A RADIOIMMUNOASSAY FOR EQUILIN IN EQUINE PREGNANCY PLASMA

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1. Introduction

It is well known that the pregnant mare produces large quantities of the ring B unsaturated steroid equilin in addition to the classical oestrogens [1]. However, the precise biogenesis of this unusual steroid remains a mystery [2,3]. To facilitate a study of the interrelationship of the steroids present during equine pregnancy we decided to develop a radioimmunoassay for measurement of equilin in peripheral plasma. As equilin is thought to be a product of the foeto-placental unit [4] such an assay may also be of use as an index of foetal well-being.

Oestrone and equilin are present in similar concentrations in equine pregnancy plasma [5] and so it was important that an antiserum was produced which could differentiate between these two steroids. We therefore synthesised an antigen in which equilin was linked to a protein carrier through the 17-position, in order that the C_7–C_8 double bond might be fully exposed for immunogenic recognition. This stratagem proved successful as the antiserum obtained gave a cross-reaction of only 7.3% for oestrone.

The use of a radioimmunoassay incorporating this antiserum is demonstrated by measuring equilin concentrations in plasma samples taken from a mare at weekly intervals from day 60 of pregnancy through to parturition. The corresponding oestrone concentrations are also recorded and demonstrate the validity of the equilin assay in this situation.

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2. Materials and methods

2.1. *Reagents*

Equilin, 17α-dihydroequilin, equilenin, 17α-dihydroequilenin and 17β-dihydroequilenin were obtained from Ayerst, McKenna and Harrison (Montreal, Canada). 7α-Hydroxydehydroepiandrosterone was a gift from the MRC Reference Collection and all other steroids were purchased from Steraloids Inc. Wilton, USA. General reagents were purchased from BDH and all solvents were redistilled prior to use. Freunds complete adjuvant was obtained from Difco Laboratories and Helix pomatia enzyme from IBF, Paris, France.

2.2. *Preparation of antigen*

A solution of equilin (0.17 g) and decarboxymethoxylamine hemihydrochloride (0.17 g) in anhydrous pyridine (8 ml) was stirred at 20°C for 24 h. After removal of the solvent under nitrogen at 37°C the oily residue was partitioned between ethyl acetate (50 ml) and water (25 ml). The aqueous phase was extracted with ethyl acetate (50 ml) and the combined organic fractions were dried (Na_2So_4) and concentrated. Recrystallisation of the product from acetone gave cream-coloured needles (0.15 g) m.p. 128–131°C. ϑ_{max} (Nujol), 1720 (carboxyl) and 1640 cm^{-1} (C_7–C_8 double bond); λ_{max} (Tris buffer pH 8.5) 230 and 270 nm; M^+ 341 ($C_{20}H_{23}NO_4$ requires 341). The derivatised steroid was coupled to bovine serum albumin [6] and the molar steroid:protein ratio determined spectrally to be 22:1.

2.3. *Immunisation and characterisation of antisera*

The conjugate was injected into four male New Zealand White rabbits, according to a previously described regimen [6] and the blood samples obtained

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nine days after the second booster injection tested as before [6].

2.4. *Preparation of the radioiodinated tracer*

An equilin 17-(*O-carboxymethyl*)oxime [^{125}I]-iodohistamine conjugate was prepared using the general method of Hunter et al. [7]. The product was purified by preparative thin layer chromatography on a silica plate (Kieselgel F 254, Merck) developed in benzene/methanol (80:20; by vol). The major radioactive band (R_F 0.36) was eluted with ethanol and stored at 4°C. The tracer could only be used for seven days after preparation.

2.5. *Radioimmunoassay*

200 μl, 100 μl or 50 μl serum samples, in duplicate, were pipetted into stoppered tubes and incubated with Helix pomatia enzyme (20 μl, 10 μl or 5 μl) for 30 min at 56°C to hydrolyse any conjugates present [8]. After extraction with diethyl ether (3 ml) the tubes were placed in an acetone–solid carbon dioxide mixture and the ether decanted into 12 X 75 mm tubes. Solvents were evaporated under nitrogen in a water bath at 37°C and the rest of the assay performed as previously described [9].

The radioimmunoassay for total oestrone will be described elsewhere [10].

3. Results

Three of the rabbits injected with the antigen produced usable antisera and the assay characteristics of the most specific (with respect to their oestrone cross-reaction) of these are presented in table 1 and the cross-reactions in table 2. The standard curve was found to be linear between the 0.5 ng point and the 20 ng point. The concentrations of equilin and oestrone in plasma samples taken at weekly intervals from day 60 of pregnancy through to parturition are shown in fig.1.

Table 2
Cross-reactions of anti-equilin-17-BSA antiserum at a working dilution of 1000 : 1

Steroid	Cross-Reaction (%)
Equilin	100
17α-Dihydroequilin	13
Equilin	0.7
17α-Dihydroequilenin	0.2
17β-Dihydroequilenin	0.3
Oestrone	7.3
17β-Oestradiol	< 0.1
Androstenedione	< 0.1
Androsterone	< 0.1
Dehydroepiandrosterone	< 0.1
7α-Hydroxydehydroepiandrosterone	< 0.1
Testosterone	< 0.1
Progesterone	< 0.1
Cholesterol	< 0.1

4. Discussion

In many cases it has been found difficult to produce antisera capable of distinguishing between

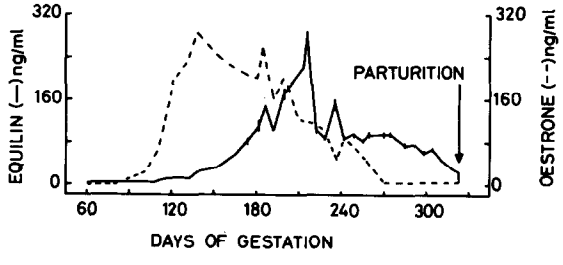

Fig.1. Concentrations of equilin and oestrone, measured by radioimmunoassay, from day sixty of pregnancy through to parturition.

Table 1

Intra assay coefficient of variation (2.4 ng) = 5.47% (n = 8)
Intra assay coefficient of variation (16.0 ng) = 6.48% (n = 8)
Inter assay coefficient of variation (2.4 ng) = 9.16% (n = 6)
Inter assay coefficient of variation (16.0 ng) = 13.09% (n = 6)
Sensitivity (least detectable point from zero) = 500 pg (p = < 0.01)
Recoveries ranged from 90.6–124%

steroids differing only by the presence of a double bond which has little influence on the shape of the steroid nuclei as a whole. For example, antisera raised against antigens in which testosterone was linked through the 3, 11 and 17 positions were unable to discriminate against dihydrotestosterone [11]. Similarly, we have raised antisera against dehydroepiandrosterone linked through the 19-position which had a high cross-reaction for epiandrosterone [9]. We therefore linked equilin through the 17-position in order to give maximum exposure to the C_7–C_8 double bond and obtained an antiserum which could discriminate reasonably well against oestrone and almost completely against equilenin. These findings correlate well with the overall shapes of the three steroids. The cross-reaction of 13% for 17α-dihydroequilin is not important as this steroid is produced in much smaller quantities than is equilin during equine pregnancy [2].

At present there is no method available for preparing tritiated equilin with the specific activity required for radioimmunoassay. We found that tritium could be introduced into the 16-position by exchange with tritiated water on activated alumina [12] but the specific activity of the product was not high enough for immunoassay purposes. Nevertheless, a radioiodinated histamine conjugate proved satisfactory, even though a homologous system [13] was employed. Using an initial percentage bound of 80%, a standard curve ranging from 480 pg–20 ng was constructed.

The assay system was tested by measuring total equilin concentrations in plasma samples taken from a pregnant mare and comparing them with corresponding oestrone concentrations. The results (fig.1) show that oestrone has little effect on the equilin concentrations measured, despite a cross-reaction of 7.3%. The general pattern of oestrone and equilin concentrations obtained are in good agreement with those recorded by Cox [5] using gas–liquid chromatography after a paper chromatographic step and support the theory of independent biosynthetic pathways for the two oestrogens [2,5].

In conclusion, we have developed the first radioimmunoassay for equilin and have used it to confirm that maximum concentrations of equilin and oestrone occur at different times during equine pregnancy. We are at present examining the relationship of equilin concentrations with those of other steroids present during equine pregnancy such as dehydroepiandrosterone.

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