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IMMUNOFLUORIMETRIC ASSAY OF OESTRADIOL-17β

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SUMMARY

Antiserum raised in a goat against 17β-oestradiol-6-(O-carboxymethyl) oxime-bovine serum albumin was fractionated to give its respective IgG fractions. Oestradiol-17β specific antibodies were isolated and their IgG_1 fraction fluorescently labelled with 4-methylumbelliferone-3-acetic acid. An immunofluorimetric assay for oestradiol-17β is described in which endogenous steroid competes with AH-Sepharose 4B-immobilised oestradiol-17β-6-(O-carboxymethyl)oxime for these fluorescently labelled antibodies.

INTRODUCTION

Oestradiol-17β in human biological fluids has been estimated by a variety of methods, chiefly by radioimmunoassay. In haptenic immunoassay, either the antibody or the hapten may be labelled, however the process of hapten labelling appears the more popular approach at the present time. New techniques such as affinity chromatography have recently made it possible to obtain antibodies in a highly purified state. Miles and Hales[1] and Addison and Hales[2] have successfully radioactively labelled antibodies, and used them for immunoradiometric techniques. Antibodies can also be labelled with non-radioactive substances. Labelling the antibody molecule with a highly fluorescent compound enables the immunoassay to be performed without the disadvantages of handling and counting radioactive material. Aalbarse [3] has successfully assayed human IgG by the use of fluorescein-labelled anti-human IgG. Using a similar technique, Burgett *et al.*[4] have recently applied the immunofluorimetric technique to the quantitation of the C_4 component of human complement.

The present communication presents an assay for oestradiol-17β, in which antibodies elicited by oestradiol-17β-6-(O-carboxymethyl)oxime-BSA are fluorescently labelled with 4-methylumbelliferone-3-acetic acid. This particular fluorescent label offers several analytical advantages over fluorescein and its derivatives.

MATERIALS AND METHODS

Materials

Assay buffer (pH 7.1) 0.1 M was prepared as described by Abraham[5]. Two stock solutions of sodium phosphate buffer were used. Solution A was 0.2 M NaH_2PO_4, and solution B 0.2 M Na_2HPO_4 in water. The assay buffer itself consisted of 195 ml of solution A, 305 ml of solution B, 1 gm of sodium azide and 9 gm of sodium chloride made up to 1 litre with water.

Assay gelatin buffer. This was prepared by dissolving 1 gm of gelatin to 1 litre of the above assay buffer.

Steroids were obtained from Koch-Light Laboratories, Colnbrook, Bucks., U.K. Radioactive [2,4,6,7-^{3}H]-oestradiol-17β (89 Ci/mmol) was obtained from the Radiochemical Centre, Amersham, Bucks., U.K. Cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluene sulphonate (CMC) was obtained from Sigma, St. Louis, U.S.A. AH-Sepharose 4B, DEAE-Sephadex A50 and Sephadex G-25 were obtained from Pharmacia, Uppsala, Sweden. Norit A charcoal was purchased from Sigma, St. Louis, U.S.A.

Steroid derivatives were prepared for conjugation to AH-Sepharose 4B as ligands in the affinity chromatography immunoadsorption and solid-phase assay.

Glossary

The trivial names and symbols mentioned in the text are given below with their corresponding I.U.P.A.C. names.

Symbol	Trivial name	I.U.P.A.C.
E_1	Oestrone	1,3,5(10)oestratriene-3-ol-17-one
E_2	Oestradiol-17β	1,3,5(10)oestratriene-3,17βdiol
4MU-3-CH_2COOH	4-methylumbelliferone-3-acetic acid	1,2-benzonpyrone-7-hydroxy-4-methyl-3-acetic acid

Symbols used for steroid derivatives in the text are:

$E_2$6CMO	Oestradiol-17β-6-(O-carboxymethyl)oxime
$E_1$6CMO	Oestrone-6-(O-carboxymethyl)oxime

The hapten oestradiol-17β-6-(O-carboxymethyl)oxime ($E_2$6CMO) was synthesized according to the method of Dean, Exley and Johnson[6]. Oestrone-6-(O-carboxymethyl)oxime ($E_1$6CMO) was prepared according to Dean, Rowe and Exley[7].

Immunoadsorbents. $E_1$6CMO-AH-Sepharose 4B and $E_2$6CMO-AH-Sepharose 4B were prepared by carbodiimide (CMC) condensation using a modification of the method of Cuatrecasas and Affinsen[8]. The non-covalently bound steroids were removed from the AH-Sepharose 4B by the washing programme devised by Exley and Avakian[9]. The concentration of functionally available conjugated hapten (mol/ml) of swollen-Sepharose 4B gel was calculated by radioimmunoassay. Immunoadsorbents were then stored in the assay buffer at 4°C until the need to use them arose.

Fluorimetric measurement. All fluorimetric measurements were made using an Hitachi Model 203 spectrofluorimeter (Perkin-Elmer Ltd., Beaconsfield, Bucks, England).

Methods

Antisera. This study made use of antisera raised in a male goat by immunisation with $E_2$6CMO-BSA [10]. The total immunoglobin fraction was obtained by precipitation with 50% saturated ammonium sulphate, a standard immunological procedure. The precipitate was dissolved in 0.5% NaCl solution containing 0.1% sodium azide and exhaustively dialysed against a large volume of this solution. The IgG component was fractionated by ion exchange chromatography using DEAE Sephadex A50. The two goat IgG subclasses (IgG_1 and IgG_2) were eluted. The IgG_2 eluted first with TRIS-acetate buffer, pH 7.0, 0.01 M, followed by IgG_1 when 3% NaCl gradient was used. The concentration of anti-E_2-17β antibodies present in the IgG_1 fraction was determined by the saturation curve technique of Abraham[5].

Isolation of anti-E_2-17β antibodies. Molar excess (in terms of $E_1$6CMO ligand) of S4B-$E_1$6CMO was allowed to react in assay buffer under gentle stirring at room temperature for 2 hr with the IgG_1. The reactants were transferred to a small 10 ml column and washed with at least 200 volume excess of the assay buffer. Washings were collected in order to estimate the amount of bound antibody. The bound anti-E_2 antibodies were competitively eluted with assay buffer containing 2 μg unlabelled E_2-17β/ml with 10 pg[^{3}H]$_2$-E_2-17β/ml as radioactive label. The eluted antibody was filtered through a Sepahdex G-25 column, pooled and ether-extracted (10 ml ether: 1 ml of antibody solution) three times. The ether was removed by gently blowing under a stream of air followed by dialysis. This treatment removed about 99.8% of E_2-17β from the antibody. The amount of recovered antibody was again estimated by saturation curve technique.

Synthesis of 4-methylumbelliferone-3-acetic acid (4MU-3-CH_2COOH). The synthesis was performed essentially as by the method of Banerjee[11]. The two-stage reaction involved first, the synthesis of the ester, 4-methylumbelliferone-3-ethylacetate, and secondly the hydrolysis of this ester with 10% methanolic KOH to give 4MU-3-CH_2COOH (Fig. 1). Recrystallisation was carried out in 20% ethanol:water and gave colourless needles m.p. 265–267°C (Literature 268°C). The compound was maximally excitated at 365 nm, and fluoresces maximally at 455 nm.

Preparation of 4-methylumbelliferone-3-acetic acid–IgG_1 conjugate. The condensation of 4MU-3-CH_2COOH with available active amino groups of the purified anti-E_2 antibody to form a peptide link was effected using the carbodiimide CMC. An active intermediate of 4MU-3-CH_2COOH and CMC was first formed in 5 ml of 0.01 M phosphate buffer, pH 5.0, by reacting 4 mg 4MU-3-CH_2COOH and 120 mg CMC (high molar excess of CMC) at 10–12°C for 45 min. This intermediate was added slowly to 2 ml solution of the same buffer containing 165 μg of the purified IgG_1, whose antibody binding sites had been protected by adding 40 μg of oestrone. The antibody site protection was considered necessary in case essential active amino acid residues in the active site were involved in 4MU-3-CH_2COOH conjugation, resulting in possible antibody deactivation. The mixture was stirred at 10–12°C for 3 h and then dialysed in 0.2 M NaCl containing 0.1% sodium azide before extracting with ether three times to remove the oestrone. The volume of the preparation after dialysis was reduced using polyethylene glycol powder. It was then layered on a Sephadex G-25 column, equilibrated with assay buffer and 1 ml fractions collected. Aliquots were removed from each column fraction and these were used to determine both fluorescence and the ability of the antibody in each fraction to bind a given load of [^{3}H]-E_2. The fluorescence unconnected with the antibodies was removed. The procedure was repeated in the case where the antibody binding sites were not protected by oestrone. The total antibody recovered after conjugation was estimated by the saturation curve technique [5] using oestradiol-17β.

Determination of 4MU-3-CH_2COOH/antibody ratio in the conjugate. A standard graph of fluorescence versus 4MU-3-CH_2COOH (10–100 ng/ml concentration) was set up by making the appropriate dilutions of the umbelliferone in pH 10.0 glycine buffer. Appropriate dilutions of the fluorescent antibody conjugate were made up in the glycine buffer to a standardised volume, and the fluorescence estimated from the standard graph.

Immunofluorimetric assay of oestradiol-17β. The amount of Sepharose 4B-linked $E_2$6CMO hapten to be used per assay tube was determined by radioimmunoassay. The amount of this material functionally equivalent to 15 pg of E_2-17β was chosen for the construction of antibody, dilution curves using the fluorescently labelled antibodies. Antibody dilution corresponding to about 65% binding of the fluorescent antibodies (bound to the immobilised $E_2$6CMO) was

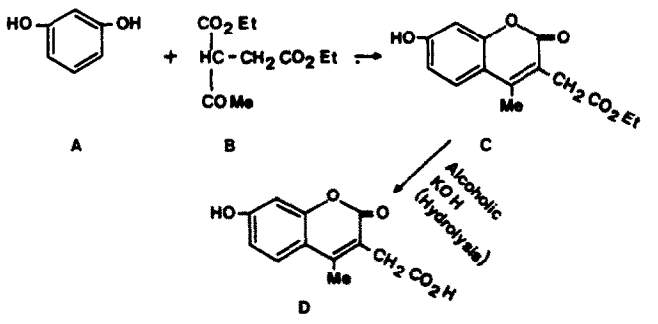

Fig. 1. Synthesis of 7-hydroxy-4-methyl coumarin-3-acetic acid (4MU-3-CH_2COOH). A. Resorcinol; B. Diethylacetylsuccinate; C. 4 methylumbelliferone-3-ethyl acetate; D. 4 methylumbelliferyl-3-acetic acid.

used in the immunofluorimetric assay. One microlitre of this antibody solution was used per assay tube.

The final assay system used 0.1 ml of $E_2$6CMO-S4B, 0.1 ml of fluorescent antibody and 0.1 ml of varying doses of E_2-17β in assay buffer containing 0.1% gelatin. The assay tubes were incubated and continually mixed in an "end-to-end" mixer at R.T. for 2 h and then the contents finally equilibrated by standing at 4°C for 45 min. The contents were then centrifuged and 0.2 ml aliquots of the supernatant transferred into tubes containing 1 ml of 0.2 M sodium–glycine buffer, pH 10.4. The fluorescence of the free (labelled) antibody was then determined.

RESULTS

Thirty percent of the anti-oestradiol-17β antibodies applied to the affinity chromatography column (AH-S4B-$E_2$6CMO) were recovered. Ether extraction permitted the preservation of over 95% of the activity of the recovered antibody. There appeared to be no loss of antibody activity, whether or not the binding sites were protected during fluorescent conjugation. In an effort to ascertain that no fluorescence quenching was induced by the conjugation process, an experiment was performed in which N-α-acetyl-L-lysine was conjugated by carbodiimide (CMC) to 4MU-3-CH_2COOH in a 1:1 molar ratio. This showed that there was virtually no loss in fluorescence due to such a conjugation process. Calculation showed that an average of 16 molecules of 4MU-3-CH_2COOH were conjugated per molecule of antibody. Harrison and Mage[12] determined the amino acid composition of ovine serum and deduced that the % lysyl composition of the IgG_1 fraction was 4.8%. If it is assumed that there is not much variation in this figure in goat IgG_1 fraction, then about 45% of available lysyl groups were on average fluorescently labelled.

Figure 2 shows a typical immunofluorimetric assay standard curve for $E_2$17β. The ordinate shows the percentage of bound fluorescently-labelled antibody. Less than 100 pg of oestradiol-17β can be measured with accuracy. Several immunofluorimetric assay standard curves were obtained from triplicate standard amounts. The fluorescence readings were found

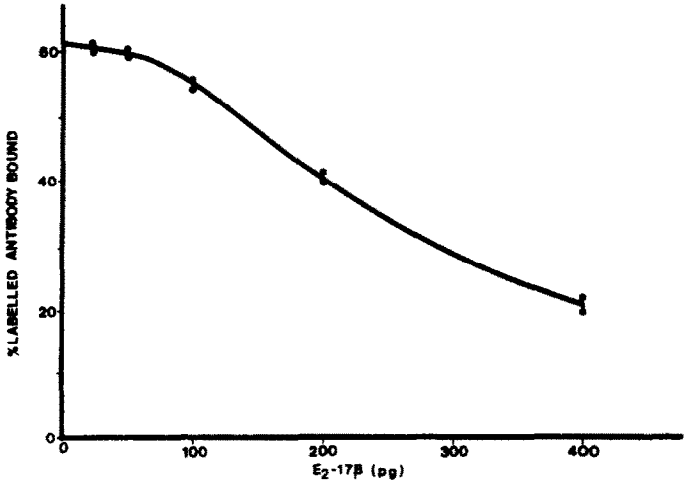

Fig. 2. Immunofluorimetric assay standard curve for E_2-17β using fluorescently (4MU-3-CH_2COOH) labelled anti-E_2-17β (IgG) antibody.

to be highly reproducible. The fluorescent antibodies were found to be stable both in buffer or when lyophilised for periods up to 6 months or more when they were stored at 4°C.

DISCUSSION

The standard curve (Fig. 2) reveals that reasonably sensitive immunofluorimetric assay has been achieved. However, when compared with radioimmunoassay this is slightly less sensitive. The slightly less sensitive nature of the assay could be due to the following reasons. The level of detectable fluorescence demanded that more antibody be used in the assay; the use of a homologous anti-serum (anti-$E_2$6CMO-BSA) and the multivalent binding of immobilized hapten with the labelled antibody clearly contributed to the insensitivity. Sensitivity for the immunofluorimetric assay of this nature could be significantly improved by using an heterologous antiserum. This was demonstrated for E_2-17β in enzyme-immunoassay by Exley and Abuknesha [13]. Using either the homologous (anti-$E_2$6CMO-BSA) serum or an heterologous antiserum to E_2-17β-3-hemisuccinyl-BSA they showed a better sensitivity with the latter antiserum.

The sensitivity of the immunofluorimetric assay was achieved because of the use of a highly sensitive fluorescent label. Fluorescently-labelled antibodies using fluorescein derivatives (notably fluorescein isothiocyanate, FITC) have a very wide application in immunohistochemical studies. Although 4MU-3-CH_2COOH possesses about 10% less molar fluorescence than FITC, it has advantages over this compound. One of the advantages of 4MU-3-CH_2COOH is that background fluorescence is minimal at neutral pH, and is maximal at alkaline pH. This is not true of FITC. This latter compound has a very high fluorescence at neutral pH; at pH 7.0 FITC exhibits about 80% of its maximum fluorescence. Its maximum molar fluorescence occurs at about pH 8.0 as shown by Burd *et al.*[14]. Such high background fluorescence is undesirable for immunofluorimetric assay. Again the activation and fluorescence spectra for FITC (490 and 520 nm respectively) are less well separated than those for 4MU-3-CH_2COOH (365 and 455 nm respectively). When the fluorescence and excitation wavelengths are closer together, the distortion due to light scattering severely limits general sensitivity (Udenfriend, 1962).[15] Hence, 4MU-3-CH_2COOH offers an improvement by limiting errors due to light scattering effect.

This is probably the first time an immunofluorimetric assay has been used for steroid estimation. A good fluorimeter is cheaper than radioactive counting equipment, thus possession of the fluorescently labelled antibodies enables the assay to be performed at less expense than radioimmunoassay since no costly scintillation reagents for radioactive assessments are involved. Again quantitation by fluorescence is rapid compared to assessment of radioactive counts making the assay slightly more rapid than radioimmunoassay.

The commercial production of fluorescently labelled antisera using this type of fluorescent label appears an attractive possibility. The use of immobilized hapten ($E_2$6CMO) as a solid phase technique for separating free from bound complex, obviated the need for a second antibody precipitation, hence reducing costs.

Finally, since anti-$E_2$6CMO antibodies are highly specific with $E_2$17β (cross reacting only 1–2% against the major interfering steroid, oestrone), a biological sample, such as blood plasma, need not be chromatographically separated before assay.

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