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SPECIFICITY OF ANTISERA TO OESTRONE DETERMINED BY DIFFERENT RADIOIMMUNOASSAY METHODS

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SUMMARY

The specificity of an antisera to oestrone which was raised with oestrone-6-(O-carboxymethyl) oxime bovine serum albumin conjugate has been tested using different radioimmunoassay methods. Cross reactivity tests were made using (i) the liquid phase radioimmunoassay technique involving dextran coated charcoal, (ii) the double antibody precipitation technique, (iii) the solid phase method using polystyrene tubes, and (iv) the solid phase procedure using Enzacryl AA. The specificity of the oestrone antisera to the various steroids tested, assessed as percentage cross reaction against oestrone, was found to vary when determined by different radioimmunoassay methods. The results suggest that a choice of radioimmunoassay technique may be made such that a particular method would enable higher specificity to be obtained towards certain cross reacting steroids.

INTRODUCTION

A recent study made in this laboratory to investigate the relationship of steroid haptenic structure of antigens to the specificity of their ellicited antisera led us to question whether the assessment of the specificity of a particular antisera is the same when determined by different steroid radioimmunoassay methods. Again since different laboratories use different steroid radioimmunoassay methods to assess the specificity of a particular antisera, and no direct information on this point appeared available, it was decided to make a study which would enable a comparison of the specificity of an antisera to be made using different radioimmunoassay procedures.

The specificity of the antisera ($LR_4(6KE_1)B_1$) raised by the antigen oestrone-6-(O-carboxymethyl) oxime albumin conjugate [1] was examined using seven steroids. The cross reactions of these steroids against oestrone were obtained by (i) the solid phase radioimmunoassay technique using polystyrene tubes [2], (ii) the solid phase system using Enzacayl AA [3], (iii) the liquid phase radioimmunoassay using dextran coated charcoal [4], and the double antibody precipitation technique. The results of the cross reactions obtained by the individual radioimmunoassay methods were compared.

METHODS AND MATERIALS

Materials

Water. All water was deionized and redistilled from glass. Ethyl alcohol: this was refluxed over calcium hydride (1 h) and redistilled.

Norit A. Charcoal was obtained from Sigma Co. Ltd., London. Dextran T40 was obtained from Pharmacia Fine Chemicals, London.

Rivanol. Rivanol (2-ethoxy-6,9-diaminoacridine lactate) pure was obtained from Koch-Light, Colnbrook, Bucks, U.K. Bovine Serum Albumin (BSA) was obtained from Sigma Co. Ltd., London. Barbital buffer: (pH 9·8) 0·07 M sodium barbitone was used for coating antibodies to polystyrene tubes.

Assay buffer. (pH 7·0) 0·1 M was prepared as described by Abrahams [5]. Two stock solutions of sodium phosphate buffer were used. Solution A was 0·2 M NaH_2PO_4 and solution B was 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 g of sodium azide and 9 g of sodium chloride made up to 1 litre of water. This assay buffer was used in the solid phase radioimmunoassay techniques, i.e. the polystyrene tube and the Enzacryl AA methods (see below).

Assay gelatin buffer. 1 g of gelatin was added per litre of the assay buffer above for the liquid phase radioimmunoassay techniques, i.e. the dextran coated charcoal and the double antibody precipitation techniques (see below).

Anti-rabbit-precipitation serum. Donkey RD17 was purchased from Burrough's Wellcome, London. Enzacryl AA was obtained from Koch-Light Laboratories, Colnbrook, Bucks, England.

Steroids. These were obtained from Koch-Light Laboratories, Colnbrook, Bucks. Radioactive [6,7-^{3}H]-oestrone (40 Ci/mmol), obtained from the Radiochemical Centre, Amersham, U.K., was used for the

radioimmunoassay studies. A solution of 8333 d.p.m. per 0·1 ml ethanol was used; this represented a radioactive load of 20·4 pg.

Dioxane scintillator. This was made up by the addition of 8 g of Butyl PBD and 100 g of naphthalene (both obtained from BDH Chemicals). Polystyrene tubes. Small disposable (LP/2) polystyrene tubes were obtained from Luckhams Ltd., Surrey, U.K.

Antisera $LR_4(6KE_1)B_1$. The first bleed taken 2 weeks after the second injection of oestrone-6-(O-carboxymethyl) oxime-bovine serum conjugate [1] was bovine serum albumin (BSA) adsorbed and Rivanol treated (see below). Packard Tri-Carb Scintillation Counter (Model 3375): Aqueous solutions were counted in dioxane scintillator, whilst toluene scintillator was used for non-aqueous solutions. The efficiency of tritium counting was 30% in dioxane and 45% efficient in toluene. All samples were counted for at least 20 min.

METHODS

Adsorption of anti-BSA antibodies and Rivanol treatment of the antisera to oestrone was carried out according to the method of Thornycroft *et al.* (b). Dilution response curves were constructed using this treated antisera for both the dextran coated charcoal and the Enzacryl AA methods. These curves were prepared by plotting the percentage of the 20·4 pg radioactive oestrone load bound to antibody at various dilutions of antisera, and comparing them with curves produced when a 150 pg non-radioactive oestrone was added. The maximum difference between the curves gave the optimal dilution of antibody required to give maximum sensitivity. The best dilution for the antisera by both methods was 1:1250, despite the fact that the sensitivity of the dextran coated charcoal radioimmunoassay was greater than the Enzacryl AA method. Past experience in this laboratory suggested that coating of tubes in the polystyrene tubes with the antisera dilution which was optimal for the dextran charcoal method gave near optimum conditions for the solid phase radioimmunoassay. This, together with the fact that the double antibody precipitation technique used identical equilibration conditions to the dextran coated charcoal method, meant that all four methods were optimized for antisera dilution.

Cross-reaction tests. The specificity of the antisera was tested by determining the percentage cross-reaction of various steroids by four different radioimmunoassay methods (see below). Various amounts of the steroids to be tested were dissolved in assay buffer (volume dependent on type of radioimmunoassay used). The binding vs mass curve for the cross-reacting steroid was compared with the standard curve given by oestrone. Seven cross-reacting steroids were tested: Oestradiol 17α ($17\alpha E_2$) oestradiol 17β ($17\beta E_2$), oestriol (E_3), progesterone, cortisol, testosterone and cholesterol. A range of concentrations of each of these steroids were made up to cover the range 0–1,000,000 pg/0·1 ml. All determinations were performed in duplicate. Calculation of the cross-reaction was made as indicated by Abraham [5], i.e. mass of oestrone to displace 50% of bound [6,7-^{3}H]-tritiated oestrone = x. Mass of cross-reacting steroid required to displace 50% of bound (6,7-^{3}H) tritiated oestrone = y. Percentage cross-reaction = $x/y \times 100$.

RADIOIMMUNOASSAY METHODS

1. The liquid phase radioimmunoassay using dextran coated charcoal. The method used was a modification of the method of Hotchkiss, Atkinson and Knobil, 1971 [4]. 0·1 ml of [6,7-^{3}H]-oestrone solution was added to an assay tube and evaporated gently to dryness under a stream of nitrogen. 0·1 ml of diluted antibody solution (1:1250) and 0·1 ml of assay gelatin buffer containing the respective steroid (standard oestrone or cross-reacting compound) was then added. After mixing briefly the tube was left to incubate at 4°C for 16 h. After incubation, 1 ml of Dextran-coated charcoal suspension was added, the tube which was shaken briefly and then left to incubate at 4° for 15 min before centrifugation at 5000 *g* for 15 min. The supernatant containing the steroid bound to antibody was then decanted into a scintillation phial and its radioactivity counted in a scintillation counter.

2. Solid phase radioimmunoassay using polystyrene tubes. This method is similar to that described by Exley *et al.*, 1971 [2] and is a modification of the method of Abraham and Odell [7].

(i) *Preparation of antibody coated tubes.* These tubes were prepared as one batch of 150 tubes. Antisera $LR_4(6KE_1)B_1$ was diluted to a final dilution of 1:1250 with barbital buffer. 0·5 ml of the diluted antisera was added to the polystyrene tubes and the tubes incubated at 4°C for 16 h overnight. After incubation and careful removal of the barbital buffer by suction, 0·5 ml of assay buffer was added which was again removed by suction. A further 0·5 ml of assay buffer was then added and the tubes covered with parafilm and stored at 4°C until use.

(ii) *Assay method.* Assay buffer solutions of steroids were made up such that 0·5 ml contained the required mass of cross-reacting steroid together with the standard mass (20·4 pg) of radioactive oestrone. The assay buffer in the coated polystyrene tubes was removed by

suction and the solution to be assayed (0·5 ml) was immediately added and incubated at 4°C for 16 h. After incubation, the liquid in the tube was quantitatively transferred to a scintillation phial together with 0·5 ml of assay buffer used to wash out the polystyrene tube and then counted for radioactivity.

3. Solid phase radioimmunoassay using Enzacryl AA. Method of Moore and Axelrod (1972) [3]. 200 mg of Enzacryl AA was diazotized and the enzacryl–antibody complex prepared as described by the above authors. The final dilution of the Rivanol treated antisera (originally 1·5) was 1:100 for the complex.

Assay. 0·1 ml of [6,7-^{3}H]-oestrone solution and 0·1 ml of cross-reacting steroid in ethanol were added to the assay tube and evaporated down to dryness under nitrogen. 0·5 ml of assay buffer was then added, followed by 1 ml of Enzacryl antibody complex (dilution 1:1250) and the contents mixed and then allowed to stand for 1 h, mixed again, and then allowed to stand for an additional hour. After centrifugation at 5000 *g* for 5 min, 0·5 ml of the supernatant representing the free fraction was aliquoted into a counting phial and counted for radioactivity.

4. Double antibody precipitation technique. 0·1 ml of [6,7-^{3}H]-oestrone and 0·1 ml of an ethanolic solution containing the standard or cross-reacting steroid was added to an assay tube and blown to dryness under nitrogen. 0·2 ml of diluted oestrone antibody solution (1:2500) was then added and left to incubate at 4°C for 16 h. 0·2 ml of (1:40) dilution of anti-rabbit precipitation sera was then added, and after shaking, tubes were incubated at 4°C for 48 h. After this latter incubation, 0·5 ml of assay buffer was added and mixed and then centrifuged at 2000 *g* for 30 min. 0·5 ml of the supernatant was counted for radioactivity.

RESULTS

Apart from the double antibody precipitation radioimmunoassay method when the average coefficient of variation for duplicate determination was ±5·5% (n = 40 duplicates), all other methods gave excellent precision. The average coefficient of variation was ±1·4% (n = 67) for the solid phase radioimmunoassay technique using polystyrene tubes, ±2·2% (n = 40) for the Enzacryl AA method and ±2·2% (n = 44) for the Dextran coated charcoal technique. This standard of accuracy enabled us to clearly distinguish small differences between the specificities of a cross-reacting steroid when assessed by the various radioimmunoassay methods.

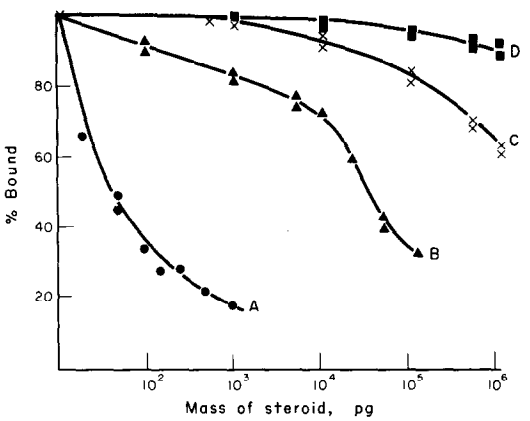

Fig. 1. Curves of % bound against mass of steroid obtained by the solid phase radioimmunoassay method using polystyrene tubes. A = oestrone. B = oestriol. C = testosterone. D = cholesterol.

Figure 1 shows typical curves obtained for assessment of cross-reactivity. Plots were made of percentage antibody bound [6,7-^{3}H]-tritiated oestrone (set at 100% for the blank) against mass of steroid.

The radioimmunoassay methods possessed different sensitivities. Using the solid phase technique with polystyrene tubes it was found that only 40 ± 5 pg of non-radioactive oestrone was required to displace 50% of the binding of [6,7-^{3}H]-oestrone, whilst the liquid phase method using dextran coated charcoal required 80 ± 10 pg, the double antibody precipitation technique required 120 ± 10 pg and the method using Enzacryl AA needed 450 ± 50 pg.

Table 1 shows the results of the cross-reaction tests obtained by all four radioimmunoassay methods. As can be seen, the specificity of the anti-oestrone sera varies with the different radioimmunoassay methods. There is a range of 0·19–0·57% for the percentage cross-reaction of oestradiol 17α (three methods compared), a range of 0·13–0·29% for oestriol (three methods compared) and a range of 0·10–0·39% for oestradiol-17β when all four methods are compared. The order of cross-reaction by three methods (unfortunately there was not enough of this particular antisera left to check this point by the double antibody precipitation technique) was oestradiol-17α > oestradiol-17β > oestriol. This occurred despite the differences in sensitivity and specificity of the radioimmunoassay methods. All the methods agree that there is virtually no cross-reaction with cholesterol, cortisol or testosterone (all less than 0·005% cross-reactivity). This lack of reactivity obviously precludes quantitative measurement and specificity comparison.

Table 1. Comparison of cross reactivity of steroids with oestrone as assessed by different radioimmunoassay methods for antisera. $LR_4(6KE_1)B_1$.

Steroid	% Cross reaction			
	Dextran coated charcoal method	Enzacryl AA method	Polystyrene tube method	Double antibody precipitation technique
Oestrone	100	100	100	100
Oestradiol-17α	0·45	0·57	0·19	—
Oestradiol-17β	0·39	0·31	0·10	0·10
Oestriol	0·13	0·29	0·14	—
Progesterone	0·06	0·05	0·05	0·05
Cortisol	<0·005	<0·005	<0·005	<0·005
Testosterone	<0·005	<0·005	<0·005	<0·005
Cholesterol	<0·005	<0·005	<0·005	<0·005

DISCUSSION

The various radioimmunoassay methods give results for the specificity of the Rivanol treated anti-oestrone sera $LR_4(6KE_1)B_1$ which on the average agree with those of Rowe, Cook and Dean [8] who used the much later bleed $LR_4(6KE_1)B_8$ and the liquid phase radioimmunoassay with charcoal to test the specificity of this antisera. This later bleed was neither BSA adsorbed nor Rivanol treated, suggesting that the specificity of the anti-oestrone sera was not dramatically changed by this treatment or with time of collection. The antibody titre however increased after the collection of the first bleed used for the present study. The antisera examined are possibly the most highly specific yet developed for any oestrogen, possessing no cross-reactivity above 1% with typical steroids normally encountered in non-pregnancy plasma. At first glance the results of this investigation could suggest that there is little difference between the specificity of the antisera when determined by the various methods. For general radioimmunoassay in clinical practice the difference between even the most divergent results obtained, i.e. the cross-reaction of oestradiol (range 0·39–0·10%), could be considered negligible and would mean that any of the four methods could be used in clinical practice. However, despite the fact there is only 0·29% difference in the above cross-reaction it means that the reaction towards oestradiol-17β is four times greater when assessed by the polystyrene tube method and the double antibody method than by the Dextran coated charcoal technique. Other differences of this kind are also apparent (Table 1), and thus since the radioimmunoassay methods all gave excellent duplicate cross-reaction curves these differences are meaningful. This suggests that the specificity of the antisera to cross-reacting steroids varies according to the type of radioimmunoassay used for the determination.

The reason for the different specificities obtained by the various methods is most probably due to the different treatments of the anti-oestrone sera. Radioimmunoassay involving binding of antibodies to polystyrene tube surfaces may partially fractionate the antisera in that certain antibodies bind in preference to others. Again in the Enzacryl AA method the preparation of water insoluble antibodies by diazotisation may slightly change the configuration of some of the active sites of the original antibodies. These factors, among many others, may account for the differences in specificity obtained by the various methods. This difference in specificity could suggest that a choice of radioimmunoassay technique may be made such that a particular method may enable higher specificity to be obtained towards certain cross-reacting steroids.

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