J. steroid Biochem. Vol. 23, No. 6A, pp. 1043–1051, 1985
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0022-4731/85 $3.00 + 0.00
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THE MEASUREMENT OF TESTOSTERONE AND OESTRADIOL-17β USING IODINATED TRACERS AND INCORPORATING AN AFFINITY CHROMATOGRAPHY EXTRACTION PROCEDURE

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(*Received* 25 *May* 1985)

Summary—The development of sensitive radioimmunoassays (RIA) for testosterone and oestradiol-17β, utilising ^{125}I-radioligands, is described. Use of an homologous bridge at the same site of attachment, for both the radioligand and the steroid–carrier protein conjugate employed in raising antibodies, normally results in a loss of assay sensitivity and precision. This was overcome in the oestradiol assay by utilising an heterologous configuration at the site of attachment (11α vs 11β). In contrast, for testosterone, even though an homologous bridge and site of attachment was used for the radioligand and the steroid–carrier protein conjugate, a very sensitive assay with extremely high antibody titres (dilution of $1:2 \times 10^6$) was achieved. This finding was repeated with a different antiserum suggesting that the "bridge binding" phenomenon may be related to the position of attachment to the steroid molecule. In addition, an antibody-Sepharose 4B affinity chromatography extraction procedure has been developed for both oestradiol and testosterone. This approach allows the measurement of very low concentrations of steroids from large volumes of a variety of biological fluids. As antibody-linked Sepharose 4B uses high concentrations of antibody, steroids of similar structure are extracted from biological fluids. However, the cross-reactivity of these related steroids are very low in the RIA's, ensuring good specificity.

INTRODUCTION

Although a number of methods have been described for the measurement of steroid hormones [1] there is still a requirement for more precise and sensitive methods to assay these hormones in plasma or serum. This is especially relevant for the study of reproduction in domestic animals where the concentrations of oestradiol-17β and testosterone in the peripheral circulation are extremely low [see reviews 2–4].

An increase in RIA sensitivity has been achieved by using the higher specific activity γ-emitting radionuclides such as ^{125}I as opposed to β-emitting radioisotopes. This approach also has the added advantage of the cost effective manner in which samples can be counted [5–8]. A serious disadvantage however, of using radioiodinated labels is the affinity that the antibody may show, not only for the hapten, but for the bridge through which it is attached to the carrier protein. As reviewed recently [5, 6] the "bridge-binding" phenomenon can be overcome by using either an homologous bridge to different configurations at the same site of attachment to the hapten [9] or by using an heterologous bridge [7, 10]. The occurrence of "bridge binding" may also be affected by the position of attachment to the steroid, even when using an homologous bridge and site of attachment. The present paper describes our results using an homologous bridge, from different configurations at the same site of attachment, in the case of oestradiol, and an homologous bridge from the same site, in the case of testosterone.

Even with the utilisation of these techniques for the development of sensitive assays, relatively large volumes of plasma or serum are still required to measure very low concentrations of steroids in the peripheral circulation of domestic animals [11, 12]. Classical extraction procedures utilise a range of solvents normally followed by chromatographic purification to remove cross-reacting substances. The large volumes of fluid (at least 3 ml) that have to be extracted necessitate the use of large quantities of solvents which, as well as being hazardous, costly and laborious, increases the risk of high assay blank values [8] and low recoveries. In this report we also describe the use of an affinity chromatography extraction procedure [13] and its incorporation into very sensitive RIA's for the measurement of low concentrations of testosterone and oestradiol-17β.

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EXPERIMENTAL

Chemicals and glassware

Testosterone-3-carboxymethyloxime (testosterone-3-CMO) was purchased from Steraloids, Croydon, U.K., and 17β-oestradiol-11α-hemisuccinate and 17β-oestradiol-11β-hemisuccinate were supplied by Upjohn Co. (Kalamazoo, Michigan, U.S.A.). Pig skin gelatin, merthiolate (thimerosal), sodium metabisulphite, chloramine-T and steroids, used in the standard curves and cross-reactivity studies, were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). Na ^{125}I (carrier free) and tritiated steroids [1,2,6,7-^{3}H]testosterone (3.4 TBq/mmol); [2,4,6,7,16,17-^{3}H]oestradiol-17β (5.4 TBq/mmol); [2,4,6,7-^{3}H]oestrone (3.0 TBq/mmol); [1,2,6,7,16,17-^{3}H]progesterone (3.9 TBq/mmol) and [1,2,6,7-^{3}H]androstenedione (3.0 TBq/mmol) were obtained from Amersham International (Buckinghamshire, U.K.). All solvents (analar grade) and scintillation fluid, except for "aristar" ethanol (BDH Chemicals, Glasgow, U.K.) were purchased from Fisons Ltd. (Loughborough, U.K.). Sephadex G-25 (finemesh), LH20 and CNBr-activated Sepharose 4B were supplied by Pharmacia Fine Chemicals (Uppsala, Sweden).

Purity of the water and cleanliness of the glassware is of particular importance when measuring low steroid concentrations. Therefore the water was coarse filtered to remove particulate material, deionised and finally double glass distilled. The glassware, except the columns used for the affinity chromatography, was washed in a Meile dishwasher, rinsed twice in distilled water and finally baked at 200°C for 2 h in a laboratory oven.

After assessing a number of different columns for use in the affinity chromatography extraction procedure the most appropriate were those using 10 mm dia (porosity 1) glass sinter discs (Schott Glass, U.K.). The sinter discs were inserted into soda glass columns 10 × 120 mm with 55 mm tapered outlets. This size of column allows a large number (≥100) to be used simultaneously.

Syntheses

Syntheses of steroid conjugates were similar to those described by Nordblom *et al.*[7].

Preparation of testosterone-3-CMO-histamine

Testosterone-3-CMO (0.0668 mmol) and triethylamine (Sigma, 0.133 mmol) were dissolved in freshly-distilled dioxane (BDH, 3 ml) and the reaction mixture was cooled to 10°C. Isobutylchloroformate (Sigma, 0.0734 mmol) was added resulting in the immediate formation of a white precipitate; the reaction mixture was stirred for an additional 30 min. Histamine (Sigma, 0.0668 mmol), dissolved in 2 ml dioxane plus 3 drops of water, was added and the mixture was stirred overnight at 10°C. The reaction mixture was poured into water (40 ml) and the aqueous layer was extracted 6 times with CH_2Cl_2 (15 ml). The combined organic layer was dried with Na_2SO_4 and concentrated to approx 500 μl *in vacuo*. The concentrate was loaded onto a 20 × 20 cm silica gel preparative thin layer chromatography (TLC) plate (60F 254, 0.25 mm thickness; Merck, Darmstadt, West Germany) and developed with CH_2Cl_2–CH_3OH–NH_4OH (90:10:1 by vol). The plate was treated with fluorescent dye and visualised with a u.v. lamp showing three bands at R_f 0.26, 0.10 and at the origin. The unreacted testosterone-3-CMO remained at the origin. The bands at R_f 0.10 (band A) and 0.26 (band B) were scraped off the plate and extracted with ethanol (10 ml). The ethanol solutions were evaporated to dryness and the resulting materials radioiodinated as described below. Radioiodinated band A was bound by antibody to testosterone (as described in the Results section) and therefore proved to be the desired product.

Preparation of 17β-oestradiol-11α-succinyl-tyrosinemethylester

17β-Oestradiol-11α-hemisuccinate (0.030 mmol) and triethylamine (0.060 mmol) were dissolved in freshly distilled dioxane (5 ml) and the mixture was cooled to 10°C. Isobutylchloroformate (0.033 mmol) was added resulting in the immediate formation of a white precipitate. After the reaction had mixed for 30 min tyrosine methyl ester (TME, 0.030 mmol), dissolved in dioxane (2 ml), was added and the reaction mixture was stirred overnight at 10°C. The mixture was poured into water (40 ml) and made slightly acidic (pH 4.0) with dilute HCl. The aqueous phase was extracted 6 times with CH_2Cl_2 (15 ml). The combined organic layer was dried with Na_2SO_4 and concentrated to about 500 μl *in vacuo*. The concentrate was loaded onto a 20 × 20 cm preparative TLC plate and developed with CH_2Cl_2–CH_3OH–CH_3COOH (95:5:1, by vol). As in the case of the testosterone-3-CMO-histamine synthesis above, three bands were found. Unreacted 17β-oestradiol-11α-hemisuccinate remained at the origin. In addition, two bands were found at R_f 0.33 (band A) and 0.63 (band B). The silica for each band was scraped from the plate and extracted with ethanol (10 ml). When the material from band A was radioiodinated as described below the label was bound by the oestradiol-17β antibody as described in the Results section.

Preparation of 17β-oestradiol-11β-succinyl-tyrosinemethylester

This was prepared as described for the 17β-oestradiol-11α-TME except that 17β-oestradiol-11β-hemisuccinate was used. As for the 17β-oestradiol-11α-TME separation by TLC following synthesis gave three bands. Unreacted oestradiol-11β-hemisuccinate remained at the origin. In addition there were two bands at R_f 0.29 (band A)

and 0.57 (band B). Furthermore, as for the 17β-oestradiol-11α-TME, when band A was radioiodinated, see below, the label was bound by the oestradiol antibody as described in the Results section.

Preparation of 17β-oestradiol-11β-succinyl-bovine serum albumin

17β-Oestradiol-11β hemisuccinate (0.023 mmol) was dissolved in dimethyl formamide (2 ml) and H_2O (1 ml). 1-Ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide (0.192 mmol) was added and the mixture stirred for 30 min. Bovine serum albumin (36 mg), dissolved in sodium phosphate buffer (0.02 M, pH 7.8, 10 ml), was added dropwise to the steroid solution and stirred for 4 days at room temperature in the dark. The reaction mixture was dialysed against 0.05 M $NaHCO_3$ (2 × 500 ml) and H_2O (2 × 500 ml) and subsequently dried by lyophilization.

Radioiodination

Iodination of the testosterone-3-CMO-histamine, 17β-oestradiol-11α-TME and 17β-oestradiol-11β-TME were carried out as described previously by Hunter *et al.* [14]. Aliquots of the steroid derivatives (2 μg) were stored in 10 μl ethanol (aristar) at −50°C. Prior to iodination the solvent was evaporated to dryness under N_2 and the steroid conjugate was then reconstituted in 40 μl phosphate buffer (0.5 M, pH 7.5).

Aqueous Na [^{125}I] (37 MBq, 10 μl) was added. The reaction was initiated by the addition of chloramine-T (10 μg/10 μl in 0.05 M sodium phosphate buffer, pH 7.5). After mixing and incubation at ambient temperature for 2 min the reaction was quenched by the addition of sodium metabisulphite (10 μg/10 μl in 0.05 M phosphate buffer, pH 7.5).

In the case of the oestradiol-17β derivatives the reaction mixture was transferred to a 10 × 200 mm Sephadex G25 (fine mesh) column, previously swollen in 0.05 M phosphate buffer (pH 7.5). The iodinated steroid was eluted under pressure using a peristaltic pump, with 0.05 M sodium phosphate buffer (pH 10.5). Approximately 1 ml aliquots were collected directly into glass tubes containing 200 μl phosphate buffer (0.5 M, pH 7.5). The first peak eluted was discarded and the second peak, normally fractions 20–30, were pooled and adjusted to pH 7.5 and stored at 4°C. The specific activity of the label was assessed as described previously [5, 7] and was found to be approx 48 TBq/mmol. The shelf-life of the material was at least 60 days.

In the case of the testosterone derivative, following iodination, the reaction volume was increased to 500 μl by addition of phosphate buffer (0.05 M, pH 7.5) and then transferred to a tapered glass stoppered centrifuge tube. The iodinated derivative was extracted by mixing for 1 min with 500 μl ethyl acetate. The solvent phase was transferred to a second glass vial and the reaction mixture extracted for a second time. The combined solvent was then evaporated to a vol of 200 μl, by incubating in a water bath at 40°C, under a stream of air. The concentrated preparation was then loaded onto a plastic backed silica gel TLC plate (20 × 20 cm) as a continuous streak. The plate was developed in CH_2CL_2–CH_3OH–CH_3COOH (90:10:1, by vol) for approx 2 h. The plate was scanned, using a minimonitor and the required band (R_f 0.8) was cut out and the iodinated steroid eluted by incubating overnight in ethanol (aristar) at 4°C. Two other bands at the origin and just behind the solvent front were discarded. The ethanol was filtered through a Pasteur pipette containing glass wool to remove particulate silica gel. The material was stored at 4°C until use, the shelf life of the label being usually at least 90 days. The specific activity of the label was found to be approx 63 TBq/mmol.

Recently one of the authors (G. D. Nordblom, pers. commun.) has developed an HPLC procedure for purifying the radiolabelled testosterone and oestradiol-11α-TME derivatives. Purification was performed on an Altech 600 RPA C_{18} reverse phase column. The entire radioiodination reaction mixture was injected on the column using a Rheodyne injector fitted with a 100 μl loop. A Spectra-Physics 8700 pump and solvent delivery system provided the following mobile phase at a flow rate of 1 ml per min: for oestradiol-17β; 0–5 min, an isocratic mixture of 20% CH_3CN (A), 80% 0.05 M potassium formate buffer, pH 4.0 (B), 5–55 min, a 20–45% gradient of A; 55–80 min, a 45–70% gradient of A; for testosterone; 0.5 min an isocratic mixture of 20% A and 80% B, 5–80 min, a 20–60% gradient of A.

The elution pattern of the oestradiol-17β label consisted of free iodine at 3 min, monoiodinated TME at 52 min and diiodinated TME at 54 min. Several other peaks at 61, 64, 69 and 74 min were also eluted. The material in these peaks was not bound by antibody to oestradiol and undoubtedly correspond to derivatives that are iodinated on the A-ring of the steroid. The elution pattern of the testosterone label consisted of free iodide at 3 min and peaks at 56, 59 and 65 min all three of which were bound by antibody to testosterone and are probably due to 2-iodo, 5-iodo and 2,5-diiodo derivatives of the imidazole ring of the histamine functional group.

Antisera

The testosterone antisera used in the RIA and also the oestradiol and testosterone antisera used for linking to the CNBr-activated Sepharose 4B were raised in sheep as described previously [16]. The oestradiol-17β antisera used in the radioimmunoassay was raised in rabbits against 17β-oestradiol-11β-succinyl-bovine serum albumin (BSA) in collaboration with H. M. Fraser and A. McNeilly (MRC, Reproductive Biology Unit, Edinburgh). Rabbits were injected intra-dermally

with 150 μg 17β-oestradiol-11β-succinyl-BSA in a 2 ml emulsion of saline:Freunds complete adjuvant (ratio 2:3). The animals were then boosted at approx 3 month intervals with 50–100 μg of oestradiol conjugate in a 2 ml emulsion of saline: Freunds incomplete adjuvant (ratio 2:3). Animals were bled at weekly intervals following boosting and the different pools of serum characterised. The anti-oestradiol-17β serum used from rabbit 32 (Table 1) was collected following the first booster injection and anti-oestradiol-17β serum from rabbit 48 (Table 1) was collected following the second booster injection. Specificity of the antisera used in the radioimmunoassays was estimated by measuring 50% displacement of the label by the other steroids [17,18].

Ovine oestradiol-17β and testosterone antisera were covalently coupled to CNBr-activated Sepharose 4B using the methodology recommended by the manufacturer. Briefly 9 g (dry weight) of Sepharose, swollen to give approx 35 ml slurry, was placed on a grade 3 sintered glass filter funnel and washed with 1800 ml of 1 mM HCl. The slurry was then transferred to a 50 ml ground glass stoppered test-tube containing 150 μl of antiserum, in 18 ml of coupling buffer ($NaHCO_3$, 0.1 M; NaCl, 0.5 M; pH 8.3). The solution was mixed overnight at 4°C on an end over end mixer. Unbound material was removed by washing with 50 ml coupling buffer on the sintered glass filter funnel. Remaining residual active groups on the Sepharose were blocked by incubating with 200 ml ethanolamine (1 M, pH 9.0) for 2 h at ambient temperature. Non-covalently bound material was removed by four alternate washes with high (borate buffer, 0.1 M, pH 8.0) and low (acetate buffer, 0.1M pH 4.0, containing 0.5 M NaCl) pH buffers. Finally the antibody bound Sepharose was washed, resuspended and stored at 4°C in 75 ml phosphate buffered saline (0.05 M, pH 7.5, 0.01% thimerosal) ready for use.

Affinity chromatography extraction procedure

Approximately 1,000 cpm of the appropriate tritiated steroid (10 μl diluted in ethanol) together with the appropriate sample (5–3,000 μl) were pipetted into screw-capped glass culture tubes (16 × 125 mm), mixed and incubated for 30 min at room temperature. A further 10 ml of double distilled H_2O was then added to each tube, followed by 500 μl of antibody-Sepharose. It was later found that as little as 50 μl of antibody-Sepharose could be used therefore negating the need for recycling. The samples were then mixed end over end, for at least 2 h at room temperature or overnight at 4°C. The contents of each tube were poured directly on to prewashed sintered glass columns and the aqueous waste discarded. The antibody-Sepharose residues in the tubes were then rinsed with 7 ml H_2O and again poured on to the respective columns. The antibody-Sepharose in each column was washed with 20 ml H_2O and the eluates allowed to run to waste. Residual water in the system was then removed by applying slight positive pressure using an aquarium pump. Bound hormone was then eluted with 3 ml CH_3OH–H_2O (90:10, v/v). Positive pressure was applied to ensure maximum elution of the solvent into glass test tubes (125 × 16 mm). The eluate was evaporated to dryness in a Buchler Vortex evaporator at 40°C (Gallenkamp). The hormone extracts were reconstituted in 1800 μl phosphate buffered saline, containing 0.1% gelatin (PBS-Gel), and then mixed for at least 20 min on a Vortex evaporator (37°C). Recovery was assessed as a percentage of total counts by taking a 500 μl aliquot of the reconstituted material and counting on a liquid scintillation counter (LKB, Rackbeta 1211). For example mean recovery for testosterone was 81.4% (c.v. = 4.0%, $n = 160$) and for oestradiol-17β 76.9% (c.v. = 6.0%, $n = 154$) over several assays.

Two 500 μl aliquots of the extracted and reconstituted steroid were then removed for radioimmunoassay. As the tritiated label used for recovery estimates does not interfere with gamma counts, individual recovery estimates were routinely made. However, because of the sensitivity of the assays (see Results section) correction was made for the weight of steroid added in the recovery counts.

After use the residual antibody-Sepharose was resuspended in 7 ml H_2O and the slurry from each column pooled in a large sintered glass funnel (porosity 3) and recycled by 3 alternate washes in CH_3OH and H_2O. The material was found to be very stable and was recycled, without loss of binding activity, at least 50 times over a 2 year period. However, it has been estimated that a 15 g batch of Sepharose provides enough material to extract over 2000 samples (50 μl antibody-Sepharose) thereby reducing the need to recycle the material.

Some of the validation experiments (see Results section) also employed a solvent extraction procedure. In these cases the samples, which had previously been equilibrated with recovery label, were extracted with either 15 ml (oestradiol-17β) or 3 ml (testosterone) diethyl ether by mixing for 15 min on a multivortexer (SMI). The aqueous phase was frozen in a dry ice–methanol bath and the solvent phase decanted into glass tubes (125 × 16 mm) and evaporated to dryness in a Buchler vortex evaporator. The sample was then reconstituted in either assay buffer or H_2O prior to assay or affinity chromatography extraction respectively.

Radioimmunoassay

The steroid standards (500 pg/ml) or unknown samples were dissolved in assay buffer (PBS-gel) and dispensed in duplicate or triplicate and made up to a constant volume of 500 μl in assay buffer. A 100 μl aliquot of iodinated steroid (approx 20,000 counts per 100 s) was then added immediately followed by a 200 μl aliquot of first antibody at an appropriate dilution (see Table 1). The assay was then incubated

Table 1. Comparison of antisera and radioligands

Antiserum	Immunogen	Iodine labelled conjugate	Final antibody Titre ($\times 10^{-3}$)	Percent of label bound (%)	Slope of inhibition curve	Mass of steroid for 50% displacement of bound label (pg/tube)
Sheep 501	oestradiol-6-CMO*	17β-oestradiol-11α-TME	1:120	49	†	200
Sheep 614	oestradiol-3-CME‡	17β-oestradiol-11α-TME	1:28	37	†	400
Rabbit 32	oestradiol-11β-H§	17β-oestradiol-11α-TME	1:200	40	−0.82[w]	20
Rabbit 48	oestradiol-11β-H	17β-oestradiol-11α-TME	1:160	38	−1.09[y]	5
Rabbit 32	oestradiol-11β-H	17β-oestradiol-11β-TME	1:200	38	−0.60[x]	125
Rabbit 48	oestradiol-11β-H	17β-oestradiol-11β-TME	1:160	62	−0.73[z]	96
Sheep 505	testosterone-3-CMO	testosterone-3-CMO-histamine	1:2800	82	−0.99	40
Sheep 506	testosterone-3-CMO	testosterone-3-CMO-histamine	1:2800	84	−1.2	28

*Carboxymethyloxime; †Binding was inhibited over a very small range (see text for details); ‡Carboxymethyl ether; §Hemisuccinate. w versus x and y versus z are significantly different ($P < 0.001$).

at room temperature for approx 2 h followed by the addition of previously optimised precipitating reagents. These included 100 μl normal sheep or rabbit serum, depending on first antibody, and 100 μl of second antibody (raised in donkeys to either sheep or rabbit IgG) diluted in PBS gel, containing 0.1 M EDTA. Following an overnight incubation at 4°C, 2 ml of ice-cold PBS gel was added and the tubes centrifuged at 800 *g* for 30 min (4°C). The supernatants were immediately decanted to waste, tubes drained for 15 min and the radioactivity in the precipitates counted on a gamma counter LKB (80,000 Series Wallac or LKB 1271 Riagamma).

Analysis of results

The assay results were computed by the ABRO RIA program package based on the method of Rodbard and Lewald [19]. Scatchard analysis [20] of results was carried out by the RIA programme package, but were also re-calculated following correction for non-specific binding [21, 22]. Significance of differences between the slopes of the standard curves and Scatchard plots were calculated by comparing the regression lines of the curves (Students *t*-test).

RESULTS

Antibody characteristics

Titres. The antibody titres and the various iodinated labels used are shown in Table 1. The most sensitive oestradiol assays were developed by using an heterologous configuration assay system consisting of 17β-oestradiol-11α-TME label with an antibody raised against 17β-oestradiol-11β-BSA. For both of the oestradiol antisera tested the use of an 17β-oestradiol-11β-TME label shifted the slope to the right, i.e. the weight of hormone required to inhibit binding was markedly increased. Also the slope of the standard curve was significantly shallower than the ideal −1.00, as would be expected from classical "bridge binding" theory [5, 6].

Although 5 antisera, raised against oestradiol-17β conjugated at the three or six positions, were tested for their ability to bind to iodinated oestradiol-17β (17β-oestradiol-11α-TME) only two antisera, sheep 501 and 614 (Table 1), were capable of binding the label. Binding of the iodinated label by these two antisera could be inhibited with unlabelled oestradiol. However, inhibition did not occur until at least 50 pg of oestradiol had been added and then occurred over a narrow range (sheep 501: between 50–100 pg; sheep 614: between 100–500 pg), thereby producing abnormal standard curves.

For the testosterone assay an homologous assay system (same chemical bridge and site of attachment) was used. Although extremely high titres were achieved (Tables 1 and 2), suggesting a "bridge binding" phenomenon, the label was capable of being displaced with unlabelled hormone (Table 2). Therefore at extremely high antibody dilutions very sensitive assays were obtained with both antisera.

From these initial studies rabbit 48 for the oestradiol-17β assay (using 17β-oestradiol-11α-TME label), and sheep 505, for the testosterone assay (using testosterone-3-CMO-histamine label), were characterised further and then incorporated with an affinity chromatography extraction procedure.

Table 2. Binding characteristics of testosterone antiserum (sheep 505) at three dilutions

Final titre ($\times 10^{-3}$)	Slope of the inhibition curve	Dissociation constant (M)	Percentage of total label bound (%)	Mass of steroid for 50% displacement of bound label (pg)
1:2,800*	−1.25	(a) 1.9×10^{-11}a	82	37
	−0.93	(b) 1.1×10^{-10}b		
1:10,000	−1.15	1.2×10^{-11}	55	10
1:20,000	−1.03	1.4×10^{-11}	48	5

*Two populations of antibodies (see Fig. 1) and overall slope of the inhibition curve = −1.07. a versus b $P < 0.001$.

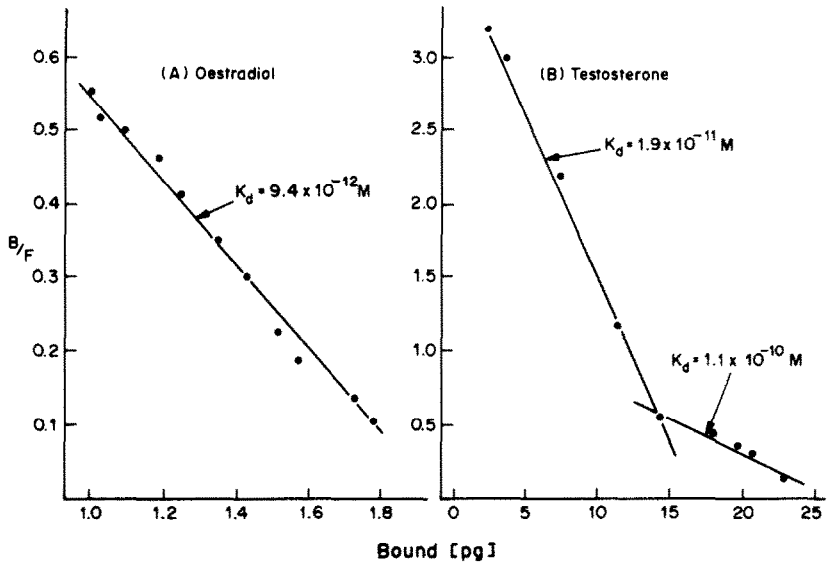

Fig. 1. Scatchard analyses of (A) oestradiol antiserum (rabbit 48) tested at a final dilution of 1:200,000 and (B) testosterone antiserum (sheep 505) tested at a final dilution of 1:2,800,000.

Antibody affinities. Scatchard analysis of the oestradiol-17β inhibition curve (rabbit 48) demonstrated that at the antibody dilution used there appeared to be only one population of binding sites with a dissociation constant (K_d) of 9.4×10^{-12} M (Fig. 1). When the testosterone antiserum (sheep 505) was tested at a lower antibody dilution (final dilution of $1:2.8 \times 10^6$) two populations of antibodies were found (Fig. 1) which produced significantly different ($P < 0.001$) slopes (Table 2). However, when tested at two higher dilutions (Table 2) only the high affinity antibodies were detected. However, the slope of the inhibition curves were similar when the antiserum was tested at three dilutions. Both the oestradiol-17β (rabbit 48) and testosterone assays [sheep 505] (Fig. 1) were very sensitive when used at the higher dilutions, giving a minimum detectable dose of approx 800 fg per tube. As the testosterone antibody was still binding 50% of the label this assay could probably be made even more sensitive by diluting the antiserum further.

Antibody specificity. All 3 antisera demonstrated good specificity (Table 3). Antiserum from rabbit 48 had a 16% cross-reactivity with oestrone, but England *et al.*[9] have previously demonstrated that very specific antisera can be obtained when oestradiol-17β is conjugated to the carrier proteins through the 11β position. This is supported by the results using antiserum from rabbit 32 which showed only a 2% cross-reaction with oestrone. Antiserum from sheep 505 also showed good specificity, with only a 12% cross-reaction with 5α-dihydrotestosterone.

Affinity chromatography

Extraction specificity. The specificity of the antibody-Sepharose was assessed by incubating with various tritiated steroids (Table 4). The results demonstrate that the method does provide some specificity, with progesterone showing very low cross-reaction for both the testosterone and oestradiol linked antibodies. However, because of the high antibody concentrations, more closely related steroids were bound by both the testosterone and oestradiol antibody-Sepharose. The results also demonstrate that 2 or 3 washes with water, of the antibody-Sepharose, removed virtually all of the unbound steroid. A 3 ml wash with 90% methanol was also very efficient at removing the antibody bound steroid. The results indicate that residual steroid contamination is negligible, therefore allowing re-use of the antibody-Sepharose if required [13].

Comparison of extraction procedures. Several methods of extraction of both testosterone (Table 5) and oestradiol (Table 6) were compared. Affinity chro-

Table 3. Antisera specificity showing percentage cross-reactivities

	Oestradiol-17β antiserum		Testosterone antiserum
Steroid tested	Rabbit 32	Rabbit 48	Sheep 505
Oestradiol 17β	100	100	0.01
Oestrone	3.2	16	0.01
Oestriol	1.6	3.0	0.01
Testosterone	3.2	1.2	100
5α-Dihydrotestosterone	0.3	0.1	12.1
Androsterone	0.1	0.01	3.3
Androstenedione	0.1	0.1	0.9
5-Androsten-3β-ol-17-one	—	—	0.6
Progesterone	0.8	0.01	0.01

Antisera 32 and 48 were tested using the heterologous system (oestradiol-11α tyrosine methyl ester label) at final dilutions of 1:200,000 and 1:160,000 respectively. Sheep 505 was tested at a final antibody dilution of $1:2.8 \times 10^6$. The following steroids also had <1.0% cross reaction with all three antisera: 5-androstan-3β,17β-diol; cortisol, corticosterone, etiocholan-3α-ol-17-one; 5-pregnen-16α-ol-3,20-dione; 4-pregnen-17α-ol-3,20 dione; 4-pregnen-20α-ol-3-one; 4-pregen-16α-ol-3,20 dione.

Table 4. Percentage recovery of various tritiated steroids following extraction with either oestradiol antibodies or testosterone antibodies linked to Sepharose 4B

Antiserum	Tritiated steroid	Non-specifically bound hormone: Eluate	First H_2O wash	Second H_2O wash	Total free hormone recovered	Bound hormone: First methanol wash	Second methanol wash	Total steroid recovered
Oestradiol-17β	Oestradiol-17β	3.7	0.9	0.6	5.2	83.5	1.5	90.2
Oestradiol-17β	Oestrone	13.8	1.5	0.7	16.0	87.1	0.6	103.7
Oestradiol-17β	Progesterone	82	11.0	3.0	96	2.0	0.0	98.0
Oestradiol-17β	Testosterone	26.3	9.8	7.2	43.3	53.2	0.3	96.5
Oestradiol-17β	Androstenedione	75.7	13.6	4.2	93.5	6.6	0.1	100.2
Testosterone	Oestradiol-17β	16.9	4.3	2.9	24.1	65.3	3.5	92.9
Testosterone	Oestrone	75.4	5.2	3.5	84.1	16.9	0.1	101.1
Testosterone	Progesterone	69.7	16.4	6.1	92.2	5.7	0.1	98.0
Testosterone	Testosterone	11.4	1.1	0.4	12.9	76.8	1.9	91.6
Testosterone	Androstenedione	15.5	1.7	1.1	18.3	72.6	1.8	92.7

Table 5. Comparison of three methods of extraction of testosterone from ovine follicular fluid

Method of extraction	Mean percentage recovery (± SEM)	Inhibition slope of sample	Mean testosterone concentrations (ng/ml ± SEM)
Solvent followed by affinity chromatography	79.6 ± 1.3	−0.98	33.3 ± 2.9
Solvent only	90.2 ± 2.3	−0.97	31.9 ± 2.4
Affinity chromatography only	92.5 ± 1.0	−0.95	30.6 ± 3.0

Each sample was assayed at 4 vol in quadruplicate, using sheep 505 antiserum, with duplicate recovery estimates at each level. The 50% inhibition point and slope of the inhibition curve was 40 pg/tube and −0.91 respectively. All samples were parallel to the standard curve.

matography gave satisfactory results, since there was no significant differences in mean hormone concentrations between the various extractions methods and the samples were parallel to the inhibition curve. Moreover, the results were in agreement with follicular fluid oestradiol-17β concentrations measured without previous extraction [23]. Use of affinity chromatography to extract known amounts of oestradiol-17β, from various volumes of ovine serum, gave good recoveries (Table 7). As a further assessment of the effect of different sample volumes the recovery of exogenous oestradiol and testosterone, added to ovine serum, was measured. For oestradiol, 100 pg/ml added to ovariectomised ewe plasma was assayed at 4 vol, (150, 250, 500 and 1000 μl) in duplicate. Mean concentrations of oestradiol recovered were 99.7, 111.9, 98.9 and 102.2 pg/ml respectively. The sample was parallel to the standard curve, the slopes being −1.15 and −1.25 respectively. For testosterone 10, 50, 100 and 200 pg was added to four 3 ml aliquots of ovine plasma and the amount of testosterone recovered was 9.4 ± 1.5; 49.8 ± 2.5; 102.5 ± 8.9; 216 ± 8.4 pg respectively. Testosterone was also assayed in 4 vol (50; 100, 250 and 500 μl) of ram plasma following affinity chromatography extraction. The amount of testosterone measured was 3.5, 3.6, 3.5 and 3.5 ng/ml respectively. The sample was also parallel to the standard curve, the slopes being −0.96 and −0.99 respectively. In both the oestradiol and testosterone assays the assays blanks were always undetectable.

Table 6. Comparison of different extraction procedures of oestradiol-17β from ovine follicular fluid

Method of extraction	Mean percentage recovery (± SEM)	Inhibition slope of sample	Mean oestradiol concentrations (ng/ml ± SEM)
Solvent followed by affinity chromatography	63.8 ± 0.9	−1.34	30.5 ± 4.4
Affinity chromatography only	70.7 ± 1.4	−1.28	27.6 ± 3.0
Direct assay	—	−1.35	30.1 ± 3.4

Each sample was assayed at 5 levels in duplicate, with individual recovery corrections, using rabbit 48 in the heterologous assay. The 50% inhibition point and slope of the inhibition curve was 5.2 pg/tube and −1.2 respectively. All samples were parallel to the standard curve.

DISCUSSION

The results described here support the previous conclusion [5, 6] that the use of an heterologous configuration at the site of attachment significantly reduces the recognition of the chemical bridge by the antibody. This was found using two antisera

Table 7. Extraction of oestradiol-17β from ovine serum using an affinity chromatography extraction procedure

Quantity of oestradiol-17β added (pg)	Sample volume (μl)	Mean concentration of oestradiol-17β recovered (pg ± SEM)
10	1000–3000	10.9 ± 0.5
50	250–900	49.5 ± 1.1
100	150–1000	103.2 ± 3.0

Each sample was assayed in duplicate using 3 or 4 vol within the range shown. The concentration of oestradiol-17β in the control serum was 1.6 ± 0.2 pg/ml.

(Table 1) raised against 17β-oestradiol-11β-BSA. [9]. Furthermore, the slope of the inhibition curve was significantly steeper when using the heterologous compared to the homologous configuration of attachment, leading to increased assay sensitivity and precision [5, 6]. However, if the site of attachment was too remote (3 and 6 positions), although two of the antisera could bind the label, the inhibition curves were not usable (Table 1).

The results described here also extend these findings and indicate that the occurrence of "bridge binding" may also be affected by the actual position of attachment to the steroid molecule. Although an homologous chemical bridge and site of attachment (3 position) were utilised in the testosterone assay a suitable slope, similar to the oestradiol-17β system, was achieved. Moreover, because similar results were obtained using antisera from two different animals this may be a common mechanism, rather than due to the individual characteristics of a single antiserum. This conclusion is supported by a previous investigation of a number of steroids [24], although exact titres and inhibition curves were not shown.

Why this phenomenon should apply to the 3 position of the steroid molecule and whether it is related to molecular folding and spatial relationships between hapten and carrier protein remains to be determined [6]. Interestingly, the extremely high titres of the testosterone antibodies (Tables 1 and 2), suggests a high affinity for the radiolabel. Previous results with androstenedione [7; unpublished data] indicated that very high antibody titres can be achieved when an homologous system is used. Normally however, the radiolabel is not easily displaced from the antibody.

The Scatchard analyses indicated the presence of a high affinity binding site in both the oestradiol-17β and testosterone antisera (Fig. 1, Table 2). However, two significantly different binding affinities were apparent (Fig. 1) in the testosterone assay when tested at high antibody concentrations. Two possible alternatives are either the presence of two pools of antibodies or an antibody with divalent characteristics [25], as described for monoclonal antibodies raised against deoxycorticosterone [26]. The Scatchard analysis and slope of the testosterone inhibition curves demonstrated that at the high antibody dilutions (Table 2) only the high affinity binding site was of importance.

The cross-reaction studies suggest that utilising the 11 position for oestradiol-17β can lead to specific antisera (Table 3) and is in agreement with the previous work of England *et al.*[9]. The testosterone assay also demonstrated good specificity (Table 3). There was some cross-reactivity with 5α-dihydrotestosterone, but antibodies have been shown to have problems in differentiating between the 4-ene and 5α reduced forms of androgens [7, 27–30]. The specificity achieved in our assay system was better than that quoted by Cameron *et al.* [24], but this could be related to antibody concentration as this does have a significant effect on steroid antibody specificity [31]. Recently monoclonal antibody techniques have been used to produce a specific testosterone antibody [32]. although monoclonal antibody affinities tend to be lower [32, 33].

Steroid antibody cross-reactivities are further highlighted by the specificity of the antibody-Sepharose (Table 4), where the antibody concentrations are high [31]. In agreement with the results of Glencross *et al.*[13] the oestradiol-17β antibody-Sepharose and also the testosterone antibody-Sepharose had low cross-reactivity with progesterone. Although Glencross *et al.*[13] reported no other cross-reactivities the results presented here (Table 4) demonstrate that steroids with a similar structure do cross-react significantly. However, this is not a problem when the extraction procedure is incorporated with a specific RIA. The elution profiles (Table 4) demonstrated that unbound steroid is removed by washing with water, while a wash with methanol is extremely efficient at removing the bound steroid. This technique is currently being investigated for the purification of radiolabelled steroid derivatives following iodination.

Unlike the previous report [13] the technique used here produced negligible assay blanks in either the testosterone or oestradiol assays. Comparison with other extraction systems and recovery estimates indicated that affinity chromatography is a reliable and repeatable technique and is therefore a suitable extraction procedure for steroids, as recently demonstrated for small molecular weight protein hormones [34]. The use of radioiodinated ligands in the radioimmunoassay, in conjunction with affinity chromatography, has a dual advantage of firstly, allowing individual recovery estimates on each sample and secondly, removes the need for extracting the standards [13]. Moreover, only one extraction step would suffice for samples being assayed for more than one hormone. Antibody-Sepharose for each of the hormones to be measured, could be pooled followed by specific RIA's for each of the hormones being measured.

In conclusion, we have demonstrated that by judicial selection of antibody and radioiodinated label very precise and sensitive assays can be obtained. In addition the incorporation of these assay systems with an affinity chromatography extraction procedure allows the measurement of low concentrations of steroids in large volumes of a range of biological fluids.

Acknowledgements—We thank, Miss J. Dawson, Mr J. Walia and Mr G. Andrews for excellent techical assistance; Mrs B. McKenzie for preparation of glassware; Mrs J. Cherrie for typing the manuscript and Mr C. Manson for art work; Mr B. Morris for the gift of ovine testosterone and ovine oestradiol antisera; Upjohn for the oestradiol 11α- and 11β-hemisuccinate and Scottish Antibody Production Unit for the second antibodies and normal serum.

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