

## Comparison of Radioimmunoassay and Gas-Liquid Chromatography Analyses of Progesterone Concentrations in Cow's Milk<sup>1</sup> (38917)

L. C. NUTI, B. C. WENTWORTH, H. J. KARAVOLAS, W. J. TYLER,  
AND O. J. GINTHER

*Departments of Veterinary Science, Poultry Science, Physiological Chemistry and Dairy Science.  
The Endocrinology-Reproductive Physiology Program and the Waisman Center on Mental Development,  
University of Wisconsin, Madison, Wisconsin 53706*

There has been considerable disagreement and confusion on the concentration of progesterone in cow's milk. Previous studies in our laboratory (1) indicated that the concentration of progesterone during days 30-210 of pregnancy was approximately four times higher in milk (21.2 ng/ml) than in blood plasma (5.3 ng/ml). The samples were taken approximately 2 hr after the morning milking. Laing and Heap (2) have reported that the progesterone concentrations in the milk of non-pregnant and late pregnant cows were similar to those in plasma, whereas in early pregnancy the concentrations were apparently much higher in milk. However, in a subsequent study these workers (3) were unable to confirm the high concentrations in milk. The possible existence of progesterone metabolites which interfere with assay procedures has been considered (2) and differences in assay techniques among laboratories may also be a source of different findings.

Another possible explanation for divergent results among laboratories may be differences in the method of obtaining milk samples. It is well known that the amount of fat in milk samples is dependent on the time during the milking process at which a sample was obtained (4). There are indications (5) that changes in milk progesterone and fat concentrations show a high positive correlation when an active corpus luteum is present. Thus some of the discrepancies among laboratories may be due to differences in the time or method of milk sampling.

<sup>1</sup> Research supported by the College of Agricultural and Life Sciences, University of Wisconsin, Madison, Grant No. 630-0505A from the Ford Foundation, and by a grant from Dairy Research Incorporated, Arlington Heights, IL.

The present experiments were conducted to compare the effects of different progesterone antisera, purification procedures and determination methods (RIA vs. GLC) for the quantitation of progesterone in cow's milk. In addition, the effect of time of sampling during the milking process on progesterone concentration was studied.

*Materials and Methods. Experiment 1.* As part of a previous study, samples of milk (40 ml) were obtained from 8 cows on each of days 30, 60, 90, 120, 150, 180 and 210 of pregnancy and were stored at  $-20^{\circ}$  (1). In the present project, samples were randomly selected from three cows on each of days 30, 120 and 210 after the samples had been stored for 10 mo. Progesterone concentration was quantitated for each sample by 3 assay methods: (a) radioimmunoassay after ether extraction and LH-20 chromatography (RIA-1) (b) radioimmunoassay after ether extraction, LH-20 chromatography and additional purification procedures (RIA-2), and (c) gas-liquid chromatography after initial processing identical to that used for RIA-2 (GLC).

Tritiated progesterone (1, 2, 6, 7-<sup>3</sup>H progesterone, New England Nuclear; RIA-1, 1,000 cpm; RIA-2 and GLC, 10,000 cpm) was added to each sample to serve as an internal tracer for estimation of procedural losses. For RIA-1, the samples were assayed as reported previously (1).

For RIA-2 and GLC, the following modifications were made prior to chromatography on LH-20 sephadex. After diethyl ether extraction the lipid was removed by a precipitation step. The dried extracts were dissolved in diethyl ether and 70% methanol (1:4) and kept at  $-20^{\circ}$  for 6 hr (4), followed by centrifugation to remove the lipid precipitate. The supernatants were then

chromatographed on LH-20 columns as described earlier (1). The progesterone fractions were further purified by the following thin-layer chromatography systems: (a) methanol:carbontetrachloride, 1:99, and (b) methanol:benzene, 1:19. After each separation the zones isopolar with the appropriate standards were scraped and eluted. Reference steroids were visualized by scanning under uv light. The eluted samples were then subjected to a liquid phase acetylation (7, 8) in order to form acetates of hydroxylated compounds which may not have been separated from progesterone. Progesterone was isolated by further chromatography on sephadex LH-20 columns using a solvent system of hexane:benzene:methanol (85:5:4). The eluted samples were dried under a stream of nitrogen gas and subsequently dissolved in ethyl acetate for quantitation by GLC. Aliquots were removed at this time for recovery estimation and determination of mass by RIA or GLC. The GLC determinations were conducted using a Hewlett-Packard (Model 402) gas chromatograph, equipped with a hydrogen flame ionization detector (8). The 6 ft.  $\times$  4 mm (i.d.) Pyrex columns were packed with either 2% Xe-60-1%Se-30 or Ov-1 3% on 80/100 mesh Gas Chrom Q (Applied Science Laboratories). Data were statistically analyzed with a  $3 \times 3$  factorial analysis of variance. The factors were day of pregnancy (30, 120 and 210) and method of assay (RIA-1, RIA-2 and GLC).

*Experiment 2.* Milk samples were taken from four cows in mid-diestrus (days 11-14) at the following times: (a) premilking (immediately prior to the morning milking), (b) milking pool (aliquot of the morning milking pool), (c) immediately postmilking, and (d) 3 hr postmilking. Each milk sample was analyzed for total fats with a Babcock test (9). In addition, two 1.0 ml milk aliquots were quantitated by RIA. The following two antisera were compared: (a) AS-1; antisera prepared in sheep to the progesterone-11 alpha-bovine serum albumin conjugate as used in the earlier report (1) and in experiment 1; and, (b) AS-2; antiserum prepared in a ewe immunized against 11-deoxycortisol-21-monoheemisuccinate-bo-

vine serum albumin (S-49 No. 6, supplied by Dr. G. E. Abraham, University of California, Los Angeles). One aliquot of each sample was extracted and chromatographed without lipid precipitation as reported earlier (1). The other aliquot was subjected to the lipid precipitation step as described for RIA-2 and GLC in experiment 1.

Data were statistically analyzed by a  $4 \times 2 \times 2$  factorial analysis of variance. The factors were time of sample collection (premilking, milking pool, immediately postmilking, and 3 hr postmilking), type of antiserum (AS-1 and AS-2) and type of purification (with and without lipid precipitation).

*Results. Experiment 1.* Progesterone concentrations in milk were not significantly different among the three assay methods (Table I). There was a day effect ( $P < 0.05$ ) apparently due to gradually decreasing values as pregnancy advanced. The interaction of day of pregnancy and method of assay was not significant.

*Experiment 2.* The means for the percent fat content were different ( $P < 0.05$ ) for all comparisons among the four times of collection (Table II). For progesterone concentration, the main effect of time and the three-way interaction (time  $\times$  antiserum  $\times$  purification) were significant

TABLE I. PROGESTERONE LEVELS IN MILK SAMPLES AT THREE DAYS OF PREGNANCY AS QUANTITATED (ng/ml) BY RIA AND GLC.

Day of pregnancy	No. cows	Assay method		
		RIA-1 <sup>a</sup>	RIA-2 <sup>b</sup>	GLC <sup>c</sup>
		Mean SE	Mean SE	Mean SE
30	3	24.68 $\pm$ 5.81	43.13 $\pm$ 12.43	30.73 $\pm$ 9.01
120	3	27.83 $\pm$ 4.24	29.60 $\pm$ 11.19	26.40 $\pm$ 3.85
210	3	16.73 $\pm$ 3.04	10.14 $\pm$ 2.51	11.13 $\pm$ 4.03

<sup>a</sup> RIA-1 = radioimmunoassay after ether extraction and LH-20 chromatography.

<sup>b</sup> RIA-2 = radioimmunoassay after ether extraction and LH-20 chromatography and additional purification procedures.

<sup>c</sup> GLC = gas-liquid chromatography after ether extraction and LH-20 chromatography and additional purification procedures.

Day effect,  $P < 0.05$ ; Assay effect, not significant; Interaction, not significant.

TABLE II. EFFECT OF TIME OF MILK SAMPLING, DIFFERENT ANTISERA AND PURIFICATION BY LIPID REMOVAL ON PROGESTERONE DETERMINATION IN MILK

Time of collection of milk sample	Progesterone concentration (ng/ml) <sup>a</sup>				
	Percent fat	Antiserum 1 (AS-1)		Antiserum 2 (AS-2)	
		Without lipid removal	With lipid removal	Without lipid removal	With lipid removal
		Mean SE	Mean SE	Mean SE	Mean SE
Immediately premilking	1.32 <sup>b</sup> ±0.08	3.20 <sup>b</sup> ±1.09	5.00 <sup>b</sup> ±1.02	4.02 <sup>b</sup> ±0.78	5.44 <sup>b</sup> ±0.77
Milking pool	2.92 <sup>c</sup> ±0.27	9.08 <sup>b</sup> ±2.20	7.43 <sup>b</sup> ±0.95	9.44 <sup>b</sup> ±1.42	12.30 <sup>b</sup> ±2.42
Immediately postmilking	10.20 <sup>d</sup> ±0.33	30.23 <sup>c</sup> ±5.50	17.22 <sup>c</sup> ±3.17	19.54 <sup>c</sup> ±3.03	24.98 <sup>c</sup> ±4.64
3 hr postmilking	7.25 <sup>e</sup> ±0.93	9.31 <sup>b</sup> ±1.53	8.50 <sup>b</sup> ±1.00	10.45 <sup>b</sup> ±2.87	12.52 <sup>b</sup> ±2.74

<sup>a</sup> The main effect of time and the three-way interaction (time × antiserum × purification) for progesterone concentration are significant  $P < 0.005$ ; all other main effects and interactions are not significant.

<sup>bcd e</sup> Within each column, means with different superscript letters are different ( $P < 0.05$ ).

( $P < 0.005$ ); all other main effects and interactions were not significant. The three-way interaction seemed due primarily to differences in progesterone determinations among the 4 assay groups (2 antisera × 2 purifications) in the samples which were taken immediately postmilking (Table II); based on a multiple-range test, mean progesterone concentration was higher ( $P < 0.05$ ) in the assay which used AS-1, without lipid removal (30.23 ng/ml) than in the assay which used AS-1 with lipid removal (17.22 ng/ml) or the assay which used AS-2, without lipid removal (19.54 ng/ml). The determinations were not significantly different among the four assay groups for the other three times of sample collection. Within each of the four assay groups the concentration of progesterone was greater ( $P < 0.05$ ) for the samples which were collected immediately postmilking than for any of the other times of collection. Over all milk samples within each assay group, the percent fat content and the concentration of progesterone were positively correlated ( $r = +0.71$ ;  $P < 0.01$ ).

*Discussion.* There has been earlier qualitative evidence (10, 11) provided by means of gas chromatography combined with mass spectroscopy indicating that progesterone and its metabolites are present in cows' milk. The earlier report (1) from this laboratory of high progesterone concentration in milk from pregnant cows was supported by the results of experiment 1;

progesterone concentrations were comparable to those reported earlier and were not significantly different when analyzed by RIA (with or without purification of samples beyond LH-20 chromatography) or GLC.

There have been conflicting reports (2, 3, 12) concerning milk progesterone concentrations and their relationship to blood plasma concentrations. There has been a tendency to imply that milk progesterone levels approach plasma levels and that the discrepancies between milk and plasma levels are due to a number of factors including: (a) contaminating metabolites, (b) sample deterioration with storage, (c) nonspecificity of antisera used in radioimmunoassay procedures, and (d) time at which milk samples were taken. These points have not been well documented.

In the present studies, progesterone measurements for samples stored at  $-10^{\circ}$  for 10 mo were similar to those obtained in an earlier study (1) prior to prolonged storage. This result failed to suggest an effect of storage at  $-10^{\circ}$  on progesterone concentrations.

It is known that milk which is drawn first during the milking process contains considerably less fat than milk which is drawn last, whereas there is no difference in the proportion of other major constituents in the milk obtained at various times during the milking process (4). In the present study fat content was different among all sampling times. Both fat content and progesterone

concentration were significantly higher for samples collected immediately postmilking, than for any of the other sampling times. Furthermore, over all samples there was a positive correlation between percent fat and progesterone concentration which is consistent with an earlier report (5). These findings may explain some of the discrepancies in progesterone concentrations reported by various investigators.

The specificity of AS-1 antiserum has been reported earlier (1). The LH-20 chromatography removed most of the commonly occurring contaminating progestins. The relative cross-reactivities of AS-2 antiserum has also been investigated (13). It was found that 17-hydroxyprogesterone and 11-desoxycorticosterone (DOC) had a cross-reactivity of 35% with antiserum AS-2. These two progestins are effectively separated from progesterone with the LH-20 chromatography system. Other steroids which were not removed by chromatography are cholesterol,  $^4\Delta$ -androstene-dione and  $^5\Delta$ -pregnenolone. Pregnenolone and androstene-dione had a cross-reaction with AS-2 of less than 0.1% and cholesterol of less than 0.01%. Thus it appears that these antisera are effective in quantitating progesterone when used in conjunction with chromatographic procedures. However, the results of experiment 2 indicate that the quantitation of progesterone in milk was affected by the purification procedures (with or without lipid removal) particularly when AS-1 was used for samples which were high in fat content.

**Summary.** Progesterone concentrations in milk were not significantly different when quantitated by different methods (RIA vs. GLC). There was a significant day effect ( $P < 0.05$ ) on milk progesterone level due apparently to gradually decreasing values as pregnancy advanced over days 30, 120 and 210. The means for the percent fat content were different ( $P < 0.05$ ) for all comparisons among four times of collection (immediately premilking, milking pool, immediately postmilking, and 3 hr postmilking). For progesterone concentration, the main effect of time and the three-way interaction (time  $\times$  antiserum  $\times$  purification method) were significant ( $P < 0.005$ ); all other main effects and interac-

tions were not significant. Within each of 4 assay groups (2 antisera  $\times$  2 purifications), the concentration of progesterone was greater ( $P < 0.05$ ) for the samples which were collected immediately postmilking than for any of the other times of collection. The three-way interaction seemed due primarily to difference in progesterone determinations among the four assay groups in the samples which were taken immediately postmilking. Over all milk samples within each assay group, the percent fat content and the concentration of progesterone were positively correlated ( $r = 0.71$ ,  $P < 0.01$ ).

Thanks are due to Donna Mears for her assistance in sample handling and RIA procedures. Also, the valuable technical assistance of Mr. Don O'Brien on the GLC equipment is gratefully acknowledged. Special thanks are due to Dr. M. Garcia for help with the statistical analysis.

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