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THE RADIOIMMUNOASSAY OF STEROID CONJUGATES

A. E. KELLIE

The Courtauld Institute of Biochemistry, The Middlesex Hospital Medical School, London W1P 5PR, England

SUMMARY

The determination of steroid hormones and their metabolites by radioimmunoassay is now well established. Conventionally the steroid is converted into a derivative which introduces a carboxylic acid group and this group is joined covalently as a hapten to a carrier protein. Many steroids, both endogenous and exogenous, are excreted as glucuronides; such conjugates already have the carboxylic acid group necessary for covalent linkage and can be linked directly to protein. Several steroid–glucuronosyl–BSA complexes have been prepared and have been used as antigens in rabbits. Antisera thus produced bind steroid glucuronides with high specificity and they can be used to determine steroid glucuronides without preliminary hydrolysis. The development of this type of assay has been limited by the non-availability of labelled steroid conjugates of high specific radioactivity and general methods for the preparation of these radioligands have now been established. Conjugates treated in this way include; oestrone-3-, oestradiol-3, oestradiol-17β-, oestriol-3-, oestriol-16α-, dehydroepiandrosterone-3β- and testosterone-17β-glucuronides. The work is currently being extended to include other steroid conjugates the determination of which has clinical importance.

INTRODUCTION

It is accepted that in humans endogenous and many exogenous steroids are excreted in urine principally as water-soluble conjugates. Derivatives in the pregnane series are almost exclusively excreted as glucuronides, but metabolites of the androgens appear mainly as glucuronides but also as sulphates ($\sim 15\%$). With oestrogens the principal excretory form is as a glucuronide although mixed conjugates containing both glucuronide and sulphate radicals have been reported [1]. Even in late pregnancy urine there is very little oestrogen sulphate [2].

Almost without exception the conventional methods of assaying these steroid forms involves the hydrolysis of the water-soluble conjugates to liberate the corresponding free steroids for which methods of assay exist. This hydrolytic step whether carried out by hot acid, by enzyme or by solvolysis is unsatisfactory in many respects. Because, in general, radioactive-labelled steroid conjugates are not available as internal standards, it is not easy to determine the loss which occurs during the hydrolysis and subsequent work-up. A more serious feature of this step is that from the outset the identity of the conjugate form is lost *e.g.* oestriol-3-glucuronide, oestriol-16α-glucuronide and oestriol-3-sulphate-16α-glucuronide, all of which are present in late pregnancy urine [1], will subsequently be processed and determined as oestriol. Moreover, when the hydrolysis is complete the assay methods available are limited in sensitivity.

There appears to be no sound reason why these conjugated forms should not be determined by the radioimmunoassay techniques which are already available for steroid assay [3]. Steroids cannot be used as haptens without modification and the conventional method is to convert them into a derivative which includes a carboxylic acid group, *e.g.* a hemisuccinate, an O-(carboxymethyl-oxime) [4] or a thioalkanoic acid [5]. Steroid glucuronides, and incidentally bile acids, already contain a carboxylic acid group; they require no modification before being joined covalently to a carrier protein. Several steroid glucuronides (Table 1), some of clinical importance, have been synthesized by established methods [6–9] and have been covalently linked to bovine serum by the mixed acid anhydride method [10]. More recently we have prepared three ring-A steroid sulphates, oestrone-3-sulphate dehydroepiandrosterone-3β-sulphate, pregnenolone-3β-sulphate [11] and have successfully joined these conjugates covalently to BSA using the carbodimide reaction [12]. At the time of writing we have immunized a number of rabbits with these steroid sulphate antigens but have not yet assayed the rabbit antisera for antibody titre.

Immunization

A conventional immunization procedure was used. The lyophilized antigen (2 mg) was homogenized in water (1 ml) in the presence of Freund's adjuvant (1 ml) and small doses equivalent to 20 μg of steroid conjugate were injected subcutaneously at multiple sites on the flanks of the rabbit. Similar booster doses were given at approximately one month intervals and test blood samples were withdrawn at monthly intervals after the sixth injection. Each test antiserum was examined for antibody titre by constructing a serum dilution curve in the presence and in the absence of non-radioactive steroid conjugate *e.g.* the anti-oestradiol-3-glucuronide serum at serial dilutions was incubated with 0·12 pmol [6,7-^{3}H]oestradiol-3-glucuronide in the absence and in the presence of 1·06 pmol of non-radioactive oestradiol-3-glucuronide. The form

Table 1. Steroid conjugates used as haptens

Glucuronides	Sulphates
Oestrone-3-glucuronide (13)	Oestrone-3-sulphate (14)
Oestradiol-3-glucuronide (9)	
Oestradiol-17β-glucuronide (28)	
Oestriol-3-glucuronide (15)	
Oestriol-16α-glucuronide (—)	
Dehydroepiandrosterone-3β-glucuronide (20)	Dehydroepiandrosterone-3β-sulphate (14)
Testosterone-17β-glucuronide (22)	
5β-Pregnane-3α,20α-diol-3α-glucuronide (10)	Pregnenolone-3β-sulphate (23)
5β-Pregnane-3α,17,20α-triol-3α-glucuronide (—)	

Figures in parenthesis indicate the mol/mol incorporation into BSA.

of presentation illustrated in Fig. 1 makes it possible to determine the change in the percentage binding of the radioligand brought about by 1·06 pmol of non-radioactive conjugate competitor at different dilutions of the antiserum. This form of assay reveals the sensitivity of the assay procedure but does not indicate the specificity.

Source of radioligands

The steroid conjugates used as radioligands had a specific radioactivity 50 Ci/mmol and with a few notable exceptions these were not available from commercial sources and had to be synthesized. For very practical reasons we have preferred to synthesize an unsaturated steroid conjugate which could be reduced with carrier-free tritium in the presence of palladium on charcoal to the required radio-labelled steroid conjugate. One of the simpler syntheses will serve to illustrate the approach Fig. 2 and in this synthesis the location of the glucuronide radical at C-3 is not in doubt. With other conjugates unambiguous routes were devised to ensure the correct position of the substitution. Figures 3 gives an indication of the blocking procedures used to achieve these ends. [6,7-^{3}H]-Oestrone-3-sulphate was prepared from 6-en-oestrone by the method of Joseph, Dusza and Bernstein[11]. Table 2 lists the radioactive steroid conjugates used as radioligands in the present study.

Calibration and specificity of antisera

Calibration graphs for the radioimmunoassay of steroid conjugates were prepared by incubating the diluted antiserum (1:72,000; 100 μl) with the radioligand (60 pg; 100 μl) and a range of the non-radioactive steroid conjugate (0–1000 pg; 100 μl) at 30°C for 0·5 h. Unbound radioligand was removed by adding Dextran-charcoal suspension and the bound ligand remaining in solution was determined by β-scintillation counting (Packard Model 3375 and 3385). Most steroid glucuronides and sulphates do not mix with toluene-based phosphors but a dioxan-xylene based media was found satisfactory. The mixture used contained 3·5 g P.P.O.; 100 g naphthalene; 230 ml ethanol; 385 ml xylene and 385 ml dioxan; the counting efficiency for tritium was about 35%. Cross reactions of steroids and steroid conjugates against the anti-steroid conjugate sera were carried out in phosphate buffer (0·1 M; pH 7·0) containing 0·9% NaCl and 1% gelatine.

RESULTS AND DISCUSSION

The method of calculating the degree of cross-reaction between antisera and the steroid conjugates tested was based on that described by Weinstein *et al.*[5] and is illustrated in the comparative graphs (Figs. 4–8). The percentage cross-reaction is defined as $100 \times X/Y$ where X is the mass of non-radioactive homologous steroid conjugate and Y is the mass of non-

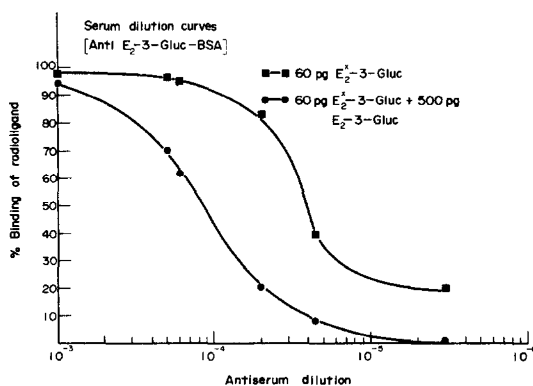

Fig. 1.

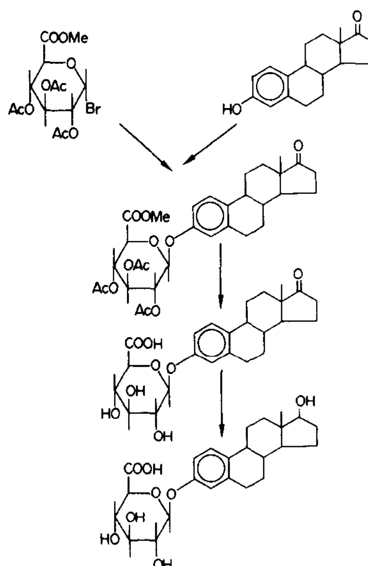

Fig. 2.

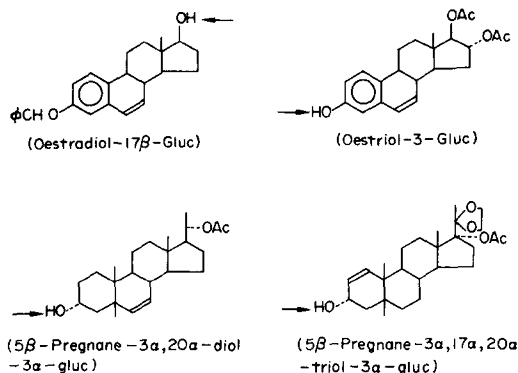

Fig. 3.

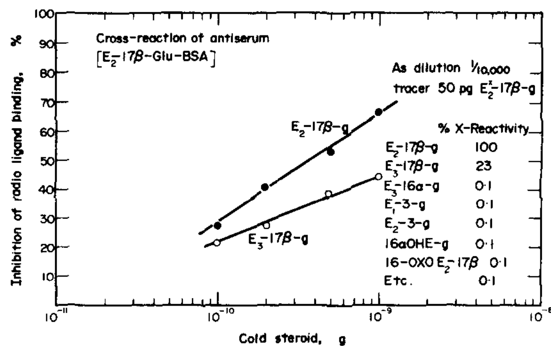

Fig. 4.

radioactive heterologous steroid or steroid conjugate required to produce 50% inhibition of binding of the radioligand by the antiserum. Values of X and Y are determined by the intersection of the appropriate calibration graph and the horizontal line representing 50% inhibition of binding of the radioligand.

Consideration of the properties and cross-reactions of the antisera produced to date by the methods described can conveniently be considered in three groups.

Group 1. Initial study: antisera to oestradiol-17β-glucuronide, testosterone-17β-glucuronide and dehydroepiandrosterone-3β-glucuronide. The haptens prepared and joined covalently to BSA are not closely related in structure and were chosen, not for comparative studies but to ascertain whether the concept of inducing antisera to steroid glucuronides was a practical possibility. They were chosen because the corresponding isotope-labelled steroid conjugates at suitable specific radioactivity were available immediately from commercial sources.

Anti-oestradiol-17β-glucuronide serum did not cross-react with any androstane or pregnane series glucuronide available nor with any ring-A oestrogen glucuronide. This antiserum was tested against three other oestrogen ring-D glucuronides but showed significant cross-reaction with only one, oestriol-17β-glucuronide which bears a close resemblance to the hapten used (Fig. 4). This cross-reacting glucuronide has an additional 16α-hydroxyl group. The closely related conjugates 16-oxo-oestradiol-17β-glucuronide and 16α-hydroxyoestrone-16α-glucuronide did not appear to compete with the radioligand.

Anti-dehydroepiandrosterone-3α-glucuronide serum did not cross-react with any of the oestrogen-glucuronides available nor was there significant cross-reaction with the 3α-glucuronides in the androstane series, androsterone-3α-glucuronide and aetiocholanolone-3α-glucuronide. There was no cross-reaction with any of the ring-D conjugates available. Significantly, 3-epiandrosterone-3β-glucuronide showed the ability to compete with the radioligand (Fig. 5) and as with the foregoing anti-oestradiol-17β-glucuronide serum there was a structural similarity between the competing conjugate 3-epiandrosterone-3β-glucuronide and the hapten dehydroepiandrosterone-3β-glucuronide. The difference in this case was the absence of a double bond (C 5–6) in the competitor.

Anti-testosterone-17β-glucuronide serum. This antiserum failed to cross-react with any of the oestrogen glucuronides available, which notably include oestradiol-17β-glucuronide. It did not cross-react with several androstane ring-A glucuronides nor with 17-epitestosterone-17α-glucuronide which is enantiomorphic with the hapten used.

It is noteworthy that these three antisera were taken from the immunized rabbits approximately 10 weeks after the first injection and with further experience it is clear that after 6–8 months much higher antibody titres are obtainable. These antisera were used in less diluted form (1:5000–1:15,000) than those

Table 2. Radioactive steroid conjugates used as radioligands

[6,7-^{3}H]Oestrone-3-glucuronide	[6,7-^{3}H]Oestrone-3-sulphate
[6,7-^{3}H]Oestradiol-3-glucuronide	
[6,7-^{3}H]Oestradiol-17β-glucuronide*	
[6,7-^{3}H]Oestriol-3-glucuronide	
[6,9-^{3}H]Oestriol-16α-glucuronide†	
[6,7-^{3}H]Androsterone-3α-glucuronide	[6,7-^{3}H]DHA-3β-sulphate
[6,7-^{3}H]Aetiocholenolone-3α-glucuronide	
[1,2-^{3}H]Testosterone-17β-glucuronide*	
[7-^{3}H]DHA-3β-glucuronide*	
[6,7-^{3}H]5β-Pregnane-3α,20α-diol-3α-glucuronide	[7-^{3}H]Pregenolone-3β-sulphate
[1,2-^{3}H]5β-Pregnane-3α,17,20α-triol-3α-glucuronide	

* Available from NEN GmbH, Dreieichenhain, Frankfurt, W. Germany.
† Available from The Radiochemical Centre, Amersham, Bucks, U.K.
Most other labelled conjugates will be made available through the MRC Labelled Steroids Reference Collection.

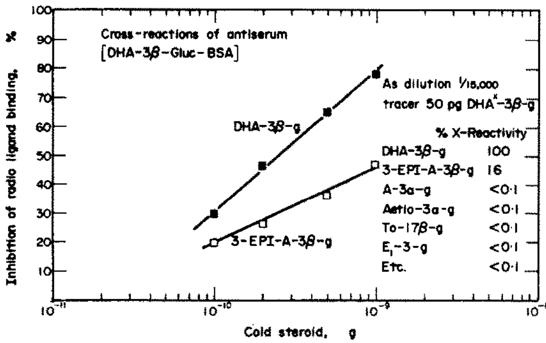

Fig. 5.

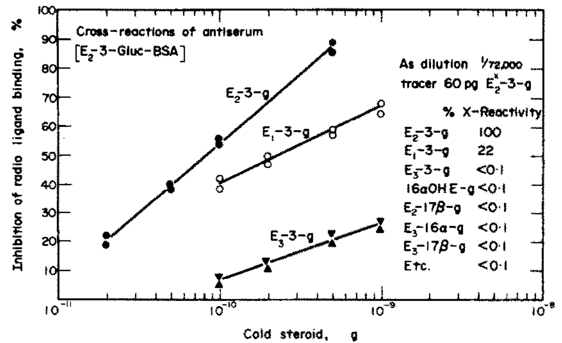

Fig. 7.

in the following comparative group and were, in general, less sensitive when used in radioimmunoassay.

The anti-testosterone-17β-glucuronide serum has been used to measure the urinary excretion of this conjugate in the urine of men and women and the method compares very favourably with alternative methods based on hydrolysis and gas–liquid chromatographic analysis [13].

Group 2. Comparative study. Antisera to oestrone-3-glucuronide, oestradiol-3-glucuronide and oestriol-3-glucuronide. These antisera did not cross-react with any glucuronide of the androstane or pregnane series nor with any of four oestrogen ring-D glucuronides available; oestradiol-17β-, oestriol-17β-, oestriol-16α-, 16α-hydroxyoestrone-16α- and 16-oxo-oestradiol-17β-glucuronides. On testing the three antisera, in turn, against oestrogen-3-glucuronides there was some degree of cross-reaction in each case although significant competition which might interfere with the radioimmunossay of oestrogen-3-glucuronides was found only in two circumstances (Fig. 6, 7) (Table 3). Clearly oestradiol-3-glucuronide competes with the radioligand of anti-oestrone-3-glucuronide and conversely oestrone-3-glucuronide competes with the binding of the radioligand of anti-oestradiol-3-glucuronide. Although there is some cross-reaction by oestrone-3- and oestradiol-3-glucuronides against anti-oestriol-3-glucuronide serum the competition is weak and unlikely to interfere with the radioimmunoassay of oestriol-3-glucuronide. Oestrone-3- and oestriol-3-glucuronides have been recognized as substantial

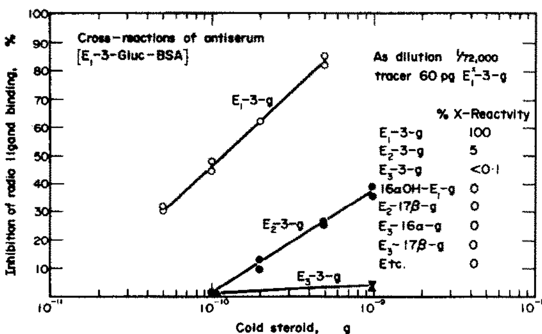

Fig. 6.

metabolites in late pregnancy urine [1]; oestradiol-3-glucuronide has been reported as being present but in much smaller amounts [14].

In each case the antiserum titre against the corresponding radioligand rose to a maximum at about six months after the initial immunization and remained high until the final bleed at 8–9 months. The antisera were used at a dilution of 1:72,000 in the presence of 50–60 pg of radioligand and were significantly more sensitive than those of the initial series.

Group 3. Antisera of clinical importance. Antisera to aetiocholanolone-3α-glucuronide, 5β-pregnane-3α,20α-diol-3α-glucuronide, 5β-pregnane-3α,17,20α-triol-3α-glucuronide, oestriol-16α-glucuronide, oestrone-3-sulphate etc. Although well advanced the work on this group is incomplete at the present time. Antisera to aetiocholanolone-3α- and androsterone-3α-glucuronides prepared by Cameron[15] show surprisingly little cross-reaction against the corresponding isomeric glucuronides, *i.e.* anti-aetiocholanolone-3α-glucuronide serum versus androsterone-3α-glucuronide and vice versa although the difference in structure between hapten and ligand is confined to isomerism at C-5. There is potential value in the application of anti-aetiocholanolone-3α-glucuronide serum for radioimmunoassay in relation to aetiocholanolone fever [16] and possibly in relation to the Bulbrook "discriminant factor" for mammary carcinoma [17]. For application in the field of obstetrics we have prepared and injected the antigens 5β-pregnane-3α,20α-diol-3α-glucuronide-BSA and oestriol-16α-glucuronide-BSA. The antisera and the corresponding radioligands are now available but the checks of specificity are still in progress. A route to 5β-pregnane-3α,17,20α-triol-3α-glucuronide and the corresponding radioligand 5β-[6,7-^{3}H]-pregnane-triol has been

Table 3. Cross-reactions of anti-oestrogen-3-glucuronide sera and oestrogen-3-glucuronides

	% Inhibition of Binding of Radioligand		
	Anti-oestrone -3-gluc.	Anti-oestradiol -3-gluc.	Anti-oestriol -3-gluc.
Oestrone-3-gluc.	100	22	0·1
Oestradiol-3-gluc.	5	100	0·1
Oestriol-3-gluc.	0·1	0·1	100

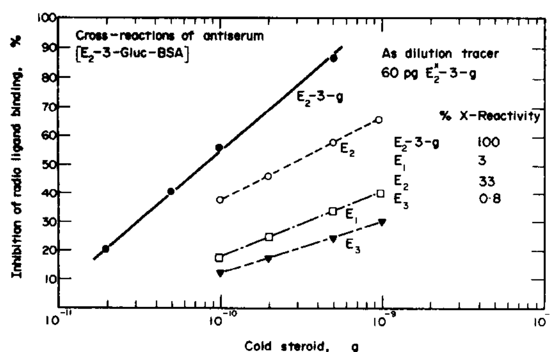

Fig. 8.

established and it is intended to apply the corresponding antiserum for the early diagnosis of adrenogenital syndrome.

As a step of considerable interest and importance we have prepared and immunized rabbits with complexes prepared by linking BSA to oestrone-3-sulphate, dehydroepiandrosterone-3β-sulphate and pregnenolone-3β-sulphate [11]. To date we have no knowledge as to whether or not these complexes are antigenic.

Cross-reactions of anti-steroid–glucuronide sera and free steroids

Without exception the antisera prepared by immunizing with the antigen "steroid–glucuronide–BSA" gave strong cross-reactions with the corresponding free steroid glucuronide, and it is possible that two kinds and oestriol with anti-oestradiol-3-glucuronide-serum (Fig. 8). This is not surprising if one compares the structure of this antigen with that of "steroid–succinyl–BSA" which is a conventional agent for raising antisera to free steroids. The hapten can be considered either to be the steroid or the steroid derivative, steroid glucuronide, and it is possible that two kinds of antibodies are produced by these entities. With each anti-steroid–glucuronide serum produced, the steroid glucuronide was more effective than the free steroid in displacing the radioligand.

The incompleteness of this study is obvious and we are aware that we have just touched the fringe of a big problem. The slow progress is a consequence of entering a new field in which neither the haptens, radioligands, nor the reference compounds were available. Nevertheless the results so far appear to establish that steroid glucuronides can be determined directly by radioimmunoassay and that this method may prove to be sensitive and specific.

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