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STEROID DETERMINATION IN MILK BY ENZYME IMMUNOASSAY (EIA)

K.-I. ARNSTADT

Institut für Physiologie der Südd. Versuchs- und Forschungsanstalt für Milchwirtschaft, Technische Universität München, 8050 Freising-Weihenstephan, F.R.G.

SUMMARY

A simple and sensitive enzyme immunoassay for progesterone using a homologous system was developed covering the range of 30–3000 fmol/test tube (9.4–944 pg) and applied to extracts from milk fat and bovine serum. By changing the incubation procedure the sensitivity of the assay was increased to measure milk from cows directly without extraction. The influence of milk fat is tolerable and does not falsify physiological results concerning corpus luteum activity or inactivity. The EIA can also be used to follow the change of progesterone under physiological, pathological and therapeutic conditions in samples of human blood and saliva after extraction and in serum directly.

The first reports of Nakane and Avrameas[1, 2] on the use of enzyme labels for immunological reactions stimulated the search for suitable non-radioactive immunoassays. Besides the fluoroimmunoassays and the more modern chemiluminescent immunoassays and fluoro-enzyme immunoassays, the almost classical enzyme immunoassay is still useful, especially for the determination of steroid hormones. This report is on a sensitive EIA for progesterone.

Horseradish peroxidase was used as the enzyme label of progesterone in a homologous immunoassay system. The antiserum was raised to 11α-hydroxyprogesterone-11-hemisuccinate-BSA in rabbits. To facilitate B/F-separation by centrifugation the antiserum was bound to cellulose. The immunological reaction was carried out for 30 min at 37°C and the unbound progesterone enzyme conjugate was washed by 0.15 M NaCl solution at 0–4°C. The bound label was measured by the peroxidase activity of the pellets using an *o*-phenylene-diamine assay.

The sensitivity and range of this EIA for progesterone of 0.1–10 pmol [3] was improved to 30–3000 fmol/test tube by using an antiserum with an association constant of $K_A = 1.5 \times 10^{10}\,l\,mol^{-1}$ [4]. The precision of the method is given by intra- and inter-assay coefficients of variation of between 4.3 and 9.2% [3, 5]. The specificity is satisfactory since of the naturally occurring steroids only 5α- and 5β-pregnane-3,20-dione give considerable crossreactions (10.0 and 19.1%) but these steroids do (nearly) not occur in cow's milk. The correlation with an established RIA is good ($r = 0.98$; [3, 5]).

The EIA was applied to extracts of milk via a milk fat procedure [6] to follow cyclicity and pregnancy for oestrous confirmation and fertility control of cows. For progesterone measurement of serum samples it was important to avoid petrolether for extraction and to use chemically better defined solvents like *n*-hexane, *n*-pentane or *i*-octane. Full advantage of such a nonradioactive technique is however to be taken only if measurements are possible without the need for prior extraction of steroids [7]. For developing a reliable direct assay for milk, attention had to be paid to constituents of the milk, especially to fat, which in RIA often prevents a good differentiation between samples without and with little progesterone to detect a rising corpus luteum activity between oestrous (day 0) and day 5–7 [8]. The problems caused by the content of milk fat can partially be solved by using fat-free milk or fore milk [9, 10]. Because of the solubility of progesterone in fat, the progesterone content of fat-free milk is low and the differences between samples of day 0 and day 5–7 is small. Furthermore, the addition of milk to the standards of a calibration curve for compensation of the unspecific influence by milk constituents causes loss of sensitivity. This loss is much higher with whole or last milk than with fore milk. The problem was solved by increasing the sensitivity of the EIA so that only a very small sample volume is necessary, with only a minor and tolerable influence on the assay system. Change from competitive to sequential incubation increases the sensitivity from the measurable range of 30–3000 fmol to 10–1000 fmol/test tube. Addition of 15 μl of progesterone-free whole milk to the standards shifts the range of 10–1000 fmol back to 30–3000 fmol/test tube. The addition of oestrous or post partum milk from different cows leads to somewhat different standard curves but they remained in a normal range of variation. Final results in ng/ml or nmol/l are obtained directly with the measurement of extinction after the enzyme reaction by using a combination of flow through cuvette photometer, on-line computer with a special EIA program and printer (EIA-Arbeitsplatz, Co. Eppendorf/München) without drawing a standard curve manually. The discriminatory level between an active and inactive corpus luteum was estimated for this assay to be 5 ng/ml. The coefficients of variation were 8.5% intra-assay and 14.2% inter-assay.

With the aid of the direct EIA, results can be obtained in 4–6 h. This relatively quick assay allows prediction of oestrous of cows after an unsuccessful first insemination when progesterone is measured at days 19–21. Milk from mares contain less fat than cow's milk. Therefore the direct estimation of progesterone causes no problem.

A direct assay also works with human serum. Saliva has to be concentrated from a volume of 0.5 ml by extraction. The progesterone concentration of saliva from women correlates with corpus luteum activity [11, 12] but with saliva from cows, because of their high and changeable saliva production and therefore unreproducible dilution of progesterone [4, 13], it does not.

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