Development of a direct and specific enzymeimmunoassay for the measurement of oestrone sulfate in bovine milk

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A 12-step procedure is described for the synthesis of an oestrone-3-sulfate-6-hemisuccinate–BSA immunogen with oestradiol as starting material and the production of specific polyclonal antibodies. A competitive inhibition-type enzymeimmunoassay has been developed based on these specific antibodies using 3-hemisuccinate-oestrone–peroxidase as conjugate for direct measurement of the hormone in body fluids. The method has a minimum sensitivity of 0.03 ng ml^{-1} in bovine milk, and satisfactory specificity, recovery and reproducibility. In a small field trial with a group of 20 pregnant cows that were followed throughout gestation, it was shown that the assay is potentially an accurate pregnancy test for assessing the viability of the fetoplacental unit at approximately 100 days after insemination. The assay is well suited for routine testing, particularly as a confirmatory bovine pregnancy test.

Introduction

Oestrone sulfate is one of the major oestrogens produced by the fetoplacental unit of many animals during pregnancy. The presence of this hormone in the blood and even in the milk of pregnant lactating animals, such as cows, is an accurate indicator of pregnancy with diagnostic applications (Heap and Hamon, 1979; Dhindsa *et al.*, 1981; Saba and Hattersley, 1981; Chaplin and Holdsworth, 1982).

Early methods for measuring oestrone sulfate required hydrolysis and extraction of the hormone to oestrone. The assays used were of the radioimmunoassay-type and measured oestrone or oestradiol (Loriaux *et al.*, 1971; Heap and Hamon, 1979).

Later, antiserum raised against an oestrone-3-glucuronide–BSA conjugate allowed direct measurement of oestrone sulfate in samples by radioimmunoassay (Saba and Hattersley, 1981; Holdsworth *et al.*, 1982; Fletcher and Worsford, 1988) and later with an enzymeimmunoassay (Power *et al.*, 1985). Because the sulfate group was replaced in the immunogen by a glucuronide group, the specificity of the antiserum was compromised. The crossreactivity of the resultant antibodies with oestrone was reported to be up to 231% in relation to oestrone sulfate (Holdsworth *et al.*, 1982).

Nambara *et al.* (1980) described an approach for the production of a specific antiserum to oestrone sulfate which was used in a direct radioimmunoassay to measure the hormone in human serum and urine (Honjo *et al.*, 1986, 1987). Recognizing the need for a non-isotopic assay that would allow specific and direct measurement of oestrone sulfate, we synthesized a 6 α -hemisuccinate-oestrone sulfate derivative which we used to produce specific antibodies to oestrone sulfate and to develop

a simple and highly accurate competitive inhibition-type enzymeimmunoassay.

Materials and Methods

Synthesis of hapten–protein immunogen

Synthesis of the immunogen involved a total of 12 reactions carried out in four major stages (Fig. 1). All organic solvents and reagents were purchased from Merck (Darmstadt), unless stated otherwise.

Stage (i). Activation of the C-6 position of the starting material, oestradiol (Steraloids, Wilton), was performed as described previously (Longwell and Wintersteiner, 1940). Briefly, oestradiol (I) was acetylated by reflux with acetic anhydride at 140°C to give 3,17 β -di-acetyl oestradiol (II), then oxidized with chromium trioxide yielding approximately 10% 6-keto-oestradiol di-acetate (III) which was de-acetylated with 5% potassium hydroxide solution in methanol to give 6-keto oestradiol (IV). Thin layer chromatography (TLC) of the product in toluene:ethyl acetate (5:1 v/v) gave a single spot. The UV absorbance spectrum gave peaks at 327 and 255 nm and the melting point was 275°C . These characteristics agreed closely with the corresponding UV peaks at 326 and 256 nm and the melting point of 280°C which were reported by Longwell and Wintersteiner (1940). The nuclear magnetic resonance (NMR) spectrum confirmed the correctness of the product structure. NMR in CD_3OD gave the following: δ p.p.m. 0.7 (3H,S,18- CH_3), 3.95 (1H,t17 α -H), 6.85–7.25 (three aromatic protons q,d,d 2-1-4H).

Stage (ii). A hemisuccinate group was introduced to the activated C-6 position according to Nambara *et al.* (1974). First,

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Received 20 July 1992.

Fig. 1. Synthesis of oestrone sulfate-BSA conjugate used for the production of antibodies.

the hydroxyl groups of the 6-keto oestradiol (1 g) were protected with 3 ml dihydropyran using a catalytic amount of *p*-toluene sulfonic acid in 16 ml ethyl acetate for 20 min. When a single spot was obtained in TLC (toluene:ethyl acetate, 1:1 v/v) with R_F value of 0.68, the reaction mixture was diluted with ethyl acetate, washed with 0.5 mol sodium hydrogen carbonate l^{-1} and evaporated yielding the diprotected derivative (V). The product was reduced to a 6-hydroxyl derivative (VI) using

sodium borohydride. Reaction of the derivative (1 g) with 1.5 g succinic anhydride and 2 ml triethylamine in 20 ml dioxane for 20 min at room temperature gave a 6-hemisuccinate product (VII). This derivative (1 g) was treated with 50 ml of 0.5 mol oxalic acid:methanol l^{-1} (1:6 v/v) at 70°C for 1 h for cleavage of the dihydropyran ethers. The final product of this stage was 6β-hemisuccinate oestradiol (6β-hs-oestradiol) (VIII) which was purified after dilution with ethyl acetate and extraction with 0.5 mol sodium hydrogen carbonate l^{-1} and acidification of the water-phase with hydrochloric acid to pH 2.0. The product was filtered and air dried. It was identified as 6β-hemisuccinate oestradiol with NMR in CD_3OD : δ p.p.m. 0.75 (3H,S,18- CH_3), 2.65 (4H,S,CO-(CH_2)₂-CO), 3.65 (1H,t,17αH), 5.95 (1H,broad,6βH), 6.6–7.2 (three aromatic protons q,d,d 2-1-4H). The physical properties of the product were: TLC in ethyl acetate:*n*-hexane:ethanol:acetic acid (7.2:1.35:0.45:1.0 v/v) gave an R_F value of 0.7 and in chloroform:methanol (5:1 v/v) an R_F value of 0.5, whereas the melting point was 165°C with a published melting point of 164–166°C (Nambara *et al.*, 1980).

Stage (iii). This series of reactions involved the conversion of the oestradiol derivative (VIII) to an oestrone 3-sulfate derivative (Nambara *et al.*, 1980). 500 mg of 6β-hs-oestradiol was selectively oxidized with 1 ml Jones reagent (1.5 mol chromium trioxide l^{-1} in 2.2 mol sulfuric acid l^{-1}) in 100 ml acetone at 0°C for 5 min to give 6β-hs-oestrone (IX). After inactivation with methanol and addition of water, the resultant precipitate was filtered and dried. The NMR in CD_3OD gave δ p.p.m. 0.9 (3H,S,18- CH_3), 2.65 (4H,S,CO-(CH_2)₂-CO), 6.0 (1H,broad,6βH), 6.6–7.2 (three aromatic protons q,d,d 2-1-4H). TLC in ethyl acetate:*n*-hexane:ethanol:acetic acid (7.2:1.35:0.45:1.0 v/v) gave an R_F value of 0.73. The product reacted with chlorosulfonic acid in pyridine to give 6β-hs-oestrone-3-sulfate (X). The final derivative was purified by silica gel column chromatography (silica gel 60, 230–400 mesh, Merck, Darmstadt; column bed volume 65 ml, flow rate 2 ml min^{-1} , fraction size 5 ml) in ethyl acetate:methanol:ammonia (100:20:1 v/v). Fractions with R_F values of 0.4 were pooled, evaporated and stored dry until further use. NMR in CD_3OD gave δ p.p.m. 0.9 (3H,S,18- CH_3), 2.65 (4H,S,CO-(CH_2)₂-CO), 6.0 (1H,broad,6βH), 6.6–7.2 (three aromatic protons q,d,d 2-1-4H).

Stage (iv). The final process involved conjugation of the oestrone sulfate derivative to BSA (Sigma, St Louis, MO). This was achieved by activating the derivative using *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide and *p*-nitrophenol in dioxane at room temperature for 2.5 h (XI) (Nambara *et al.*, 1980). The reaction mixture was developed by preparative TLC in chloroform:methanol (5:1) and the fractions with R_F values of 0.4 were eluted and evaporated to dryness. Conjugation of the activated derivative with BSA was achieved by mixing the two in a mixture of pyridin:phosphate buffer (0.1 mol l^{-1} , pH 7.0) at 1:1 ratio, for 12–15 h.

The hapten-BSA conjugate (XII) was dialysed extensively, freeze-dried and used for immunization.

Antibodies

Antiserum was raised in New Zealand rabbits (2 kg body weight). The immunogen was dissolved in sterile phosphate-

buffered saline (0.5 mol l^{-1} , pH 7.4) at a concentration of 1 mg ml^{-1} (0.5 ml) and emulsified with an equal volume of complete Freund's adjuvant. The emulsion was injected into the rabbits subcutaneously at multiple sites. The procedure was repeated a further three times at intervals of 3 weeks but with $0.1 \text{ mg immunogen ml}^{-1}$ in incomplete Freund's adjuvant. Blood samples were taken one week after the last booster injection. Boosting and blood sampling cycles were repeated at monthly intervals for approximately 12 months. The sera were separated by centrifugation at $3000 g$ for 5 min, purified with caprylic acid (Steinbuch and Audran, 1969) and stored at -20°C until use.

Preparation of hapten–peroxidase conjugate

Horseradish peroxidase was coupled to 3-hemi-succinate-oestrone by the standard mixed anhydride reaction (Munro and Stabenfeldt, 1984) to give a molar ratio of hormone:enzyme of 1:1 as determined according to Erlanger *et al.* (1957).

Enzymeimmunoassay procedure

Flat-bottomed microtitration plates (Maxisorb, Nunc, Kamstrup, Denmark) were coated with $100 \mu\text{l}$ per well ($1 \mu\text{g ml}^{-1}$) hyper-immune IgG in carbonate–bicarbonate buffer (50 mmol l^{-1} ; pH 9.6), overnight at 4°C (Krambovitis *et al.*, 1986). Excess antibody was washed off with distilled water containing 0.05% Tween 20 (Merck, Darmstadt) and the coated wells were blocked with $200 \mu\text{l}$ per well of 1% w/v BSA in saline for 1 h at room temperature. Test milk samples, $50 \mu\text{l}$, or corresponding volumes with standard concentrations of oestrone sulfate were added to wells in duplicate, $150 \mu\text{l}$, respectively, of hapten–peroxidase conjugate at working strength ($1/50\,000$). The mixture was incubated at room temperature for 1 h. After three washings with 0.05% Tween-20 in distilled water, the antibody–antigen reaction was revealed by adding $100 \mu\text{l}$ of substrate ($0.25 \text{ mol } 3,3',5,5'$ tetramethylbenzidine l^{-1} , 0.03% v/v hydrogen peroxidase in $50 \text{ mmol sodium acetate l}^{-1}$ buffer pH 5.2) to each well. The reaction was stopped after 15 min by the addition of $50 \mu\text{l}$ per well of $2 \text{ mol sulfuric acid l}^{-1}$. The absorbance was measured at 450 nm (Multiskan, Flow, Finland).

Results

Assay characteristics

The assay we developed for measuring oestrone sulfate directly was based on the principle of direct competition of native hormone with the conjugate against the immobilized antibody. A key factor that influences the sensitivity of such an assay is the affinity of the antibody for the native hormone. Studies were made initially to identify antibodies with acceptable affinity by titration of immobilized rabbit anti 6β -hs-oestrone-3-sulfate (rabbit anti-oestrone sulfate antibodies), from different serum samples, against the conjugate alone (3-hs-oestrone–peroxidase, 2 mg ml^{-1} , diluted 1:50 000) or in the presence of 5 ng authentic oestrone sulfate ml^{-1} (Fig. 2). The results showed that antibodies

Fig. 2. Titration of hyperimmune rabbit anti- 6β -hemi-succinate-oestrone sulfate against 3-hemi-succinate-oestrone-peroxidase alone ($\square$ — $\square$) and with constant 5 ng authentic oestrone sulfate ml^{-1} ($\blacksquare$ — $\blacksquare$) showing the differences in competitive inhibition of the first three serum samples taken during immunization; antiserum from the (a) first serum sample; (b) second sample, and (c) third sample.

from the first serum sample gave excellent reaction with the conjugate but poor inhibition (40%) (Fig. 2a). A marked increase of the inhibitory effect of the antibodies was noted with the second sample (73%) (Fig. 2b), which reached a maximum inhibition in the third sample (81%) (Fig. 2c) onward. This effect was observed in the antibodies from all five animals immunized, although the maximum inhibition varied: two animals gave 81% inhibition with 5 ng authentic hormone ml^{-1} , one 65%, one 53% and one 51%.

Competitive inhibition studies were carried out to assess the degree of conjugate displacement in the assay. Titration of authentic oestrone sulfate in $10 \text{ mmol potassium phosphate buffered saline l}^{-1}$ pH 7.4 containing 0.1% BSA and in the presence of constant conjugate concentration (at working strength) against rabbit anti-oestrone sulfate antibodies from the third sample showed that 12.5 pg per well of authentic hormone was required to inhibit the reaction of the conjugate against the antibody by 50% (Fig. 3).

Oestrone sulfate standards in bovine milk were prepared for the calibration curve by adding appropriate amounts of hormone to milk taken 10 days after birth. Six standards were made

Fig. 3. Standard curve for oestrone sulfate in milk enzyme immunoassay from four different batches of standards (vertical bars represent SD).

Table 1. Specificity of the antiserum with selected steroids

Steroid	% Crossreactivity*
Oestrone sulfate	100
Oestrone	11
Oestradiol sulfate	6.3
Oestradiol	0.3
Oestradiol 3(β-D glucuronide)	0.2
Oestriol sulfate	0.1
Oestriol	<0.1
Oestriol 3(β-D glucuronide)	<0.1
Testosterone	<0.1
Androstenedione	<0.1
Dehydroepiandrosterone	<0.1
Dehydroepiandrosterone sulfate	<0.1

*Crossreactivity was defined as the amount of steroid, relative to oestrone sulfate, required to reduce conjugate binding by 50%.

covering the intended range (<0.01 – 1.0 ng ml $^{-1}$ oestrone sulfate) which were sufficient to discriminate between pregnant and nonpregnant animals (Fig. 3).

Sensitivity of the assay, calculated as the concentration of oestrone sulfate that could be detected three standard deviations above the value of the zero standard, was 0.03 ng ml $^{-1}$ ($n = 16$).

Specificity experiments with twelve commercially available steroids (Steraloids, Wilton), indicated 11% crossreactivity with oestrone, 6.3% with oestradiol sulfate and less than 1% with all other steroids tested (Table 1).

Precision of the assay was assessed by replicate measurements of three unknown milk samples taken from pregnant cows at approximately 100 days of gestation. The intra-assay mean values obtained were 0.81 ng ml $^{-1}$ (CV = 5.0%, $n = 16$), 0.51 ng ml $^{-1}$ (CV = 5.6%, $n = 16$) and 0.138 ng ml $^{-1}$ (CV = 5.6%, $n = 16$). The corresponding interassay values using four different batches of reagents were 0.83 ng ml $^{-1}$ (CV = 9.1%,

Table 2. Recovery of authentic oestrone sulfate added to bovine milk

Basal oestrone sulfate (ng ml $^{-1}$)	Authentic oestrone sulfate added (ng ml $^{-1}$)	Expected value (ng ml $^{-1}$)	Observed value (ng ml $^{-1}$)	Recovery (%)
0.02	0.2	0.22	0.25	113
0.02	0.5	0.52	0.55	105
0.07	0.2	0.27	0.29	107
0.07	0.5	0.57	0.60	105
0.5	0.2	0.7	0.7	100
0.5	0.5	1.0	1.0	100

Fig. 4. Oestrone sulfate concentration in the milk of 20 cows during pregnancy as measured by enzyme immunoassay (values are means $\pm$ SD).

$n = 4$), 0.50 ng ml $^{-1}$ (CV = 8.8%, $n = 4$) and 0.140 ng ml $^{-1}$ (CV = 8.5%, $n = 4$).

Recovery of oestrone sulfate was investigated by adding authentic hormone to milk samples. The results (Table 2) indicated a recovery close to 100%.

Application of the assay in measuring oestrone sulfate during gestation

The assay was used with milk samples taken from 20 pregnant cows at regular intervals throughout gestation. All the animals in the experiment subsequently calved normally. The results (summarized in Fig. 4) showed a low oestrone sulfate concentration in the first 90 days (≤ 30 pg ml $^{-1}$) followed by a sharp rise after day 100. During months 4–6 the average oestrone sulfate in the milk was 1 ng ml $^{-1}$, followed by a rise to 2 ng ml $^{-1}$ in month 7 and then a drop to 1.5 ng ml $^{-1}$ in month 8. The concentration of oestrone sulfate in 25 nonpregnant cows was 30 pg ml $^{-1}$ (SD = 20).

Discussion

In this study we have described a method for producing specific antibodies to oestrone sulfate as well as the development and evaluation of an immunoenzymatic method for measuring the hormone directly in bovine milk. Previous methods for measuring oestrone sulfate either required hydrolysis and extraction with organic solvents of the sample and estimation of free oestrone (Robertson *et al.*, 1978), or estimated directly with anti-oestrone glucuronide antibodies (Wright *et al.*, 1978) but with substantial crossreactions against related steroid hormones (Saba and Hattersley, 1981). A direct radioimmunoassay with specific antibodies was described later (Honjo *et al.*, 1986) which was evaluated only with human samples.

The immunogen we prepared was designed to allow the specificity-determining sulfate group in the C-3 position to be exposed for maximum immune response (Nambara *et al.*, 1980). The final product was essentially the same as that reported previously (Nambara *et al.*, 1980), although we described the complete sequence of 12 reaction steps starting from oestradiol. We preferred BSA as the protein carrier, despite our intention to use the antibodies with bovine samples, because of its high degree of purity which allowed us to control the coupling reaction more accurately. We avoided any potential interference in the bovine milk assays by including BSA in both the blocking and assay buffers. The assay we developed was based on the principle outlined below:

The conjugate consisted of oestrone with a hemi-succinate bridge in the C-3 position (for enzyme coupling) instead of the sulfate group. When a mixture of natural oestrone sulfate and conjugate was exposed to immobilized oestrone sulfate specific antibodies, there was a greater affinity for oestrone sulfate, thus enhancing the competitive inhibition of the conjugate (Hatzidakis *et al.*, 1993) and increasing the sensitivity of the assay to a useful range. The observed sensitivity was 0.03 ng ml^{-1} in the milk. The assay was optimized to give the required discrimination necessary to study the concentrations of oestrone sulfate in the milk mainly during days 80–140 of pregnancy. The quality of the antibodies played a crucial role in the effectiveness of the assay. Acceptable inhibition was obtained from the second hyperimmune serum sample onwards. The overall specificity of the antibodies was satisfactory, although crossreaction with oestrone (11%) was higher than that reported by Nambara *et al.* (1980) (under 1.5%). The crossreaction was attributed to the conjugate we elected to use to maximize sensitivity without affecting the specificity greatly. As free oestrone in body fluids is normally at very low concentrations, we did not experience any problems in specificity with test samples.

It is well established that detection of oestrone sulfate in animals is clearly associated with pregnancy. An immunoassay such as the one we have described could be used to provide strong evidence for the presence of a viable fetoplacental unit, thus giving the basis of a direct pregnancy test. Our assay was evaluated prospectively in a limited-scale field trial with milk from dairy cows. The results showed that the concentrations of oestrone sulfate in the milk of pregnant cows rose dramatically at approximately 100 days after insemination, remaining high throughout the remainder of the pregnancy, in agreement with a previous report (Holdsworth *et al.*, 1982). As oestrone sulfate

has been reported to be of diagnostic value in sows (Robertson *et al.*, 1978; Wright *et al.*, 1978; Saba and Hattersley, 1981), the assay has also been adapted to measure oestrone sulfate in the serum and urine of sows and is currently under evaluation.

The importance of oestrone sulfate concentrations in human body fluids and tissues as an indicator of normal and certain pathological conditions has been gaining momentum. This is supported by reports on the concentrations of oestrone sulfate during the menstrual cycle (Honjo *et al.*, 1987), last trimester and delivery periods (Honjo *et al.*, 1986), age-related conditions (Myking *et al.*, 1980; Honjo *et al.*, 1989) and oestrogen-dependent neoplasia (Naitoh *et al.*, 1989). The simplicity, satisfactory sensitivity/specificity makes our assay a very useful tool with which to study and evaluate further the role and significance of oestrone sulfate in humans.

The authors would like to thank M. Papadopoulos of the University of Crete for his advice on the preparation of the immunogen.

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