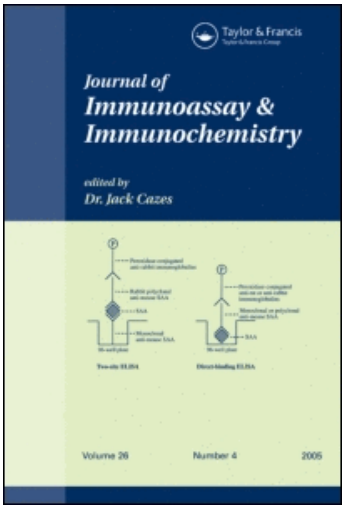

Journal of Immunoassay and Immunochemistry

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

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To cite this Article Seth, R. , Samarajeewa, P. and Coulson, W. F.(1992) 'Production and Utilisation of Antibodies Directed Against Oestrone Sulphate Using Specific Hapten Synthesis', Journal of Immunoassay and Immunochemistry, 13: 2, 297 — 314

To link to this Article: DOI: 10.1080/15321819208021233

URL: http://dx.doi.org/10.1080/15321819208021233

JOURNAL OF IMMUNOASSAY, 13(2), 297-314 (1992)

PRODUCTION AND UTILISATION OF ANTIBODIES DIRECTED AGAINST OESTRONE SULPHATE USING SPECIFIC HAPTEN SYNTHESIS

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(Key words: Hapten, Oestrone sulphate, antibodies, RIA, Milk)

ABSTRACT

Oestrone-3-sulphate ($E_1$3S) is an important metabolite of oestrone. Studies in cattle had previously shown that it is synthesised in the gravid uterus by the foetus, but not by the corpus luteum. Progesterone measurement in milk by radioimmunoassay (RIA) is routinely carried out in some laboratories as a pregnancy test for cattle. The major drawback of progesterone measurement in milk, by RIA, as a pregnancy test was the failure to detect the lack of conceptus in those cows where early embryonic death had occurred but the corpus luteum still persisted thereby giving false positive results.We have developed a direct RIA for $E_1$3S by raising antibodies to an immunogen prepared from a specific hapten synthesised by an unambiguous chemical synthesis.The sensitivity of

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the RIA in milk was found to be 0.368 nmol/l. The levels of $E_1$3S in non-pregnant cows are undetectable but during a viable pregnancy, the levels are elevated to >0.40 nmol/l by day 100. There was no cross-reactions in the assay with any free oestrogens. The measurement of this metabolite of oestrone promises to provide an accurate marker for the detection of a viable conceptus in cattle.

INTRODUCTION

Production of $E_1$3S by the conceptus was first established in the pig (1, 2). It was observed that the pig blastocyst tissue was capable of synthesising high concentrations of oestrone which was converted to $E_1$3S by sulphokinases present in the endometrium (3, 4)

It was also shown that in cows and swamp buffaloes there was a rise in concentration of $E_1$3S in peripheral circulation as pregnancy progressed (5) Importantly, in the animals where pregnancy was not established, there was no elevation of the circulating plasma $E_1$3S indicating that it was derived from the conceptus. The pattern of $E_1$3S in milk was similar to that reported in plasma except that small amounts were detectable in whey quite early (6,7). Similar findings were also reported in goats' milk (8).

The measurement of progesterone in milk by RIA is routinely carried out in some laboratories as a pregnancy test for cattle. Normally, about day 25 after insemination, the presence of progesterone in milk has been used as an index of pregnancy in lactating cows (4, 5). However the major drawback of this assay is the failure to detect the absence of embryo in cows where early embryonic death has occurred but the corpus luteum still persists. The corpus luteum is the main site of progesterone production during early stages of pregnancy and for this reason, it is difficult to eliminate the false positives obtained by this technique.

The original assays for $E_1$3S have been carried out by the lengthy procedure of enzymic hydrolysis of the conjugate to oestrone and then extracting the free oestrone which is assayed by RIA. Early attempts to raise antibodies by various

groups had failed (9, 10). Direct measurement of $E_1$3S in cow's milk by RIA employing an antibody raised against oestrone 3-glucuronide-BSA has been possible (11) and it has also been reported that antibodies to $E_1$3S were produced when oestrone 3-glucuronide-thyroglobulin was used as an immunogen (12). These antibodies were used to measure $E_1$3S in human plasma (12).

The objectives of the present work were to raise and characterise antisera specific for $E_1$3S in terms of sensitivity, affinity and specificity and to establish a direct immunoassay for this metabolite in milk. The stability of the hapten was also studied using dual isotope labelling technique.

MATERIALS AND METHODS

Solvents were obtained from BDH, Poole, Dorset, U.K., and were redistilled before use. Isobutyl chloroformate and tri-n-butylamine were obtained from Kodak, Kirkby, Liverpool, U.K. Oestradiol-17β was obtained from Steroloids, Surrey, U.K. Oestrone 3- sulphate and BSA were purchased from Sigma Chemical company, Kingston, Surrey, U.K. Sephadex-G25 was obtained from Pharmacia (G.B.) Ltd, Paramount House, London, U.K. (1,2,6,7,^{3}H) oestradiol-17β (85–110 Ci/mmol) and Chloro [^{35}S] sulphonic acid (32 Ci/mmol) were purchased from Amersham International, U.K. [6,7 ^{3}H] Oestrone 3-sulphate was purchased from New England Nuclear, Southampton, U.K.

Infra-red spectra were carried out with a Perkin Elmer model (237) grating spectrophotometer, using potassium bromide discs. NMR spectra were obtained using Variant HA100 at 100mHz at Westfield college, University of London (MRC steroid reference collection). Melting points were determined on a microheating stage. Protein measurements were performed on a Gilford Spectrophotometer. Radioactivity was measured on a Packard Tricarb scintillation counter (model 3385).

Synthesis of the hapten, oestradiol 3-sulphate, 17β hemisuccinate.

Stage 1. Synthesis of oestradiol 17β -hemisuccinate

The first stage of the synthesis involved the formation of oestradiol 17β–hemisuccinate according to the method of Kohen, Bauminger & Lindner (13). Tracer amounts of labelled oestradiol-17β were added to monitor the reaction and estimate the yield of the product. Oestradiol-17β ($E_2$17β) (0.8g, 2.94 mmol) and [2,4,6,7 ^{3}H] $E_2$17β (100 μCi) were mixed and added to the succinic anhydride (2.94g, 29.4mmol). Freshly redistilled pyridine (20ml) was added to the reagents and mixed on a magnetic stirrer for 1h at room temperature. A clear solution was obtained which was placed in a light-tight box for 4 days. At the end of the incubation time, the reaction mixture was added to a mixture of cold HCl (100ml) and ice (to a total volume of 500 ml) and extracted 3 times with ether (250 ml) to remove the pyridine and excess succinic anhydride. The ether layer was extracted three times with 5% (w/v) sodium bicarbonate solution to remove the free $E_2$17β. The required product was then present as a sodium salt in the aqueous phase. The aqueous phase was poured over ice-HCl to neutralise the solution which was re-extracted with ether. The required product in the ether layer was dried over anhydrous magnesium sulphate for 15 min, filtered and evaporated on a rotary evaporator. A pale yellow oil was obtained which was dissolved in toluene and recrystallised. Three consecutive recrystallisations were carried out to obtain pure white needle-shaped crystals. N.M.R. and I.R. spectroscopy confirmed the presence of a hemisuccinate group on carbon-17 and a hydroxyl group on carbon-3 position.

Stage 2. Synthesis of oestradiol,3-sulphate,17β-hemisuccinate

Chlorosulphonic acid (1ml) and (2mCi) chloro[^{35}S] sulphonic acid was gently added to pyridine (4ml) at 0°C to form the reactive pyridinium sulphur trioxide complex.

To this complex, the above prepared oestradiol 17β-hemisuccinate (94.1 mg, 0.20 mmol) dissolved in pyridine was added and left to stand at room temperature overnight. At the end of the reaction, ammonium hydroxide (10 ml, 7M) was added to the reaction mixture and extracted with ether to remove the pyridine. To the aqueous phase, acetone was added to precipitate the excess ammonium sulphate. The mixture was filtered through a Buchner funnel and the filtrate was extracted with butanol (10 ml) three times. The organic layers were pooled and dried on the rotary evaporator. Recrystallisation was not attempted due to the labile nature of the product but it was dried and stored in a dessicator. I.R. and N.M.R. spectroscopy confirmed the presence of a sulphate moeity on C-3 and a hemisuccinate group on C-17 position.

Stage 3: Synthesis of oestradiol,3-sulphate, 17β-hemisuccinate-BSA

The coupling reaction was carried out using the mixed anhydride method of Erlanger, Borek, Beiser & Lieberman (14). Oestradiol 3-sulphate,17β-hemisuccinate (50mg, 3.2μCi) was dissolved in dimethylformamide (1.28 ml) and dimethylsulphoxide (0.025 ml) and cooled in ice for 20 mins. To this solution, tri-n-butylamine (0.02 ml) was added followed by isobutyl chloroformate (0.01ml). This solution was stirred and chilled in ice for 30 minutes. The formation of the mixed anhydride was assumed to be completed after this period of time. BSA (128 mg) was dissolved in equal volumes (3.30 ml) of distilled water and dimethylformamide and chilled on ice for 10 minutes. The BSA solution was added to the above reaction and the mixture was stirred for 4 h at 4°C. The pH of the reaction mixture was monitored and maintained at 7 by adding sodium hydroxide (1M) dropwise.

At the end of the reaction time, the resulting turbid solution was applied to a Sephadex G-25 column (50 cm x 2 cm) prepared in water. The column was developed with water and thirty (10 ml) fractions were collected. The BSA -coupled

steroid eluted in the void volume (fractions 7,8 and 9) and the unreacted hapten eluted in the later fractions. Protein was measured in each fraction by the method of Lowry, Rosebrough, Farr & Randall (15). The amount of radioactivity present in each fraction was also measured in the β-scintillation counter. From these two parameters, steroid incorporation to BSA was estimated by the method of Samarajeewa (16). The molar ratio i.e. the number of steroid molecules attached to the lysine residues in the BSA was found to be 4. This immunogen was used to raise antibodies.

Hapten stability studies

Since the aim of the study was to raise specific antibodies to oestrone 3-sulphate, it was necessary to confirm the presence of the sulphate group on the hapten and subsequently on the immunogen. Tracer amounts of ^{35}S labelled chlorosulphonic acid was used in the sulphation reaction. The starting material ($E_2$17β) already contained tracer amounts of [^{3}H]$E_2$17β, therefore it was possible to monitor loss of [^{35}S] before and after the hapten conjugation.

Production of antisera

Active immunisation was carried out in 6-7 week old New Zealand white, half-lopped male rabbits using the conventional method (16). The immunogen (4mg/rabbit) was emulsified with complete adjuvant containing heat-killed mycobacteria.

The immunogen complex was administered intra-muscularly into each flank of the animal and subcutaneously on several sites on its back. After the primary injection, a booster dose of the same composition was given after two weeks and subsequently at one month intervals. The animals were bled from the marginal ear vein after three months of the primary injection and ten days after each booster.

The sera were separated by centrifugation at 1000g for ten minutes and stored at -20°C. Antibody titres, defined as the dilution of antibodies which in a

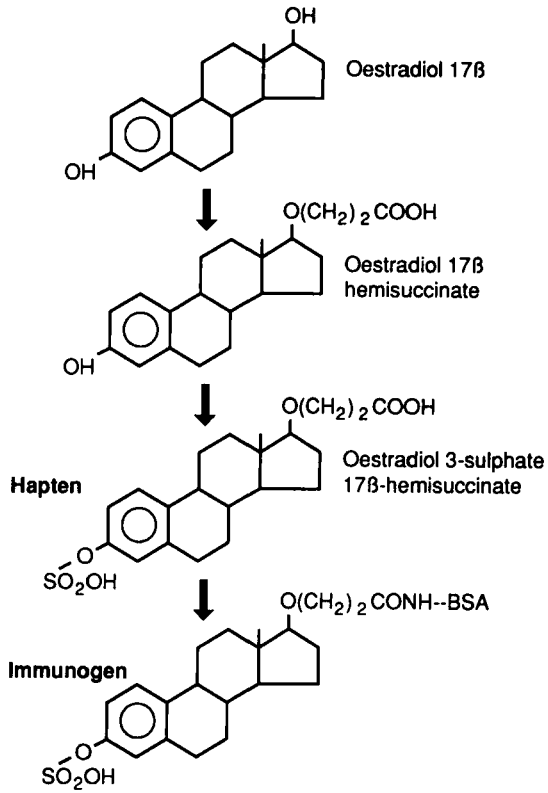

FIGURE 1: Synthesis Of The Hapten And The Immunogen

radioimmunoassy (RIA) gives rise to a 50% displacement of the radioactive tracer, were determined using [^{3}H]oestrone 3-sulphate as follows. Each antiserum was serially diluted with phosphate buffer to give a dilution range from 1:10 to 1:32,000. The reagents were added in the following order. Duplicates from each dilution of antiserum (100 ul) were incubated with radioligand (100ul) and phosphate buffer (100 ul) at 37°C for 1 hour.

An identical assay was set up where the buffer was replaced by a standard solution of oestrone 3-sulphate (100µl, 500pg). In the assay, tubes were included which contained radioligand (100µl) and buffer (200µl) to moniter non-specific

binding (NSB). At the end of the incubation time, the tubes were chilled in ice for 30 minutes. Well stirred dextran-charcoal suspension (200µl) was added and the tubes incubated for a further 20 minutes at 4°C. The unbound reagents bound to charcoal were separated in a centrifuge at 1000g for 15 minutes at 4°C. The supernatant (250µl) which contained the antibody bound complex was added to the scintillation fluid (4ml) and the radioactivity was measured in a beta-scintillation counter.

The antibody titre was determined as the reciprocal of the dilution which binds 50% of the radioligand in the absence of 'cold' oestrone sulphate (see table 1).

Titres were also determined, as above, for the antibodies which were raised against oestrone 3-glucuronide. Oestrone was used as the radioligand, but the rest of the methodology was identical to the above. The titres obtained are shown in table 1.

Calibration graph for the measurement of oestrone sulphate was prepared by incubating the diluted serum (100 µl; 1:2800 which bound 50% of the radioactive oestrone sulphate) in phosphate buffer with radioligand (100µl) and a range of standard oestrone sulphate solution (100 µl; 10pg, 20pg, 50pg, 100pg, 200pg, 500pg, 1000pg and 2000pg/tube) at 37°C. The rest of the procedure was identical to that used for the determination of antibody titres. The results obtained were used to prepare a calibration graph of the percentage of binding of labelled oestrone 3-sulphate against the concentration of added non-radioactive standard oestrone sulphate.

The specificity of each antiserum was determined as follows. A calibration graph was set up with increasing concentrations of oestrone sulphate. In addition, a range of structurally related steroids were incubated with the antisera and radioligand at three different doses (100pg, 500pg, 1000pg/tube). The degree of cross-reaction was calculated by the method of Thornycroft, Tilson, Abrahams &

TABLE 1

Titres And Affinity Constants Of Antibodies Produced

Animal Identification number	Animal species	Immunogen	Titre	Affinity constant moles/litre
R1/83	Rabbit	$E_2 3SO_4 17\beta$ HS BSA	1:3800	4.67×10^{-10}
R2/83	Rabbit	$E_2 3SO_4 17\beta$ HS BSA	1:7400	1.08×10^{-9}
R3/83	Rabbit	$E_2 3SO_4 17\beta$ HS BSA	1:2000	1.09×10^{-9}
R8/82	Rabbit	Eβ3Gluc BSA	1:25000	4.08×10^{-9}
RG/29	Sheep	Eβ3Gluc OA	1:74000	8.6×10^{-9}
Y12	Sheep	EβGluc OA	1:48000	3.65×10^{-10}

TABLE 2

Cross-reactivities Of The Antibodies

Steroid	% Cross-reactivity using ($^3HE_1SO_4$)			% Cross-reactivity using($^3HE_1$)		
	R1/83	R2/83	R3/83	R8/82	RG/29	Y12
Oestrone, 3-sulphate	100	100	100	100	100	100
Oestrone	nil	nil	nil	100	100	100
Oestradiol, 17β	nil	nil	nil	14.4	nil	76
Oestriol	nil	nil	nil	0.1	0.1	0.1
Oestradiol, 3-glucuronide	0.1	0.1	0.1	100	100	100
Oestradiol, 17β-glucuronide	63.6	27.3	108	100	100	100
Oestriol, 3-glucuronide	0.1	54.3	0.1	3.0	11	4.9
Oesrtriol, 16α-glucuronide	1.0	nil	0.1	97	100	100
Oestradiol, 17β-hemisuccinate	nil	nil	0.1	1.0	1.0	1.0
Oestrone, 3-glucuronide	nil	nil	nil	---	---	---
Progesterone	0.1	6.8	0.1	1.0	1.0	1.0

Scaramuzzi, 1970 (17). Table 2 shows the number of steroids investigated for cross-reactivity with antisera raised against oestrone 3-sulphate and oestrone 3-glucuronide.

The sensitivity of the assay which is defined as the ability of the antibody to detect the least amount of ligand present was determined as follows: zero standard was determined ten times in an assay. The mean and three standard deviations were calculated and marked on the standard curve.

Precision of the assay was determined by measuring the inter and intra-assay variation by replicate measurement of the same samples in one assay and in ten different assays. The coefficient of variation was determined.

The affinity constant of each antiserum was determined by means of a Scatchard plot (18).

Measurement of oestrone 3-sulphate in milk.

The measurement of oestrone 3-sulphate in whey has already been reported previously using an antiserum raised against oestrone,3-glucuronide (11). The direct measurement of oestrone 3-sulphate in milk in the present study was established using antiserum designated R1/83. Whole milk was obtained from non-pregnant dairy cows (AFRC, Babraham,Cambridge). The milk had been preserved by the addition of potassium dichromate (30 mg) obtained in a tablet form known as 'Lactab' (Thompson and Cooper Ltd, Cheshire, England) and was stored at 4°C. Fat-free milk was prepared by centrifuging whole milk at 30,000g for one hour followed by the removal of butter fat. Standards were prepared in the range 0 - 5000 pg/100µl by the addition of fat-free milk to dry residues of pure oestrone 3-sulphate. The assay procedure was similar to that described previously. An aliquot (100µl) of the calibration standard was mixed with labelled oestrone (100µl) and antiserum (100µl; final dilution 1:2800).

The tubes were incubated at 37°C for one hour. After a further incubation at 4°C for 30 minutes, dextran coated charcoal (200µl) was added and incubated for 15 minutes. The tubes were then centrifuged at 1000g for 20 minutes. The supernatant (250µl) containing antiserum-bound radioligand was added to the scintillation fluid and radioactivity was measured in a scintillation counter.
Each assay was validated in terms of sensitivity and precision.

RESULTS

Stage 1 Synthesis of Oestradiol 17β -hemisuccinate (see fig 1)

Yield	462 mg
i.r KBr	3400 cm^{-1}(O-H stretching)
γ Max	2800–3000 cm^{-1} (C-H stretching 1700 cm^{-1} (Carboxylic group-as in COOH) 1275 cm^{-1} (C-O stretching as in phenolic 3-hydroxyl group)
^1H n.m.r. δ (CD_3OD + C_5D_5N)	0.95 (3H, s, C-18 methyl group) 2.84 (4H, s, $(CH_2)_2$ of hemisuccinate 4.78 (1H, t, C-17 α H) 6.85 (1H, s, C-4) 6.90 (1H, dd C-2) 7.20 (1H, d, C-2) ppm

Stage 2 Synthesis of Oestradiol 3-sulphate, 17β -hemisuccinate (see fig 1)

Yield		15 mg
i.r.	KBr	3700-3200 cm^{-1} (N-H stretching)
	γ	3200-2850 cm^{-1} (OH and C-H
	Max	stretching
		1700-1600 cm^{-1} (COOH carboxyl group)
		1400-1360 cm^{-1} (sulphate group)
		1100-1050 cm^{-1} (sulphate group)
^1H n.m.r. δ (CD_3 OD + C_5D_5N)		0.95 (3H, s, C-18 methyl group)
		1.04 - 2.40 (methylene envelope)
		2.84 (4H, s, $(CH_2)2$ of hemisuccinate)
		4.78 (1H, t, C-17α H)
		6.80 - 7.60 (3H, aromatic protons)

Stage 3 Synthesis of oestradiol 3-sulphate,17β-hemisuccinate-BSA

Yield of immunogen was 124 mg: Molar ratio was 4.

Hapten stability studies

The radioactivity of the dual-labelled [^3H] oestradiol,3-[^{35}S]sulphate,17β-hemisuccinate was determined on a Beckman multichannel radioactive scintillation counter (LS 7500, microprocessor controlled).

The isotope ratio of the hapten before the mixed acid anhydride was 0.65 where as after the coupling reaction, it was raised to 0.83, suggesting that during the coupling reaction, the loss of the sulphate moiety in the order of 22% had occurred due to hydrolysis of the sulphate bond.

Characterisation of antisera

Titres: Table 1 shows the antibody titres obtained using different immunogens for the immunisation protocols.

Specificity : Table 2 shows the crossreactivity of the antisera with related and non-related steroids. There was no cross-reaction with free oestrogens. This is as would be expected since the structural features present in the free steroids were absent in the hapten. There was considerable cross-reaction with conjugated oestrogens due to close resemblance between the sulphate and the glucuronide moiety in terms of size and ionic charge.

The affinity constants of these antibodies agreed with the data published for antibodies raised against similar steroid conjugates by other workers (10).

Calibration Graph

Calibration graph for the radioimmunoassay of oestrone 3-sulphate in the milk was constructed as shown in fig 2 using antiserum from rabbit R1/83. The limit of detection of the assay was 15 pg/tube (0.368 nmol/l) (n=10 assays). The coefficient of variation within assay was 6% (n=4) and between assay was 18% (n=10). The concentration of oestrone 3-sulphate in non-pregnant cows is less than 0.08nmol/l and is elevated to 0.4 nmol/l by day 100 of pregnancy. So, it would be possible to confirm the presence of a viable foetus by measuring the oestrone sulphate level after day 100 using this antibody.

CONCLUSION

The measurement of oestrone 3-sulphate as a marker to confirm the presence of a viable conceptus has become increasingly important since it is synthesised in the gravid uterus by the foetus and not by the corpus luteum. The aim of the present study was firstly, to prepare a stable hapten and an immunogen in order to raise specific antisera with high affinity for oestrone 3-sulphate and

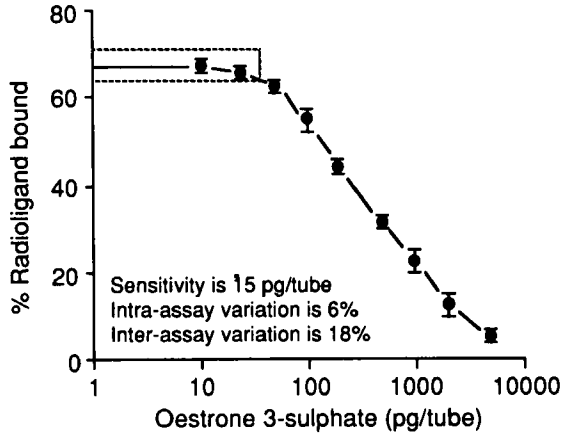

FIGURE 2: Calibration Graph Of Oestrone 3-sulphate In Milk

secondly, to use the antisera to develop an immunoassay for the measurement of this metabolite of oestrone in milk.

The immunogen was synthesised by an unambiguous chemical synthesis. Using oestradiol-17β as a starting material a hemisuccinoxy bridge was introduced at C17 position.The sulphate group was then added on C3 (see figure 1). During the course of the study, it had become clear that the sulpho-conjugate of oestrone was not stable and that significant hydrolysis was taking place during the purification and conjugation steps. The stability of the sulphate moiety was studied using dual isotope labelling technique whereby [^{35}S] and [^{3}H] were incorporated into the hapten. The 35S/3H ratio was measured before and after the conjugation. The results showed that there was a loss of the sulphate groups from the steroid molecule in the order of 22%.

Animals immunised with this immunogen did respond by producing antibodies reactive against oestrone 3-sulphate. However the titres of the antibodies were not as high as expected as shown in table 1. This could be due to the fact that

rapid hydrolysis may be taking place *in vivo* which may have eliminated majority of the immunogen.

Inspite of the low titres obtained in this study, the antibodies were found to be specific for oestrone 3-sulphate. It was interesting to find that the antisera showed no cross-reactivity with oestradiol-17β-hemisuccinate although this compound was the immediate precursor of the hapten during its chemical synthesis.

There was no cross-reaction with free oestrogens which is what one would expect since the functional groups present in the free steroids were absent in the immunogen (see table 2). The only conjugates which showed cross-reactivity were oestradiol 17β glucuronide and to some extent oestradiol-3-glucuronide presumably due to the close resemblence between the sulphate and the glucuronide moiety.

There was little cross-reaction with progesterone (R2/83 6.8%) and it is likely that this antibody will be useful in determining the levels of oestrone 3-sulphate in milk.

The RIA of oestrone 3-sulphate in milk was established using the antibody from rabbit R1/83). The assay was reasonably sensitive (0.368 nmol/l) in the sense that it could be used to measure oestrone 3-sulphate in milk. In non-pregnant cows the level of oestrone 3-sulphate is less than 0.08 nmol/l but by day 100 of pregnancy, the levels are elevated to 0.4 nmol/l. It would therefore be possible to confirm the presence of a viable foetus using this particular assay.

It is possible to improve the sensitivity of the immunoassay in the near future by using the ELISA-type format and setting up a competitive immunoassay where an alkaline phosphatase -labelled hapten can be used as the competing ligand with oestrone sulphate for the limited antibody sites. A method of enzyme amplification (19) can then be incorporated to enhance the sensitivity greatly as it was done for calcitonin (20 and 21) and CGRP (22). This form of enzyme amplified immunoassay should allow sensitive and fast determination of oestrone sulphate in milk from cattle.

This work has highlighted a major problem involved with this particular metabolite of oestrone. It has shown that the sulphate moiety is unstable thereby making the preparations of the hapten and hence the immunogen very difficult, but not impossible, and useful antibodies were produced giving a workable RIA.

ACKNOWLEDGMENTS

The work carried out in this report was supported by the AFRC council, U.K. by grant to P.S. and W.F.C. The authors would like to acknowledge Dr Heap and Dr Hamon of ARC Institute of Animal Physiology , Babraham, Cambridge for supplying the milk samples used in this study and Dr D. Farrant of Westfield College, University of London, for performing the n.m.r.

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