assay in this study because it is very widely used by researchers for determining the P4 concentration in bovine milk or plasma. The 3 assays did not differ in their ability to determine the P4 concentration in bovine plasma, skim milk, or whole milk samples when overall means were considered. However, there were differences within and among assays as related to P4 concentration and sample type and their interactions.

Progesterone is a fat-soluble hormone. Waldmann et al (14) reported that the ratio of P4 in skim milk to that in milk fat was 1:147. Hence, the P4 concentration is expected to be higher in whole milk than in skim milk. In the present study, overall, P4 concentrations in diestrous and pregnant cows were higher in plasma than in milk, mainly owing to the lower P4 determinations in skim and

Table I. Mean concentrations (and standard error) of progesterone (P4) in 24 lactating dairy cows expected to have high concentrations (those in diestrus or pregnant) or low concentrations (those in estrus or anestrus)

Expected P4 concentration and sample	Assay; mean concentration (and standard error), ng/mL		
	RIA	SPEIA	DEIA
High concentration			
Plasma	8.1 (1.0)[a,e,g]	10.9 (2.0)[b,e]	8.9 (0.4)[a,e]
Skim milk	4.1 (0.7)[a,f]	2.7 (0.5)[b,f,g]	4.6 (0.5)[a,f]
Whole milk	9.8 (1.0)[a,e,h]	4.5 (1.0)[b,f,h]	5.6 (0.5)[b,f]
Low concentration			
Plasma	0.2 (0.06)[c]	0.4 (0.1)[c]	1.1 (0.2)[d]
Skim milk	0.2 (0.03)[c]	0.4 (0.2)[c]	1.4 (0.2)[d]
Whole milk	0.2 (0.09)	0.6 (0.2)	0.8 (0.1)

RIA — radioimmunoassay; SPEIA — solid-phase enzyme immunoassay; DEIA — direct EIA.

[ab] Within a row, means without a common superscript differed ($P < 0.05$).

[cd] Within a row, means without a common superscript tended to differ ($P < 0.1$).

[ef] Within a column, means without a common superscript differed ($P < 0.001$).

[gh] Within a column, means without a common superscript tended to differ ($P < 0.09$).

whole milk samples than in plasma by both EIAs. However, the RIA determined higher P4 concentrations in whole milk than in plasma or skim milk, in agreement with results obtained by Dobson et al (2), in whose study the mean P4 concentrations measured by RIA during the luteal phase were 7.5 and 10 ng/mL for plasma and whole milk, respectively. The reason for the differences among assays is unclear but undoubtedly related to the assay type.

One of the purposes of measuring the P4 concentration is to monitor ovarian activity (4). It is well accepted that P4 concentrations $\geq$ 1 ng/mL indicate a functional corpus luteum, whereas concentrations $<$ 1 ng/mL indicate the lack of a functional corpus luteum or noncyclicity (2). Hence, in samples from diestrous or pregnant cows (when a functional corpus luteum is expected), we were interested in determining, for each assay, the proportion of those samples with P4 values $<$ 1 ng/mL and $\geq$ 1 ng/mL. We observed some differences among assays, which again depended on sample type and P4 concentration. Interestingly, whereas none of the samples that were expected to have a high P4 concentration (obtained from pregnant or diestrous cows) was found to have a value lower than 1 ng/mL by RIA and DEIA, 8% of the samples (including all the plasma and milk samples) had values lower than 1 ng/mL by SPEIA. The SPEIA included an extraction procedure, and some milk samples jelled at the freezing step; this may have resulted in the lower P4 values for some cows expected to have high P4 concentrations.

Both EIAs tended to overestimate the P4 concentration, mainly in plasma and skim milk from animals in estrus or anestrus; the SPEIA and DEIA determined P4 values $\geq$ 1 ng/mL in 14% and 44%, respectively, of the samples. Only 1 of these animals was determined by RIA to have P4 values $>$ 1 ng/mL. Our findings are in partial agreement with those from a previous study by Nagy et al (8), who, after comparing 1155 plasma samples from mares in diestrus or estrus, reported that DEIA resulted in more elevated P4 values than did RIA. We believe that a reason for this discrepancy between RIA and EIA results might be insufficient assay sensitivity to discriminate among very low P4 values in cows in estrus and anestrus. However, the proportion of cows with P4 concentration $\geq$ 1 ng/mL as determined by DEIA was lowest in whole milk; therefore, the use of DEIA to determine P4 concentrations in bovine whole milk would be acceptable.

In terms of rapidity, DEIA was better than the other 2 techniques. Another important attribute of this assay is that results could be read visually with some degree of accuracy to differentiate between samples of high and low P4 concentration. The 2 EIAs were less expensive than the RIA and involved no radioactive material.

In summary, in whole milk samples, the P4 concentrations were higher with RIA than with the EIAs, whereas both EIAs determined higher concentration of P4 in plasma. In estrous or anestrous cows, the DEIA tended to overestimate the P4 concentration in plasma and skim milk, but all 3 assays were comparable in determining the P4 concentration in whole milk. From these findings we conclude that both EIAs could be used for determining the P4 concentration in bovine whole milk with an acceptable level of precision relative to RIA. However, determining the P4 concentration with DEIA in plasma and skim milk samples expected to have a low concentration could have a considerable margin of error.

Acknowledgments

We thank Tanya Lewandoski (Brandon), Fiona Brown (Lethbridge), Randy Wilde (Lethbridge), and Pavol Zalkovic (Edmonton) for technical assistance. This research was partially supported by the Alberta Science and Research Authority (Alberta Agricultural Research Institute), Alberta Livestock Industry Development Fund (grant 2004L020R to Dr. Ambrose), Alberta Milk, and Alberta Agriculture and Food.

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