# RADIOIMMUNOASSAY FOR MEDROXYPROGESTERONE ACETATE (PROVERA®) USING THE 11α-HYDROXY SUCCINYL CONJUGATE

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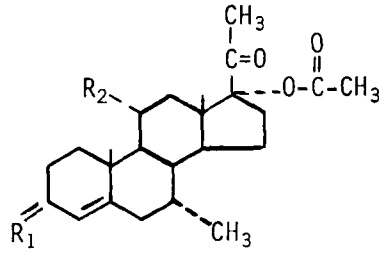
## ABSTRACT

A radioimmunoassay was developed for medroxyprogesterone acetate (MPA; Provera®) employing rabbit antibodies made against a bovine serum albumin (BSA) conjugate of the hemisuccinate ester of 11α-hydroxy MPA. The 11α-hydroxyl group was introduced into MPA by a series of chemical and microbiological transformations. The acid succinate MPA derivative was coupled to BSA *via* reaction with N,N'-carbonyldiimidazole. Cross-reactivities of several MPA analogs were compared using rabbit antisera developed against the C-11 conjugate of MPA and goat antiserum developed against a C-3 conjugate of MPA. Antibodies formed against the C-11 conjugate showed increased specificity to steroid analogs which changes in the C-3, C-5 and C-6 positions but less specificity to changes in the C-21 side chain. The profiles of serum MPA levels *vs.* time, measured with both the C-3 and C-11 antisera, after oral drug administration to the monkey were similar. After intramuscular drug administration to dogs, serum MPA levels measured with the C-3 antisera were consistently greater as compared to those with the C-11 antisera. Factors affecting (a) precipitation of the antibody-antigen complex, (b) assay precision and (c) assay sensitivity were evaluated.

## INTRODUCTION

Medroxyprogesterone acetate (MPA, Provera®) is an effective agent for treatment of malignancies and an effective injectable contraceptive agent (Figure 1). An additional method for determination of MPA in biological systems was desired since the gas-liquid chromato-

FIGURE 1: NOMENCLATURE OF MPA DERIVATIVES



| $R_1$ | $R_2$ | Name |
|---|---|---|
| =O | -H | 17-hydroxy-6α-methylpregn-4-ene-3,20-dione 17-acetate (Medroxyprogesterone acetate) (MPA) |
| =O | -OH | 11α,17-dihydroxy-6α-methyl-pregn-4-ene-3,20-dione 17-acetate (11α-hydroxy-MPA) |
| =O | $O-C(=O)(CH_2)_2COH$ | 11α,17-dihydroxy-6α-methyl-pregn-4-ene-3,20-dione 11-acid succinate (11α-hydroxy-MPA-acid succinate) |
| =O | $O-C(=O)-(CH_2)_2C(=O)-NH-BSA$ | (C-11 conjugate) |
| $=N-O-CH_2C(=O)-OH$ | -H | MPA-3-(O-carboxymethyl)-oxime |
| $=N-O-CH_2C(=O)-NH-BSA$ | -H | (C-3 conjugate) |

graphic assay (1) lacked sufficient sensitivity at the low levels present in humans and the radioimmunoassay using antibodies prepared from the MPA-3-(O-carboxymethyl)oxime (2) showed some cross reactivity with potential A-B ring metabolites of MPA.

It has been supposed that steroid conjugates with linkages which occupy functional groups of the hapten would diminish the specificity of

the corresponding antibody (3). Highly specific radioimmunoassays have now been developed using conjugates with linkages to steroids at the C-6, C-7,and C-11 positions remote from the functional groups at C-3 and C-20 (4-9).

In this study the key intermediate, 11α-hydroxy-MPA, was synthesized by two routes (10-15). The 11α-hydroxyl group was converted to its hemisuccinate ester and coupled to bovine serum albumin (BSA). Rabbits were injected with this material to form antibodies. The specificity of rabbit antisera to the C-11 conjugate was compared to that of goat antisera to the C-3 conjugate. A number of steroid analogs with single functional group changes in strategic positions were analyzed for cross-reactivity with both antisera to MPA.

## EXPERIMENTAL

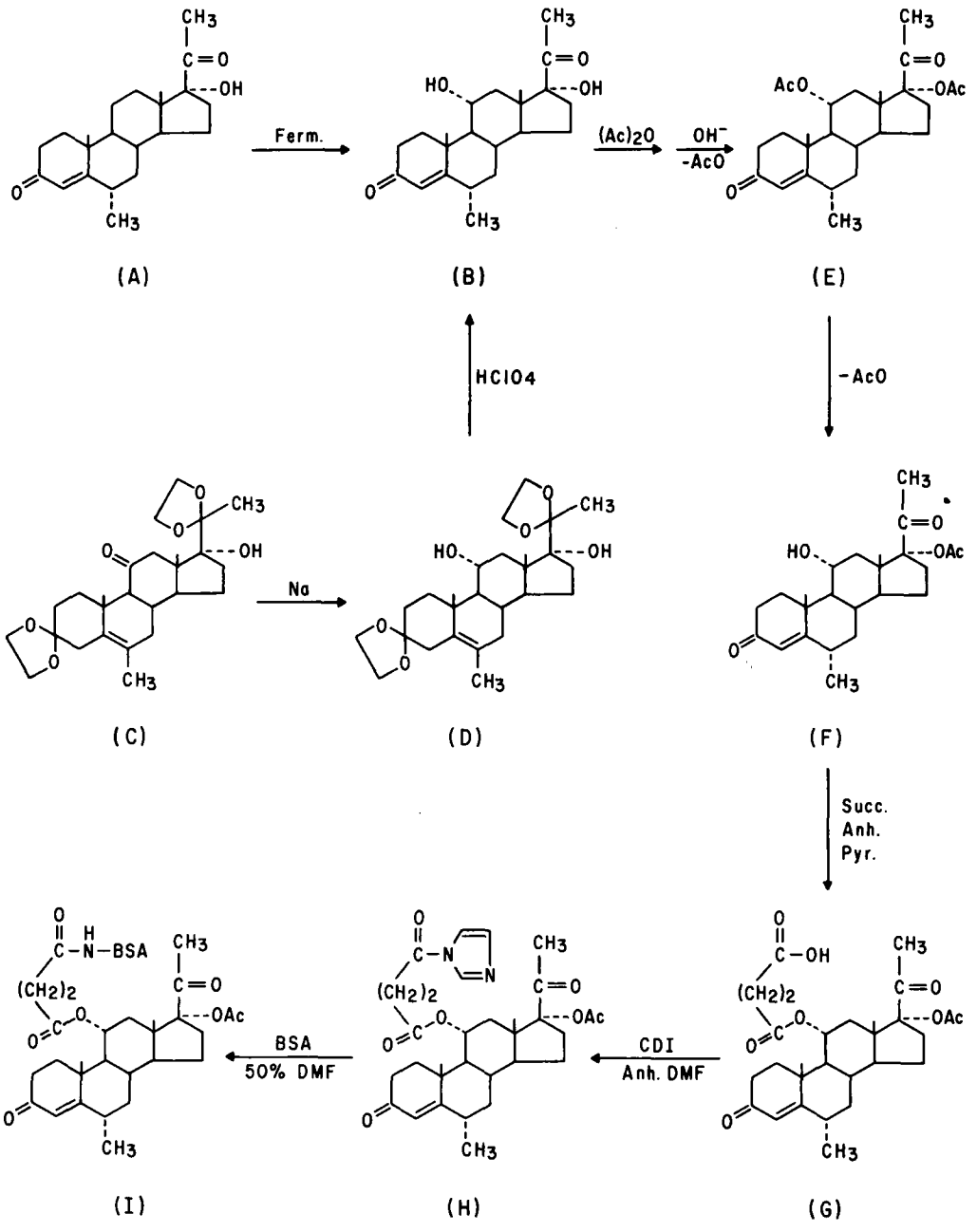
The general synthetic scheme for preparation of the MPA-BSA conjugate is shown in Figure 2.

### I. Biosynthesis of 11α,17-Dihydroxy-6α-methylpregn-4-ene-3,20-dione (D) from 17-hydroxy-6α-methylpregn-4-ene-3,20-dione (A)

One hundred and twenty-five liters of medium consisting of 1% glucose, 0.25% soy flour and 2.0% liquid corn steep was adjusted to pH 5.0 and sterilized. This medium was innoculated with a 72 hour vegetative growth of *Rhizopus nigricans* (ATCC 62276). Three hundred ml of Ultrawet 30 DS Surfactant (ARCO Chemical Co., Chicago) and 20 g of 17-hydroxy-6α-methylpregn-4-ene-3,20-dione (A) in 300 ml of dimethylformamide were added. After 72 hours, thin-layer chromatography (TLC) indicated a small amount of starting material remained and considerable diol (B) was present. The culture was filtered through 2 kg Celetone FW 40 (Eagle Pitcher Co., Cincinnati), and the clear beer was extracted several times with methylene chloride. The filter cake was stirred with 20 liters of acetone and then with methylene chloride. The two extracts were combined and evaporated to dryness.

The residue was chromatographed through silica gel and recrystallized from acetone with charcoal treatment to give 7.88 g of the diol (B).

FIGURE 2. PREPARATION OF THE MPA-BSA CONJUGATE



| | |
|---|---|
| M.P.: | 233-243°C |
| U.V.: | $\lambda_{max}$ (ethanol) 241 nm<br>$\epsilon_{max}$ 14,950 |
| Anal. | calc. for $C_{22}H_{32}O_4$: C, 73.30%; H, 8.95%<br>found: C, 73.37%; H, 8.89% |

II. Chemical Synthesis of 11α,17-Dihydroxy-6α-methylpregn-4-ene-3, 20-dione (B) from 17-hydroxy-6-methylpregn-5-ene-3,11,20-trione 3,20-bis(ethylene acetal)(C)

A mixture of 20 g of 17-hydroxy-6-methylpregn-5-ene-3,11,20-trione 3,20-bis(ethylene acetal)(C) prepared from 17-hydroxy-6α-methylpregn-4-ene-3,11,20-trione (11, 12), was vigorously stirred in 1 liter of n-propyl alcohol.

A few pea-sized pieces of sodium were added and the mixture was heated to reflux. Sodium was continually added in small pieces and the reaction followed by TLC. After addition of 28 g of sodium, the starting material had all been consumed. The mixture was cooled and diluted with ice-water. The product was extracted with ether, washed, concentrated and triturated with ether to give 17 g of the 11α-hydroxy bis-acetal (D).

A mixture of 16 g of the acetal (D), 250 ml of tetrahydrofuran, 25 ml of water and 21 ml of 1N perchloric acid was stirred under $N_2$ for 3 days. The product was extracted with ether-ethyl acetate, chromatographed through silica gel, and recrystallized from acetone-water to give 4.6 g of the diol (B).

| | |
|---|---|
| M.P.: | 204-225°C |
| U.V.: | $\lambda_{max}$ (ethanol) 242 nm<br>$\epsilon_{max}$ 14,450 |

Infrared and NMR analyses showed the product was the same as the product produced by fermentation. However, TLC showed small amounts of polar impurities which accounted for the lowered M.P.

III. Acetylation of 11α,17-Dihydroxy-6α-methylpregn-4-ene-3,20-dione (B)

One g of 11α,17-dihydroxy-6α-methylpregn-4-ene-3,20-dione (D) was added to a solution of 87.5 ml of carbontetrachloride, 12.5 ml of acetic anhydride and 0.16 ml of 70% perchloric acid. The solution was stirred for 1 hour, at room temperature, washed with water, dilute sodium bicarbonate, dried, and concentrated to dryness. The crude triacetate was dissolved in a $N_2$ purged solution of 0.4 g of potassium carbonate and 5 ml of water in 50 ml of methanol. After about 15 minutes the product was extracted with ether, washed, dried, chromatographed through silica gel and crystallized from ether. The yield of the diacetate (E) was 0.359 g.

M.P.: 190-195°C
U.V.: $\lambda_{max}$(ethanol) 239 nm
$\varepsilon_{max}$ 16,000
I.R.: 1725, 1715 and 1675 (C=O), 1610 (C=C) $cm^{-1}$
M.S.: m/e = 444 ($M^+$) 401, 384
NMR: $CDCl_3$ 0.71 (C-18), 1.0 (d, J=6, C-6-$CH_3$) 1.23 (C-19), 1.98 and 2.01 (acetate $CH_3$), 2.08 (C-21), 5.21 (q, J=9, C-11), 5.78 (d, J=2, C-4)

## IV. Selective Microbiological Hydrolysis of the 11-Acetate

A 40-hour growth of *Flavobacterium dehydrogenans* (UC 955) in antibiotic broth #2 (Difco) was used to innoculate 40 flasks containing 100 ml each of the same medium. The flasks were aerated by shaking for 48 hours and 40 mg of 11α,17-hydroxy-6α-methylpregn-4-ene-3,20-dione diacetate was added. The aeration was continued for 72 hours in a lighted room, then the flasks were pooled and adjusted to pH 3.0 with acid. The coagulated cells were removed by filtration and the filtered beer and washings were extracted four times with about one-half volume aliquots of methylene chloride. Thin-layer chromatography with a system of acetone:chloroform (20:80) was used to detect the completeness of the hydrolysis.

The methylene chloride extract was concentrated to an oil, taken up in ether and washed with water, dilute hydrochloric acid and again with water. The product was chromatographed through alumina and recrystallized from ether to give 11α-hydroxy-MPA (F).

M.P.: 205-210°C
U.V.: $\lambda_{max}$(ethanol) 242 nm
$\varepsilon_{max}$ 15,300
M.S.: m/e = 402 ($M^+$), 359, 342, 317 and 299
I.R.: 3550 (OH), 1720 and 1670 (C=O), 1605 (C=C)
NMR: $CDCl_3$ δ 0.71 (C-18), 1.09 (d, J=6, -6-$CH_3$), 1.32 (C-19) 2.07 and 212 (C-21 and acetate $CH_3$), 4.0 (m, C-11), 5.80 (d, J=2, C-4)

## V. Formation of 11α,17-Dihydroxy-6α-methylpregn-4-ene-3,20-dione 11-acid succinate 17-acetate (G)

A solution of 0.55 g of 11α,17-dihydroxy-6α-methylpregn-4-ene-3,20-dione 17-acetate (F) and 0.6 g of succinic anhydride in 8 ml of pyridine was heated at reflux under $N_2$ for 20 hours. The reaction mixture was diluted with water and ether and filtered to remove some black insoluble residue. The ether layer was washed with dilute hydrochloric acid and water, dried ($Na_2SO_4$), concentrated, and chromatographed through silica gel. The succinate was eluted with 50:50:3 ethyl acetate:Skelly-solve B:acetic acid. The product was further purified by preparative TLC and crystallized from moist ether.

M.P.: 147-152°C
U.V.: $\lambda_{max}$(ethanol) 239 nm
$\varepsilon_{max}$ 15,500
I.R.: 3590, 3500 (acid OH), 3170 (OH); 1735, 1705, 1670 (C=O), 1605 (C=C), 1295 (C-O)
M.S.: m/e = 502 ($M^+$), 460, 417, 299
NMR: $CDCl_3$ δ 0.73 (C-18), 1.03 (d, J=6, 6-$CH_3$), 1.26 (C-19), 2.02, 212 (C-21, acetate $CH_3$), 2.66 (-$CCH_2$-$CH_2$-C-) 5.30 (m, C-11), 5.86 (C-4)

Anal. calc. for $C_{28}H_{38}O_8$: C, 66.91%; H, 7.62%
found: C, 66.39%; H, 7.57%

VI. Preparation of the Bovine Serum Albumin Conjugate of the Hemisuccinate Ester of 11α-hydroxy MPA

Ninety-two mg of N,N'-carbonyldiimidazole (0.56 millimoles) was added to 143 mg (0.28 millimoles) of 11α,17-dihydroxy-6α-methylpregn-4-ene-3,20-dione 11-acid succinate 17-acetate (G) in 5 ml of dimethylformamide (DMF). After 20 minutes the reaction mixture was added to 200 mg of BSA in 40 ml of 1:1 DMF:$H_2O$ adjusted to pH 9.5 with triethylamine (TEA). Five ml of water was added and the pH brought to 10.5 with TEA. After stirring overnight, the solution was dialyzed for 24 hours against running water, 6 hours against deionized water, and then freeze-dried (C-11 conjugate, I).

VII. Preparation of Rabbit Antisera

Thirty-two mg of the C-11 conjugate was mixed with 4 ml of isotonic saline and emulsified with an equal volume of Freund's complete adjuvant (Difco). Rabbits were injected at weekly intervals in the front foot pads, hind pads or by intradermal injections in twenty sites in the back. The schedule for immunizing rabbits is shown in Table 1. Blood was collected from the ear by cutting the vein and applying gentle suction. The blood was allowed to clot a half an hour and the serum collected. The antisera was stored at -10°C. All rabbits produced adequate titers of antibody. Rabbit #32 produced the highest titer and the bleeding at 88 days was used for all subsequent experiments. The C-3 goat antiserum was prepared by J. C. Cornette (2).

VIII. Assay Procedure

PBS (phosphate buffered saline) - 17.19 g NaCl, 0.812 g $NaH_2PO_4$, 20 ml of 1:1000 Merthiolate, NaOH to pH 7.0, dilute to 2000 ml.

EDTA-PBS - 18.612 g (ethylenedinitrilo)tetraacetic acid disodium salt, 800 ml of PBS, NaOH to pH 7.0, dilute to 1000 ml.

Counting Solution - 250 ml BioSolv (Beckman), 85 ml Liquifluor (New England Nuclear), 2000 ml toluene (Burdick and Jackson).

TABLE 1

IMMUNIZATION SCHEDULE

| Day | Bleeding or Injection Site | Total Conjugate Injected | Diluent |
|---|---|---|---|
| 0 | Hind Paws | 2 mg | 0.5 ml CFA[a] |
| 7 | Fore Paws | 2 mg | 0.5 ml CFA |
| 14 | ID Back | 1 mg | 1.0 ml CFA |
| 42 | IM | 1 mg | 0.25 ml CFA |
| 56 | Bleed | | |
| 77 | Bleed | | |
| 77 | IM | 1 mg | 0.25 Saline |
| 88 | Bleed | | |
| 97 | All rabbits sacrificed except #32 | | |

[a] CFA = 1.1 Complete Freund's adjuvant and isotonic saline.

Secondary Antibody - Rabbit antigoat antisera, goat antirabbit antisera.

These were titrated and diluted with PBS to give maximum precipitation.

All MPA antisera were first diluted 1:400 with EDTA-PBS then diluted further with 1:400 normal serum of the same species. The antisera to the C-11 conjugate was diluted 1:400 and 0.2 ml was used per sample. The goat antisera made from the C-3 conjugate was diluted 1:12000.

One-tenth ml (12,000 DPM) of 7-$^{3}$H-MPA (specific activity 26.1 $\mu$Ci/$\mu$g) in PBS was added to a 12 X 75 mm glass culture tube followed by 0.2 ml of diluted primary antibody solution, the sample, and PBS diluent to make a total volume of 0.8 ml. The standards were measured in the same manner as the unknowns except normal serum was added in the same amount as used for the sample (0.02-0.1 ml).

After 2-25 hr., 0.2 ml of secondary antibody was added and the mixture was incubated overnight at 5°C. The resulting solution and

precipitate were centrifuged at 2000xg for 30 minutes at 5°C. The supernatant was decanted into counting vials and the tubes were left inverted in the vial 20-30 minutes. The inverted tubes were touched to the side of the counting vial to remove remaining liquid and then discarded. Fifteen ml of counting solution was added, and the samples were counted in a Packard Tri-Carb liquid scintillation counter. The counting efficiency was 42%.

A Micromedic Automatic Pipette (model 25004) was used for analyses with large numbers of samples. With an Eppendorf pipette, 50 µl of PBS was added to the sample tubes and 50 µl of normal serum to the standard tubes. To each tube 50 µl of sample (or standard) plus 700 µl of a mixture containing $^{3}$H-MPA, primary antibody, and PBS were added by the Micromedic Automatic Pipette.

Estimates of MPA concentrations were determined from a plot of the cpm recovered in the supernatant *vs.* the log of the standard MPA concentration. When large junbers of samples were analyzed, estimates were calculated by computer.

## RESULTS AND DISCUSSION

### A. Synthesis of 11α-hydroxy-MPA Acid Succinate

The 11α-hydroxy-MPA intermediate (B) for preparing the succinate could not be produced by direct fermentation of MPA. The free alcohol (A) (Figure 2), however, was converted to the desired 11α-hydroxy compound with *Rhizopus nigricans* (10). Since the yields were low and inconsistent, an alternate source of the 11α-hydroxy steroid was developed. The 3 and 20 keto groups were protected as their ethylene acetals (C) while the 11-ketone was reduced to the 11α-hydroxy group with sodium-alcohol (11-13).

Acetylation of this diol (B) under forcing conditions with acetic anhydride and perchloric acid (14) afforded the 3-enol-11,17-triacetate which readily hydrolyzed to the 11,17-diacetate (E). Attempts to selectively hydrolyze the 11-acetate (E) by the usual acid or base hydrolysis caused either extensive decomposition of the steroid mole-

cule or non-selective hydrolysis. Fermentation of the diacetate with *Flavobacterium dehydrogenans* (15) selectively hydrolyzed the 11-acetate to yield the 11α-hydroxy-MPA (F).

The reaction of succinic anhydride with 11α-hydroxy-MPA could not be driven to completion, so the desired hemisuccinate derivative (G) of 11α-hydroxy-MPA required isolation by silica gel chromatography.

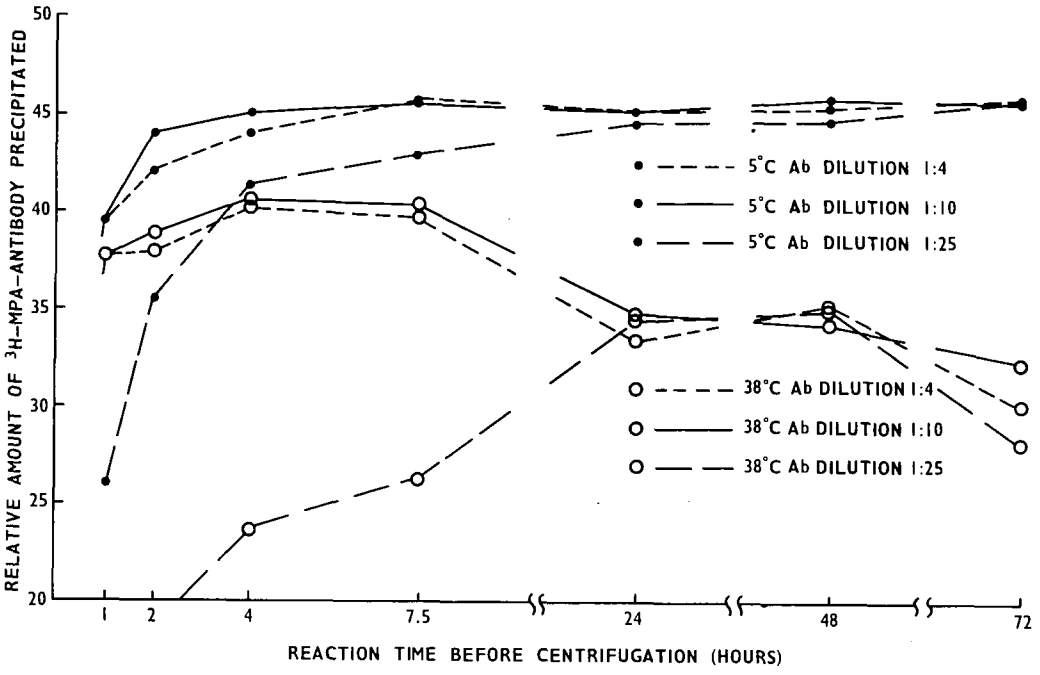
B. Formation of the BSA Conjugate

Carbonyldiimidazole was chosen to couple the hemisuccinate ester of 11α-hydroxy-MPA to BSA since its use did not expose the compound or the protein to extremes in pH (16). The first reaction of the carboxylic acid with carbonyldiimidazole requires nonaqueous conditions. Any excess carbonyldiimidazole was probably hydrolyzed on addition to the aqueous BSA solution. The imidazolide intermediate (H), however, is sufficiently stable in water to react preferentially with the free amino groups of BSA (17). The conjugate (I) was soluble in water at about pH 9. Assuming an $\varepsilon$ of 14,850 at 238 nm for 11α-hydroxy-MPA, 9.4 moles were conjugated per mole of BSA.

C. Conditions for Secondary Antibody Precipitation

The effect of the variables of reaction time, temperature, and secondary antibody concentration on precipitation of the primary antibody are shown in Figure 3. Although the precipitation of the primary antibody by secondary antibody was generally allowed to proceed overnight at 5°C, 4 hours appears sufficient to give maximal precipitation with the appropriate antibody concentration. At 38°C, the precipitation was never as complete as at 5°C. The rate and completeness of precipitation is also a function of secondary antibody concentration; however,

FIGURE 3: EFFECT OF TIME, TEMPERATURE AND DILUTION OF SECONDARY ANTIBODY ON PRECIPITATION OF ANTIGEN – ANTIBODY COMPLEX




the concentration effect was less pronounced at longer precipitation times.

D. Equilibration of Tritium-Labeled and Unlabeled MPA with Primary Antibody

The time required for equilibrium to be reached between the labeled and unlabeled MPA was investigated. Unlabeled MPA was added to primary antibody which had previously been equilibrated for 7 days at 5°C with labeled MPA. The mixture was then maintained at room temperature until secondary antibody was added. Secondary antibody was added at various times with respect to unlabeled MPA addition and incubated overnight at 5°C to precipitate the primary antibody. There was no

statistical difference in the concentration of supernatant label if the secondary antibody was added at the same time as the unlabeled MPA or if the secondary antibody was added after 4 hours of equilibration. Even when the secondary antibody was added 1 hour previous to the addition of unlabeled MPA, the same amount of label was observed in the supernatant. Equilibrium thus is either reached very quickly and/or equilibration continues effectively even during the precipitation of primary antibody by secondary antibody.

E. Decantation of the Supernatant

Since the specific activity of the $^{3}$H-MPA was quite low, it seemed advisable to decant the total supernatant as opposed to pipetting off a portion (18). As a measure of the reproducibility and efficiency of this procedure, the amount of sample solution remaining in the culture tubes was weighed after being inverted in the counting vial for 5 or 30 minutes. The percentage of supernatant left in the tube (no precipitate) after 5 minutes of draining was 1.33 ± 0.16% and after 30 minutes 0.69 ± 0.18% (S.D.). Assuming that care is taken to avoid dislodging the precipitate, it would seem that this nearly quantitative method of removing the supernatant would compare quite well with the usual method of removing an aliquot of the supernatant with a pipette.

F. Assay Statistics

The sensitivity of the assay was estimated as the lowest level on a standard curve which was significantly different (at the 95% confidence limits) from the zero value (19). At the level of antibody, labeled MPA, and sample used, this occurred at 0.5 ng MPA per ml. This figure is probably low since serum blank values obtained from untreated

animals was in the range of 0-1 ng and the extent to which this blank varies is uncertain after injection of MPA.

The average percentage recovery of known amounts of MPA added to serum (MPA = 32 ng/ml) was 108.2 ± 5.8% (S.D.). The within day and between day coefficients of variation (C.V.) of MPA added to serum were used as a measure of assay precision. The C.V. were ± 5.0% and ± 6.0%, respectively, at the 32 ng/ml level and 3.4% and 12.2%, respectively, at the 6 ng/ml level.

G. Specificity of the Antibody

It has been well established that specificities of steroid antibodies are influenced by the linkage to the protein steroid conjugate (5,7,8,9).

A radioimmunoassay had previously been developed for MPA by using the C-3 conjugate to make antibodies in goats (2). The cross-reactivities of steroids similar to MPA were analyzed using antisera to both the C-11 conjugate and the C-3 conjugate. As can be seen in Table 2, both antibodies show considerable cross-reaction with steroid analogs with alterations at C-5 and C-4. As might be expected, the C-11 MPA antibodies showed little cross-reactivity with analogs in which the C-3 carbonyl is replaced by a 3-β-hydroxyl group or in which C-6 is hydroxylated. Strangely, neither 11α-hydroxy-MPA nor 11α-hydroxy-MPA-acid succinate bind to the C-11 antibody as well as MPA.

H. Measurement of Serum MPA Levels in Dogs

The C-11 and C-3 MPA antisera were diluted to the same level so that their standard curves were nearly identical (Figure 4). The MPA levels in dogs given a single intramuscular injection of MPA

TABLE 2

CROSS-REACTIVITY OF MPA ANALOGS

| Steroid Change from MPA | % Cross-Reactivity[a] C-11 MPA | C-3 MPA |
|---|---|---|
| None | 100 | 100 |
| Loss of 6α-Methyl | 26 | 47 |
| 4,5β-Dihydro | 19 | 39 |
| Reduced to 3β-Hydroxy | 10 | 80 |
| Add 21-Hydroxy | 7 | < 1 |
| Loss of 17-Acetate | < 1 | < 1 |
| Add 6β-Hydroxy | < 1 | 34 |
| Add 6β and 21-Hydroxy | < 1 | < 1 |
| Add 11α-Hydroxy | 34 | 2 |
| Add 11α-Hemisuccinate | 35 | < 1 |

[a] Defined as: $\frac{100 \text{ X The Mass of Unlabeled MPA Where 50\% is Free}}{\text{The Mass of Compound Tested Where 50\% is Free}}$

(30 mg/kg) were measured using both antisera as shown in Figure 5. The levels measured with the C-3 antisera were consistently higher than those measured with the C-11 antisera. Similar results were obtained in nine other dogs.

I. Measurement of Serum MPA Levels in Monkeys

A 1 mg dose of MPA was administered orally to four monkeys. Serum MPA levels were measured using both the C-11 and C-3 antisera at the times indicated in Figure 6. The serum MPA pattern of each monkey measured with the C-11 antisera was similar to that measured with the

FIGURE 4: STANDARD CURVES FOR MEASUREMENT OF MPA IN THE DOG USING BOTH THE C-11 AND C-3 ANTIBODIES

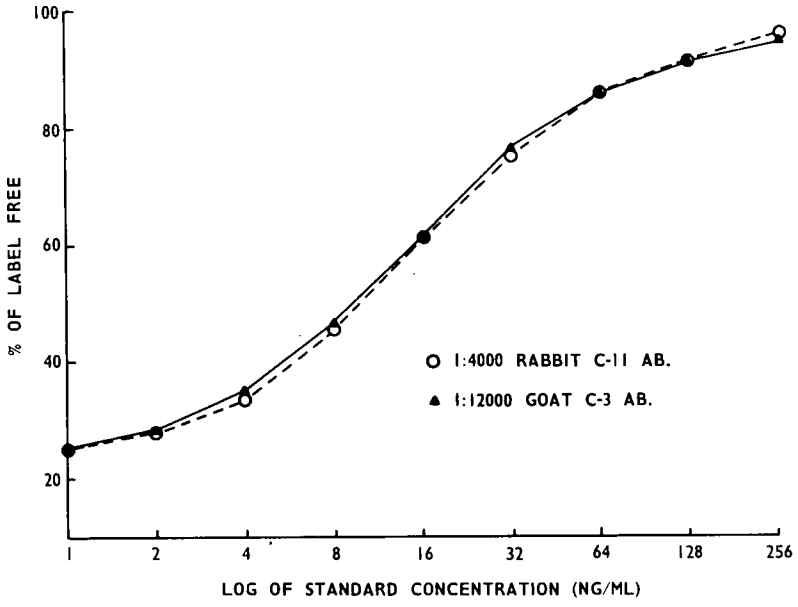



FIGURE 5: MPA LEVELS IN DOG SERUM AFTER INTRAMUSCULAR INJECTION

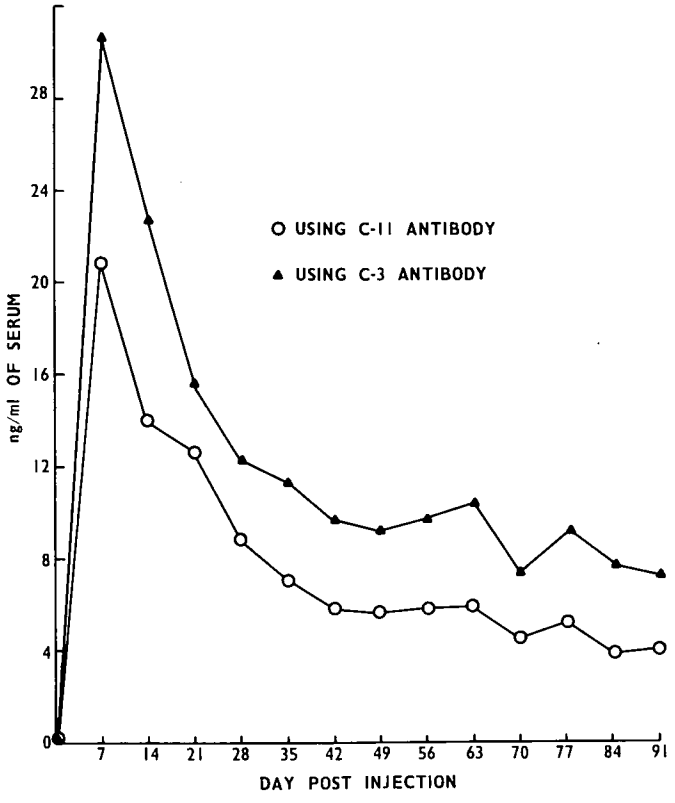
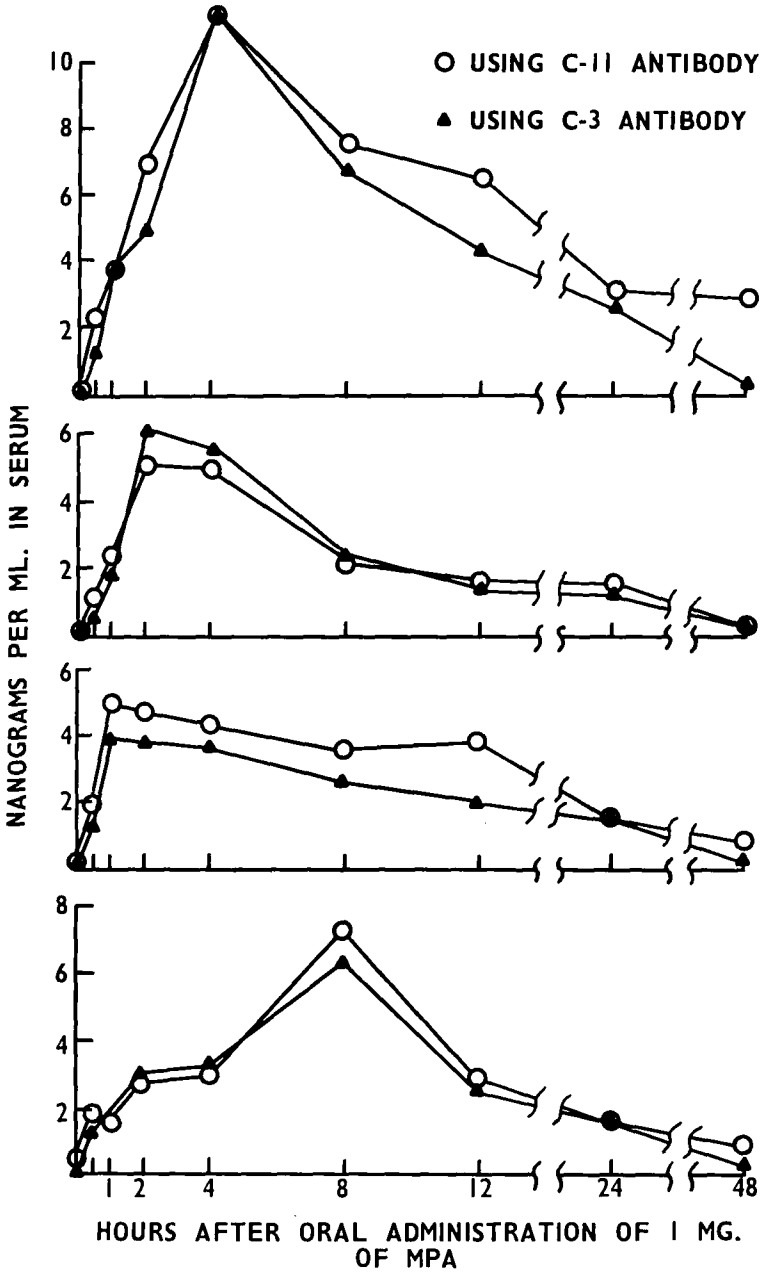


FIGURE 6: SERUM MPA LEVELS IN RHESUS MONKEYS



C-3 antisera. This may imply that any MPA metabolites present that are detected by the C-11 antisera are also detected with similar efficiency by the C-3 antisera.

The differences in MPA levels observed in the dog may be due to MPA metabolite(s), possibly in the A ring, which are produced in this species. Alternatively, the similarities observed in the monkey's MPA levels may be due to the short term nature of this experiment. Further studies will be required to elucidate the nature of the materials which are being measured by both antisera.

## ACKNOWLEDGMENTS

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## REFERENCES

1. Kaiser, D. G., Carlson, R. G., and Kirton, K. T., J. PHARM. SCI., (In Press).

2. Cornette, J. C., Kirton, K. T., and Duncan, G. W., J. CLIN. ENDOCRINOL. METABOL., 33, 459 (1971).

3. Kuss, E. and Goebel, R., STEROIDS, 19, 509 (1972).

4. Spieler, J. M., Webb, R. L., Saldarini, R. J., and Coppola, J. A., STEROIDS, 19, 751 (1972).

5. Niswender, G. D. and Midgley, A. R. Jr., IMMUNOLOGIC METHODS IN STEROID DETERMINATION, Editors, F. G. Péron and B. V. Caldwell, Appleton-Century-Crofts, New York, 1970, p. 149.

6. Lindner, H. R., Perel, E., Friedlander, A., and Zeitlin, A., STEROIDS, 19, 357 (1972).

7. Walker, C. S., Clark, S. J., and Wotiz, H. H., STEROIDS, 21, 259 (1973).

8. Weinstein, A., Lindner, H. R., Friedlander, A., and Bauminger, S., STEROIDS, 20, 789 (1972).

9. Niswender, G. D., STEROIDS, 22, 413 (1973).

10. Meister, P. D., Peterson, D. H., Murray, H. C., Spero, G. B., Eppstein, S. H., Weintraub, A., Reineke, L. M., and Leigh, H. M., J. AM. CHEM. SOC., 75, 416 (1953).

11. Lincoln, F. H., Schneider, W. P., and Spero, G. B., U. S. Patent 2,867,633, January 6, 1959.

12. Johnson, W. S., Rogier, E. R., Szmuszkovicz, J., Hadler, H. I., Ackerman, J., Bhattacharyya, B. K., Bloom, B. M., Stalmann, L., Clement, R. A., Bannister, B., and Wynberg, H., J. AM. CHEM. SOC., 78, 6300 (1956).

13. Heusser, H., Anliker, R., and Jeger, O., HELV. CHIM. ACTA, 35, 1537 (1952).

14. Barton, D. H. R., Evans, R. M., Hamlet, J. C., Jones, P. G., and Walker, T., J. CHEM. SOC., 747 (1954).

15. Charney, W., Weber, L., and Oliveto, E., ARCH. BIOCHEM. BIOPHYS., 79, 402 (1959).

16. Staab, H. A., ANGEWANDTE CHEMIE (I.E.), 1, 351 (1962).

17. Axen, U. F., PROSTAGLANDINS, 5, 45 (1974).

18. Krzeminski, L. F., Personal Communication.

19. Abraham, G. E., Swerolloff, R., Tulchinsky, D., and Odell, W. D., CLIN. ENDOCRINOL. METAB., 32, 619 (1971).