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Dexamethasone Concentrations in Plasma and Milk of Cows following the Injection of Long- and Short-acting Dexamethasone Esters

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Abstract

A radioimmunoassay has been developed for the measurement of dexamethasone in plasma and milk of cows injected with long- and short-acting dexamethasone esters. Dexamethasone antiserum was produced by injecting cows with a dexamethasone-21-hemisuccinate–human serum albumin complex. The antisera was highly specific for dexamethasone, cross-reacting less than 0·7% for all endogenous steroids tested.

Plasma concentrations of dexamethasone in cows injected intramuscularly with either 20 mg dexamethasone-21 trimethyl acetate ($n = 2$) or the tributyl derivative ($n = 2$) reached a peak level of 0·6–1·1 ng/ml in 2–6 days then declined to undetectable levels (<0·15 ng/ml) by 14 days after injection. In general, dexamethasone concentrations in milk were 0·3–0·5 times the plasma concentrations but showed the same pattern of values.

Plasma dexamethasone concentrations were also determined in three lactating dairy cows injected intramuscularly with tritiated dexamethasone-21 trimethyl acetate. In these cows plasma dexamethasone concentrations, as determined by isotopic dilution, reached maximal levels of 1·1–1·6 ng/ml in 1–3 days then declined to levels of around 0·05 ng/ml within 30 days. The concentrations of dexamethasone in milk of two of these cows were, in general, similar to those found in plasma.

In three cows injected intramuscularly with 20 mg dexamethasone sodium phosphate the concentrations of dexamethasone in plasma rose sharply to maximum levels of 24–70 ng/ml within 2–20 min after injection and fell to undetectable levels (<0·15 ng/ml) after 72 h.

Extra keywords: radioimmunoassay; parturition; residues.

Introduction

Administration of a soluble dexamethasone (9-fluoro-11,17,21-trihydroxy-16-methyl-1,4-pregna-diene-3,20-dione), preparation to cows during late gestation provokes premature calving within 2–3 days (Adams and Wagner 1970; Jöchle 1973) whereas insoluble ester derivatives of dexamethasone such as Opticortenol (dexamethasone-21 trimethyl acetate) induces delivery within 10–14 days (Bailey *et al.* 1973; Welch *et al.* 1973). Some evidence that these differences in the actions of the soluble and insoluble esters are due to differences in the effective duration of the drug in blood has been obtained from studies which estimate the time the drug takes to elevate blood glucose concentrations (Heindrich *et al.* 1963; Woollett and Evans 1971).

In general, the soluble or short-acting dexamethasone esters cause an elevation in plasma glucose levels for 3–4 days (Maplesden *et al.* 1960; Woollett and Evans 1971)

whereas the insoluble or longer-acting dexamethasone esters cause the plasma glucose to remain raised for 12–14 days (Box, personal communication, 1974). One disadvantage of this approach is that the plasma glucose levels provide only an indirect measure of the activity of dexamethasone in blood. There was a need therefore to develop a sensitive, relatively specific assay for monitoring concentrations of dexamethasone in biological fluids directly. This information was also required by licencing agencies such as the Animal Remedies Board in New Zealand so that they could set withholding times after injection for meat and milk products.

This paper describes the development of a specific radioimmunoassay for determining plasma and milk concentrations of dexamethasone in cows injected with long- and short-acting dexamethasone esters. Plasma levels obtained by the radioimmunoassay method were compared with those obtained using an isotopic-dilution technique following the administration of tritiated Opticortenol to cows during the last 6 weeks of gestation. A summary of some of the results presented in this paper have been published previously in abstract form (McGowan *et al.* 1975).

Materials and Methods

Production of Antisera

Dexamethasone-21-hemisuccinate was conjugated to human serum albumin by the method of Erlanger *et al.* (1958). Then 5 mg of the conjugate was dissolved in 4 ml saline and the mixture emulsified with 5 ml Freund's complete adjuvant; 2 ml of this mixture was injected subcutaneously into five sites on two cows at 14-day intervals for 6 weeks and then at monthly intervals. The animals were bled 7–10 days after each immunization. The antiserum used in this study was obtained from one cow, 12 months after the primary injection. The dexamethasone antisera cross-reacted 16, 0·6, 0·5 and 0·3% with Opticortenol (Ciba–Geigy, Ltd), corticosterone, 11-desoxycortisol and cortisol respectively and 0·1% with progesterone and oestradiol-17β.

Radioimmunoassay of Dexamethasone

Plasma or milk samples (usually 0·5 or 1·0 ml respectively) were extracted in duplicate with 4 vol. ethyl acetate in disposable polypropylene test tubes (100 by 15 mm) by using a horizontal shaking machine. After extraction the tubes were centrifuged to break any emulsion formed. The aqueous layer was frozen in an acetone–dry ice mixture and the organic phase decanted into polypropylene test tubes. The extracts were then taken to dryness under nitrogen and the residue dissolved in 0·1 ml of solution containing dexamethasone antiserum diluted 1:150 with 0·05 M phosphate-buffered saline (PBS). After incubating at room temperature for 30 min, 0·1 ml PBS containing 6 nCi [1,2-^{3}H]dexamethasone (specific activity 20 Ci/m mole, Amersham Radiochemical Co., England) was added to all tubes followed by 0·1 ml of a 3% (w/v) bovine gammaglobulin solution. The tubes were incubated at 30°C for 30 min and at 4°C overnight. Free and protein-bound dexamethasone were separated by adding 1 ml of an ice-cold charcoal solution, prepared by dissolving 25 mg charcoal and 2·5 mg dextran in 100 ml PBS. After centrifugation at 5000 *g* for 10 min the supernatant was decanted directly into counting vials containing 5 ml Triton X-100 scintillation fluid. A standard curve was constructed for each assay by plotting the percentage radioactivity bound against the amount of dexamethasone added (0·1–2 ng). The results were corrected for an average recovery from plasma of 85% and sample volume.

The accuracy of the dexamethasone radioimmunoassay was assessed by adding 0·1, 0·25 and 0·5 ng of dexamethasone to 0·5 ml bovine plasma and assaying for dexamethasone content as described. The amount of dexamethasone recovered for eight samples at each concentration was 0·13 $\pm$ 0·02 (s.d.); 0·26 $\pm$ 0·03 and 0·43 $\pm$ 0·7 ng respectively. The precision of the assay determined from 41 duplicate analyses of samples varying in concentration from 0·25 to 0·9 ng/ml dexamethasone present was 4·7%. The sensitivity of the standard curve, calculated from the variance of the control tubes according to the method of Ekins and Newman (1970), was 0·06 ng.

For practical purposes, however, taking into account the sample volume and procedural losses, the limit of sensitivity of the method is estimated at 0·15 ng/ml plasma.

Plasma corticosteroid concentrations were determined by a competitive protein binding assay of Fairclough *et al.* (1975). The sensitivity of this assay was 1 ng for a plasma sample of 1 ml.

Animals and treatments

Experiment 1

Four multiparous lactating cows which formed part of a milking herd were used for this experiment. During the trial the cows were grazed on pasture and allowed free access to water. Two of the cows were injected intramuscularly with 20 mg Opticortenol as an insoluble suspension and two others were given an intramuscular (i.m.) injection of 20 mg dexamethasone tributyl acetate.

Blood samples (20 ml) were withdrawn once a day from the jugular vein into heparinized syringes. Milk was withdrawn at the afternoon milking from the collection jar of a pipeline milking system. The blood was kept at 4°C and centrifuged within 10 min of collection. Plasma was stored at −15°C and milk at 4°C until analysis.

Experiment 2

An i.m. injection of 20 mg Dexadreson (dexamethasone sodium phosphate) in 10 ml phosphate buffer was administered to three non-lactating Jersey cows. Blood samples (20 ml) were withdrawn at frequent intervals on the day of injection and then at least twice daily for 4 days. Plasma concentrations of dexamethasone were determined by radioimmunoassay.

Experiment 3

Three pregnant Jersey cows in the last 6 weeks of gestation were used. Throughout the trial the cows were kept in pens and fed approximately 15 kg of hay per day and were given water *ad lib.* Before the trial they were introduced to the pens once a week so they could become accustomed to the hay diet and sampling procedure. A catheter was inserted into the jugular vein of the cows one day before the start of the trial. Each cow was injected intramuscularly with an insoluble suspension of 25 mg [1,2,4-^{3}H]dexamethasone trimethyl acetate (specific activity 80 μCi/mg) to induce premature calving. Blood samples (50 ml) were withdrawn once daily for 30 days into ice-cold heparinized syringes. After calving the cows were machine-milked and a 500-ml aliquot from each milking was stored at −15°C until analysis.

Milk (15 ml) and plasma (10 ml) samples were extracted with 5 vol. ethyl acetate. The organic phase was removed, washed successively with 0·1 vol. 0·1 M NaOH and 0·1 vol. water and then taken to dryness under nitrogen. The dried extracts were transferred to a counting vial with acetone, taken to dryness and 10 ml scintillation fluid added. The counts were corrected for quenching by the channel-ratio method.

Results

Experiment 1

The pattern of dexamethasone in milk and plasma of two cows following an i.m. injection of Opticortenol is shown in Fig. 1. For cow 1, the plasma concentrations of dexamethasone rose to peak levels of around 0·5 ng/ml on days 3–4 after injection and then declined to undectable levels (<0·15 ng/ml) on day 14. The plasma levels of dexamethasone in cow 2 rose sharply to 0·5 ng/ml by day 3 and then declined by day 8.

The plasma concentrations of dexamethasone in the two cows injected intramuscularly with dexamethasone tributyl acetate showed a similar pattern with maximum levels of 0·7–1·1 ng/ml occurring 1–7 days following injection (Fig. 1). In both cows dexamethasone levels in both plasma and milk were undetectable (<0·15 ng/ml) 14 days after injection. Changes in milk concentrations of dexa-

methasone were similar to those observed in plasma except that the absolute levels in milk were 0·3–0·5 times the plasma concentrations.

Plasma corticosteroid concentrations in the one cow (cow 1) examined fell sharply after injection, reaching undetectable levels within 4 days (Fig. 1).

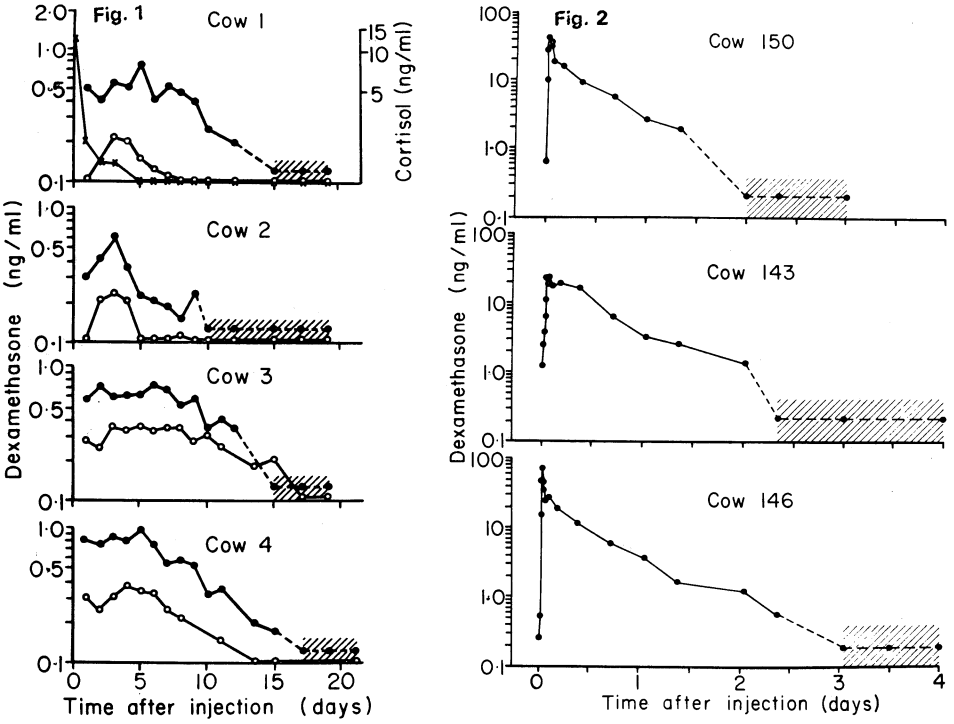

Fig. 1. Concentrations of dexamethasone in plasma (●) and milk (○) following an intramuscular injection of 20 mg dexamethasone trimethyl acetate (Opticortenol) to cows 1 and 2, and 20 mg dexamethasone tributyl acetate to cows 3 and 4. Plasma cortisol levels (×) in cow 1 are also shown. All cows were lactating during the experiment. In Figs 1 and 2 each point represents the mean of duplicate determinations. Samples whose values were below the limit of the sensitivity are shown within the stippled area.

Fig. 2. Changes in plasma concentrations of dexamethasone in three cows following an intramuscular injection of dexamethasone sodium phosphate.

Experiment 2

The plasma concentrations of dexamethasone in three cows given an i.m. injection of 20 mg dexamethasone sodium phosphate is shown in Fig. 2. In all three cows peak concentrations of dexamethasone occurred 2–20 min after injection although the peak heights showed considerable variation ranging from 22 to 70 ng/ml. In all cows plasma concentrations of dexamethasone declined sharply over the 24 h following treatment and were undetectable (<0·15 ng/ml) after 72 h.

Experiment 3

The calculated concentration of [^{3}H]dexamethasone in plasma and milk of three cows treated with 25 mg tritiated Opticortenol are shown in Fig. 3. Maximum levels

of dexamethasone in plasma of 1·1–1·6 ng/ml were attained within 1–3 days of the injection. The concentrations of [^{3}H]dexamethasone in milk and plasma were similar except for the first few days after calving when the levels in milk were higher in one of the cows. The concentrations of [^{3}H]dexamethasone in both milk and plasma were still detectable at around 0·05 ng/ml, 30 days after treatment. One cow (No. 187) became sick during the course of the experiment and was put out to pasture for 10 days after calving to recover. Plasma samples were not collected during this period.

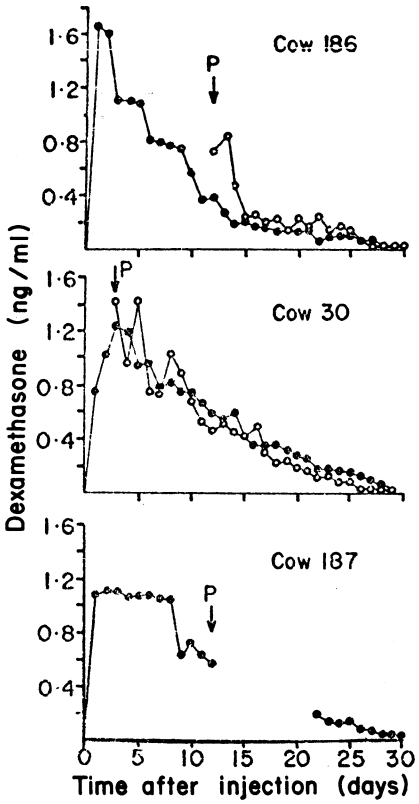

Fig. 3. Radioactivity levels in plasma (●—●) and milk (○—○) of three pregnant cows following an intramuscular injection of 25 mg tritiated Opticortenol (2 mCi). P, time of parturition.

Discussion

The accuracy, sensitivity and reproducibility of the dexamethasone radioimmunoassay described in this paper compare favourably with radioimmunoassays reported by Meikle *et al.* (1973) and Hitchens and Hogan (1974). The specificity of the assay depends on the low cross-activity of the antisera with endogenous steroids and also on the method used to extract the plasma samples. The antisera used for this study showed little cross-reactivity with all endogenous steroids tested. The moderate cross-reactivity of the dexamethasone with Opticortenol and the observation that Opticortenol is rapidly hydrolysed to dexamethasone by an esterase enzyme present in blood (unpublished observations) suggests that interference in the assay from Opticortenol is minimal. However, we cannot discount the possibility that metabolites formed from either Opticortenol or dexamethasone would cross-react with the dexamethasone antisera.

In general, the concentrations of immunoreactive dexamethasone in plasma and milk of cows treated with Opticortenol were lower than the concentrations of radioactive dexamethasone determined by the isotopic-dilution method. These differences in levels between the two methods may reflect the greater specificity of the radioimmunoassay compared with the non-specific isotopic-dilution methods. An alternative explanation for the difference in milk dexamethasone levels is that pregnant cows were used for the isotopic-dilution experiment so it is possible that dexamethasone may have accumulated in milk before calving. This may have been the cause of the high amounts of dexamethasone observed in milk at the start of the lactation. Such an accumulation would not have occurred in cows used for the radioimmunoassay determinations since these cows were lactating throughout the trial.

To our knowledge no data on plasma concentrations of dexamethasone have been reported in cows following the administration of dexamethasone esters. The only data available on the activity of dexamethasone in blood of cows have been obtained indirectly by monitoring the plasma glucose concentrations (Maplesden *et al.* 1960; Heindrich *et al.* 1963; Woollett and Evans 1971) or changes in undifferentiated white cells in cows after injecting various dexamethasone formulations.

In the study of Maplesden *et al.* (1960) an i.m. injection of 20 mg dexamethasone resulted in an elevation of glucose concentrations for 72 h following injection. Similar results were obtained by Woollett and Evans (1971) who observed an increase in plasma glucose levels for 96 h after administering dexamethasone sodium phosphate to cows. The pattern of release of both immunoreactive and radioactive dexamethasone into plasma of cows injected with the dexamethasone ester, Opticortenol, are compatible with the findings of Box (personal communication, 1974) who found that the plasma glucose concentrations were elevated for 12–14 days following an i.m. injection of 20 mg Opticortenol. Somewhat different results, however, were reported by Heindrich *et al.* (1963) who injected cows with the same dose of Opticortenol as that used by Box (personal communication, 1974). These investigators found that the plasma glucose levels were elevated for only 4–6 days.

Overall, it would seem that plasma glucose levels provide a reasonably good assessment of dexamethasone activity in blood although, as noted above, discrepancies can occur. This is, perhaps, not too surprising since it is known that other factors, such as insulin, play a major role in regulating glucose homeostasis.

The finding in this study in cows that insoluble ester formulations of dexamethasone led to a sustained release of dexamethasone into the systemic circulation from i.m. injections is in accord with a similar comparison made in humans by Melby and Dale (1969). These workers showed that plasma dexamethasone levels were elevated for 3–4 days and 7–8 days following i.m. injections of the phosphate and acetate esters of dexamethasone respectively. The marked differences in release pattern between the soluble and insoluble dexamethasone esters probably explains why the former preparation can provoke parturition in cows in 2–3 days (Adams and Wagner 1970; Jöchle 1973) whereas the latter preparation takes 10–14 days (Bailey *et al.* 1973; Welch *et al.* 1973).

On submission of the data reported in this paper on plasma and milk dexamethasone concentrations in pregnant cows to the Animals Remedies Board in New Zealand this agency has made the recommendation that cows treated with long-acting dexamethasone esters to induce calving should be withheld from slaughter for a period of 4 days after calving. Since Welch *et al.* (1977) have shown that calving

normally occurs at a mean 10 days after administering insoluble dexamethasone esters to cows during the latter stages of gestation the average withholding time for induced cows would be around 14 days post-injection.

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