PREPARATION OF 17β-OESTRADIOL-6-(O-CARBOXYMETHYL) OXIME - BOVINE SERUM ALBUMIN CONJUGATE

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ABSTRACT

A 17β-oestradiol hapten has been prepared in which conjugation has been made at the 6 position, which being distal to the functional groups of the oestrogen may elicit a more specific immune response than that produced by present day haptens. The preparation of 17β-oestradiol-6(O-carboxymethyl) oxime-bovine serum albumin conjugate is described. The compound was prepared via 6-keto-17β-oestradiol and its O-carboxymethyl oxime derivative, which was coupled using isobutyl chloroformate to bovine serum albumin. The compounds were characterised by melting points, ultra violet spectra, nuclear magnetic resonance and mass spectrometry.

INTRODUCTION

A number of radioimmunoassay systems have recently been developed for the determination of steroid hormones[1-3]. In order to elicit an immune response for steroids, however, one requires the synthesis of steroid-protein conjugates. Antisera for steroid radioimmunoassay have been obtained using sheep and rabbits immunised with steroid derivatives

conjugated at various positions to bovine or rabbit serum albumin.[4-7] The specificity of the respective antibodies appeared to be dependent on the position of conjugation, and data have suggested that the major portion of the steroid is recognised as a single immunoreactive unit. In general, structural differences in the steroid molecule distal to the conjugation had the greatestinfluence on relative activity.[1] This has led to the suggestion that conjugates prepared through a position distal to structurally unique regions would enable these functional groups to be recognised, and that this would lead to the production of more specific antisera.

Highly specific antibodies now exist for testosterone and progesterone[1], but as yet there is no truly specific antibody available for 17β-oestradiol. Present antibodies for the latter steroid have been produced using either of the functional 3 or 17 groups and there is a serious cross reaction with oestrone and 17α-oestradiol. This communication describes the preparation and characterisation of a steroid hapten which involves coupling serum bovine albumin via the 6 position. Details of the specificity and sensitivity of the antibody produced by this antigen will be given in a subsequent publication.

APPARATUS

Melting points were obtained using a Kofler block and are uncorrected. Infra red spectra were obtained with nujol emulsions using a Perkin Elmer, (Beaconsfield,

England), Model 137 double beam spectrophotometer.

Mass spectra were recorded with a Perkin Elmer Model MS9. The probe temperature was 180°. Nuclear magnetic resonance spectra were recorded using a Perkin Elmer Model R12 spectrometer. Spectra were time accumulated on a digital signal averaging accessory (100 times).

Ultra violet spectra were recorded on a Pye Unicam SP 800 (Cambridge, England).

MATERIALS

All solvents used were of analytical grade. O-carboxymethyl hydroxylamine hemihydrochloride was obtained from K & K Laboratories (New York). Tri-n-butylamine was purchased from British Drug Houses (Poole, Dorset). Bovine serum albumin was purchased from Armour Pharmaceuticals Ltd. Laboratories (Eastbourne, England).

17β-oestradiol was obtained from Koch-Light (Colnbrook, England). An authentic sample of 6-keto-17β-oestradiol diacetate was obtained from the Medical Research Council reference collection.

EXPERIMENTAL - METHODS

Preparation of 17β-Oestradiol-diacetate

17β-oestradiol was recrystallised from ethanol (needles m.p. 73-4°) and dried. Purified 17β-oestradiol (10·0 g) was acetylated by dissolving in pyridine (140 ml) and heating the solution with acetic anhydride (35 ml). The mixture was heated under reflux for 1 hr, cooled, poured into ice-water and filtered. The precipitate was

dried and recrystallised from ethanol-water to give plates m.p. 123-126° (Lit. m.p. 126-128°). The yield was 12·6 g (97%).

Preparation of 6-keto-17β-oestradiol-diacetate

The purified diacetate (11 g) was dissolved in glacial acetic acid (38 ml) and chromium trioxide (9·3 g) in aqueous acetic acid (7.5: 56) added. The mixture was stirred at room temperature for 24 hr, diluted to 500 ml with water and the mixture extracted with ether (4 x 500 ml).

The combined ethereal extracts were washed with saturated $NaHCO_3$ solution until a pink colour developed in the aqueous phase. The ethereal layer was then washed with a solution containing 1N Na_2CO_3 and saturated $NaHCO_3$ (3:1) and finally with water. The washed ethereal extract was dried ($MgSO_4$) and evaporated to dryness under reduced pressure. The yield of crude 6-keto-17β-oestradiol diacetate was 6·6 g (60%).

The crude product was dissolved in ethyl acetate-petroleum ether (b.p. 60-80°) [1:4] and chromatographed on a column of silica gel (400 ml). The column was eluted with ethyl acetate-hexane [1:4] and 250 ml fractions collected. (The fractions were analysed by thin layer chromatography (TLC) using systems 1 and 2 (see below). After 2500 ml had been passed through the column, fractions containing 6-keto-(3)17β-oestradiol diacetate were pooled (750 ml). The purity of these pooled fractions was checked by the use of two silica gel TLC systems: only one spot was observed which corresponded identically with the authentic marker. The

solvent from the fractions containing this steroid was removed under reduced pressure and the residue recrystallised from ethanol to give needles m.p. 173.5-175° (lit. ref. 173-175°).

Preparation of 6-Keto-17β-oestradiol

The 6-keto-17β-oestradiol diacetate was hydrolysed as follows: The diacetate (1 g) was dissolved in 20% (v/v) methanolic KOH (30 ml). The mixture was left at room temperature for 24 hr and then poured into water and extracted with ether. The ether was removed and the residue recrystallised from absolute ethanol to give plates m.p. 280-82° (yield 520 mg = 67%); $\lambda_{max.}$ 256 (ϵ=8500), 327 (ϵ=3000) nm. ; The compound moved as one spot on thin layer chromatography.

Preparation of 17β-oestradiol 6-(O-carboxymethyl) Oxime

A mixture of 6-keto-17β-oestradiol (0·5 g) and O-carboxymethyl hydroxylamine hemihydrochloride (0·5 g) was dissolved in a solution containing 20% (v/v) aqueous methanol (60 ml) and 1 M sodium acetate (40 ml). The solution was left at room temperature overnight. The volume of the solution was reduced by rotary film evaporation and water added to 200 ml. The pH of the solution was adjusted to pH 8·5 with 2N NaOH and the mixture extracted with ethyl acetate (4 x 200 ml). The combined ethyl acetate extracts were washed with dilute sodium hydroxide (pH 8·5) (2 x 300 ml) and then with water (2 x 500 ml). The extracted aqueous phase was acidified to pH 3·0 with dilute HCl, and extracted with ethyl acetate (4 x 500 ml).

The latter ethyl acetate extracts were combined and evaporated underreduced pressure and the residue recrystallised from acetone to give 17β-oestradiol-6-(O-carboxymethyl) oxime as needles m.p. 199-200°. The alkali-washed ethyl acetate extracts contained starting material which was recovered in a similar way to the above.

Spectral data

$\lambda_{max.}$(tris buffer):260 nm (ϵ= 9300), 311 nm (ϵ= 3450).

$\nu_{max.}$: 5·78, 5·91, 6·03 (shoulder), 6·18, 6·31, 9·11.

The product moved as one spot on TLC in the following two solvent systems:

1) Ethyl acetate-benzene-ethanol-acetic acid (30:30:10:0·3) R_f, 0·3 (R_f of 6-keto oestradiol = 0·8).

2) Ethyl acetate-benzene-ethanol-hexane (30:20:20:20). R_f, = 0·4 (R_f of 6-keto oestradiol = 0·62).

Mass Spectra

The mass spectrum is shown in Fig. I where it is compared with the corresponding spectra of both oestradiol and 6-keto oestradiol. All three compounds give molecular ions of very high relative abundance as expected.[8] The latter decreased in the order: oestradiol > 6-keto oestradiol > 6-(-O-carboxymethyl oxime).

All three spectra illustrate the two cleavages X-59 ($-C_3H_6OH$) and X-54 ($-C_4H_6$) thus:

Oestradiol M^+ gives M-59 = 213 and M^+-59,-54 = 159

6-Keto " M^+ gives M-59 = 227 and M^+-59,-54 = 173

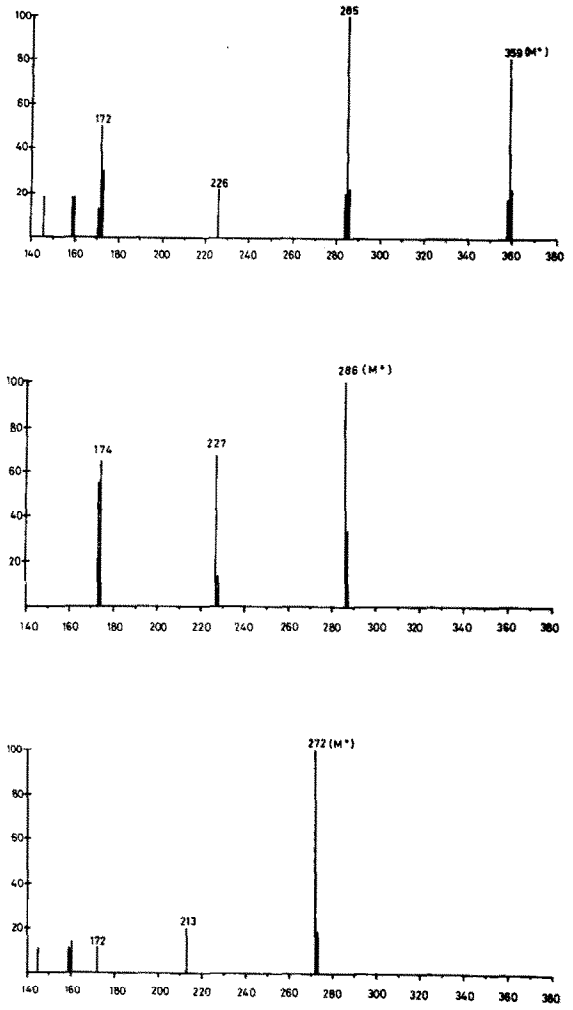


Fig. 1

The Mass Spectra of (a) oestradiol (b) 6-keto oestradiol (c) 6-(O-carboxymethyl oxime)-keto oestradiol.

The 6-(O-carboxymethyl oxime) first loses 74 ($C_2H_2O_3$) and then:

M^+-74,-59 = 226 and M^+-74, -59, -54 = 172.

Nuclear Magnetic Resonance Spectra

Nuclear magnetic resonance spectra were recorded in $(CO_3)_2CO$ solution. The following peaks, δ values: 8·48 (d, J = 6·6, H); 7·35 (d, J = 3, Ha) (see Fig. II); 7·16 (d, J = 9, Hc); 6·77 (q, J = 9·6 and 3, Hb); 4·66 (S, 2H); were clearly resolved from the bulk of the proton resonances (δ:4→0).

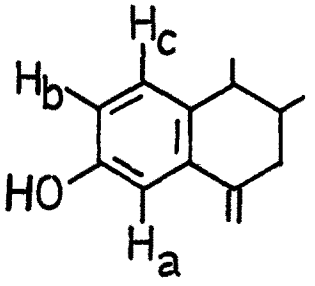


Fig II

Preparation of 17β-oestradiol-6-(O-carboxymethyl) oxime B.S.A.

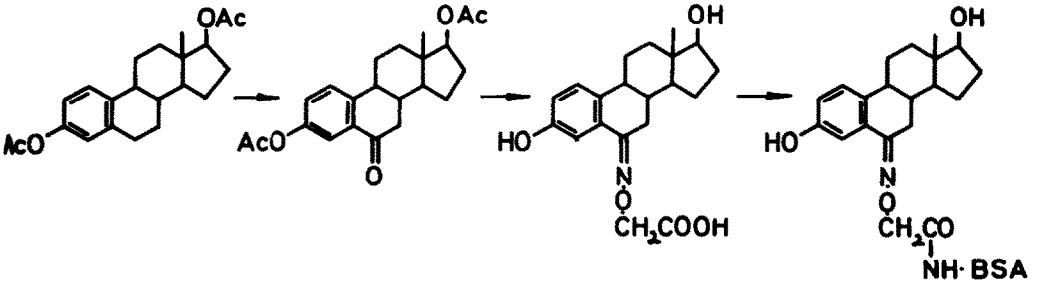
A mixture of 17β-oestradiol-6-(O-carboxymethyl) oxime (180 mg) and tri-n-butylamine (0·22 ml) was dissolved in dry dioxan (7·5 ml) and cooled in ice. To this mixture isobutylchlorocarbonate (55 µl) was added and the mixture left for 30 minutes, keeping the temperature below 10°C. A solution of Bovine Serum Albumin (0·58 g) in a mixture of water (15·3 ml), Dioxan (10·3 ml) and 1N NaOH (0·58 ml) was added and the reaction mixture left for 4 hrs at 5-10° and maintained at pH 8 with NaOH.

Low molecular weight materials were removed from reaction mixture using a Sephadex G-25 column (30 x 2·5 cm) equilibrated in 0·9% NaCl solution, by eluting with 0·9% NaCl solution.

The purified conjugate was acidified with dilute HCl (pH 4·6) and left for 4 days at 4°C.

The precipitate was centrifuged, suspended in a small volume of water and dissolved by adding enough solid $NaHCO_3$ to redissolve. The solution was then lyophylised.

A solution of the conjugate (5 mg/10 ml ≡ 7.14 μ molar) in 0.05 M tris-HCl buffer (pH 8.5) showed the following UV maxima: 225, 260 (OD = 0.94) and 311 nm. From the spectrum of the unconjugated oxime taken under similar conditions of pH etc. a solution of the oxime (5 mg/100 ml ≡ 0.14 m molar) gave an optical density of 1.29 at 260 nm), it was calculated that 14.4 moles of steroid were bound per mole of protein.



Coupling of 17β-Oestradiol to Bovine Serum Albumin via the B ring. The diacetate is oxidised to the 6-keto derivative and thence to the 6-(O-carboxymethyl) oxime. The latter is coupled to BSA by formation of a peptide bond with ε-amino groups in the BSA and the carboxyl group of the steroid.

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