A comparison of tritiated and iodinated tracers in the radioimmunoassay of progesterone in cow milk

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Summary. Progesterone levels in the milk of cows were determined by two radioimmunoassay methods. Excellent correlation ($r = 0.95$) was found between the method using an iodinated radioligand and that using a conventional tritiated tracer.

Introduction

The determination of milk progesterone levels by radioimmunoassay to provide an early indication of pregnancy status in the cow is now a well established technique (Heap, Holdsworth, Gadsby, Laing & Walters, 1976; Dobson & Fitzpatrick, 1976). These radioimmunoassays, in common with most other steroid assays, incorporate a tritium-labelled steroid and therefore suffer from the disadvantages of β -liquid scintillation counting. As an alternative, the introduction of a γ -emitting radioisotope (^{125}I or ^{75}Se) has been suggested, although the development of suitable assay systems poses considerable technical problems (Allen & Redshaw, 1978a). For human serum progesterone assays, several papers have been published describing the use of ^{125}I -labelled progesterone in place of the tritiated steroid (Hunter, Nars & Rutherford, 1975; Scarisbrick & Cameron, 1975). The increasing importance of the bovine pregnancy test has prompted us to extend these ideas to the development of a suitable γ -labelled milk progesterone assay. The purpose of the present paper is to report this work, comparing the results of the iodinated radioimmunoassay with a conventional tritiated system.

Materials and Methods

Milk samples

Daily samples (~20 ml) were taken from the bulk whole milk (p.m. milking) of 6 Friesian cows in a commercial herd. The intervals between calving and the start of the study were 74 days for Cows 116 and 201, 72 days for Cow 219, 53 days for Cow 166, 37 days for Cow 151 and 27 days for Cow 270. The milk samples were preserved with potassium dichromate-mercuric chloride (Lactabs MKII, Thompson and Capper Ltd, Runcorn), and stored at 4°C until analysis. Before analysis the samples were vortex-mixed to ensure homogeneity.

Iodinated assay

A conventional radioimmunoassay was employed with a charcoal separation step. Standards were prepared by the double dilution of an ethanolic progesterone solution and addition to 5% (w/v) aqueous skimmed milk powder solution. The range was 0–32 ng progesterone/ml milk. The antiserum, tracer and charcoal reagent (Norit OL) were prepared in phosphate-buffered saline, pH 7.4. The antiserum (78/9) was raised by us in rabbits against an 11 α -hydroxy-

progesterone 11-succinyl-bovine serum albumin conjugate and used at a final dilution of 1 in 5000. The labelled steroid derivative, progesterone-3-(O-carboxymethyl)oxime-[^{125}I]iodohistamine, was prepared and iodinated by the methods described by Allen & Redshaw (1978b). Cross-reactivity data for the antiserum with this tracer are shown in Table 1.

Table 1. Cross-reactions of various steroids with the antisera used in the iodinated and tritiated progesterone assays

Steroid	% Cross-reaction* in:	
	^{125}I assay	^3H assay
Progesterone	100	100
11 α -Hydroxyprogesterone	100	117
11 β -Hydroxyprogesterone	33.0	35.0
5 α -Pregnane-3,20-dione	5.0	35.3
5 β -Pregnane-3,20-dione	6.0	2.5
3 β -Hydroxy-5-pregnen-20-one	12.5	<0.1
Deoxycorticosterone	<0.1	12.5
Corticosterone	4.0	3.8
20 α -Hydroxy-4-pregnen-3-one	<0.1	0.2
17-Hydroxyprogesterone	1.3	<0.1
Testosterone	<0.01	<0.1
Oestrone, oestradiol-17 β , oestriol	<0.01	<0.1
Cholesterol	<0.01	<0.1

* The amount of test compound introduced into the milk progesterone assay which is required to displace 50% (10 pg) of bound ^{125}I -labelled progesterone or 50% (29 pg) of bound [^3H]progesterone.

Assays were set up in duplicate and a total and a quality control sample were included. To each assay tube was added, in turn, 20 μl standard, control or milk sample, 200 μl ^{125}I -labelled progesterone (20 pg), and 200 μl antiserum. After vortex mixing, the tubes were incubated at room temperature for 2–3 h. Charcoal suspension (20 μg in 500 μl) was added to the tubes, the contents were vortex mixed and incubated for a further 10 min. The charcoal was compacted by centrifugation (1000 g), the supernatants were decanted and the charcoal pellets were counted in a γ -counter for 60 sec.

The limit of detection of the assay is 30 pg (twice the s.d.). The mean coefficient of variation between standard curves is 7.1% ($n = 15$) and the mean within-assay variation between quadruplicates, 2.2%. Labelled progesterone added to milk samples (pooled dairy milk or milk from ovariectomized cows) showed an average recovery of 99.3% (± 14).

Tritiated assay

A semi-automated technique, closely based on that described by Heap *et al.* (1976), was used, employing an LKB 2071 sample processor to make the first series of dilutions. Standards were prepared by addition of progesterone in ethanol to milk from an ovariectomized cow to give concentrations ranging from 0 to 30 ng progesterone/ml milk. Dilutions of antiserum, tracer and dextran-coated charcoal were made in phosphate-buffered saline, pH 7.4. Antiserum (BF 465/6 supplied by Dr B. J. A. Furr) was raised in a goat immunized against 11 α -hydroxyprogesterone 11-succinyl-bovine serum albumin and used at 1 in 15 000 initial dilution. The cross-reactivity data for the antiserum is shown in Table 1. A solution of [^3H]progesterone (80 c.p.m./ μl : The Radiochemical Centre, Amersham; sp. act. 49 Ci/mmol) was added to the antiserum solution in the ratio 1:6 (v/v). To each tube was added 10 μl of standard or sample and 750 μl of the tritiated progesterone/antiserum mixture. The tubes were incubated at 4°C for

3½–4½ h. A suspension of dextran-coated charcoal was added to each tube and the contents incubated at 4°C for 15 min before centrifugation. Supernatants were decanted into scintillation vials, to each of which was added 5 ml toluene-PPO scintillation cocktail. After an 8-h equilibration period, each sample was counted for 60 sec in a β -liquid scintillation counter. Because of the cost, phase combining agents are not used and therefore an equilibration period is included to allow extraction of the antibody-bound tritiated progesterone into the scintillator.

The limit of detection of the assay is 20 pg (twice the s.d.). The mean coefficient of variation between standard curves is 6% ($n = 6$) and the mean within-assay variation between quadruplicates 3.9%. Full details of the assay have been published elsewhere (Holdsworth, Chaplin & Booth, 1979).

Text-fig. 1. Progesterone levels in 3 cows providing daily milk samples for assay by an iodinated progesterone (—) or tritiated progesterone (---) method. The arrows indicate mounting by a co-habiting fertile bull.

Results

The daily progesterone concentrations in the milk of 3 of the 6 animals used in the study are shown in Text-fig. 1. In Cow 219 (Text-fig. 1b) progesterone concentrations were low (<2 ng/ml) at the start of the cycle and then rose steadily from Day 5 to Day 10 to reach 10 ng/ml, fluctuating around this figure until Day 17 and then falling to basal levels by Day 20. Text-figure 1(a) shows that throughout gestation the concentration of progesterone is similar to that in the mid-luteal phase of the cycle; levels began to rise 6 days after mating and remained around 12 ng/ml for the duration of the study. In Cow 201 (Text-fig. 1c) the first post-partum insemination was not successful and the progesterone peak was of short duration, which may indicate luteal insufficiency in this cycle. Lamming & Bulman (1976) have previously reported low progesterone levels in cows returning to oestrus after calving. The progesterone levels of Cows 151 and 166 were similar to those of Cows 116 and 219 respectively. Cow 270 had basal values for the 40 days of study and may have been anoestrous.

Statistical evaluation of the two methods gave a correlation coefficient of 0.95 ($P < 0.001$). The regression equation for the iodinated assay (y) against the tritiated method (x) was $y = 0.9147x + 0.6704$. The standard error of the slope was 0.025.

Discussion

Pregnancy detection by progesterone measurement is generally based on direct analysis of the milk sample. The use of the ^{125}I -labelled progesterone derivative in the assay system described eliminates the problems inherent in β -liquid scintillation counting. Excellent correlation was found between the iodinated and tritiated methods. The values obtained with the iodinated system were slightly higher than those obtained with the tritiated method, probably because the standards for the two assays were prepared in different media. The skimmed milk powder used in the iodinated assay almost certainly contained some progesterone (<300 pg/ml) but it is readily obtainable and convenient to use. The combination of an antiserum raised to the 11-position and an iodinated 3-derivative as radioligand has previously been described by Scarisbrick & Cameron (1975), Scott, Stanczyk, Goebelsmann & Mishell (1978), and Allen & Redshaw (1978b). The sensitivity of this particular heterologous site combination is equivalent to that of the tritiated system.

Although progesterone can be determined in bovine serum by radioimmunoassay, it is more appropriately measured in milk where the levels not only reflect ovarian activity but are also appreciably higher (Dobson, Midmer & Fitzpatrick, 1975). The oestrous cycle in dairy cows normally varies from 18 to 24 days with a mean of 21 days. The progesterone levels are low during oestrus and high throughout the luteal phase of the cycle and pregnancy. However, it is possible to differentiate between pregnant and non-pregnant animals by measuring the concentration of progesterone in the milk, approximately 22–26 days after mating, and this forms the basis of the bovine pregnancy test (Heap *et al.*, 1976; Dobson & Fitzpatrick, 1976; Booth & Holdsworth, 1976). If the progesterone concentration is low (<6–7 ng/ml) the cow can be classified as not pregnant. The pregnancy test is now in widespread use and negative (non-pregnant) results are nearly 100% accurate. The accuracy of positive (pregnant) results (i.e. values > 7 ng/ml) is lower, at 85–90%, largely because of embryo loss and insemination at times other than true oestrus. There is increasing evidence to show that the measurement of milk progesterone levels can aid the effective monitoring of bovine infertility (van de Wiel, van Eldik, Koops, Postma & Oldenbroek, 1978). Although the number of animals involved in the present study is small, a single milk sample taken 22–24 days after mating and analysed for progesterone would have given the correct pregnancy status in all the cows.

The results of this study indicate that the tritium- and iodine-labelled methods are

comparable for direct estimations of progesterone in milk. The use of an iodinated tracer for the radioimmunoassay therefore provides a viable alternative to the widely used tritium-labelled method.

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