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# Application of Enzymeimmunoassay to Measure Oestrone Sulphate Concentrations in Cows' Milk During Pregnancy

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**The characteristics of antigen- and antibody-coated enzymeimmunoassay (EIA) formats to measure oestrone sulphtae (OS) were studied using a murine monoclonal antibody as the primary antibody. In an antigen-coated format the most sensitive EIA (9 fmol/well) was achieved using 6-ketoestrone-6-*O*-carboxymethyloxime (OCMO) coupled to bovine serum albumin (BSA), as the coating antigen, and horseradish peroxidase (HRP), as the enzyme label. In an antibody-coated format, comparable sensitivity could be achieved using HRP conjugated to either OCMO, oestrone-3-glucuronide (OG) or oestrone-3-hemisuccinate (OHS) as the steroid 'tracer'. In both the antigen- and antibody-coated formats replacing HRP with alkaline phosphatase (AP) markedly aggravated the assay sensitivity. The antigen-coated EIA format was used to measure OS concentrations in cows' milk directly without an initial defatting step, and a progressive increase in OS concentrations in milk as pregnancy progressed was observed. Median OS concentrations rose from 1.1 nmol/l at days 70–99 of pregnancy ($n = 44$) to 3.2 nmol/l at days 140–160 ($n = 92$). Oestrone sulphate concentrations in milk from non-pregnant cows ($n = 51$) ranged from non-detectable to 1.3 nmol/l with a median value of 0.4 nmol/l. Only 5% of cows 120 or more days pregnant had milk OS concentrations within the range of values found in milk from non-pregnant cows. Accurate discrimination of non-pregnant and pregnant cows can thus be achieved on the basis of OS concentrations in milk samples taken at least 120 days after mating/insemination. This EIA for OS may have a role in the dairy industry as an alternative non-invasive means of determining pregnancy status in cows.**

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## INTRODUCTION

Accurate determination of pregnancy status is a significant component in the effective management of dairy herds. Not only is early identification of pregnant cows necessary, but later in the gestational period confirmation of pregnancy and identification of 'empty' cows is important for proper management decisions to be made. Pregnancy is usually routinely diagnosed by rectal palpation of the uterine contents. While this can be a very accurate technique, it requires considerable expertise on the part of the veterinarian. It is also time consuming if whole herds are to be tested, and because of its invasive nature may be stressful to the cows.

Several studies have shown that the concentration of oestrone sulphate (1,3,5(10) oestratrien-3-ol-17-one sulphate; OS) increases in the milk of cows as pregnancy progresses, and that values are generally higher than those in milk of non-pregnant cows by days 100–120 of pregnancy [1–6]. In New Zealand, many farmers present their cows for veterinary pregnancy testing 4–6 months after mating. Thus, measurement of OS concentrations in milk samples taken from these cows offers a potential, non-invasive, complementary means of determining their pregnancy status. Oestrone sulphate is generally measured in milk by radioimmunoassay (RIA). However, RIA methodology has several disadvantages such as its dependence on radioactive materials, which may pose a potential health and environmental hazard, and the need for a specialized laboratory with suitable handling and disposal

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facilities. In addition, the equipment required to quantitate radioactivity at the assay end-point is sophisticated and expensive and RIAs are not readily automated. As a result, assay costs per sample can be high. In contrast, enzymeimmunoassay (EIA) has several advantages. These include the use of non-radioactive, comparatively inexpensive reagents and a colour change assay end-point which can be easily measured by relatively simple equipment. Enzymeimmunoassays may also be readily automated, and generally assay costs per sample can be kept low, relative to RIA. Only one EIA has been described for measuring OS concentrations in cows' milk [7] and it uses an antibody-coated format. At present no information is available regarding the relative merits of antibody vs antigen-coated EIA formats for measuring OS, or the suitability of different enzyme labels. The purpose of this study was to examine different EIA formats to measure OS and assess the suitability of using EIA to measure OS concentrations in cows' milk during pregnancy.

## EXPERIMENTAL

### *Reagents*

Unless stated otherwise, all reagents were obtained from the Sigma Chemical Co., St Louis, MO, U.S.A. 1,3,5(10)-estratrien-3-ol-6,17-dione 6-*O*-carboxymethyloxime (6-ketoestrone 6-CMO; OCMO) was obtained from Steraloids Inc, Wilton, NH, U.S.A; [6,7-$^3$H(N)]-oestrone sulphate, ammonium salt and [6,7-$^3$H(N)]-oestrone from New England Nuclear, Wilmington, U.S.A.; anti-mouse immunoglobulin (IgG) conjugated to horseradish peroxidase (HRP) or alkaline phosphatase (AP) from Silenus Laboratories Pty Ltd, Hawthorn, Victoria, Australia; horseradish peroxidase and alkaline phosphatase (both enzymeimmunoassay grade) from Boehringer Mannheim GmBH, Germany; sodium borohydride, dimethylformamide, diethyl ether and ethyl acetate from BDH Ltd, Palmerston North, New Zealand; Sephadex G25 and MAb Trap G from Pharmacia LKB, Uppsala, Sweden; bovine serum albumin (BSA) from Immunochemical Products Ltd, Auckland, New Zealand and microtitre plates (Maxisorp, C12) from Nunc, Kamstrup, Denmark.

### *Antibody to oestrone sulphate*

The antibody used in this study was a murine monoclonal antibody, prepared in-house at AgResearch, Wallaceville, which has previously been shown to be suitable for measuring OS concentrations in cows' milk by RIA [5]. Briefly, the antibody was generated by immunizing Balb/c mice with 1,3,5(10)-estratrien-3-ol-17-one 3-glucuronide (oestrone 3-glucuronide; OG); coupled to human globulins (Cohn-Fraction IV-1) and administered in Freund's adjuvant. Antibody titres were monitored in plasma samples prepared from fortnightly tail bleeds by RIA using $^3$HOS as the tracer and dextran-coated charcoal to separate free and antibody bound $^3$HOS [5]. Once a suitable antibody titre to OS had developed, a selected donor mouse was given a final booster of antigen intravenously in phosphate buffered saline. Three days later the mouse was sacrificed, spleenocytes prepared and fused with the myeloma cell line NS-1 using polyethylene glycol. Hybrid cells were selected through hypoxanthine–aminopterin–thymidine (HAT) supplemented medium and culture supernatants were screened for antibody to OS by RIA. Hybridomas from positive wells were cloned twice by limiting dilution and a specific clone secreting monoclonal antibody to OS was grown up in culture and injected into pristane primed Balb/c mice to produce ascites fluid containing a high concentration of antibody. The ascites fluid was collected and stored frozen at $-20$°C until required. Cells from the selected OS antibody secreting clone were also cryopreserved. When required, a purified IgG fraction of the monoclonal antibody was prepared from ascites fluid by affinity chromatography using a protein G affinity column (MAbTrap G). Determination of the class of the monoclonal antibody showed it was an IgG1 isotype. In the RIA for OS the monoclonal antibody cross-reacted 31% with oestrone; 0.2–0.4% with 17$\beta$-oestradiol, 17$\alpha$-oestradiol and testosterone and $<0.01$% with oestriol, 16-epioestriol, d-equilenin and progesterone (cross-reaction with OS was defined as 100%) [5].

### *Preparation of conjugates*

1,3,5(10)-Estratrien-3-ol-17-one hemisuccinate (oestrone 3-hemisuccinate, OHS), OCMO and OG were coupled to BSA, HRP and AP using a modification of the active ester method [8]. Five $\mu$M of steroid, *N*,*N*-dicyclo-hexylcarbodiimide and *N*-hydroxysuccinimide, each in 0.05 ml dimethylformamide were mixed together and incubated for 1 h at room temperature. The solution was added to 5 mg of BSA, HRP or AP dissolved in 0.5 ml of aqueous sodium bicarbonate (0.13 M), and the mixture stirred slowly at room temperature for 2 h. The reaction mixture was dialysed overnight against distilled water and purified by column chromatography on Sephadex G-25 (1.5 × 30 cm) using saline as the mobile phase. Protein fractions were pooled. Steroid–BSA conjugates were stored frozen at $-20$°C. Steroid–enzyme conjugates were stabilized by the addition of thimerosal (0.01%) and BSA (1%) then diluted 1:1 with glycerol and stored at 4°C. Attempts to couple OS to BSA, HRP or AP by this methodology were unsuccessful.

The purified IgG fraction of the OS monoclonal antibody was conjugated to HRP using periodate [9]. Four mg HRP in 1 ml water was stirred at room temperature for 20 min after adding 0.2 ml of 0.1 M sodium m-periodate, and the mixture dialysed overnight at 4°C against 1 mM sodium acetate buffer,

pH 4.4. The pH of the HRP solution was adjusted to 9.5 and added to 8 mg of IgG dissolved in 1 ml of 10 mM carbonate buffer, pH 9.5. The reactants were stirred for 2 h at room temperature, 0.1 ml of aqueous sodium borohydride (4 mg/ml) was added and the mixture stirred for 2 h at 4°C before being dialysed overnight at 4°C against 10 mM phosphate buffered saline, pH 7.4. The IgG–HRP conjugate was stabilized by the addition of thimerosal (0.01%) and BSA (1%) and stored at 4°C.

*Protein determinations*

Protein content of chromatography fractions was estimated by UV absorption at 280 nm. Other protein determinations were done by the method of Lowry *et al.* [10].

*Enzymeimmunoassay (EIA) procedures*

In the coated antigen EIA format, wells of microtitre plates (Maxisorp C12) were coated by overnight incubation at 4°C with 0.1 ml of steroid–BSA conjugate (usually 10 $\mu$g/ml) in coating buffer (50 mM carbonate–bicarbonate buffer, pH 9.6). The wells were emptied by inversion and remaining active sites saturated by incubation for 30 min at room temperature with 0.25 ml blocking buffer (coating buffer containing 0.5% gelatin). Wells were emptied and washed 3 times with washing buffer (10 mM phosphate buffer containing 0.8% NaCl and 0.05% Tween 20, pH 7.4). 0.05 ml aliquots of OS standard in assay buffer (washing buffer containing 0.1% gelatin and 0.01% thimerosal, pH 7.4) were added to replicate antigen coated wells and followed with 0.15 ml monoclonal antibody to OS. The antibody was added at an appropriate dilution in assay buffer as either crude ascites fluid or IgG–HRP conjugate. The plates were incubated for 2 h at 37°C, emptied and washed 3 times with washing buffer. In instances when crude ascites fluid was used, the amount of antibody bound to the antigen-coated wells was quantitated indirectly by the addition of secondary antibody. Dilutions of anti-mouse IgG–HRP (1/5000) or anti-mouse IgG–AP (1/1000) were prepared in assay buffer, and 0.1 ml of either added to the wells. (These secondary antibody dilutions were selected on the basis that they were the lowest dilution of antibody which consistently produced an absorbance value of $<0.01$ in 'blank' wells.) The plates were incubated for 1 h at 37°C before being emptied and washed 3 times. The enzyme activity in each well was then quantitated by measuring the colour intensity generated following the addition of 0.1 ml/well of substrate–chromogen solution. In instances when the IgG–HRP conjugate was used the secondary antibody step was omitted and the amount of IgG–HRP bound to the antigen-coated wells quantitated directly. After addition of 0.1 ml/well of substrate–chromogen solution, colour formation was allowed to proceed at 37°C for 15–30 min before the enzymic reaction was stopped. Colour intensity was quantitated by reading the absorbance of each well using a Bio-Tek EL311 microplate autoreader (Bio-Tek Instruments Inc, Winooski, VT, U.S.A.). For HRP, the substrate–chromogen solution was 50 ml of 0.05 M phosphate–citrate buffer, pH 5.0, containing 20 mg *o*-phenylenediamine and 0.02 ml 30% $H_2O_2$. The stopping solution was 2 M $H_2SO_4$ (0.05 ml/well) and colour intensity was read at 490 nm. For AP, the substrate–chromogen solution was 1 mg/ml *p*-nitrophenyl phosphate prepared in 1 M diethanolamine buffer containing 0.5 mM magnesium chloride, pH 9.8. The stopping solution was 2 M NaOH (0.05 ml/well) and colour intensity was read at 405 nm.

The antibody-coated EIA was performed in a similar fashion to the antigen-coated format. The monoclonal OS antibody, in the form of affinity purified IgG, was coated onto wells in place of the steroid–BSA conjugates. After the blocking and washing steps, 0.05 ml of OS standard and 0.15 ml of steroid–enzyme conjugate were added to replicate wells. After 2 h incubation at 37°C the plates were emptied, washed and the steroid–enzyme conjugate bound to the coated antibody quantitated by measuring colour formation after addition of the appropriate substrate–chromogen. In both the antigen-coated and antibody-coated formats, standard curves were generated by plotting percentage ($E/E_0$), or logit percentage ($E/E_0$) vs the logarithm of OS standard concentration, where $E/E_0$ is defined as the mean absorbance reading of the OS standard/mean absorbance reading of the zero OS standard. Assay sensitivity was defined as the amount of OS producing a percentage ($E/E_0$) value two standard deviations below that of the zero standard.

OS concentrations in cows' milk were measured using the coated-antigen EIA format, with OCMO–BSA (1 $\mu$g/well) as the coating antigen. After coating the wells, blocking and washing, 0.05 ml of milk sample or OS standard was added to duplicate wells, followed by 0.15 ml of IgG–HRP conjugate. The OS standards were prepared in pooled milk obtained from non-pregnant cows in which the concentration of OS was undetectable. The rest of the assay procedure was as described above for the coated-antigen format. The OS concentration in "unknown" milk samples was determined by interpolation from a standard curve. Within- and between-assay variation was monitored by the inclusion of internal standards (IS) in every plate in each assay. The IS were prepared by making 3 pools of cows' milk from samples containing low ($<1.0$ nmol/l), medium (1.4–2.3 nmol/l) or high ($>3.0$ nmol/l) concentrations of OS. The pools were stored frozen in 1 ml aliquots for inclusion in each assay. The extent of cross-reactivity of other steroids in the EIA was determined by comparing the relative amounts of OS and cross-reacting steroid required to produce an absorbance value 50% that of the zero standard. The specificity of the EIA in measuring

OS concentrations directly in intact, untreated milk samples was examined by comparing the values obtained from direct measurement by EIA with values obtained by EIA following isolation of OS from milk using solvent partitioning. Solvent partitioning was performed similarly to that described previously for human plasma [11]. Briefly, milk samples (0.4 ml) were extracted with 5 ml diethyl ether by vortexing for 60 s. The ether layer containing estrone and any other unconjugated steroids was removed. The residual milk layer was then extracted twice with 5 ml ethyl acetate, vortexing for 60 s on each occasion. The ethyl acetate fractions, containing the extracted OS, were separated from the aqueous milk layer, pooled and dried under nitrogen. The extracted OS was redissolved in 0.4 ml EIA assay buffer and replicate 0.05 ml aliquots assayed for OS by EIA. The efficiency of extraction was monitored by determining the recovery of tritiated OS added to the milk samples prior to the first extraction. The mean ± SEM recovery of OS was 77 ± 2%. The efficiency of the solvent partitioning procedure in separating OS from estrone glucuronide and oestrone, both of which cross-reacted substantially in the EIA was also determined. For oestrone this was done by measuring the recovery of tritiated oestrone added to separate aliquots of milk prior to diethyl ether extraction; >90% of the tritiated oestrone was removed by the ether extraction. For oestrone glucuronide, varying concentrations of this steroid (0–15 pmol/ml) were prepared in non-pregnant cows milk and replicate 0.4 ml aliquots subjected to the solvent partitioning procedure. Only 18 ± 3% (mean ± SEM) of the added oestrone glucuronide could be measured in the ethyl acetate fraction, the remainder being located in the residual milk aqueous fraction.

*Milk samples*

Milk samples were obtained from commercial dairy farms, from cows at various days of pregnancy. The days of insemination and of calving were recorded for each cow. Samples were also obtained from cows known to be not pregnant. Samples of whole milk (20–30 ml) were taken from the milk reservoir collected from each cow and stored frozen (−20°C) until assayed.

## RESULTS

*Coated antigen EIA format*

OG–, OHS– and OCMO–BSA were tested as coating antigens. Initial studies used crude ascites fluid as the source of OS primary antibody and HRP or AP conjugated anti-mouse IgG was used as secondary antibody to quantitate OS antibody binding to the coated antigens. Preliminary checkerboard titration studies [12] indicated that the optimum coating concentration of the steroid–BSA conjugates was 10 μg/ml. Figure 1(A) shows the OS antibody titration curves for the steroid–BSA conjugates at this coating concentration. Higher working dilutions of OS antibody (i.e. a dilution producing an absorbance in the range of 0.5 to 1.5) were obtainable using HRP–conjugated rather than AP-conjugated secondary antibody. Coated OG–BSA allowed the highest working dilution of OS antibody and OCMO–BSA the lowest. However, when standard curves were run using coated OG– or OHS–BSA, in conjunction with either secondary antibody, the sensitivity and mid-point ($ED_{50}$) values were most unsatisfactory, ranging from 0.2 to 0.9 pmol and 2.3 to >3.0 pmol/well respectively. In contrast, highly satisfactory OS standard curves were achieved using OCMO–BSA as coating antigen and HRP-conjugated secondary antibody [Fig. 1(B)], the mean ± SEM sensitivity, $ED_{50}$ and $ED_{20}$ values of 8 separate standard curves being 9 ± 3, 137 ± 11 and 611 ± 100 fmol/well respectively. Coupling HRP directly to the OS monoclonal antibody to eliminate the secondary antibody step had no significant effect on the standard curves generated. Using the monoclonal IgG–HRP conjugate, sensitivity, $ED_{50}$ and $ED_{20}$ values of 8 separate standard

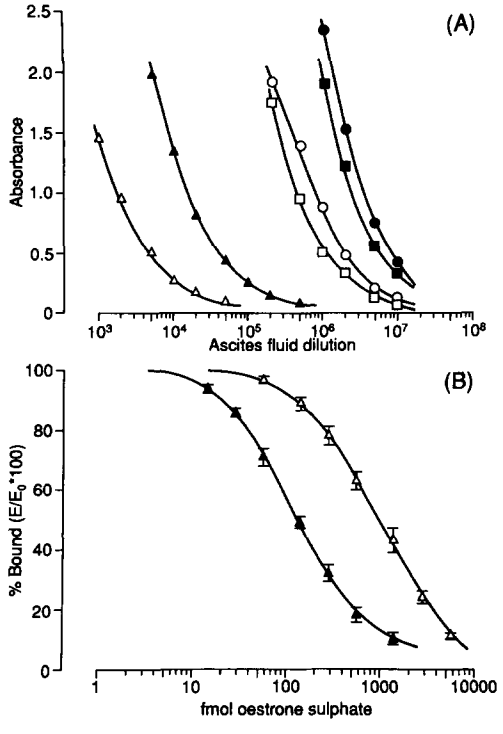


**Fig. 1. (A) Titration curves for the OS monoclonal antibody in ascites fluid using different coating antigens (10 μg/ml of OG–BSA, ○, ●; OHS–BSA, □, ■; OCMO–BSA, △, ▲) and either anti-mouse IgG–HRP (1/5000, closed symbols) or anti-mouse IgG–AP (1/1000, open symbols) as secondary antibody. Values are means of triplicates. (B) Standard curves for OS with OCMO–BSA (10 μg/ml) as coating antigen and using ascites fluid at 1/20,000 dilution in combination with anti-mouse IgG–HRP (▲, $n = 8$) or ascites fluid at 1/2000 dilution in combination with anti-mouse IgG–AP (△, $n = 4$). Values are mean ± SEM of $n$ separate curves.**

*Table 1. Characteristics of OS standard curves generated using coated antibody EIA format*

| Steroid–enzyme conjugate | OS antibody coating conc. (μg protein/well) | Steroid–enzyme tracer conc. (ng/well) | Standard curve: Sensitivity (fmol/well) | Standard curve: Mid-point $ED_{50}$ (fmol/well) | Standard curve: High-point $ED_{20}$ (fmol/well) | Standard curve: Slope |
|---|---|---|---|---|---|---|
| HRP– | | | | | | |
| OG | 0.25 | 10 | $11 \pm 3^a$ | $231 \pm 34^a$ | $1069 \pm 103^a$ | $-0.90 \pm 0.04^a$ |
| OHS | 0.25 | 10 | $14 \pm 6^a$ | $360 \pm 71^a$ | $1668 \pm 234^b$ | $-0.89 \pm 0.04^a$ |
| OCMO | 0.5 | 100 | $9 \pm 3^a$ | $303 \pm 20^a$ | $1580 \pm 191^b$ | $-0.85 \pm 0.03^a$ |
| AP– | | | | | | |
| OG | 0.25 | 30 | $103 \pm 15^b$ | $1420 \pm 243^b$ | $7462 \pm 1400^c$ | $-0.88 \pm 0.02^a$ |
| OHS | 0.25 | 150 | No standard curves generated | | | |
| OCMO | 0.25 | 200 | No standard curves generated | | | |

Standard curve values are mean ± SEM of 5 separate curves. Mean values with different letter superscripts in the same column are significantly different ($P < 0.05$, analysis of variance in conjunction with Newman–Keuls multiple range test).

curves were $9 \pm 6$, $163 \pm 17$ and $874 \pm 140$ fmol/well respectively. When AP-conjugated secondary antibody was used in conjunction with OCMO–BSA, the standard curves generated were less satisfactory than when HRP was used as the enzyme label [Fig. 1(B)]. The mean ± SEM sensitivity, $ED_{50}$ and $ED_{20}$ values of 4 separate standard curves were $69 \pm 11$, $949 \pm 66$ and $3311 \pm 89$ fmol/well respectively.

*Coated antibody EIA format*

Checkerboard titrations were performed to optimize the coating dilution of OS antibody and the dilution of steroid–enzyme conjugate 'tracer'. Appropriate combinations of antibody and tracer were selected and used in the generation of OS standard curves, the characteristics of which are summarized in Table 1. Standard curves generated using the three steroid–HRP conjugates all had similar characteristics. Assay sensitivity was comparable to that obtained in the coated antigen format, using HRP as the enzyme label, but the $ED_{50}$ and $ED_{20}$ values were somewhat higher. Although the three steroid–AP conjugates interacted with coated antibody, no standard curves could be generated with the OHS- and OCMO-conjugates. Standard curves generated using OG–AP were less sensitive and had higher $ED_{50}$ and $ED_{20}$ values than those obtained using the steroid–HRP conjugates.

*Measurement of OS in milk*

The antigen coated EIA format incorporating OCMO–BSA as the coating antigen and HRP-labelled OS monoclonal antibody was selected for measuring OS concentrations in cows' milk. The extent of cross-reaction of other steroids in this EIA format is shown in Table 2. Both oestrone glucuronide and oestrone cross-reacted substantially. The intra- and inter-assay coefficients of variation, calculated from measurements of the internal standards, composed of pooled milk samples, were $\leqslant 7.3\%$ and $\leqslant 11.7\%$ respectively. Dose–response lines of OS standards and a range of volumes of milk samples containing different concentrations of OS ran parallel, as shown in Fig. 2.

Previous immunoassays to measure OS concentrations in cows' milk have generally used defatted samples. However, comparison of the concentrations of OS measured in intact milk with values obtained following solvent partitioning, to extract the OS, showed a strong, highly significant ($P < 0.001$) correlation between the two (Fig. 3). On average, OS concentrations measured after extraction were 84% of the values measured directly in whole milk. Interestingly, there were generally undetectable amounts of cross-reacting steroid in the milk aqueous fraction following ethyl acetate extraction and, when measurable, they could be accounted for by residual OS which had not been extracted into the ethyl acetate fraction. As this aqueous fraction would have contained the bulk of any oestrone glucuronide present in milk, this finding indicates that estrone glucuronide concentrations in milk are negligible relative to those of OS. This

*Table 2. Cross-reactivity of steroids in the oestrone sulphate enzymeimmunoassay*

| Steroid | %Cross-reactivity* |
|---|---|
| Oestrone sulphate | 100 |
| Oestrone 3-glucuronide | 134 |
| Oestrone | 42 |
| d-Equilenin | 6 |
| 17 β-Oestradiol sulphate | 0.8 |
| 17 β-Oestradiol | <0.3 |
| 17 α-Oestradiol | <0.3 |
| Oestriol | <0.3 |
| Oestriol sulphate | <0.3 |
| 16-Epioestriol | <0.3 |
| 2-Hydroxyoestrone | <0.3 |
| Testosterone | <0.3 |
| Androstenedione | <0.3 |
| Progesterone | <0.3 |

*%Cross-reactivity was defined as 100 × OS/S where OS and S are the molar amounts of oestrone sulphate and steroid respectively required to produce an absorbance value 50% that of the zero standard. Cross-reactivities were determined using the antigen coated EIA format incorporating OCMO–BSA as the coating antigen and HRP-labelled OS monoclonal antibody.

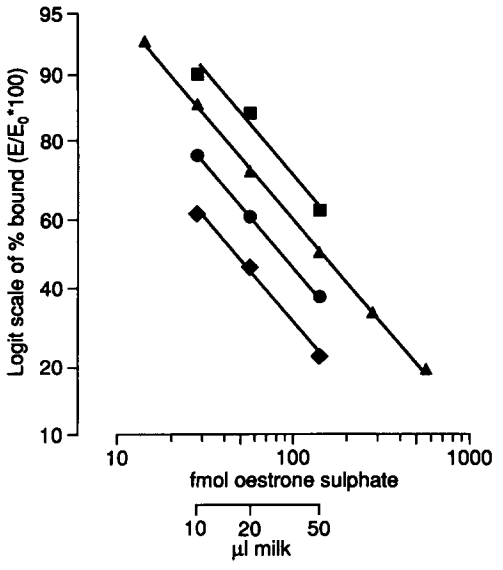


Fig. 2. Dose–response lines for OS standards (▲) and 3 milk samples containing different concentrations of OS (■, ●, ◆). The coated antigen EIA format was used with OCMO–BSA (10 µg/ml) as the coating antigen, and HRP-conjugated monoclonal antibody to OS at 1/10,000 dilution. Values are means of 4 replicates. The coefficients of variation of the replicates at each point was <6%.

is consistent with previous findings that OS is quantitatively the major conjugate in the milk of cows [3]. Given this high specificity of the EIA in measuring OS directly in whole milk, pretreatment of the milk samples prior to assay was considered unnecessary. Figure 4 shows the median concentrations of OS in milk samples from non-pregnant cows and cows 70–160 days pregnant. At 70–99 days of pregnancy the median value was already significantly higher than that of non-pregnant cows and there were further significant increases in median values as pregnancy progressed ($P < 0.05$, Kruskal–Wallis test in conjunction with multiple comparisons of rank sums [13]). The frequency distribution of OS concentration values

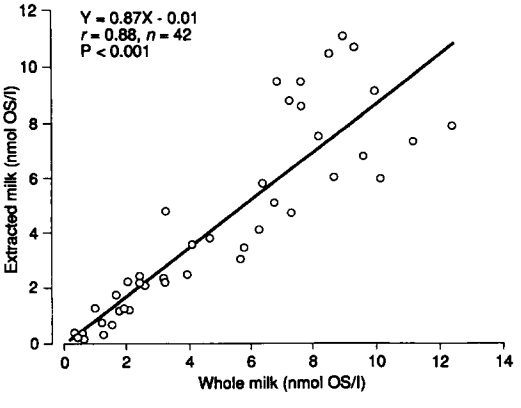


Fig. 3. Correlation between OS concentrations in milk samples before (whole milk) and after extraction of OS by solvent partitioning.

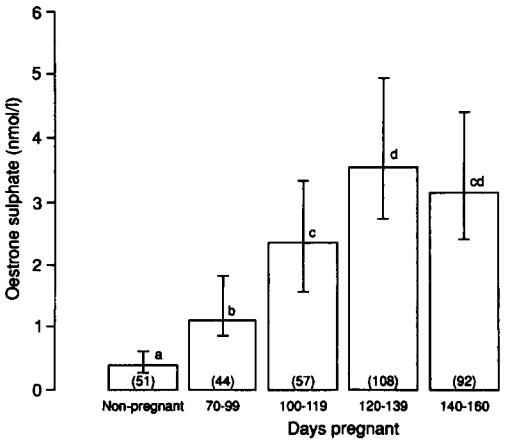


Fig. 4. Oestrone sulphate concentrations in milk samples from non-pregnant dairy cows and cows 70–160 days pregnant. Values are medians of ($n$) cows with 95% confidence limits indicated by the vertical lines. Median values with different letter superscripts are significantly different ($P < 0.05$; Kruskal–Wallis test in conjunction with multiple comparisons of rank sums).

is shown in Table 3. All the milk samples from non-pregnant cows had OS concentrations $\leqslant 1.3$ nmol/l and 96% had concentrations $< 1.1$ nmol/l. In contrast, 97% of samples from cows 120 or more days pregnant had OS concentrations $\geqslant 1.1$ nmol/l while 95% of samples had values $> 1.3$ nmol/l. While only 5% of samples obtained at 120 days of pregnancy or later had OS concentrations in the range found in non-pregnant cows, this figure increased to 57% at days 70–99 of pregnancy.

## DISCUSSION

Preparation of protein–enzyme conjugates of OS are demanding. The sulphate group is difficult to activate for efficient coupling, while the chemistry to synthesize a conjugate at a point distal to the functional groups of OS is relatively complex [14, 15]. Synthesis of conjugates of OCMO, OHS and OG is, however, straightforward. Sulphate and glucuronide moieties are similar in terms of size and ionic charge and polyclonal antisera satisfactory for use in immunoassays to measure OS can be generated using OG–protein conjugates as the immunizing antigen. Indeed several immunoassays to measure OS concentrations in cows milk have incorporated a polyclonal antibody generated against an OG–protein conjugate [3, 4, 7, 16, 17]. The murine monoclonal used in this study to study EIA formats for measuring OS was generated using OG–globulin as the immunogen. This antibody has previously been found suitable for measuring OS concentrations in cows milk by RIA [5]. Using this antibody, an antigen-coated EIA giving adequate sensitivity for measuring OS concentrations in cows milk was established using OCMO–BSA as the coating antigen [Figs 1(B) and 2]. Although

*Table 3. Frequency distribution showing the numbers of milk samples from non-pregnant and pregnant cows with particular concentrations of oestrone sulphate. Percentage values are shown in parentheses*

| OS Conc. in milk (nmol/l) | Non-pregnant | Day of pregnancy | | | |
|---|---|---|---|---|---|
| | | 70–99 | 100–119 | 120–139 | 140–160 |
| <1.1 | 49 (96) | 22 (50) | 12 (21) | 4 (4) | 3 (3) |
| 1.1 to 1.3 | 2 (4) | 3 (7) | 5 (9) | 3 (2) | 1 (1) |
| >1.3 | 0 (0) | 19 (43) | 40 (70) | 101 (94) | 88 (96) |
| *n* | 51 | 44 | 57 | 108 | 92 |

using OG–BSA or OHS–BSA as coating antigens allowed the monoclonal antibody to be used at a higher dilution [Fig. 1(A)], bound antibody could not be readily displaced with OS. Difficulty in displacing antibody from immobilized hapten in antigen-coated EIA formats has been noted previously [18]. It is probably as a consequence of a combination of the avidity of the antibody for the immobilized hapten and the high local concentration of immobilized hapten that the antibody is exposed to in this type of EIA format. It is likely that the monoclonal antibody has a lower avidity for OCMO than for the other two haptens and so can be more readily displaced by OS. Thus, in developing an antigen-coated EIA format, the choice of an appropriate coating antigen is crucial in determining the ultimate sensitivity of the EIA. Antigen-coated EIAs have the advantage that the use of purified primary antibody is not required if an additional step using enzyme-conjugated secondary antibody, widely available commercially, is employed. Using the OS monoclonal antibody in the form of diluted (1/20,000) crude ascites fluid was quite satisfactory, when used in combination with coated OCMO–BSA and anti-mouse IgG–HRP [Fig. 1(B)]. Directly coupling HRP to an affinity purified IgG fraction of the monoclonal antibody reduced the assay to 2 incubation steps which could be completed within 3 h, if pre-coated plates were used.

In the antibody-coated EIA format, standard curves having similar characteristics were obtained when HRP-conjugates of either OCMO, OHS or OG were used as the 'tracer' (Table 1). In this format, the choice of steroid–enzyme tracer appeared less crucial in determining assay sensitivity than the choice of steroid–protein conjugate in the coated EIA format. However, the coated-antibody format produced no improvement in sensitivity relative to the coated antigen format incorporating coated OCMO–BSA, while the $ED_{50}$ and $ED_{20}$ values were also higher.

Compared with HRP, alkaline phosphatase was less satisfactory as an enzyme label. In the antigen-coated format more OS antibody was required when IgG–AP was used as secondary antibody and standard curves generated were less sensitive and had higher $ED_{50}$ and $ED_{20}$ values compared to using HRP (Fig. 1). In the antibody-coated format standard curves could only be generated using the OG–AP conjugate, but again the curves were less sensitive and had higher $ED_{50}$ and $ED_{20}$ values compared to using steroid–HRP conjugates (Table 1). The inferior performance of AP may be due, at least in part, to its higher detection limit relative to HRP [19].

The coated-antigen EIA format incorporating coated OCMO–BSA in conjunction with HRP-labelled monoclonal antibody was selected to measure OS concentrations in milk. This format produced the most satisfactory standard curves and was technically straightforward to perform once the IgG–HRP conjugate had been prepared. Coated antibody EIA formats also have the advantage that the competing antigens (i.e. the antigen coated on the well of the microtitre plate and the antigen in the milk sample) can be exposed to subsequently added antibody simultaneously. This is more difficult to achieve in a coated-antibody format, which can aggravate "assay-drift" if the interval between adding the reagents to each well is not monitored closely. Both oestrone (42%) and oestrone glucuronide (134%) cross-reacted substantially in the EIA (Table 2). However, these high cross-reactivities do not present a problem when assaying pregnant cows milk for OS. Oestrone sulphate is the principal oestrogen produced by the foetal–placental unit and present in milk [3]. The concentration of oestrone in the maternal circulation during pregnancy is <10% that of OS and consequently oestrone concentrations in milk during pregnancy are low relative to those of OS [1, 20]. Results from the present study also show that oestrone glucuronide concentrations in milk are negligible relative to those of OS. Progesterone is present in milk during pregnancy at a concentration of 20–50 nmol/l, and is the only other steroid present at a concentration of comparable magnitude to that of OS. However, progesterone shows negligible cross-reactivity (<0.3%) in the EIA (Table 2). That the EIA showed good specificity in the measurement of OS directly in whole milk was indicated by the parallel dose–response lines of samples and standards (Fig. 2), and by the strong correlation between values obtained by direct assay of intact milk and values obtained following solvent partitioning to

extract the OS (Fig. 3). However, owing to the high cross-reaction with oestrone glucuronide and oestrone, this EIA may be less suitable for measuring OS directly in other biological fluids where concentrations of oestrone glucuronide and oestrone are high relative to OS. A preliminary step to isolate the OS would be required before assay in those circumstances.

An EIA to measure OS concentration in cows' milk has been described previously [7]. However, that EIA involved a coated-antibody format, using polyclonal antibody generated against OG–BSA, and required the milk samples to be pretreated by defatting prior to assay. This is the first report of an EIA for measuring OS in cows' milk utilizing a coated-antigen format and a monoclonal antibody and which does not require the milk samples to be defatted before being assayed, thereby simplifying the assay considerably. A monoclonal antibody may also have some advantage over a polyclonal antibody as a diagnostic reagent by virtue of its potentially unlimited supply and relative ease of purification. Using the coated-antigen EIA to measure OS concentrations in cows' milk confirmed the progressive rise in OS concentration which occurs during pregnancy (Fig. 4) and range of values reported by others previously [1–7]. After 120 days of pregnancy, 95% of samples analysed had OS concentrations $>1.3$ nmol/l and were outside the range of concentrations found in non-pregnant cows (Table 3). Indeed only 4% of non-pregnant cows had milk OS concentrations $\geqslant 1.1$ nmol/l compared to 97% of cows 120 or more days pregnant. Thus, accurate discrimination of non-pregnant and pregnant cows on the basis of OS concentrations in milk samples taken at least 120 days after mating/insemination is achievable with this EIA. Before 120 days of pregnancy an appreciable proportion of cows had milk OS concentrations $<1.1$ nmol/l and so were in the range of values found in the majority of samples from non-pregnant cows. Thus, while pregnancy could perhaps be confirmed for cows sampled $<120$ days after mating if milk OS concentrations were $>1.3$ nmol/l, an accurate diagnosis would not be possible if OS concentrations were $<1.1$ nmol/l. The straightforward nature of this EIA for measuring OS concentrations in milk gives it considerable potential for automation and high sample throughput at low cost. Such an assay might have a role in the dairy industry as an alternative, non-invasive means of determining pregnancy status in dairy cows.

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