An enzymeimmunoassay to measure oestrone sulphate in cows' milk

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ABSTRACT

An enzymeimmunoassay (EIA) for measuring oestrone sulphate (OS) concentrations in cows' milk is described. The EIA utilizes a coated antigen format in conjunction with a monoclonal antibody to OS, and is performed in microtitre dishes. An enzyme label allows OS concentrations to be quantitated by measuring changes in colour intensity. The EIA can be completed in under 3 h, has a sensitivity of <5 pg, and allows OS concentrations to be measured directly in small quantities (0.05 ml) of whole milk. Measurement by EIA of OS concentrations in milk samples collected throughout pregnancy confirmed the progressive rise in OS concentrations described previously using radioimmunoassay. In addition, comparison by EIA of OS concentrations in milk samples from pregnant and non-pregnant cows indicated that the EIA could be used to accurately identify pregnancy status in cows sampled 120 or more days after mating/insemination. The EIA may have a role in the dairy industry for monitoring pregnancy status.

Keywords: Oestrone sulphate, enzymeimmunoassay, cow, milk, pregnancy.

INTRODUCTION

Measurement of oestrone sulphate (OS) concentrations in cows' milk sampled about 110 days or more after mating/insemination allows an accurate determination of pregnancy status (Hamon *et al.*, 1981; McCaughey *et al.*, 1982; Henderson *et al.*, 1992). OS is generally measured in cows' milk by radioimmunoassay (RIA). While this is an accurate and robust assay technique, it does suffer from some drawbacks. Notably, RIAs are dependent upon radioisotopes which may pose a potential health hazard, are increasingly difficult to dispose of and can only be used in specially 'licensed' laboratories. RIAs are not readily automated and the end-point analysers to quantitate radioactivity in RIAs are expensive. Non-isotopic assays, such as enzymeimmunoassay (EIA), can provide comparable sensitivity to RIA and have the advantage that radioactive reagents are not required. The equipment to quantitate the end-point colour changes found in EIAs are relatively inexpensive, and the assays may be automated. An EIA for OS, ultimately in a kit format, may have some use in the dairy industry for monitoring pregnancy status. The purpose of this study was to develop an EIA capable of measuring OS concentrations in cows' milk.

MATERIALS AND METHODS

OS antibody

A murine monoclonal antibody recognizing OS, which has been described and characterized previously (Henderson *et al.*, 1992), was used in the EIA. A purified IgG fraction of the monoclonal antibody was prepared from ascites fluid by affinity chromatography using a protein G affinity column. Horseradish peroxidase (HRP) was conjugated to the purified IgG fraction using periodate (Tijssen, 1985). The anti-OS IgG-HRP conjugate was stabilized by the addition of bovine serum albumin (BSA, 1%) and thimerosal (0.01%), and stored at 4°C.

Assay procedure

Wells of microtitre plates (Maxisorp C12, Nunc, Kamstrup, Denmark) were coated by overnight incubation at 4°C with 0.1 ml of oestrone-protein conjugate (10 µg/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 0.2 ml blocking buffer (coating buffer containing 0.5% gelatin). Wells were emptied and washed 3x with washing buffer (10 mM phosphate buffer containing 0.8% NaCl and 0.05% Tween 20, pH 7.4). OS standards for the EIA were prepared in milk pooled from non-pregnant cows in which the concentration of OS was undetectable. Replicate 0.05 ml aliquots of milk sample or OS standard (0 to 500 pg) were added to the coated wells, and followed with 0.15 ml of anti-OS IgG-HRP conjugate in assay buffer (washing buffer containing 0.1% gelatin and 0.01% thimerosal, pH 7.4). (The amount of anti-OS IgG-HRP conjugate added was such that the assay end-point absorbance reading of the zero standard was between 0.5 and 1.0). The plates were incubated for 2 h at 37°C to equilibrate. The plates were again emptied and washed 3x with washing buffer. Finally, the peroxidase activity in each well was quantitated by the addition of 0.1 ml/well of substrate-chromogen solution (50 ml of 0.05M phosphate-citrate buffer, pH 5.0, containing 20 mg o-phenylenediamine and 0.02 ml 30% H202). The plates were incubated for 15 to 30 mins at 37°C before the enzymic reaction was terminated by adding 0.05 ml/well of 2M H2SO4. Colour formation was quantitated by reading the absorbance of each well at 490 nm

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using a Bio-Tek EL311 microplate autoreader. The intensity of the colour formed is inversely proportional to the concentration of OS in the milk sample/standard being assayed. Standard curves were generated by plotting percentage (E/Eo) versus the logarithm of OS standard concentration, where E/Eo is defined as the mean absorbance reading of the OS standard/mean absorbance reading of the zero OS standard. The OS concentrations in the milk samples were then calculated by interpolation.

Milk samples

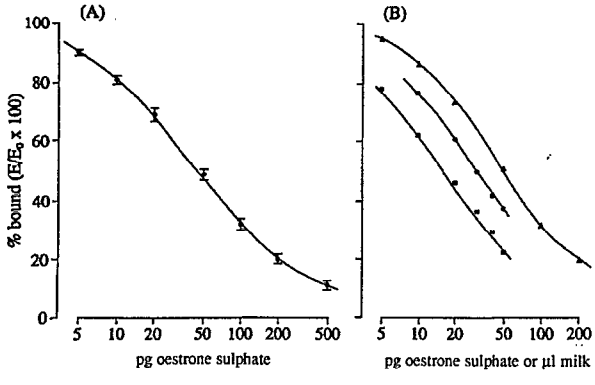
Milk samples were obtained from commercial dairy farms, from cows at various days of pregnancy. The days of insemination and of calving were recorded for each cow. Samples were also obtained from cows known to be not pregnant. Samples of whole milk (20 to 30 ml) were taken from the milk reservoir collected from each cow, and stored frozen (-20°C) until assayed. In instances when defatted milk samples were required, the milk fat was allowed to rise to the top of samples during overnight incubation at 4°C, or by centrifugation at 1000 g for 30 min at room temperature. The milk fat layer was then removed under suction.

RESULTS

OS EIA

The mean standard curve of 10 individual curves (each with duplicate or triplicate standards) set up on different occasions is shown in Fig. 1a. The sensitivity of the EIA was <4 pg OS/well and the mean ED50 and ED20 values were 44 and 210 pg OS/well respectively. The coefficient of variation between replicate determinations within each standard curve was <6%. Assaying a range of volumes of 2 milk samples with high endogenous OS concentrations produced dose-response curves parallel to the standard curve (Fig. 1b).

FIGURE 1: (a) Standard curve for OS EIA. Values are mean ± s.e.m. of 10 separate assays. (b) Dose-response curve for OS standards (▲- - -▲) and 2 milk samples with high endogenous OS concentrations (●,■). Values are means of 4 replicates and s.e.m. values were smaller than the size of the symbols.



OS concentrations in milk

Immunoassays to measure OS concentrations in milk generally use defatted milk samples. However, comparison of the OS concentrations in milk samples before and after being defatted showed a strong correlation between the OS concentrations in each milk type (Fig. 2). Defatting of milk samples before assay was therefore considered unnecessary. Fig. 3 shows geometric mean concentrations of OS in milk samples from non-pregnant dairy cows and cows 70 to 160 days pregnant. After 100 days of pregnancy, geometric mean concentrations of OS were significantly higher than those found in non-pregnant cows ($P<0.001$), and after 120 days of pregnancy only one out of seventy-nine milk samples was found to have an OS concentration within the range found in non-pregnant cows.

FIGURE 2: Correlation between OS concentrations in milk samples before (whole milk) and after being defatted.

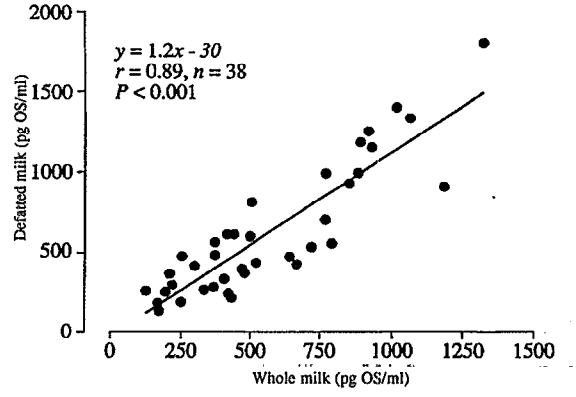
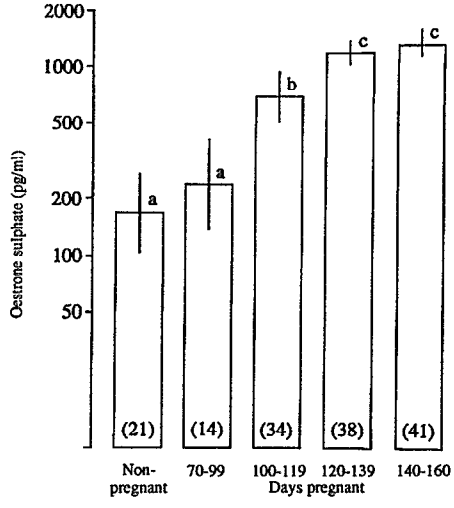


FIGURE 3: Oestrone sulphate concentrations in milk from non-pregnant and pregnant cows. Values are geometric means of (n) cows with 95% confidence limits indicated by the vertical lines. Geometric mean values with different letter superscripts are significantly different ($P<0.05$, analysis of variance in conjunction with Newman-Keuls multiple range test).



DISCUSSION

Although several radioimmunoassays to measure OS concentrations in cows' milk have been described previously (Heap & Hamon, 1979; Holdsworth & Chaplin, 1982; McCaughey *et al.*, 1982; Henderson *et al.*, 1992) no EIAs for OS have been reported other than that of Power *et al.*, 1985. However that EIA incorporated a coated antibody format and

utilized a polyclonal antibody. This is the first report of an EIA for OS in a coated antigen format and utilizing a monoclonal antibody. Coated antigen EIAs 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. Monoclonal antibodies may also have some advantage over polyclonal antibodies as diagnostic reagents by virtue of their potentially unlimited supply, and relative ease of purification.

The EIA for OS described in this report is straightforward to perform and can be completed in about 3 h, if precoated plates are used. The assay also has the advantage that whole milk may be used. The microtitre plate format allows large numbers of samples to be assayed conveniently, and automation of the assay is potentially possible. Using this EIA to measure OS concentrations in milk from pregnant cows confirmed the progressive increase in OS concentrations in milk occurring during pregnancy described previously (Heap & Hamon, 1979; Holdsworth *et al.*, 1982; Heap *et al.*, 1983; Henderson *et al.*, 1992). In addition, the OS concentration in >98% of milk samples obtained from cows at least 120 days pregnant was above the range of values found in non-pregnant cows. Thus, measurement by EIA of OS concentrations in milk samples taken at least 120 days after mating/insemination could provide an accurate indication of pregnancy status in New Zealand dairy cows, as has been suggested previously from data obtained by RIA (Henderson *et al.*, 1992). This is currently being verified in a more comprehensive trial of the EIA.

The present microtitre plate based format of the OS EIA is suitable for use in a laboratory setting. The next advance would be to produce a format suitable for use "on farm", e.g. a dipstick format. Dipstick assays for low molecular weight haptens such as steroids, having only a single epitope and present in biological fluids in very low concentrations are currently very difficult to format and awkward to use. However, the recent development of idiometric assays (Barnard & Kohen, 1990) and EIAs incorporating steroid dimers (Ali *et al.*, 1992) increases the probability that developing a simple user-friendly dipstick assay for OS may be achievable.

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