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A RADIOIMMUNOASSAY FOR THE REGULATORY ALLYLIC STEROID, 3α-HYDROXY-4-PREGNEN-20-ONE (3αHP)

J. P. WIEBE, K. J. BARR and K. D. BUCKINGHAM
Hormonal Regulatory Mechanisms Laboratory, Department of Zoology, University of Western Ontario, London, Ontario, Canada N6A 5B7

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Summary—The allylic steroid, 3α-hydroxy-4-pregnen-20-one (3αHP), found in gonadal and brain tissues by radiotracer and chemical methods, had been shown to play a role in gametogenesis, gonadotropin secretion and brain excitability. Since no simple assay was available, a radioimmunoassay for 3αHP was developed using [^{3}H]3αHP and an antiserum raised against 3αHP-20-CMO conjugated to bovine serum albumin. The specificity of the assay for the 3α allylic configuration of 3αHP was confirmed by examining 32 other steroids; cross-reaction with steroids containing different configurations (including metabolites of 3αHP such as progesterone) was less then 0.9%. A Scatchard plot indicated a K_a of 1.56×10^9 M^{-1}. Inter- and intra-assay coefficients of variation were 13.1 and 4.5%, respectively. The sensitivity of the assay was 6 pg and the 50% intercept of the standard curve was approx. 123 pg. The measurement by RIA of 3αHP from standard solutions and HPLC purified tissue extracts was confirmed qualitatively and quantitatively by GC/MS methods. The RIA method was employed to determine 3αHP levels in cultured Sertoli cells and in serum of intact and ovariectomized adult rats. Although for most uses, chromatography would not be necessary, two possible methods are presented to enable the separation of 3αHP from other interfering steroids prior to RIA.

INTRODUCTION

The allyllic steroid, 3α-hydroxy-4-pregnen-20-one (3αHP) was initially found to be a metabolite of progesterone produced by isolated rat Sertoli cells [1, 2]. It has also been shown to be produced by domestic hen thecal [3] and granulosa cells [4], rat granulosa cells [5], and human breast and brain tissue (J. P. Wiebe *et al.*, unpublished). The biological significance of 3αHP lies in its ability to selectively suppress the secretion of follicle stimulating hormone *in vivo* in intact and castrate male rats [6] and *in vitro* by cultured rat anterior pituitary cells [7]. The secretion of LH is not suppressed in either case. In addition, 3αHP influences brain excitability [8], probably by interacting with γ-aminobutyric acid receptor-gated chloride ion channels [9] and it can stimulate spermatocyte development in prepubertal rats at levels much lower than those shown to be effective for androgens [10].

Previous studies of 3αHP production were based on chromatography (TLC and HPLC) of metabolites of [^{14}C]- or [^{3}H]progesterone. There was no routine assay available for the measurement of endogenous levels and for determining hormonal regulation of 3αHP. The present study describes a radioimmunoassay (RIA) which was developed for the measurement of 3αHP in serum, tissues and cell cultures.

EXPERIMENTAL

Reagents

Solvents were obtained from BDH (Toronto, Canada). Chemicals were obtained from BDH or Fisher (Don Mills, Ontario, Canada). Freund's adjuvant was purchased from Sigma (St Louis, Mo., U.S.A.). [^{3}H]progesterone was supplied by DuPont Canada (Mississauga, Ontario). Unlabelled 3α-hydroxy-4-pregnen-20-one (3αHP) was synthesized according to Wiebe *et al.* [11]. Other unlabelled steroids came from Sigma or Steraloids (Wilton, N.H., U.S.A.). The preparation of [^{3}H]3αHP was from [^{3}H]progesterone using potassium trisiamylborohydride (KS-Selectride; Aldrich, Milwaukee, Wis.) as reducing agent [11].

Preparation of 3αHP-20-CMO

A solution of 18 mg 3αHP, 18 mg carboxymethoxylamine hemihydrochloride and

0.5 ml dry pyridine was kept at room temperature for 15 h. After evaporating pyridine, the residue was taken up in ether, washed with water, dried and concentrated to a solid. Recrystallization from ether-petroleum ether gave 14.9 mg of 3αHP-20(*o*-carboxymethyl)oxime as a colorless crystalline solid, with a melting point of 94–96°C. The structure was confirmed by i.r., ^{1}H-NMR and by ^{13}C-NMR.

Preparation of [^{14}C]3αHP-20-CMO

A solution of 3.0 mg [^{14}C]3αHP and 1.2 mg unlabelled 3αHP, 6.0 mg carboxymethoxylamine hemihydrochloride and 0.3 ml dry pyridine was allowed to stand at room temperature for 16 h. After evaporating the pyridine at room temperature, the residue was taken up in ether, washed thoroughly with water, dried and concentrated to give 4.1 mg of 3αHP-20-CMO (154,500 dpm/mg) as a colorless crystalline solid.

Preparation of 3α-HP-20-CMO-BSA conjugate

A solution of 10.0 mg 3αHP-20-CMO in 4.5 ml benzene and 0.5 mg [^{14}C]3αHP-20-CMO in 0.5 ml benzene was mixed thoroughly, the solvent was evaporated and the solid was kept over drying agent at reduced pressure (0.1 mm Hg) for 24 h. To a solution of 9.4 mg of this labelled 3αHP-CMO in 94 μl dry dimethylformamide was added a solution of 2.8 mg *N*-hydroxysuccinimide in 50 μl dimethylformamide followed by a solution of 5.6 mg dicyclohexylcarbodiimide in 50 μl dimethylformamide and kept at 4°C overnight. Precipitated dicyclohexylurea was separated and half of the succinimide ester solution was added slowly to a stirred solution of 24 mg bovine serum albumin (Fraction V, Boehringer Mannheim, Dorval, Quebec, Canada) in 0.48 ml of 0.2 M phosphate buffer (pH 8) followed by 0.2 ml 1 N NaOH and 0.1 ml water. After stirring at room temperature for 30 s, the remaining succinimide ester solution was added, followed by 0.2 ml 1 N NaOH and 0.1 ml water. After stirring at room temperature for 1 h, the material was dialyzed using ammonium bicarbonate solution (0.005 M, pH adjusted to 8.5 with NH_4OH) and lyophilized giving 31 mg of the conjugate as a foamy solid. Spectral and radiometric analyses showed that the conjugate contained 26 mol of steroid per mole of BSA.

Immunization

Three female New Zealand white rabbits, 4 months of age, were immunized with 2 mg 3α-HP-20-CMO-BSA dissolved in 2 ml sterile phosphate buffered saline and emulsified with Freund's complete adjuvant (2 ml). Each rabbit was injected with 0.4 ml of the emulsion per leg. The first six injections were given every 4 weeks, and thereafter boosters were given every 3–12 months. Rabbits were bled via the ear using Innovar-Vet, 14 days after each injection or boost, and the titer was determined. Serum was stored at −70°C or lyophilized and stored at −20°C.

Extractions

Serum (25–1000 μl) was placed in a tube (16 × 100 mm with teflon lined caps) along with ^{3}H-3αHP (4000 dpm) for calculation of recovery, and allowed to equilibrate for 1 h before extraction. A volume of 0.5–1.0 ml of 0.05 N NaOH was then added per tube and the sample extracted with 3 ml diethyl ether using vigorous shaking. Aqueous phases were frozen at −20°C and the organic phase poured off and washed with 1 ml 0.05 N NaOH. Extracts were dried under nitrogen and brought up in 0.2 ml methanol. Duplicate 90 μl aliquots were placed in assay tubes, dried under nitrogen and redissolved in 100 μl assay buffer (phosphate buffered saline, 0.1 M, pH 6.8, with 0.1% gelatin).

Assay procedure

Standards (5–1280 pg per tube) or unknowns were dissolved in 100 μl assay buffer. To each tube was added 100 μl of assay buffer containing approx. 2500 cpm (8000 dpm) of [^{3}H]3αHP and 100 μl assay buffer containing antiserum at a final tube dilution of 1:2400. After incubation at 4°C for 16 h, 1.0 ml of a cold 0.25/0.025% dextran T-70/charcoal suspension in assay buffer was added to each assay tube. Tubes were incubated at 4°C for 15 min and then centrifuged at 1800 *g* for 15 min. Supernatants were decanted into scintillation vials and counted using a PPO/POPOP/Triton X-100/xylene cocktail in a Philips PW4700 scintillation counter.

Chromatographic methods

In order to separate 3αHP from other steroids which might be present in extracts and which showed 1.0% or greater cross-reactivity with the antiserum, two chromatographic methods were used.

(a) C-18 Minicolumns. Dried extracts were brought up in 100 μl methanol:water (3:1) and loaded on 6 × 42 mm columns of C_{18} bonded

silica gel (40 μ). Fractions (1 ml) were eluted with methanol/water (3:1) at a flow rate of 0.3 ml/min using a low vacuum. They were then dried under N_2, brought up in methanol and aliquoted for use in the assay. Columns were characterized by eluting mixtures of standards, concentrating the fractions and analyzing these concentrates by HPLC. An aliquot of [^{3}H]3αHP was also eluted from a column and radioactivity was measured in the fractions.

(b) HPLC. Extracts were dissolved in methanol (20 μl) and purified using a Beckman Model 332 gradient liquid chromatograph with an Altex Ultrasphere ODS (C_{18}) column. The liquid phase was methanol:water (72:28) with a flowrate of 1.0 ml/min. Fractions were collected at 0.5 or 1.0 min intervals, dried under N_2, dissolved in methanol and aliquoted for use in the assay.

Verification by GC/MS

Ovaries from 4 mature, random cycling rats were homogenized (Polytron, 15 s at 23,500 rpm) in PBS (one pair of ovaries per 2.0 ml). Each homogenate was extracted with 9 ml of diethyl ether. The ether extract was backwashed with 1.0 ml of 0.05 N NaOH, dried down under N_2, brought up in 70% aqueous methanol and stored for 20 h at −20°C to remove lipids. The supernatant was removed and stored overnight at −20°C, then again removed, dried down under N_2 and brought up in a minimal volume (20 μl) of methanol and run on HPLC using methanol/water (75:25) at 0.8 ml/min. Fractions were collected at 15-18 min and at 23–26 min; retention times for progesterone and 3αHP standards were 16.1 and 23.9 min, respectively. The fractions were dried and brought up in methanol. Aliquots were used to measure 3αHP by the RIA. The remainder was silylated with 50 μl of *n*-methyl-*n*-trimethylsilyltrifluoroacetamide (MSTFA; Pierce Chemical Co., Rockford, Ill.) for 15 min at room temperature. A standard amount of authentic 3αHP was also similarly derivatized. The solvent was evaporated under a gentle stream of N_2 and the derivative was taken up in 20 μl of hexane. The hexane was reduced under N_2 to 10 μl, and 5 μl was injected for each GC/MS analysis. Quantitation was calculated automatically in the selected ion mode and the ovarian 3αHP was compared to the known amounts of standard 3αHP in two separate injections each.

To establish the identity and quantity of the ovarian putative 3αHP fraction, a Hewlett–Packard GC-Mass Spectrometer (Model 5970A with 5792A GC) was used in the selected ion mode with a 12.5-m cross-linked methyl silicone capillary column. The conditions were as follows: splitless mode, 0.7 kg/cm^2 helium, 200°C injection temperature, column temperature at 150°C (initial) to 220°C at 15°C/min, and scan speed of 690 amu/sec at an electron multiplier setting of 2200 V. Derivatized authentic 3αHP was run in standard mode and indicated that ions with *m/e* of 105, 127, 142, 143, 298, 388 were the major ions.

Progesterone radioimmunoassay

The RIA for progesterone was as described previously [12,13], using essentially the same assay procedures as for 3αHP. The antiserum was obtained from D. Armstrong, MRC Group in Reproduction, Dept of Physiology, University of Western Ontario.

In vitro studies: Sertoli cell cultures

Sertoli cell cultures were prepared from testes of 17 day old rats using the method of Welsh and Wiebe [14]. Cells were placed in 35 mm culture dishes (6×10^6 cells; 430 μg protein per dish) with 2 ml modified MEM containing 5% fetal bovine serum (Gibco). Leydig cell contamination of the preparations was found to be 0.15% using the method of histochemical detection of 3β-hydroxysteroid dehydrogenase [15]. After 48 h incubation at 32°C, media were removed and replaced with 2 ml media (per dish) containing 1 μg progesterone, with or without 0.5 μCi of [1,2,6,7-^{3}H]progesterone (101.3 Ci/mmol; DuPont, Canada). Following a 6 h incubation, media and cells were removed from the dishes and extracted. HPLC separation of extracts from incubations without ^{3}H-labeled precursor produced 3αHP and progesterone fractions, each of which were analyzed by both 3αHP and progesterone RIAs. Some of the fractions were derivatized and analyzed by GC/MS. Extracts from dishes with ^{3}H-precursor were separated on TLC as described previously [16], following the addition of standards of known major Sertoli cell progesterone metabolites. Following TLC, the standards were visualized using u.v. or iodine vapours, spots were scraped from the plates, extracted with ether and radioactivity quantitated.

In vivo studies

Injections. Mature male Sprague–Dawley rats were injected with 0.19 mg per day of 3αHP

(0.8 mg/kg body wt per day in saline, 1% Tween 80, 10% ethanol vehicle) for 4 consecutive days. Control rats received injections of vehicle only. On the fifth day rats were sacrificed by decapitation under CO_2 sedation and trunk blood was collected.

Ovariectomy. Mature female Sprague-Dawley rats were ovariectomized under Ketamine/Xylazine anesthesia, and sacrificed 13 days later by decapitation following CO_2 sedation.

RESULTS

Specificity and titer of the antiserum

Only one of the three immunized rabbits produced an antiserum that was judged to be acceptable after the sixth injection, on the basis of both titer and specificity. Final dilution of the antiserum per assay tube was 1:2400. At this concentration, 50% of the added label was bound to the antibody. Specificity was determined by incubating antiserum and [^{3}H]3α HP with varying quantities (10–10,000 pg) of various steroids. Crossreactivity was calculated as previously defined [17] and is shown in Table 1 and Fig. 1. Specificity is lower with 4-ene-3α-hydroxy steroids than for other steroids tested. It is not known how significantly the crossreactivity of 4-ene-3α-hydroxy steroids and 4-pregnene-3,11,20-dione affects the assay, as data on serum and tissue levels of these steroids have not been published. A chromatographic method can be used to separate 3α HP from these steroids and from progesterone. Although progesterone has a cross-reactivity of less than 1%, under certain conditions, such as incubations in which progesterone is added as precursor, it would be preferable to separate 3α HP from progesterone to eliminate interference.

Table 1. Specificity of the antiserum

Steroid	Cross-reactivity (%)
4-P-3α-ol-20-one	100.00
4-P-3,20-dione (progesterone)	0.85
4-P-3β-ol-20-one	0.40
4-P-20α-ol-3-one	0.24
4-P-3,11,20-trione	2.20
4-P-17α-ol-3,20-dione	0.02
4-P-11β,17α,21-triol-3,20-dione	<0.01
4-P-21-ol-3,20-dione	0.88
4-P-17α,21-diol-3,11,20-trione	<0.01
4-P-3β,20α-diol	0.03
4-P-3α,20α-diol	10.00
4-P-3α,20β-diol	22.80
4-P-3β,20β-diol	0.02
Cholesterol	0.03
4-P-3β-ol-20-one	<0.01
5α-P-3β-ol-20-one	<0.01
5α-P-3α-ol-20-one	0.43
5α-P-20α-ol-3-one	<0.01
5α-P-3α,20α-diol	0.06
5α-P-3β,20α-diol	<0.01
5α-P-3,20-dione	0.02
5α-P-3α,17α-diol-20-one	0.34
4-A-3,17-dione	0.23
4-A-17β-ol-3-one	0.13
4-A-7α,17β-diol-3-one	0.16
4-A-3α-ol-17-one	2.70
4-A-3β-ol-17-one	0.63
5α-A-3α,17β-diol	0.30
5α-A-17β-ol-3-one	0.14
5α-A-3β-ol-17-one	<0.01
5α-A-3α-ol-17-one	0.10
Estradiol-17β	<0.01
Estradione	0.34

[^{3}H]3α HP was incubated with antiserum and 10–10,000 pg of each of the listed steroids. Standard curves were generated for each steroid and cross-reactivity was calculated according to [17]. P: pregnene or pregnane. A: androstene or androstane.

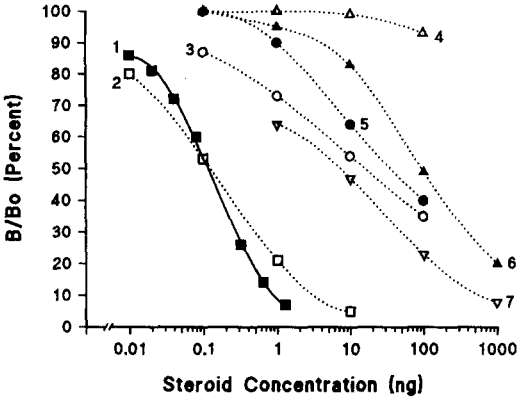

Fig. 1. Cross-reactivities of steroids in the 3α HP RIA. Curves were generated as described in Table 1. The curves are representative of the following steroids: 1: 3α HP; 2: 4-pregnene-3α,20β-diol; 3: progesterone, 21-hydroxy-4-pregnene-3,20-dione and 3α-hydroxy-4-androsten-17-one; 4: estradiol-17β and 3β-hydroxy-4-androsten-17-one; 5: 3α-hydroxy-5α-pregnan-20-one and 3β-hydroxy-4-pregnen-20-one; 6: testosterone and 4-pregnene-3β,20α-diol; 7: 20α-hydroxy-4-pregnen-3-one and 4-pregnene-3,11,20-trione.

Standard curve

A standard curve is shown in Fig. 2. The 50% intercept was located at approx. 123 pg.

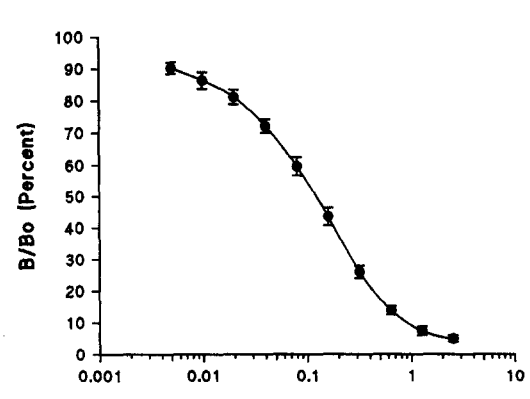

Fig. 2. Standard curve obtained for the 3α HP RIA using anti-3α HP-20-CMO-BSA serum and [1,2,6,7-^{3}H]3α HP. Values are the mean ± SEM, $n = 6$.

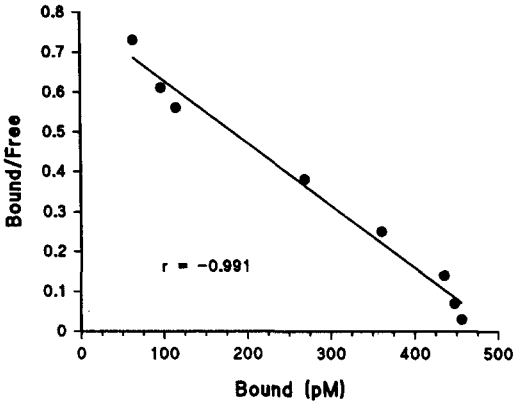

Fig. 3. Scatchard plot for 3αHP antiserum, using the data from Fig. 2.

Precision

The interassay coefficient of variation was 13.1% ($n = 5$) and the intraassay coefficient of variation was 4.5% ($n = 5$). These were calculated using extracts of pooled sera from 3αHP injected rats.

Sensitivity

The sensitivity of the assay, defined as the least amount of 3αHP distinguishable from zero sample at 95% confidence limit, was 6 pg/tube. A Scatchard plot of the data from the standard curve is shown in Fig. 3. The antibody appears to have a single binding site and shows a K_a of $1.56 \times 10^9\ M^{-1}$.

Chromatographic separation of 3αHP from other steroids

The specificity of the 3αHP RIA in the presence of progesterone was tested in mixtures

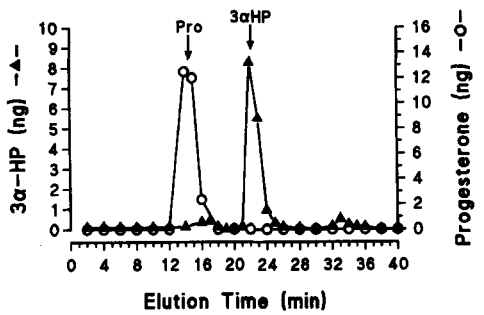

Fig. 4. Progesterone and 3αHP analysis by RIA of rat serum extracts following HPLC separation. Sera, to which varying amounts (1–5 μg) or progesterone and 3αHP had been added, were extracted, aliquots were run on HPLC (methanol/water, 72:28; 1.0 cc/min), 1.0 min fractions were collected and RIAs for the two steroids were conducted on each fraction. The curve shows results of one typical separation and assay. Arrows indicate retention times of progesterone (14.1 min) and 3αHP (22.3 min) standards determined by u.v. absorbance (210 nm) of parallel runs under identical conditions.

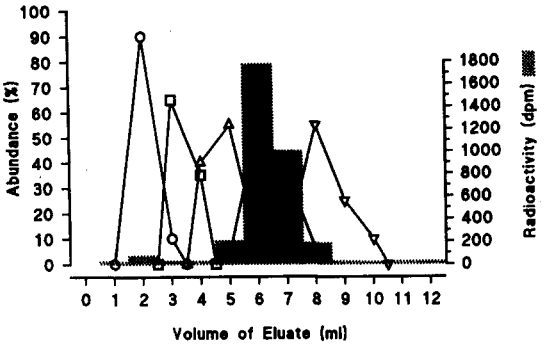

Fig. 5. Separation of mixtures of steroids by C_{18} minicolumns. The following steroid standards and 5000 dpm [^{3}H]3αHP were added to male rat serum: 4-pregnene-3,11,20-trione (○), 3α-hydroxy-4-androsten-17-one (□), progesterone (△), 3αHP (●) and 4-pregnene-3α,20α/β-diols (▽). The serum extracts were eluted from minicolumns into 1.0 ml fractions, monitored by u.v. absorbance and scintillation spectrometry.

(1–5 μg each) of the two steroids. HPLC fractions corresponding to the elution times of progesterone (12–16 min) and 3αHP (21–24 min) were obtained from mixtures of these steroids and each was analyzed by the two RIAs (Fig. 4). The RIA results confirmed that the two steroids are well separated by HPLC in a methanol/water (72:28) system and show absence of reactivity for progesterone in the 3αHP fraction.

Attempts were made to separate mixtures of steroids by means of C_{18} minicolumns. Figure 5 shows the results of one such elution and indicates that 3αHP can be reasonably well separated by minicolumn from other steroids that may show some crossreaction in the 3αHP RIA.

Validation of RIA

To validate serum measurements of 3αHP, serum from either control or 3αHP injected rats was extracted and extracts were purified either by minicolumn or HPLC or used in the RIA without chromatography (Table 2). Immunoassayable 3αHP activity was present in those

Table 2. Relative amounts (ng/ml) of immunoassayable 3αHP in rat serum with/without chromatography on minicolumn or HPLC

Treatment	No chromatography	Minicolumn	HPLC
Saline injected	2.72	2.35	2.33
3αHP injected	8.61	7.83	9.24
Charcoal treated	—	—	—

Serum was pooled from 6 male rats which had been injected either with saline or 0.19 mg 3αHP per day for 4 consecutive days. One ml of each serum was extracted as described under Experimental. 3αHP in the extracts was measured by RIA either without chromatography or after chromatography by minicolumn or HPLC. As assay blanks, charcoal treated extracts of serum from gonadectomized rats were used in the RIA and showed undetectable amounts (—) of 3αHP. The values represent the mean of two measurements made on each pooled serum extract.

Table 3. Identification and relative abundance of 3α HP in rat ovaries determined by RIA and GC/MS

Source	RIA (ng)	GC/MS (ng)	GC R_T (min)	Molecular mass (m/z)	Major ions (m/z)
3α HP-Si (Std)		5.0	15.89	388	105, 127, 143, 298, 388
A1 + A2	7.1	5.75	15.93	388	105, 127, 143, 298, 388
A3 + A4	8.6	6.83	15.91	388	105, 127, 143, 298, 388

Ovaries from four mature, random cycling rats (A1, A2, A3, A4) were homogenized and extracted as described under Experimental. An aliquot was used to measure 3α HP by RIA and the remainder was silylated. A standard amount of authentic 3α HP was also similarly derivatized (3α HP-Si). The solvent was evaporated under a gentle stream of N_2 and the derivative was taken up in 20 μl of hexane. The hexane was reduced to 10 μl under N_2 and 5 μl was applied to the GC/MS. The values are the means of two measurements and are given as ng per animal (pair of ovaries). Also shown is the retention time of the 3α HP quantitated in the standard and the two samples, and the molecular mass and major ions of the standard and samples.

fractions representing areas of elution of 3α HP from either minicolumns or HPLC. The levels of 3α HP following chromatography are similar to those without chromatography. A marked increase in serum 3α HP levels was seen following injections of 3α HP. Control samples, in which steroids had been removed by charcoal gave readings below the level of detectability (Table 2).

To validate measurements of 3α HP in tissues, rat ovaries were extracted and 3α HP was measured by RIA and GC/MS. The RIA values were in the same range as extracts determined by GC/MS (Table 3). The identity of 3α HP in these extracts was confirmed by capillary GC and selected ion mode mass spectrometric monitoring of derivatized fractions of the extracts (Table 3).

As an addition test of validity of the 3α HP RIA, the amount of 3α HP produced by Sertoli cells in culture was measured by RIA and by TLC/radiotracer methods. Table 4 shows that there is good agreement in quantitation by the two methods and the GC/MS determinations of the masses of 3α HP in the HPLC fractions further validate these readings.

Table 4. Amounts of 3α HP produced by Sertoli cells in culture as measured by the 3α HP RIA and by TLC/radiotracer techniques

Method	n	3α HP (ng) (±SEM)
(A) TLC	5	8.58 (±0.44)
(B) RIA	4	8.96 (±1.32)
(C) GC/MS	2	7.55

Sertoli cells from 17-day-old rats were isolated and cultured. Five culture dishes received 1.0 μg [^{3}H]progesterone (0.5 μCi) per dish (A) and five dishes received 1.0 μg unlabelled progesterone (B). After 6 h, media were extracted. Group A extracts were run on 2-D TLC along with appropriate standards and the area coinciding with authentic 3α HP was extracted and the extract quantitated by scintillation spectrometry after appropriate corrections for procedural losses. Group B extracts were purified by HPLC and the eluate corresponding to authentic 3α HP elution time, was quantitated by the 3α HP RIA (RIA). Aliquots of Group B HPLC fractions were derivatized and two determinations were made by GC/MS (C). Values are expressed as ng 3α HP (±SEM for A and B; mean of 2 measurements for C), corrected per culture dish.

Accuracy

Unlabelled 3α HP (250–2000 pg) and [^{3}H]3α HP (6800 dpm), both dissolved in methanol, were added to extraction tubes, dried under N_2 and 0.5 ml rat serum was added to each tube. After allowing 1 h for the steroid to dissolve and equilibrate, the samples were extracted. Extracts were allowed to dissolve overnight in assay buffer and replicate aliquots were taken for scintillation counting and RIA. Final recovery of the [^{3}H]3α HP was found to be 55.6 ± 2.2% (mean ± SD), with 15.6 ± 5.2% of the activity remaining in the original aqueous portion. The line of regression for measured vs added unlabeled 3α HP was $y = 0.995x + 0.925$ and had an $r = 0.996$, indicating a very high correlation between expected and determined values.

These results were confirmed by observations on aliquots of different volumes (0–80 μl) of a plasma sample obtained from 3α HP injected male rats. Linear regression analysis of these results gave the equation $y = 1.03x - 11.26$ ($r = 0.998$) where y represents the measured values and x the corresponding values calculated from the levels measured in the 5 μl sample.

3α HP levels in ovaries and serum of female rats

Table 5 shows that serum progesterone and 3α HP both decrease markedly (92 and 79%

Table 5. Comparison of changes in serum progesterone and 3α HP following ovariectomy

	3α HP	Progesterone
Control	13.10 ± 2.45	25.74 ± 1.47
Ovariectomized	2.79 ± 0.65	1.96 ± 0.55

Mature rats were ovariectomized and sacrificed 13 days later. Each serum sample was analyzed for both steroids by RIAs and corrections were made for cross-reactivity. Results show the mean ± SEM of 3 replicates and are expressed as ng steroid per ml serum.

respectively) following ovariectomy indicating that the ovary may be a major source of 3αHP in the female.

DISCUSSION

The antiserum developed for this radioimmunoassay appears to have a high degree of specificity for 3αHP. Out of 32 different steroids tested, comprising C_{18}, C_{19}, and C_{21} steroids of various categories, only 3 showed cross-reactivities in excess of 1%. The highest cross-reactivities occur with 4-pregnene-3α,20α/β-diols (10.0 and 24.6% respectively). The cross-reactivity of the 4-pregnen-3α-ol compounds is not surprising, given their structural similarities in the A ring. However, the importance of the cross-reactivity of these diols is difficult to assess. Although these compounds have been found in various tissues including gonadal and mammary tissues [18], little is known of their endogenous levels. Fractionation of rat serum by HPLC and subsequent testing of fractions corresponding to retention times of the diols indicated little or no reactivity in the RIA, even in 3αHP injected rats, where conversion of 3αHP to the diols could be a possibility. Therefore, interference from these diols may not be a problem.

Progesterone shows less than 1% cross-reactivity. Under most conditions that we have examined, progesterone levels appear to be present at about the same or at only slightly higher concentrations than 3αHP and it should not create significant interference in the assay. However, in cases of exceptionally high progesterone levels, such as in ovarian tissue, or in incubations with a progesterone precursor, chromatographic separation should be considered.

Frequently, other steroid RIAs have employed various chromatographic methods for partial purification. In many of these assays, chromatographic separations are a prerequisite due to either cross-reactivity problems (e.g. 16α-hydroxypregnenolone [19]) or due to low circulating levels of the particular steroids in the blood (e.g. 21-deoxycortisol [20]). Methods that have been used include paper [21, 22], adsorption [23], partition [22], or thin layer [24] chromatography. These methods tend to be inconvenient for routine assay; also, we have found that very small quantities (picogram amounts) of 3αHP may not be stable under some of these chromatographic conditions Sephadex LH-20 is also frequently used to partially purify biological extracts [25]. However, in direct comparisons between C_{18} columns (HPLC and/minicolumns) and LH-20, we find that ease of column preparation, reproducibility, speed and quality of separation by HPLC and/or C_{18} minicolumn generally exceed the Sephadex LH-20. In addition, C_{18} columns do not require noxious solvents such as benzene or methylene chloride which are commonly used with LH-20 columns. These attributes have made the HPLC and C_{18} minicolumn the methods of choice in our laboratory.

The levels of 3αHP measured in 100 μl of serum are well within the working range of the assay, 5 pg being the lowest standard used, although the formal sensitivity is 6 pg. Incubations of cultured cells using serum-free media can be measured without extraction of the media. Concentrations as low as 10^{-9} M can be measured without changes in assay protocol. 3αHP appears to be present in significant amounts in serum from both male and female rats. In male rats, serum 3αHP levels are 1–2 ng per ml (unpublished), while serum progesterone levels are 2–4 ng per ml [26]. 3αHP appears to be present in higher quantities in serum from mature female rats (Table 5) where levels are 13 ng/ml and progesterone levels are 26 ng/ml.

Because the allylic steroid, 3αHP, has been shown to exhibit gonadotropin regulating, gametogenic and CNS action in rats, it is important to determine its levels in various tissues at different physiological and endocrine states and to study the control of its synthesis. The method we have described provides the first RIA for 3αHP and should enable studies of this regulatory steroid during various reproductive stages and in various species, including human.

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