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Production and Characterization of Antisera Against Steroids in Rabbits

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Abstract.- Antibodies against testosterone, progesterone and estradiol were raised by immunization of rabbits with testosterone-3-(o-carboxymethyl) oxime-bovine serum albumin (testosterone-3-BSA), progesterone-11-(o-carboxymethyl) oxime-bovine serum albumin (progesterone 11-BSA), and estradiol-6-(O-carboxymethyl) oxime-bovine serum albumin (estradiol-6-BSA). The antisera have shown a high titer of 48-54% binding at the final dilution of 1:24,000. These antisera, therefore, have shown high affinity. The affinity constant (Ka) calculated for testosterone, progesterone and estradiol antisera were 6.5×10^9 l/mol, 3.2×10^9 l/mol, and 0.29×10^9 l/mol, respectively. The specificity and cross-reactivity results have shown a high degree of specificity toward their respective homologous steroids. The mean percent recovery of unlabelled hormones added to samples was 105±10.63 for testosterone, 92.75±2.77 for progesterone and 107±5.38 for estradiol antisera. The sensitivity of testosterone, progesterone and estradiol standard of testosterone, progesterone and estradiol standard curves was 10, 25 and 15 picograms per 200 µl, respectively, when calculated at 95% confidence limit

Key words: Antibodies against steroids, rabbit, antisera against steroids, radioimmunoassay.

INTRODUCTION

Specific antisera are the most important ingredients of any immunoassay (Franchimont *et al.*, 1983). The specificity and affinity of antibodies not only determine the specificity and sensitivity of the assay but also its practicability (Naegele and Drahovsky, 1980). Erlanger *et al.* (1957) employed for the first time a technique in which steroids were coupled with bovine serum albumin (BSA) to produce stable conjugates capable of eliciting antibodies formation in animals. Several workers later followed the same method to produce a variety of stable steroid-protein conjugates for testosterone, progesterone and estradiol using BSA (Abraham and Grover, 1971; Niswender, 1973; Pang and Johnson, 1974; Grover and Odell, 1976).

The specificity of antisera is greatly influenced by cross reaction, which can be avoided by a suitable choice of the site of attachment on the particular steroid molecule. It appears from several studies of cross-reactions that the antisera possess specificity mainly against that part of the steroid molecule which is remote from the point of attachment to protein (Niswender, 1973). This principle has been used with success in the production of suitable antisera with low cross-reactivities in case of testosterone by coupling at carbon-3 (Anneke *et al.*, 1974), use of progesterone-11-conjugate (Niswender, 1973) and estradiol of coupling at carbon-6 position (Youichi *et al.*, 1987).

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We in the present study have described the production of antisera raised against conjugates of steroid hormones namely testosterone-3-BSA, progesterone-11-BSA and estradiol-6-BSA. The antisera produced were then characterized for their titer, affinity, cross-reactivity and specificity. As the antiserum is an important constituent of radioimmunoassay (RIA), the practicability of antisera in RIA has also been highlighted.

MATERIALS NAD METHODS

Chemical

All chemicals used in the present study were of analytical grade. Testoterone-3-BSA, progesterone-11-BSA, estradiol-6-BSA, Freund's complete adjuvant and unlabelled hormones, were obtained from Sigma Chemical Co., ST. Louis, Mo, USA. (1,2,6,7-^{3}H) testosterone, 98 Ci/m Mol., (1,2,6,7-^{3}H) progesterone, 98 Ci/m mol., and (2,4,6,7-^{3}H) estradiol, 98 Ci/m mol; were obtained from Amersham International, PLC, England.

Immunization

Immunogens were prepared according to the method of Bauminger *et al.* (1973). Two adult male rabbits were immunized with each immunogen (testosterone, progesterone and estradiol). The emulsified immunogen (2 ml) containing 500 µg steroid conjugate was injected into each rabbit subcutaneously at previously shaven thighs, several sites in the loin, at either side of spine and foot pads. The injections were

repeated every fourteen days. Altogether seven injections were given to each animal.

Collection and storage of antisera

The first blood sample was obtained five weeks after the first injection. The rabbits were bled from a marginal ear vein and serum was obtained by allowing blood to clot at room temperature for one hour, further keeping overnight at 4°C and then by centrifuging at 3000 rpm for 30 minutes at 4°C in a refrigerated centrifuge (Kokusan, Model H-103RS). The serum, in presence of 1% sodium azide, was stored in 500 ul aliquots at -20°C.

Titer and cross-reactivity determination

Serial dilutions of antisera were made using phosphate buffer saline (PBS) containing 1% bovine serum albumin, pH 7.2. Aliquots of 500 µl from each antiserum dilutions were incubated with 500 µl of the respective tritiated tracer (12000-16000 cpm) and 200 µl PBS and left overnight at 4°C. The unbound steroid was separated out by previously described dextran coated charcoal method (Dufau *et al.*, 1978) and was counted using 5 ml scintillation fluid (5 g permablend/l toluene) in Scintillation counter, Beckman LS 1801.

The cross-reactivity of testosterone, progesterone and estradiol antisera was determined according to the method described by Abraham (1969). The dilution of antisera (final dilution 1:24,000) which could bind 48-54% with their respective ^{3}H-tracer (12000-16000 cpm/500 µl) in absence of unlabelled hormone was used in the assay. Aliquots of 500 µl of antisera dilution were incubated overnight with 500 µl of the respective ^{3}H-tracer in the presence of 200 µl aliquots of cold hormone of various concentrations (1, 10, 100, 500, 1000, 5000 and 10,000 ng/200 µl). The methods for separation of unbound fraction and counting were same as described in previous paragraph. The percent cross-reactivity was calculated by using the following formula:

$$\% \text{ cross-reactivity} = \frac{\text{Mass of authentic steroid needed to displace 50\% of label}}{\text{Mass of competing steroid needed to displace 50\% of label}} \times 100$$

Recovery assays

Aliquots of 500 µl of antisera dilution of the steroids (final dilution 1:24,000) were incubated with 500 µl of respective ^{3}H-tracer in the presence of either the cold hormones ranging 0.1, 1, 5, 10, 100, 1000 and 10,000 ng/200 µl or the unknown/control at 4°C overnight. After overnight incubation at 4°C, the unbound fractions were separated by using dextran-coated charcoal and radioactivity was determined as described earlier. The percent recovery was calculated from a standard curve.

Radioimmunoassay (RIA)

For the determination of assay sensitivity, 500 µl aliquots of antisera (final dilution 1:24,000) were incubated with 500 µl of their respective tritiated tracer in the presence of respective standard ranging from 15-4000 pg/2000 µl. The tubes were also set for total counts and non-specific binding. After overnight incubation at 4°C, 200 µl dextran-coated charcoal was added into each tube except tubes having total counts and processed further as described earlier. The sensitivity of the assay was determined from a standard curve at 95% limit of confidence.

RESULTS

All immunized rabbits have produced antibodies against the respective steroid conjugates within three months. An increase in binding capacity (titer) was observed after each injection (Fig. 1). After three months of injections (seven injections), the antisera showed highest titer for each steroid conjugate. In the antiserum raised against testosterone-3-BSA, a titer of 54% binding was obtained, with tritiated testosterone at a final dilution of 1:24,000 (initial dilution 1:10,000). Similarly the serum from the rabbits treated with progesterone-11-BSA and estradiol-6-BSA showed a titer of 48% and 50% binding respectively with ^{3}H-progesterone and ^{3}H-estradiol at a final dilution of 1:24,000 (initial dilution 1:10,000).

The binding capacity of antisera raised against steroid conjugates were also investigated using iodinated tracer. Therefore, the results suggest a higher binding capacity of antisera raised against testosterone-3-BSA (initial dilution 1:10,000 and final dilution 1:24,000) with iodinated tracer: 62% as compared to tritiated: 54% (Fig. 2A). Similarly the antisera raised against progesterone-11-BSA and estradiol-6-BSA showed a titer of 52% and 56% binding respectively with their respective iodinated tracter when compared to their respective tritiated tracers :48% and 50% at a initial dilution of 1:10,000 and final dilution of 1:24,000 (Fig. 2B-C).

The affinity constant (Ka) was calculated from Michaelis-Menton Plot, which is the reciprocal of the

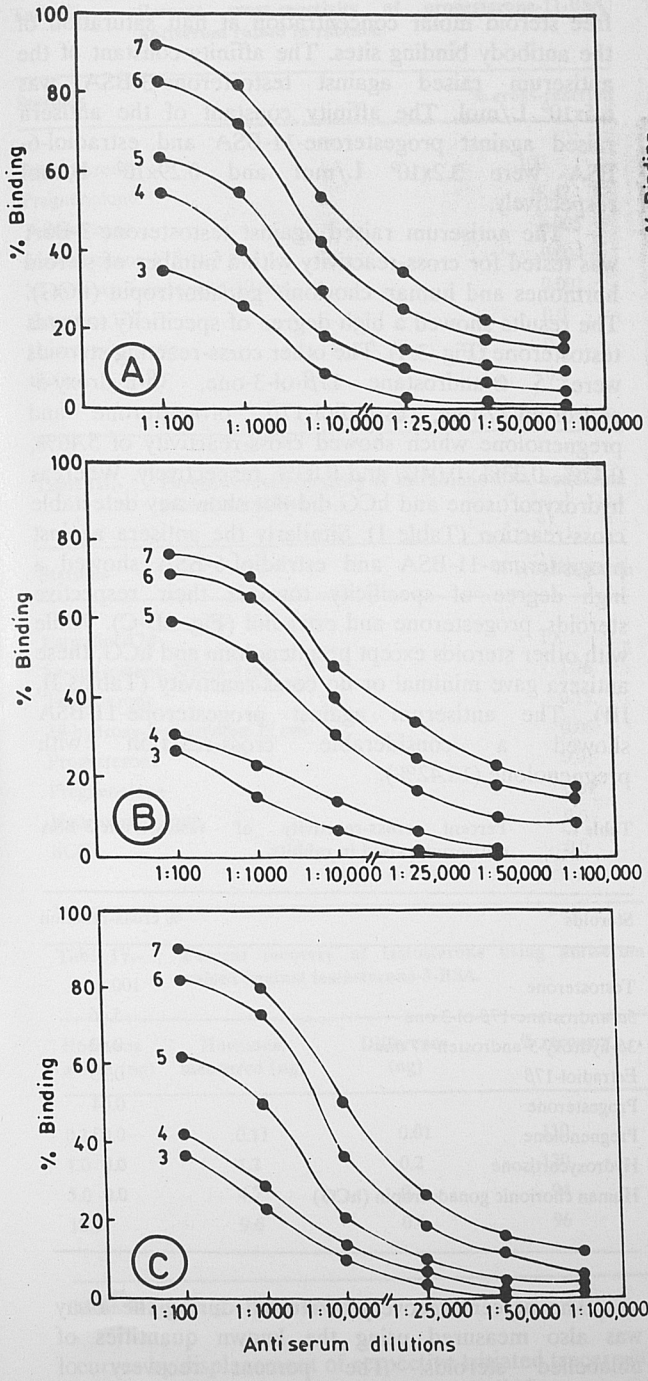

Fig. 1. The effect of repetitive immunization on the titer of testosterone-3-BSA antiserum (A), progesterone-11-BSA antiserum (B), and estradiol-6-BSA antiserum (C). The number of each curve represents the number of injection.

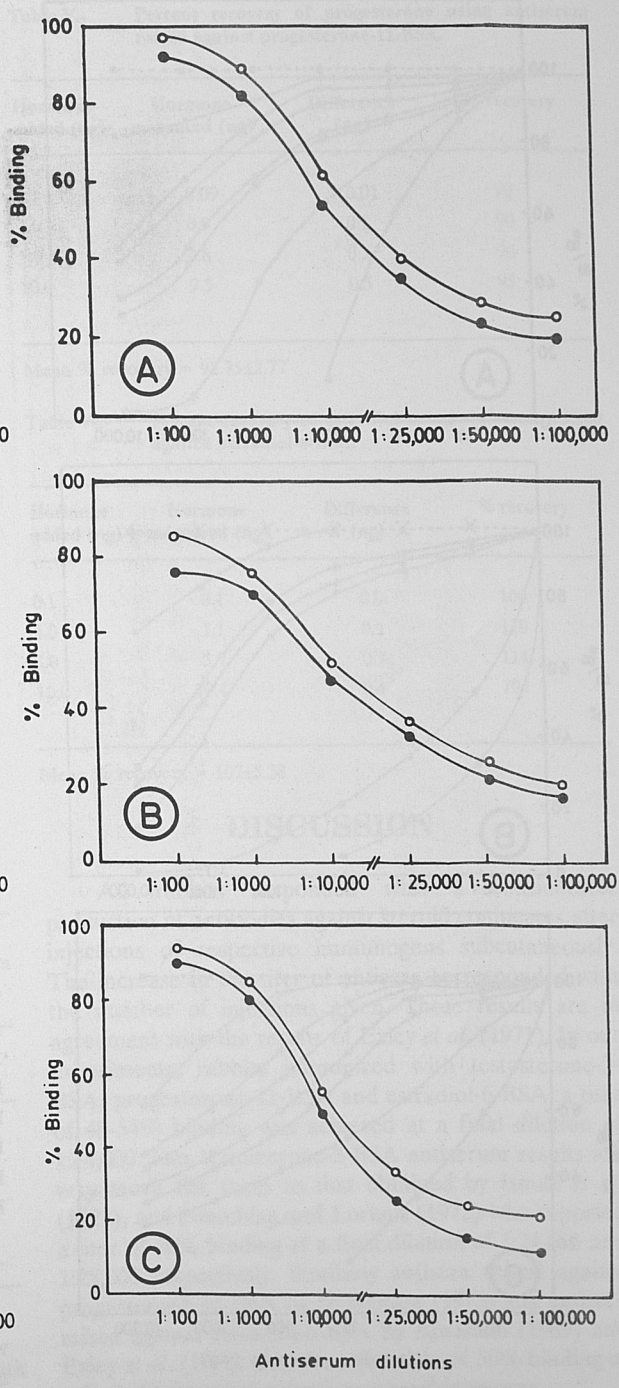

Fig. 2. Testosterone antiserum titer curves (A), progesterone antiserum titer curves (B), and estradiol antiserum titer curves (C) obtained after seven injection using tritiated and iodinated testosterone (A), progesterone (B) and estradiol (C), repsectively.

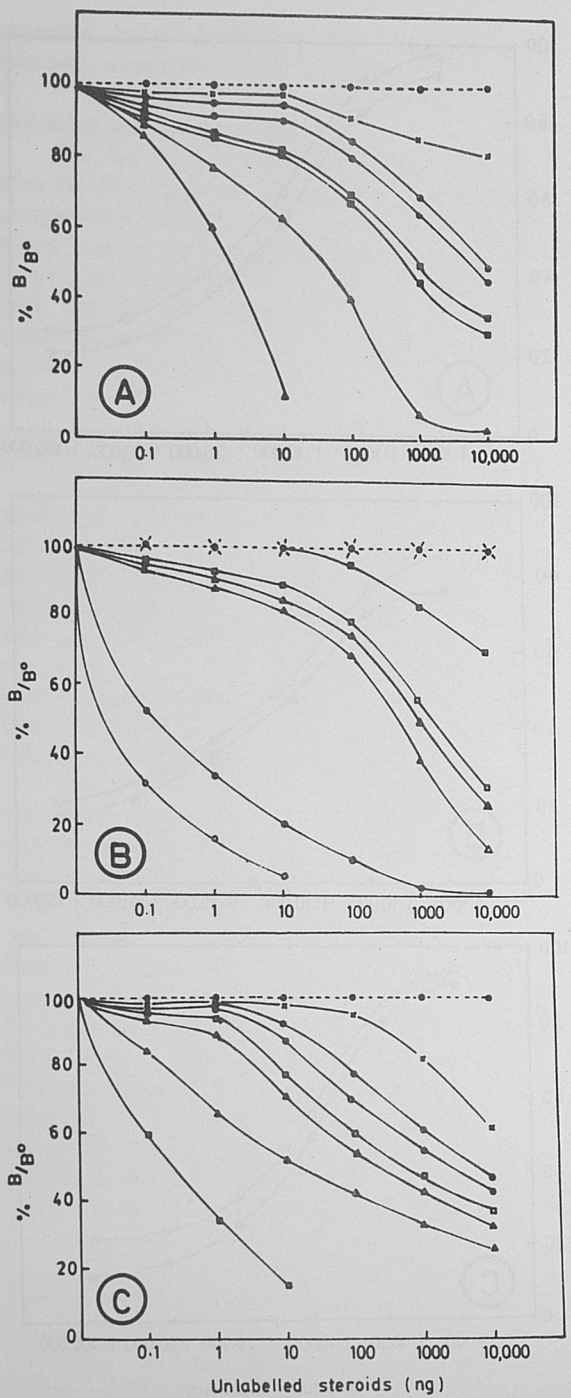

Fig. 3. Cross-reactions of testosterone and various other steroids with testosterone-3-BSA antiserum (A), of progesterone and various other steroids with progesterone-11-BSA antiserum (B), and of estradiol and various steroids with estradiol-6-BSA antiserum (C). The final dilution of antisera was 1:24,000.

free steroid molar concentration at half saturation of the antibody binding sites. The affinity constant of the antiserum raised against testosterone-3-BSA was 6.5×10^9 L/mol. The affinity constant of the antisera raised against progesterone-11-BSA and estradiol-6-BSA were 3.2×10^9 L/mol and 0.29×10^9 L/mol respectively.

The antiserum raised against testosterone-3-BSA was tested for cross-reactivity with a number of steroid hormones and human chorionic gonadortropin (hCG). The results showed a high degree of specificity towards testosterone (Fig. 3A). The other corss-reacting steroids were 5 α-androstane 17β-ol-3-one, 3β-hydroxy-5-androsten-17-one, estradiol-17β, progesterone and pregnenolone which showed cross-reactivity of 5.46%, 0.42%, 0.33%, 0.04% and 0.03% respectively. Whereas hydroxycortisone and hCG did not show any detectable cross-reaction (Table I). Similarly the antisera against progesterone-11-BSA and estradiol-6-BSA showed a high degree of specificity towards their respective steroids, progesterone and estradiol (Fig. 3B-C). While with other steroids except pregnenolone and hCG, these antisera gave minimal or no corss-reactivity (Tables II, III). The antiserum against progesterone-11-BSA showed a considerable cross-reaction with pregnenolone (18.42%).

Table I.- Percent cross-reactivity of testosterone-3-BSA antiserum raised in rabbits.

Steroids	% cross-reaction
Testosterone	100
5α-androstane-17β-ol-3 one	5.46
3β-hydroxy-5-androsten-17 one	0.42
Estradiol-17β	0.33
Progesterone	0.04
Pregnenolone	0.03
Hydroxycortisone	0.0
Human chorionic gonadotropin (hCG)	0.0

The percent recovery of steroid during the assay was also measured using the known quantities of unlabelled steroids. The percent recovery of testosterone, progesterone and estradiol using their antisera were 105±10.63, 92.74±2.77 and 107±5.38 respectively (Table IV-VI). Furthermore, the antisera raised against testosterone-3-BSA, progesterone-11-BSA and estradiol-6-BSA were used to obtain standard

Table II.- Percent cross-reactivity of progesterone-11-BSA antiserum raised in rabbits.

Steroids	% cross-reaction
Progesterone	100
Pregnenolone	18.42
Testosterone	0.005
5α-androstane-17β-ol-3 one	0.004
3β-hydroxy-5-androsten-17 one	0.001
Estradiol-17β	0.0
Hydroxycortisone	0.0
hCG	0.0

Table III.- Percent cross-reactivity of estradiol-6-BSA antiserum raised in rabbits.

Steroids	% cross-reaction
Estradiol-17β	100
5α-androstane-17β-ol-3 one	1.76
Testosterone	0.15
3β-hydroxy-5-androsten-17 one	0.06
Progesterone	0.01
Pregnenolone	0.01
Hydroxycortisone	0.0
hCG	0.0

Table IV.- Percent recovery of testosterone using antiserum raised against testosterone-3-BSA.

Hormone added (ng)	Hormone measured (ng)	Difference (ng)	% recovery
0.1	0.11	0.01	110
1.0	1.2	0.2	120
5.0	4.7	0.3	94
10.0	9.6	0.4	96

Mean % recovery = 105±10.63

curves by displacement of respective tritiated tracer with increasing concentration of unlabelled respective hormones. The sensitivity of standard curve of testosterone, progesterone and estradiol, calculated at 95% confidence limit was 10, 25, and 15 picograms per 200 μl, respectively.

Table V.- Percent recovery of progesterone using antiserum raised against progesterone-11-BSA.

Hormone added (ng)	Hormone measured (ng)	Difference (ng)	% recovery
0.1	0.09	0.01	90
1.0	0.9	0.1	90
5.0	4.8	0.2	96
10.0	9.5	0.5	95

Mean % recovery = 92.75±2.77

Table VI.- Percent recovery of estradiol using antiserum raised against estradiol-6-BSA.

Hormone added (ng)	Hormone measured (ng)	Difference (ng)	% recovery
0.1	0.1	0.0	100
1.0	1.1	0.1	110
5.0	5.7	0.7	114
10.0	10.4	0.4	104

Mean % recovery = 107±5.38

DISCUSSION

All rabbits responded with a considerable production of antibodies against steroid conjugates after injections of respective immunogens subcutaneously. The increase in the titer of antisera corresponded with the number of injections given. These results are in agreement with the results of Exley *et al.* (1971). In our experiments, rabbits immunized with testosterone-3-BSA, progesterone-11-BSA and estradiol-6-BSA, a titer of 48-54% binding was achieved at a final dilution of 1:24,000. Our testosterone-3-BSA antiserum results are very much the same as that obtained by Ismail *et al.* (1972), and Nieschlag and Loriaux (1972) who reported a titer of 50% binding at a final dilution of 1:24,000 and 1:25,000 respectively. Similarly antisera raised against progesterone-11-BSA by Niswender (1973) and antisera raised against estradiol-6-BSA by Abraham (1969) and Exley *et al.* (1971) also showed a titer of 50% binding at a final dilution in the range comparable to ours.

The binding capacity of antisera were determined separately using tritiated as well as iodinated tracers. Our results indicated that antisera have high binding capacity with iodinated tracer as compared to tritiated

tracer. A plausible explanation of this observation, as suggested by Jeffcoate (1980) is that ^{125}I-steroids also react with the antibodies raised against the bridges that hold the steroids and the carrier proteins. This is, however, only true in homologous systems, in which the structure of the steroid derivative, that is, the position, the orientation and the nature of the bridges between steroids and carriers are the same, both in the label as well as in the immunogen. These results also indicate that the bridges may be involved in the determination of the specificity of the antibody binding site and the subsequent binding of antigen by the antibody *in vitro*. The affinity of an antibody to its specific antigen depends upon the affinity constant (Ka). Several workers who obtained the antisera for steroid radioimmunoassay by immunizing rabbits or mice, have shown the affinity constant of the antisera in the range 10^9 to 10^{10} l/mol (Exley *et al.*, 1971; Bauminger *et al.*, 1973; Tateishii *et al.*, 1978; Malvano and Rolleri, 1980). Our results of affinity constant were also close to the earlier described values and provided an evidence of suitability of our antisera for its use in steroid radioimmunoassay. Moreover, the validation of steroid radioimmunoassays requires an adequate proof of specificity. Therefore, the results given in the Tables I, II and III clearly indicate a high degree of specificity of antisera produced in our laboratory. In general this is comparable to other studies reported by Abraham *et al.* (1971), Exley and Woodhames (1976), Attanasio and Gupta (1980) and Moreno *et al.* (1980). Further, our results of cross-reactivity of antisera with various steroids support the previous finding that a higly specific antisera can be obtained by coupling testosterone at C-3; progesterone at C-11; and estradiol at C-6, position (Jeffcoate and Searle, 1972; and Niswender, 1973).

Like others, we have also carried out recovery studies, like others for the determination of accuracy of radioimmunoassay (Abraham *et al.*, 1971; Bodley *et al.*, 1973). The percent of recoveries were slightly higher in case of testosterone (105%) and estradiol (107%) when compared with already reported percent of recoveries (80-103%). This as described by Abraham (1980) may be caused due to the presence of excess lipids and proteins which interfere with the adsorption of the free steroid molecules with dextran-coated charcoal during separation. This interference would result in an increase in the amount of free steroid molecules left behind in the supernantant. Since the supernatant was evaluated for the bound fraction, therefore, an increase in the radioactivity of the bound fraction was observed. In contrast to overestimation, the underestimation was observed in the case of progesterone (93%) may be the result of two principal factors, *i.e.*, contamination and loss of the steroid. It is not completely known which organic/inorganic compounds may intefere with the estimation, but a number of compounds have been reported to be involved. In all cases these contaminations either compete with binding sites present both on the antigen as well as antibody, thus preventing mutual binding between these two or by causing some damage either to the antibodies or to the steroid molecules, thus resulting in a decrease in the fraction of radioactivity labelled steroid bound to the anti-steroid antibodies.

The results of sensitivity of our antisera raised against testosterone-3-BSA, progesterone-11-BSA, and estradiol-6-BSA were comparable to the sensitivity values calculated by different investigators for testosterone, progesterone and estradiol (Ismail *et al.*, 1972; Wickings and Nieschlag, 1978). The sensivitiy of the standard curve is defined as the smallest amount of steroid standard that is significantly different from zero at the 95% confidence limit. We on the basis of our studies suggest that the antisera, we produced in our laboratory, have sufficient sensitivity and specificity and therefore may be used for routine steroids radioimmunoassay.

REFERENCES

ABRAHAM, G.E., 1969. Solid-phase RIA of estradiol-17β. *J. Clin. Endocrinol. Metab.*, **29**: 866-870.

ABRAHAM, G.E., 1980. Reliability criteria for steroid radioimmunoassay. In *Radioimmunoassay of steroid hormones* (ed. D. Gupta), pp. 9-17. Verlag Chemie, Weinheim, Deerfield Beach, Florida.

ABRAHAM, G.E. AND GROVER, P.K., 1971. Covalent linkage of hormonal haptens to protein carrier are used in radioimmunoassay. In *Principles of competitive protein binding assay* (ed. W.G. Odell and W.H. Daughaday). pp. 134-140. J.B. Lippincott. Philadelphia.

ABRAHAM, G.E., HOOPER, K., TULCHINSKY, D., SWERDLOFF, R.S. AND ODELL, W.D., 1971. Simultaneous measurement of plasma progesterone, 17-hydroxy progesterone and estradiol-17β by radioimmunoassay. *Anal. Letter*, **4**: 325-335.

ANNEKE, M.G.B., HOLLANDER, F.C.D. AND WOODS, G.F., 1974. Specificities of antisera against testosterone linked to albumin at different positions (C3, C11, C17). *Steroids*, **23**: 699-711.

ATTANASIO, A. AND GUPTA, D., 1980. Simultaneous radioimmunoassay of estrogens and androgens in plasma of prepubertal children. In *Radioimmunoassay of steroid hormones* (ed. D. Gupta), pp. 117-125. Verlag Chemie, Weinheim.

BAUMINGER, S., LINDER, H.R. AND WEINSTEIN, A., 1973. Properties of antisera to progesterone and to 17-hydroxy progesterone elicited by immunization with the steroids attached to protein through position 7. *Steroids*, **21**: 847-856.

BODLEY, F.H., CHAPDELAINE, A., FLICKINGER, G., MIKHAIL, G., YAVERBAUM, S. AND ROBERTS, K.D., 1973. A highly specific RIA for progesterone using antibodies covalently linked to acrylamine glass particles. *Steroids*, **21**: 1-16.

DUFAU, M.L., TSURUHARA, T., HORNER, K.A., PODESTA, E. AND CATT, K.J., 1977. A highly sensitive *in vitro* bioassay for luteinizing hormone and chorionic gonadotropin: testosterone production by dispersed Leydig cells. *J. Clin. Endocrinol. Metab.*, **39**: 610-613.

ERLANGER, B.F., BOREK, F., BEISER, S.M. AND LIBERMAN, S., 1957. Steroid-protein conugates. 1-preparation and characterization of conjugates of bovine serum albumin with testosterone and with cortisone. *J. biol. Chem.*, **228**: 713-727.

EXLEY, D., JHONSON, M.W. AND DEAN, P.D.G., 1971. Antisera highly specific for 17β-estradiol. *Steroids*, **18**: 605-620.

EXLEY, D. AND WOODHAMS, B., 1976. The specificity of antisera raised by estradiol-17β-3-hemisuccinyl-bovine serum albumin. *Steroid*, **27**: 813-820.

FRANCHIMONT, P., HANDRICK, J.C. AND REUTER, A.M., 1983. Antibody production for immunoassay. In *Principles of competitive protein-binding assay* (eds. W.D. Odell and P. Franchimont), pp. 33-25. John Wiley and Sons, Inc.

GROVER, P.K. AND ODELL, W.D., 1976. Specificity of antisera of sex steroids. *J. Steroid Biochem.*, **8**: 121-125.

ISMAIL, A.A.A., NISWENDER, G.D. AND MIDGLEY, A.R., 1972. RIA of testosterone without chromatography. *J. Clin. Endocrinol. Metab.*, **34**: 177-184.

JEFFCOATE, S.L., 1980. Use of ^{125}I tracers in steroid radioimmunoassays. In *Radioimmunoassay of steroid hormones* (ed. D. Gupta), pp. 209-219. Verlag Chemie Weinheim.

JEFFCOATE, S.L. AND SEARLE, J.E., 1972. Preparation of a specific antiserum to estradiol-17β coupled to protein through the B-ring. *Steroid*, **19**: 181-188.

MALVANO, R. AND ROLLERI, E., 1980. Methodological aspects of steroid radioimmunoassay. In *Radioimmunoassay of steroid hormones* (ed. D. Gupta), pp. 27-53. Verlag Chemie, Weinheim.

MORENO, M.C., WICKINGS, E.J. AND NIESCHLAG, E., 1980. Methodology of RIA for testosterone. In *Radioimmunoassay of steroid hormones* (ed. D. Gupta), pp. 101-115. Verlag Chemie, Weinheim.

NAEGELE, W. AND DRAHOVSKY, M., 1980. Production of steroid antisera. In *Radioimmunoassay of steroid hormones*. (ed. D. Gupta), pp. 54-72. Verlag Chemie, Weinheim.

NIESCHLAG, E. AND LORIAUX, D.L., 1972. Radioimmunoassay for plasma testosterone. *Z. Klin. Chem. Klin. Biochem.*, **10**: 164-168.

NISWENDER, G.D., 1973. Influence of the site of conjugation on the specificity of antibodies to progesterone. *Steroids*, **23**: 203-218.

PANG, C.N. AND JOHNSON, D.C., 1974. A method for the preparation of steroid-protein antigens for use in immunoassay of steroids. *Steroids*, **23**: 203-218.

TATEISHI, K., KATO, A., YAMMATO, H., ITAYASHI, C. AND KITAGAWA, M., 1978. Anti-testosterone antiserum produced in mice of different strains and sexes. *Steroids*, **32**: 233-243.

WICKINGS, E.J. AND NIESCHLAG, E., 1978. The effect of active immunization with testosterone on the pituitary-gonadal feedback in male Rhesus monkey (*Macaca mulatta*). *Biol. Reprod.*, **18**: 602-607.

YOUICHI, F., TERANISHI, M., IKEDA, Y., YAMAZAKI, M., KISHIDA, S. AND MLYABO, S., 1987. Characterization of antisera of 2-hydroxy estradiol and 4-hydroxy-estradiol using 6-(o-carboxymethyl) oxime and 17-hemisuccinate-BSA conjugates in RIA. *Steroids*, **46**: 857-866.

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