# PREPARATION OF ANTISERA SPECIFIC FOR 6β-HYDROXYCORTISOL

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## 1. Introduction

Cortisol can be metabolised by reduction of the A ring, reduction of the 20-ketone function or by 6β-hydroxylation to 6β-hydroxycortisol [1,2]. The latter process may be stimulated by agents such as drugs, pesticides or polycyclic hydrocarbons which induce the hydroxylating enzymes of the hepatic microsomes [3]. Therefore the measurement of 6β-hydroxycortisol in urine may provide an index of enzyme induction in the liver microsomes. Furthermore, measurement of this steroid may be useful in the diagnosis of Cushing's syndrome, in which the increase in the production of 6β-hydroxycortisol is disproportionately large relative to that of cortisol [4]. To date, large scale investigations of the effect of various agents on the urinary content of this steroid have been restricted by the complexity of the methods of measurement. Assays developed previously have employed either a long and tedious work-up prior to a non-specific colour reaction [4] or oxidation of the sample prior to gas-liquid chromatography [5].

We therefore decided to develop a specific radioimmunoassay for this steroid. In this communication we report the synthesis of a protein conjugate of 6β-hydroxycortisol and the characterisation of antisera raised against it.

## 2. Materials and methods

### 2.1. *Reagents*

Tritiated 6β-hydroxycortisol (sp. act. 35 Ci/mmol) was obtained from the Radiochemical Centre, Amersham, UK. Samples of 6β-hydroxycortisol, 6β-hydroxycortisone, 3α, 11β, 17α, 21-tetrahydroxy-5β-pregnan-20-one and 11β, 17α, 20α, 21-tetrahydroxypregn-4-en-3-one were gifts from the MRC Reference Collection. All other steroids were purchased from Steraloids Inc., Wilton, USA. Scintillant (NE 260) was obtained from Nuclear Enterprises, Edinburgh and all samples were counted in 4 ml of this material. General reagents were purchased from BDH, Poole, UK and all solvents were redistilled prior to use.

### 2.2. *Synthesis of Antigen*

To a well stirred suspension of powdered cortisol 21-acetate (1.8 g) in anhydrous tetrahydrofuran (20 ml) containing absolute ethanol (2 ml) and triethylorthoformate (3 ml) was added 4-methylphenylsulphonic acid (0.04 g). After 50 min the reaction was quenched with pyridine (1 ml) and then poured onto ethyl acetate (200 ml) and saturated aqueous sodium bicarbonate (100 ml). The organic phase was separated, dried ($Na_2SO_4$) and concentrated to give 21-acetoxy-11β, 17α-dihydroxy-3-methoxypregna-3,5-dien-20-one as a pale yellow oil which, without further purification, was dissolved in ethanol (100 ml) and shaken in direct sunlight for 5 h. Removal of the solvent left a glass which, on trituration with ethyl acetate left a crystalline mass which was recrystallised five times from acetone to give 6β-hydroxycortisol 21-acetate as white needles (0.62 g) which had a m.p. 216–220°C (lit. m.p. 219–221°C [6]). Removal of the acetate in methanolic sodium methoxide [6] gave 6β-hydroxycortisol which was identical with an authentic specimen.

The residue from the recrystallisation of the crystalline mass was separated by preparative thin layer chromatography on silica plates, developed in ethyl acetate–cyclohexane–methanol 70:30:4 to give 6α-hydroxycortisol 21-acetate (0.08 g) which,

after treatment with methanolic sodium methoxide, gave 6α-hydroxycortisol as white plates m.p. 216–219°C (lit. m.p. 216–217°C [6]).

A solution of 6β-hydroxycortisol 21-acetate (0.21 g) and carboxymethoxylamine hemihydrochloride (0.058 g) in pyridine (5 ml) was stirred at 5°C for 24 h. Removal of the solvent in vacuo left a gum which was taken up in ethyl acetate (50 ml), washed with saturated brine (10 ml × 2), dried ($Na_2SO_4$), concentrated and recrystallised from methanol–water to give 6β-hydroxycortisol 21-acetate 3(*O*-carboxymethyl)oxime (0.16 g) as white plates m.p. 220–223°C.

$\nu_{max}$ (Nujol); 3500 (broad; hydroxyls), 1738 (acetate), 1720 (20-ketone) and 1708 $cm^{-1}$ (carboxyl).

δ ($d_6$-DMSO); 0.78 (3 H, s, 18-$CH_3$), 1.38 (3 H, d, 19-$CH_3$), 2.06.

(3 H, s, 21-acetate), 4.07 (1 H, m, 6α-H), 4.19 (1 H, m, 11α-H), 4.45.

(2 H, d, O.$CH_2$.CO), 4.90 (2 H, AB system, 20-$CH_2$) and 5.70 and 6.34 (1 H, d, 4-H).

The splitting of the peaks for 19-$CH_3$, O.$CH_2$.CO and 4-H protons are similar to those observed in the n.m.r. spectrum of cortisol 3(*O*-carboxymethyl)oxime [7] and show that the compound is a 2:5 mixture of *syn* and *anti* isomers. The chemical shift and splitting pattern of the peak for the 6α-proton are identical to those observed, for the corresponding proton, in the n.m.r. spectrum of 6β-hydroxycortisol 21-acetate.

Mass spectrum; $M^+$ *f*493 ($C_{25}$ $H_{35}$ N $O_9$ requires 493).

The derivatised steroid was coupled to bovine serum albumin (BSA) using the mixed anhydride technique [8] and the molar steroid: protein ratio of the conjugate was determined by ultraviolet spectroscopy to be 32.

### 2.3. *Immunisation and characterisation of antisera*

Antisera were raised and characterised as described previously [8].

Table 1
Specificity and working dilutions of anti-6β-hydroxycortisol-3-BSA antisera

| Steroid | Antiserum | | | |
|---|---|---|---|---|
| | R1B2 | R2B2 | R3B2 | R4B2 |
| 6β-Hydroxycortisol | 100 | 100 | 100 | 100 |
| 6α-Hydroxycortisol | 1.9 | | 3.7 | 5.7 |
| Cortisol | 1.4 | 58.1 | 1.6 | 3.6 |
| Cortisone | < 0.05 | | < 0.05 | 0.05 |
| 6β-Hydroxycortisone | < 0.05 | | 0.13 | 72 |
| 6β-Hydroxycortisol 21-acetate | 9.0 | 0.56 | 3.3 | 3.0 |
| 3α, 11β, 17α, 21-tetrahydroxy -5β-pregnan-20-one | < 0.05 | | < 0.05 | < 0.05 |
| 11β, 17α, 20α, 21-tetrahydroxypregn--4-en-3-one | < 0.05 | | < 0.05 | < 0.05 |
| Corticosterone | 0.09 | | 0.18 | 0.10 |
| 11-Desoxycortisol | < 0.05 | | 0.08 | 0.02 |
| 21-Desoxycortisol | 0.05 | | < 0.05 | 0.10 |
| 21-Hydroxyprogesterone | < 0.05 | | < 0.05 | < 0.05 |
| Progesterone | < 0.05 | | < 0.05 | < 0.05 |
| Cholesterol | < 0.05 | | < 0.05 | < 0.05 |
| Oestradiol | < 0.05 | | < 0.05 | < 0.05 |
| Testosterone | < 0.05 | | < 0.05 | < 0.05 |
| Working dilution (Reciprocal) | 2500 | 1000 | 350 | 550 |

Coding of antisera: R1–R4 refers to four individual rabbits and B2 refers to blood samples removed after the third booster injection.

## 3. Results

The working dilutions of the blood samples removed from the four rabbits following the second booster injections were as follows: R1B1 120:1, R2B1 < 100:1, R3B1 100:1, and R4B1 < 100:1. After the third booster injections the titres were increased considerably and the blood samples obtained at this time were fully tested; the cross reactions and working dilutions being presented in table 1. The detection limit for 6β-hydroxycortisol was 20 pg using antiserum R1B2 (distinguishable from zero with 99% certainty).

## 4. Discussion

Steroid-specific antisera for radioimmunoassay can be prepared by linking the steroid to a carrier using existing functional groups of the steroid molecule. The specificity of the resulting antisera is high only when the accessible part of the steroid is structurally unique [9,10]. In particular, it has been shown that corticoid-specific antisera may be obtained using antigens in which the steroid is linked to the immunogenic carrier through the 3 position [9] and so we elected to synthesise a 6β-hydroxycortisol-3-BSA conjugate.

6β-Hydroxycortisol 21-acetate was prepared by photolytic autooxidation of the corresponding 3,5-dienol 3-ethyl ether [11]. The selective formation of the 3(*O*-carboxymethyl)oxime via an enamine [12] was not possible in this case because of the presence of the 6β-hydroxyl function, however we found that, in cold pyridine, the 21-acetate provided sufficient protection of the 20-ketone to allow selective conversion of the 3-ketone. Inspection of the n.m.r. spectrum of the product confirmed the presence of the carboxymethylene group and indicated there to be a mixture of *syn* and *anti* isomers. The chemical shift and splitting pattern of the 6α-proton in the spectrum show that the stereochemistry of the 6β-hydroxyl had not been disturbed. Previous reports have shown that there is no obvious advantage in removing the acetate groups prior to coupling a derivatised steroid to BSA [9,13] and the low cross-reactions for 6β-hydroxycortisol 21-acetate shown in table 1 confirm this.

Three of the four antisera tested showed good discrimination against cortisol and were fully characterised. The best of these (R1B2) showed good all-round specificity and should be of value in the measurement of 6β-hydroxycortisol in urine, where the compounds that did give some cross-reaction are, at most, only present in relatively low concentrations. As the normal daily production of 6β-hydroxycortisol is approximately 300 μg/day [14], it should be possible to measure this steroid in a fraction of a microlitre of urine. Thus it may be possible to assay unextracted urine, since such a tiny volume of urine is unlikely to interfere in an assay [15].

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