PREPARATION OF ANTISERUM SPECIFIC FOR OESTRADIOL 17-GLUCURONIDE

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1. Introduction

Considerable attention has been recently focused on the physiological significance of steroid hormone conjugates. Usually the conjugated steroids are determined indirectly after hydrolysis by enzymatic and/or chemical means. Such procedures have inevitable disadvantages of lack of reliability due to incomplete hydrolysis and formation of artifacts, and in loss of information about the conjugate forms. Consequently several investigators have attempted to prepare anti-steroid glucuronide sera for use in radioimmunoassays [1–5]. However, antisera so far obtained are not satisfactory in respect of specificity. We have undertaken the development of a radioimmunoassay method specific for oestrogen glucuronides without prior hydrolysis. In this communication we report the synthesis of oestradiol 17-glucuronide–bovine serum albumin (BSA) conjugate in which the hapten is linked to protein through the C-2 position and characterisation of antiserum raised against it.

2. Materials and methods

2.1. *Reagents*

[6,7-^{3}H]Oestradiol 17-glucuronide (spec. act. 45.9 Ci/mmol) was supplied by New England Nuclear, Boston, USA. Methyl (3-hydroxy 1,3,5(10)-oestratrien-17β-yl-2,3,4-tri-*O*-acetyl-β-D-glucopyranoside)uronate (oestradiol 17-glucuronide acetate-methyl ester) and other conjugated steroids were prepared in these laboratories. All other steroids were kindly donated from Teikoku Hormone Co., Tokyo, Japan. General reagents were purchased from Nakarai Chemicals, Ltd, Kyoto, Japan and all solvents were redistilled prior to use.

2.2. *Preparation of antigen*

To a solution of oestradiol 17-glucuronide acetate-methyl ester (I) (400 mg) in acetic acid (10 ml)–chloroform (5 ml) was added conc. HNO_3 (0.07 ml) dropwise and stirred at room temperature for 20 min. The resulting solution was poured into ice-water, neutralised and extracted with ethyl acetate. The organic phase was washed with water, dried (Na_2SO_4) and evaporated. An oily residue was chromatographed on silica gel. Elution with benzene–ether (95:5) and recrystallisation of the eluate from methanol gave 2-nitro-oestradiol 17-glucuronide acetate–methyl ester (II) (159 mg) as pale-yellow needles, m.p. 201–204°C, $[\alpha]_D^{20}$ + 23.6°C (c = 0.89, $CHCl_3$).

Nmr ($CDCl_3$)δ:
0.78 (3H, s, 18–CH_3)
2.00 (6H, s, –$COCH_3$)
3.74 (3H, s, –$COOCH_3$)
4.60 (1H, d, J = 7 Hz, pyranose–C_1–H)
4.85–5.35 (4H, m, pyranose–CH–OAc)
6.82 (1H, s, 4–H)
7.95 (1H, s, 1–H).

To a solution of II (150 mg) in methanol (10 ml) was added 1 N NaOH (1.4 ml) and the mixture allowed to stand at 4°C for 3 days. The resulting precipitate was collected by filtration and recrystallised from methanol to give the sodium salt of 2-nitro-oestradiol 17-glucuronide (III) (50 mg) as red plates, m.p. $>$300°C, $[\alpha]_D^{20}$ –7.9°C (c = 0.81, H_2O).

Nmr (D_2O)δ:
1.19 (3H, s, 18–CH_3)
3.90–4.50 (4H, m, pyranose–CH–OH)

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4.93 (1H, d, J = 7 Hz, pyranose–C_1–H)
6.92 (1H, s, 4–H)
8.12 (1H, s, 1–H).

To a solution of III (80 mg) in 0.1 N NaOH (5 ml) was added $Na_2S_2O_4$ (240 mg) and the mixture stirred at room temperature for 1 h. The resulting solution was passed through a column of Amberlite XAD-2 resin. After thoroughly washing the resin with distilled water the glucuronide was eluted with methanol. The eluate was further purified by chromatography on a Sephadex G-25 column. The desired fractions were collected and lyophilised to give 2-amino-oestradiol 17-glucuronide (IV) (65 mg).

Nmr (pyridine–d_5)δ:
1.15 (3H, s, 18–CH_3)
3.91–4.60 (4H, m, pyranose–CH–OH)
4.90 (1H, d, J = 7 Hz, pyranose–C_1–H)
6.92 (1H, s, 4–H)
8.02 (1H, s, 1–H)

The derivatised steroid (IV) (50 mg) was coupled to BSA (110 mg) by condensation with glutaraldehyde [6] followed by reduction with $NaBH_4$. The reaction mixture was processed in the usual manner to provide a steroid–BSA conjugate (V) (130 mg) as a fluffy powder. The molar steroid:protein ratio of the conjugate was determined spectrometrically (at 295 nm) to be 15.

2.3. *Immunisation and characterisation of antiserum*

A domestic strain male albino rabbit was immunised with the conjugate and antiserum raised against it was characterised as previously reported [7].

3. Results

Five months after initial injection serum from the immunised rabbit showed a remarkably increased binding activity to oestradiol 17-glucuronide. The association constant (K_a) was determined to be 2.58×10^9 M^{-1} by a Scatchard plot [8]. A standard curve was constructed with a 1:20 000 dilution of the rabbit serum using tritiated oestradiol 17-glucuronide as labelled antigen. The cross-reactivity of anti-oestradiol 17-glucuronide antiserum with selected steroids is listed in table 1.

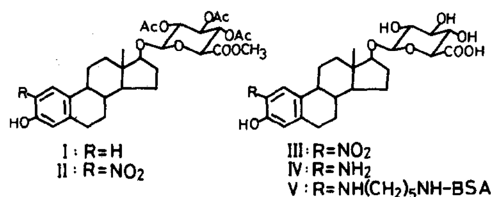

Fig.1

Table 1
Per cent cross-reaction of anti-oestradiol 17-glucuronide antiserum with selected steroids

Steroid	Cross-reaction (%)
Oestradiol 17-glucuronide	100
Testosterone 17-glucuronide	2.94
Oestrone glucuronide	0.10
Oestradiol 3-glucuronide	0.10
16-Epioestriol 3-glucuronide	0.19
Oestriol 3-glucuronide	< 0.001
Oestriol 16-glucuronide	< 0.001
Oestriol 17-glucuronide	0.06
2-Hydroxyoestradiol 2-glucuronide	< 0.001
Androsterone glucuronide	< 0.001
Pregnanediol 3-glucuronide	< 0.001
Oestradiol 17-sulphate	< 0.001
Oestriol 3-sulphate	< 0.001
2-Hydroxyoestradiol 3-sulphate	< 0.001
Dehydroepiandrosterone sulphate	< 0.001
Oestrone	< 0.001
Oestradiol	< 0.001
Oestriol	< 0.001
16-Epioestriol	< 0.001
2-Hydroxyoestradiol	< 0.001
2-Methoxyoestrone	< 0.001
Testosterone	< 0.001
Progesterone	< 0.001
Cortisol	< 0.001

4. Discussion

Several investigators have attempted to develop a radioimmunoassay method for the steroid glucuronide not involving prior hydrolysis [1–5]. These approaches, however, failed due to insufficient specificity of the antiserum used. The unsatisfactory results appeared to be ascribable to the structure of the antigens, in which the steroid was coupled to protein through the carboxyl group of the glucuronyl moiety.

We have used 2-amino-oestradiol 17-glucuronide,

derivable from oestradiol 17-glucuronide acetate-methyl ester in three steps, coupled to BSA using glutaraldehyde as a cross-linking agent [6]. It is evident from the data in table 1 that antiserum raised against this conjugate is highly specific for oestradiol 17-glucuronide, exhibiting no significant cross-reaction with oestrogen ring D and A glucuronides. No cross-reactivity was found with free oestrogens, their sulphates and other steroids.

It should be emphasised that antiserum elecited against the antigen in which the hapten is linked to the immunogenic carrier through the C-2 position remote from ring D, is capable of discriminating oestradiol 17-glucuronide from its closely related free and conjugated oestrogens. The design of similar hapten-carrier conjugates may be further applicable to the preparation of antisera specific for other steroid conjugates.

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References

[1] Kellie, A. E., Samuel, V. K., Riley, W. J. and Robertson, D. M. (1972) J. Steroid Biochem. 3, 275–288.
[2] Samarajeewa, P. and Kellie, A. E. (1975) Biochem. J. 151, 369–376.
[3] Niswender, G. D., Nett, T. M., Meyer, D. L. and Hagerman, D. D. (1975) in: Steroid Immunoassay, Proc. Fifth Tenovus Workshop (Cameron, E. H. D., Hillier, S. G. and Griffiths, K. eds) pp. 61–66, Alpha Omega Publishing, Cardiff.
[4] Adlercreutz, H., Lehtinen, T. and Tikkanen, M. (1976) J. Steroid Biochem. 7, 105–107.
[5] Soares, J. R., Zimmermann, E. and Gross, S. J. (1976) FEBS Letters 61, 263–266.
[6] Avrameas, S. (1969) Immunochemistry 6, 43–52.
[7] Nambara, T., Takahashi, M., Tsuchida, Y. and Numazawa, M. (1974) Chem. Pharm. Bull. (Tokyo) 22, 2176–2180.
[8] Scatchard, G. (1949) Ann. NY Acad. Sci. 51, 660–672.