IMPROVED ANTISERA FOR THE SPECIFIC RADIOIMMUNOASSAY OF OESTETROL

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1. Introduction

We are currently developing steroid radioimmunoassays [1] which may be useful for monitoring foetal well-being in late pregnancy, as there is, at present, no single hormone assay which is entirely adequate for this purpose. Oestetrol (15-hydroxyoestriol) offers a possible solution to this problem, as its production is almost entirely dependent upon a functioning foetal liver [2] and initial investigations have indeed indicated that the measurement of this steroid may provide a useful index of foetal viability [3,4]. The antisera used in these earlier investigations were raised against an antigen in which the steroid was linked through the three-position and showed reasonable specificity for the D ring; the cross-reaction for oestriol being 2.5% [5]. In this communication we report that more specific antisera may be obtained using an antigen in which the steroid is linked through the four-position.

2. Materials and methods

2.1. *Reagents*

[6,7-^{3}H]Oestetrol (50 Ci/mMole) was purchased from New England Nuclear. Non-labelled steroids were obtained from Steraloids Inc. Wilton, USA. General reagents were purchased from B.D.H. and all solvents were redistilled prior to use. Freund's complete adjuvant was obtained from Difco Laboratories. All samples were counted in 5 ml scintillant (NE 260, purchased from Nuclear Enterprises).

2.2. *Preparation of Antigen*

To a solution of 4-aminobenzoic acid (0.137 g) in M hydrochloric acid (2 ml) at 5°C was added a solution of sodium nitrite (0.083 g) in water (1 ml) dropwise over ten min. The solution was left to stand for a further ten min at 5°C and then excess nitrous acid was removed by the addition of sulfamic acid.

To a solution of oestetrol (0.030 g) in methanol (7.5 ml), containing M sodium hydroxide (0.4 ml) under nitrogen at 0°C was added dropwise a solution of diazotised 4-aminobenzoic acid (0.3 ml), over fifteen min. The resulting Burgundy-coloured solution was stirred at 0°C for thirty min and then acidified (pH 5) with 0.1 M hydrochloric acid. The resulting suspension was centrifuged and the pale yellow supernatant discarded. The precipitate was washed with water (50 ml) in this manner several times. The final precipitate was separated by preparative thin-layer chromatography on silica plates, developed in ethyl acetate–cyclohexane–acetic acid–methanol (70:30:2:1; by vol). Elution of the major coloured band, with tetrahydrofuran, gave 4-(4′-carboxyphenyl-azo)-oestetrol which separated from methanol as an amorphous red powder (0.024 g). A reproducible melting point could not be obtained because of decomposition above 250°C

υ_{max} (Nujol); 3350 (broad; hydroxyls), 1695 (carboxyl, 1605 (aromatic) and 1580 cm^{-1} (azo).
λ_{max} (MeOH); 346 nm (ϵ 1.4 × 10^4).
δ (d_6-DMSO); 0.70 (3H, s, 18-CH_3), 6.78 (1H, d, J = 8 Hz, 2-H), 7.38 (1H, d, J = 8 Hz, 1-H) and 8.00 (4H, AA′ BB′ system, aromatic protons).

Mass spectrum of methyl ester (obtained by treatment of the acid with ethereal diazomethane): M^+ 466 ($C_{26}H_{30}N_2O_6$ requires 466).

The derivatised steroid was coupled to bovine serum albumin using a method previously described [6] except that the crude product was purified only by dialysis against running water. The molar steroid: protein ratio of the conjugate was determined spectrally to be 34:1.

2.3. *Immunisation and characterisation of antisera*

The conjugate was injected into four adult male New Zealand White rabbits (2.5 kg) as previously described, except that the initial dose was only 1 mg/rabbit [7]. Blood was removed from the marginal ear vein ten days after the second and subsequent booster doses.

The cross-reactions of various steroids which might compete with oestetrol for binding sites were measured as previously described [7].

3. Results

Blood samples removed after the second booster injection showed virtually no binding of labelled oestetrol. The characteristics of blood samples obtained following the third booster are shown in table 1.

Table 1
Specificity, working dilutions and sensitivity of anti-oestetrol-4-BSA antisera

Steroid	Antiserum R1 B2	R2 B2	R3 B2	R4 B2
Oestetrol	100	100	100	100
Oestriol	0.7	0.7	0.35	0.38
Oestradiol	0.02	<0.02	<0.02	0.09
Oestrone	<0.02	<0.02	<0.02	<0.02
Testosterone	<0.02	<0.02	<0.02	<0.02
Dehydro-epiandrosterone	<0.02	<0.02	<0.02	<0.02
Progesterone	<0.02	<0.02	<0.02	<0.02
Working dilution	1:500	1:2500	1:1000	1:2500

With these antisera 20 pg of oestetrol was detectable (distinguishable from zero point with 99% certainty). Coding of Antisera: R1-R4 refers to four individual rabbits and B2 refers to blood samples removed after the third booster injection.

4. Discussion

In the past we have raised specific antisera to oestrogens using haptens in which the steroid linked through the 6-position. The antisera thus obtained were highly specific and this was attributed to the fact that both of the structurally unique A and D rings were free to elicit the immune response [7,8]. However we were unable to apply this methodology to oestetrol because the D ring proved to be unstable to the strongly alkaline conditions required for deacetylation. We therefore chose to conjugate the steroid through the 4-position, as models indicated that this should leave all structurally unique hydroxyl functions available for immune recognition. In this context Katagiri et al. have obtained specific antisera for oestriol using a similar type of linkage [9].

Oestetrol was coupled to diazotised 4-aminobenzoic acid using a method similar to that described by Gross et al. [10]. The n.m.r. spectrum corresponded closely to that of 4-(4'-carboxyphenylazo)-oestriol, indicating that it was indeed 4-linked [11]. The coupling constant for the two protons in the A ring is particularly important since it proves the assigned ortho relationship [12].

The antisera from all four rabbits only showed useful binding after fourteen weeks of injections which is an unusually long period in our experience [1,8]. Katagiri et al. [9] investigating the response to a similarly linked oestriol antigen also found very little binding in five out of six rabbits after twelve weeks of injections, suggesting that in the rabbit, antigens containing this type of linkage may generally give a slow immune response.

The detection limit for oestetrol, using these antisera was sufficiently low (20 pg) to permit the assay of this steroid in 100 μl of plasma at least as early as the twentieth week of gestation [3].

The described antisera were consistently more specific than those previously reported [5]. In normal pregnancy plasma the ratio oestriol: oestetrol is approximately 4:1; so that with our antisera, cross-reacting oestriol would introduce an overestimation of oestetrol of around 1% and with existing antisera this overestimation is still only 10% [5]. However if oestetrol is indeed a better indicator of foetal distress than is oestriol then it is possible that in those cases of greatest clinical interest there may be an especially

high ratio of oestriol: oestetrol. In such cases the greatest possible specificity would be essential.

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