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SIMULTANEOUS MEASUREMENT OF FOUR TESTICULAR Δ^4-3-KETOSTEROIDS BY ISOCRATIC HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ON-LINE ULTRAVIOLET ABSORBANCE DETECTION

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SUMMARY

A method is described for the simultaneous measurement of testosterone, androstenedione, 17α-hydroxyprogesterone and progesterone in venous effluent from *in vitro* perfused rat testes. The assay uses a non-radioisotopic internal standard (11β-hydroxyandrostenedione), isocratic high-performance liquid chromatography (HPLC) and UV absorbance detection at 240 nm. Either of two isocratic HPLC systems described in this report (tetrahydrofuran–methanol–water, 16:28:56; methanol–acetonitrile–water, 9:36:55; v/v/v) may be used, and assay specificity is the same in each. The separation and measurement of all four steroids are completed in 25 min. Sensitivity of the method is 10 ng for testosterone, androstenedione and 17α-hydroxyprogesterone and 25 ng for progesterone. The linear range of the assay extends through 1600 ng which is the upper amount of each steroid tested. Average inter-assay coefficient of variation was 3.3% and average intra-assay coefficient of variation was 3.6%. This rapid, specific and reliable method requires minimal sample preparation and may be performed by inexperienced personnel.

INTRODUCTION

In 1979, a method was developed in this laboratory for the measurement of testosterone (T)* in biological samples by monitoring of UV absorbance after a rapid, isocratic high-performance liquid chromatography (HPLC) separation[1]. During the intervening years, this technique has proven to be reliable, accurate, precise and specific for the measurement of T in biological samples from several species[2–5]. The

* The following abbreviations and common names are used for the steroids: 17β-hydroxy-4-androsten-3-one = testosterone (T); 4-androstene-3,17-dione = androstenedione (ADIONE); 11β-hydroxy-4-androstene-3,17-dione = 11β-hydroxyandrostenedione (11β-DIONE); 17α-hydroxy-4-pregnene-3,20-dione = 17α-hydroxyprogesterone (17-PROG); 4-pregnene-3,20-dione = progesterone (PROG); 3β,17α-dihydroxy-5-pregnen-20-one = 17α-hydroxypregnenolone (17-PREG); 3β-hydroxy-5-pregnen-20-one = pregnenolone (PREG).

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method is also rapid, and easy to teach to inexperienced personnel. Unfortunately, this method was not applicable to the measurement of Δ^4-3-ketosteroid T biosynthetic intermediates including ADIONE, 17-PROG and PROG because these compounds were not separated by the single, isocratic HPLC system employed.

Advances in solvent mixture-design since 1979 (refs. 6–8) have allowed us to develop two ternary solvent mixtures capable of separating T, ADIONE, 17-PROG, PROG and 11β-DIONE internal standard in single isocratic chromatographic separations of 25 min. With this technology, we now report a simultaneous assay for T, ADIONE, 17-PROG and PROG in rat testicular venous effluent via isocratic HPLC with on-line UV absorbance detection.

MATERIALS AND METHODS

Materials

A Waters Associates Model 6000A pump, U6K injector, 30 cm × 3.9 mm I.D. μBondapak C_{18} column (Waters Assoc., Milford, MA, U.S.A.) and a 7.0 cm × 2.0 mm I.D. Whatman CO:PELL ODS precolumn (Whatman, Clifton, NJ, U.S.A.) were used for the chromatography. Detection of Δ^4-3-ketosteroids was accomplished by monitoring absorbance at 240 nm with a Spectroflow Model 770 UV analyzer (Schoeffel, Westwood, NJ, U.S.A.). Peak areas were estimated by a Spectra-Physics Model SP4100 computing integrator (Spectra-Physics, Stirling, NJ, U.S.A.) which also carried out the post run calculations for mass estimation. Liquid scintillation counting was performed on a Beckman Model L5250 counter (Beckman, Irvine, CA, U.S.A.). T, ADIONE, 11β-DIONE, 17-PROG, PROG, 17-PREG and PREG were purchased from Steraloids (Wilton, NH, U.S.A.). Each steroid was recrystallized to constant melting point before use. [^{3}H]T, [^{3}H]ADIONE, [^{3}H]-17-PROG, [^{3}H]PROG, [^{3}H]-17-PREG and [^{3}H]PREG were obtained from New England Nuclear (Boston, MA, U.S.A.), and each was purified by thin-layer chromatography (TLC) on pre-coated plates (250 μm; Analtech, Newark, DE, U.S.A.) within 1 week of use. Reagent grade diethyl ether (Mallinckrodt, St. Louis, MO, U.S.A.) was used for sample extraction. Nanograde benzene and ethyl acetate (2:1, Mallinckrodt) were used to develop the TLC plates. Commercially prepared scintillation fluid, OCS™, was purchased from Amersham (Arlington Heights, IL, U.S.A.). The HPLC solvents used were acetonitrile, tetrahydrofuran, methanol, ethanol and benzene (Photrex grade; J. T. Baker, Phillipsburgh, NJ, U.S.A.). Mixtures of the HPLC solvents with double glass distilled water were filtered, vigorously stirred and vacuum degassed for at least 1 h before use. Standard solutions of steroids were prepared by weighing phosphorus pentoxide (Fisher Scientific, Fairlawn, NJ, U.S.A.) desiccated crystals on a Cahn Electro-balance (Ventron Instruments, Paramount, CA, U.S.A.) and dissolving them in Photrex grade benzene.

Biological material

Mature male Sprague–Dawley rats were obtained from Dominion Labs. (Dublin, VA, U.S.A.), housed in a light-controlled (14:10, light:dark) room and given food and water *ad libitum*. Testes were removed and perfused *in vitro*[9,10] with an artificial medium consisting of Krebs–Ringer bicarbonate solution with 3% (w/v) fraction V bovine serum albumin (KRB-BSA) and 25% bovine red blood cells. In addition, the medium contained 20 μM PREG to serve as substrate for the testicular production of

all the Δ^4-3-ketosteroid intermediates (PROG, 17-PROG, ADIONE and T)[11]. The testicular venous effluent was centrifuged, the plasma decanted and stored frozen.

HPLC method development

Binary mixtures (v/v) of tetrahydrofuran–water (35:65), acetonitrile–water (50:50) and methanol–water (60:40) were prepared. The resolution of 11β-DIONE, T, ADIONE, 17-PROG and PROG was tested in each binary mixture as well as three ternary blends and one quaternary blend of these mixtures as described by Lehrer[8].

Simultaneous Δ^4-3-ketosteroid assay

For preparation of each sample, a 200- or 400-ng amount of the internal standard (11β-DIONE) in benzene was pipetted into a 12-ml disposable, glass, screw-cap tube with an automatic pipette (Micromedic Systems, Huntsville, AL, U.S.A.). When biological material was to be extracted, the benzene was evaporated with nitrogen, and 50 μl of ethanol added to redissolve the 11β-DIONE. One ml of either testicular venous effluent or KRB-BSA was pipetted into the tube followed by 10 ml of cold diethyl ether. Tubes were then capped, shaken vigorously for at least 1 min, centrifuged at 1500 *g* for 10 min and placed into a freezer. Prior to assay, tubes were removed from the freezer, centrifuged at 1500 *g* for 30 sec to pellet loose ice crystals and decanted into a second disposable glass tube. The ether was then dried under nitrogen and the sample dissolved in 15 μl of ethanol; all of this solution was then injected into the chromatograph. Peaks of UV absorbance were converted to area units by the SP4100 integrator and compound mass calculated according to the formula

$$\text{ng of compound} = \frac{\text{Area of compound peak}}{\text{Area of 11}\beta\text{-DIONE peak}} \cdot (\text{RF})(\text{ng of 11}\beta\text{-DIONE added})$$

where RF is the response factor for the particular compound relative to 11β-DIONE. For determining the response factors, tubes were prepared with each assay series by pipetting 200 ng of 11β-DIONE, T, ADIONE, 17-PROG and PROG into each tube, drying it under nitrogen and injecting it into the chromatograph dissolved in 15 μl of ethanol.

Partition into diethyl ether

To assess the ability of each compound to partition from an aqueous phase into diethyl ether, samples for the determination of response factors were either injected as described above or extracted as described for biological samples with KRB-BSA as the aqueous phase, and then injected. These results were compared with a paired t-test.

Sensitivity and accuracy

The sensitivity, accuracy and detector linearity were tested by adding 5, 10, 25, 50, 100, 200, 400, 800 or 1600 ng each of T, ADIONE, 17-PROG and PROG to the appropriate tubes with either 200 ng (5–200 ng standard samples) or 400 ng (400–1600 ng standard samples) of 11β-DIONE. These were then processed as biological samples with KRB-BSA as the aqueous phase.

Precision

Assay precision was checked by measuring samples containing 75, 150 or 600 ng each of T, ADIONE, 17-PROG and PROG. Five samples of each of the three pools were assayed on each of four consecutive days.

Specificity

The specificity of the assay for biosynthesized steroids in biological material when tetrahydrofuran–methanol–water was used as the HPLC separation system was determined by extracting four aliquots of testicular venous effluent from testes with PREG containing medium. No 11β-DIONE was added to these tubes, instead 2.0×10^5 dpm of either [^{3}H]T, [^{3}H]ADIONE, [^{3}H]-17-PROG or [^{3}H]PROG were added to each tube so that each of the sample tubes contained one radioactive compound. These samples were chromatographed in the tetrahydrofuran–methanol–water mobile phase and the eluate corresponding to the peak of interest (*e.g.*, T, ADIONE, 17-PROG or PROG) was collected. A small aliquot was pipetted from the total peak eluate, evaporated to dryness and prepared for scintillation counting. The remainder was purged of organics (tetrahydrofuran and methanol) in a 45°C water-bath under nitrogen and the aqueous residue reextracted and rechromatographed with acetonitrile–water (40:60). The material was again collected, counted, extracted and chromatographed a third time in ethanol–water (40:60). This final material was collected and counted. The mass of compound was determined from mass to area curves constructed in each of the mobile phases used, and specific activities were calculated at each step.

The specificity of the assay for biosynthesized steroids in biological material when methanol–acetonitrile–water was used as the HPLC separation system was determined by comparing the results of duplicate Δ^4-3-ketosteroid assays when the two separation systems were used. Samples of venous effluent from six normal rat testes perfused *in vitro* with medium containing 20 μM PREG were assayed in duplicate, half after separation in the tetrahydrofuran–methanol–water system and half after separation by the methanol–acetonitrile–water system.

RESULTS

HPLC separation

From the solvent mixtures described in the methods section, two ternary mixtures were discovered that separated all five steroids. The results in Fig. 1 show the separations achieved when T, ADIONE, 17-PROG, PROG and the internal standard, 11β-DIONE are injected into the chromatograph and eluted with tetrahydrofuran–methanol–water (16:28:56, v/v/v) (Fig. 1A) or eluted with methanol–acetonitrile–water (9:36:55, v/v/v) (Fig. 1B). Table I contains the physical parameters of the two HPLC separations. The capacity factors (k') are within the optimal range of 1–10 for all compounds except PROG eluted in the methanol–acetonitrile–water system. The resolution (R_s), of adjacent peak pairs was calculated according to the formula

$$R_s = \frac{1}{4}\left(\frac{\alpha - 1}{\alpha}\right) N^{1/2}\left(\frac{k'}{1 + k'}\right)$$

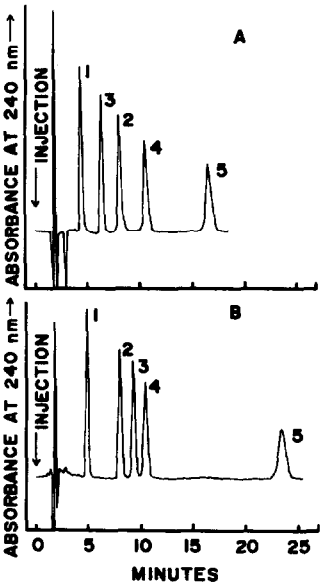

Fig. 1. HPLC separation of five Δ^4-3-ketosteroids by two different mobile phases; A, tetrahydrofuran–methanol–water (16:28:56, v/v/v); and B, methnaol–acetonitrile–water (9:36:55, v/v/v); at 2 ml/min on a μBondapak C_{18} column. Aliquots containing 200 ng of each standard were injected onto the column. Peaks: 1 = 11β-DIONE; 2 = T; 3 = ADIONE; 4 = 17-PROG and 5 = PROG. Retention times in system A for compounds 1–5 are 4.43, 8.03, 6.34, 10.45 and 16.34 min respectively and 4.76, 7.82, 9.10, 10.19 and 22.87 min respectively in system B.

where α equals k' of the later peak divided by k' of the earlier. The column efficiency (N) was experimentally determined to be 2500. R_s values ranged from a low of 1.42 for the resolution of ADIONE and 17-PROG to a high of 6.79 for the resolution of 17-PROG and PROG in the methanol–acetonitrile–water separation system (Table I). R_s values ≥ 1.25 indicate complete resolution[12]. Therefore, all five steroids are completely resolved in both HPLC solvent systems presented.

TABLE I

PHYSICAL PARAMETERS OF THE TWO Δ^4-3-KETOSTEROID SEPARATIONS

Compounds were eluted from the μBondapak C_{18} column at 2 ml/min with the indicated solvent systems.

Tetrahydrofuran–methanol–water (16:28:56, v/v/v)				*Methanol–acetonitrile–water (9:36:55, v/v/v)*			
Compound	k'	α	R_s	*Compound*	k'	α	R_s
11β-DIONE	1.67			11β-DIONE	1.78		
		1.71	3.60			2.05	4.68
ADIONE	2.86			T	3.64		
		1.39	2.71			1.21	1.74
T	3.98			ADIONE	4.40		
		1.39	2.90			1.16	1.42
17-PROG	5.54			17-PROG	5.11		
		1.67	4.42			2.52	6.79
PROG	9.26			PROG	12.87		

Partition into diethyl ether

It was of initial importance in development of the assay to determine whether all the steroids partitioned to the same degree as the internal standard from an aqueous phase into the ether phase. Paired t-tests revealed no statistically significant difference ($P > 0.05$) between extracted and unextracted values for any steroid, nor did analysis of variance reveal any difference between estimations of the response factors due to the HPLC separation system used ($P > 0.05$).

Sensitivity and accuracy

The lower limit of sensitivity was 10 ng for T, ADIONE and 17-PROG regardless of the HPLC separation system used. Due to longer retention and therefore broader peak shape, PROG sensitivity was 25 ng after separation in either tetrahydrofuran–methanol–water or methanol–acetonitrile–water.

The results in Fig. 2 show the regression lines for ADIONE, T, 17-PROG and PROG when the steroids were separated via the tetrahydrofuran–methanol–water HPLC system and mass was measured by UV absorbance. In each case the linear correlation between steroid added and steroid measured was equal to 1.0, slopes of the lines were 1.01, 1.01, 1.00 and 1.06 and the calculated intercept values were −0.6, −0.5, 2.1 and −3.7 ng (well below the limits of assay sensitivity) respectively for T, ADIONE, 17-PROG and PROG. Similar results were obtained (data not shown) when the steroids were separated with the methanol–acetonitrile–water system.

Precision

The results of assaying five replicates from each of three sample pools containing 75, 150 or 600 ng of each of the Δ^4-3-ketosteroids on 4 different days after separation in the tetrahydrofuran–methanol–water or the methanol–acetonitrile–water are shown in Table II. Assay variability was low, regardless of the HPLC separation system used, as depicted in two ways. First, the standard error of the means of each of the groups of twenty determinations (five per day for 4 days) was very low (Table II). Second, the intra (within days) —and inter (between days)— assay coefficients of variation ranged from 0.5 to 6.1%. Averaging over the three concentrations, for each steroid measured, the highest average intra-assay coefficient of variation was 3.6% for 17-PROG and the highest average inter-assay coefficient of variation was 3.3% for T, both in the tetrahydrofuran–methanol–water separation system.

Specificity

The specificity of the simultaneous assay for T, ADIONE, 17-PROG and PROG depends upon three selective factors. First, with diethyl ether extraction only lipophilic molecules partition into the sample. Second, only steroids with the Δ^4-3-keto configuration have high absorptivities at 240 nm. Third, the HPLC system resolves the UV absorbing steroids into discrete, homogenous peaks.

Homogeneity of the UV absorbing peaks was assessed when the steroids were separated by tetrahydrofuran–methanol–water by adding authentic ^{3}H-labeled steroid to testicular venous effluent from *in vitro* perfused testes, and determining the specific activity for each compound after the initial, and two subsequent HPLC separations. Specific activities for each compound in the three systems are given in Table

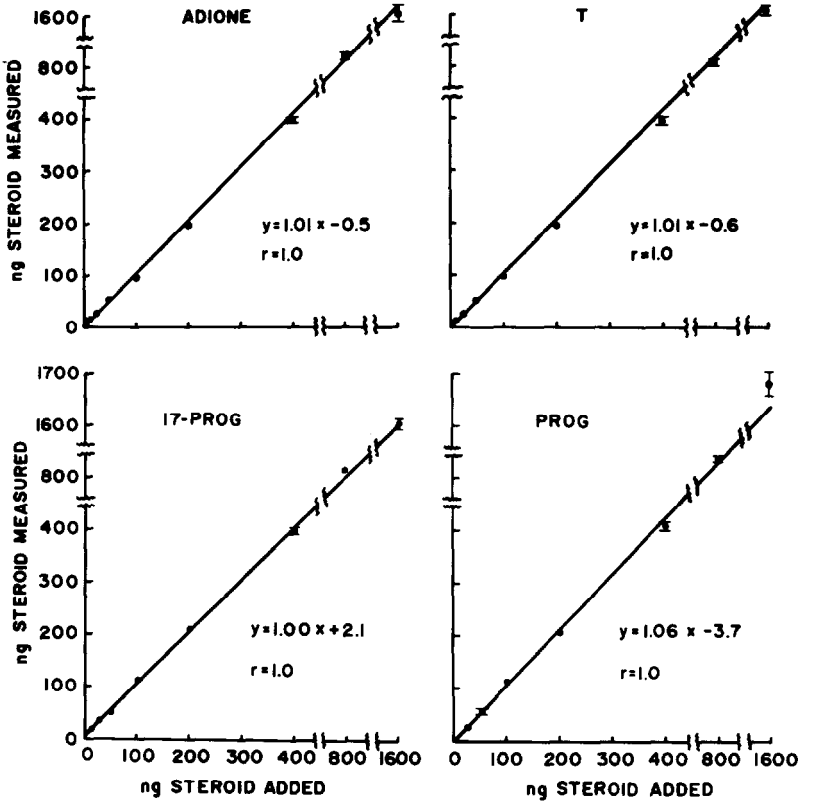

Fig. 2. Accuracy of the simultaneous assay for measurement of 10–1600 ng of T, ADIONE, 17-PROG and PROG after separation by the tetrahydrofuran–methanol–water (16:28:56, v/v/v) HPLC system. Each point is the average of five replicate determinations. The bars about some points represent the standard error of the mean. For points with no error bars, the error may be considered equal or less than the size of the point.

III. The UV absorbance of each peak was found to be associated only with the compound being measured since the relative standard deviation of the three specific activity determinations was less than 5% for T, ADIONE, 17-PROG and PROG.

The specificity of the measurement of T, ADIONE, 17-PROG and PROG when separated by methanol–acetonitrile–water was assessed subsequently by comparing the amount of each steroid in testicular venous effluent with that measured when the steroids were separated via the tetrahydrofuran–methanol–water system. The average amount of T, ADIONE, 17-PROG and PROG (μg/testis · h $\pm$ S.E.M.) secreted by the six testes was 11.6 $\pm$ 0.7, 2.4 $\pm$ 0.3, 6.6 $\pm$ 0.5 and 4.0 $\pm$ 0.5 respectively after tetrahydrofuran–methanol–water separation and 10.9 $\pm$ 0.7, 2:5 $\pm$ 0.3, 6.1 $\pm$ 0.5 and 3.8 $\pm$ 0.6 respectively when separated by methanol–acetonitrile–water. There were no statistically significant differences ($P > 0.05$) as measured by paired t-tests for any of the steroids. We conclude from these results that the simultaneous measurement of T, ADIONE, 17-PROG and PROG is specific regardless of the solvent system used to resolve the four steroids and the internal standard.

TABLE II

RESULTS OF SIMULTANEOUS Δ^4-3-KETOSTEROID ASSAYS PERFORMED TO DEMONSTRATE ASSAY PRECISION

Five samples were assayed from each of three different pools on 4 different days.

Steroid added		*Steroids separated via tetrahydrofuran–methanol–water*			*Steroids separated via methanol–acetonitrile–water*		
Amount	*Steroid*	*Steroid* estimate*	*% Intra-assay** coef. var.*	*% Inter-assay*** coef. var.*	*Steroid estimate*	*% Intra-assay coef. var.*	*% Inter-assay coef. var.*
75 ng	T	72.1 ± 1.1	5.1	5.2	73.2 ± 0.4	2.4	0.6
	ADIONE	79.1 ± 0.6	2.9	2.3	79.1 ± 0.6	2.4	2.7
	17-PROG	73.8 ± 1.1	5.7	4.7	76.0 ± 0.4	2.3	1.0
	PROG	72.0 ± 1.1	6.1	3.4	71.4 ± 0.7	3.6	2.9
150 ng	T	147 ± 1.3	1.8	3.9	148 ± 0.5	1.2	1.3
	ADIONE	154 ± 1.0	1.8	2.3	153 ± 0.8	2.0	1.3
	17-PROG	146 ± 1.2	3.4	1.7	151 ± 0.7	2.3	0.4
	PROG	149 ± 0.9	2.3	1.9	150 ± 1.3	3.9	1.4
600 ng	T	596 ± 2.1	1.6	0.7	583 ± 1.6	1.0	0.9
	ADIONE	615 ± 1.7	1.1	0.9	613 ± 2.0	1.1	1.2
	17-PROG	593 ± 2.3	1.8	0.7	592 ± 1.1	0.8	0.5
	PROG	615 ± 2.2	1.6	0.8	605 ± 1.3	0.9	0.5

* Each number is the mean from twenty measurements (five per day for 4 days) ± the standard error of the mean.
** % Intra-assay coefficient of variation = (standard deviation/mean) × 100, within days.
*** % Inter-assay coefficient of variation = (standard deviation/mean) × 100, between days.

DISCUSSION

Two recent papers[13,14] have reported separation of T, ADIONE, 17-PROG and PROG using a gradient elution HPLC system. However, neither of these reports attempt to exploit the separations as the basis of a simultaneous assay for these four steroids. Because of favorable results with measuring T by UV absorbance after isocratic HPLC separation[1], we decided to develop isocratic conditions capable of resolving these four steroids with the idea of developing a simultaneous assay tech-

TABLE III

RECHROMATOGRAPHY OF Δ^4-3-KETOSTEROIDS TO CONSTANT SPECIFIC ACTIVITY (cpm/ng)

Authentic ^{3}H-labeled steroid was added to the biological sample. The specific activity for each compound was determined after the initial tetrahydrofuran–methanol–water, and two additional HPLC separations.

HPLC system	*Steroid*			
	T	*ADIONE*	*17-PROG*	*PROG*
Tetrahydrofuran–methanol–water (16:28:56)	139	117	96	113
Acetonitrile–water (40:60)	138	117	91	114
Ethanol–water (40:60)	140	121	89	111

nique. Isocratic conditions were preferred over gradient elution because less sophisticated equipment is required, operation is simpler and gradient elution requires extra time to return to starting conditions (*i.e.*, stable baseline).

We have described in this paper two isocratic HPLC separations for T, ADIONE, 17-PROG, PROG and the internal standard, 11β-DIONE. Development of these separations was based on solvent optimization procedures described in several recent technical reports[6–8]. Each peak pair was completely resolved in both separation systems. The time required for elution of all compounds of interest was 20 min with the tetrahydrofuran–methanol–water system and 25 min with the methanol–acetonitrile–water system. These values compare well with the 20-min and 35-min gradient elutions of Walters *et al.*[13] and Kessler[14] respectively for T, ADIONE, 17-PROG and PROG. It should be noted that the tetrahydrofuran–methanol–water system requires longer mixing and degassing than does the methanol–acetonitrile–water system in order to avoid baseline aberrations.

The two isocratic HPLC separations have been used as the basis for simultaneous measurement of T, ADIONE, 17-PROG and PROG by monitoring of absorbance at 240 nm. The assay was specific for the four steroids in rat testicular venous effluent regardless of which HPLC separation system was used. This was demonstrated by the rechromatography to constant specific activity experiments described with ^{3}H-labeled steroids. Since specificity will depend on the biological fluid assayed, we have presented both separations to aid in the application of the method to different biological materials.

The sensitivity (10–25 ng), linear accuracy from the limit of sensitivity through 1600 ng and precision (average intra- and inter-assay coefficients of variation from 0.6 to 3.6%) are not different from those obtained for the measurement of T alone in our previously published method[1]. These parameters also compare favorably with the gas–liquid chromatographic (GLC) method (sensitivity 20 ng, assay coefficient of variation 3.3%) previously used in this laboratory to measure T, ADIONE, 17-PROG and PROG[11], and with other conventional GLC techniques reported for androgen measurement[15]. Capillary GLC with electron capture or nitrogen sensitive (thermal) detectors can potentially measure picogram amounts of plasma steroids[16,17]. However, to date these procedures have not been widely applied at least in part due to the extensive sample preparation required. Only radioimmunoassay offers substantial increases in sensitivity (low picogram) with only nominal loss of precision (assay coefficients of variation 3.7–18.4%) and moderate sample preparation[18].

The simultaneous assay for T, ADIONE, 17-PROG and PROG described herein requires only the quantitative addition of the non-radioisotopic 11β-DIONE, diethyl ether extraction and a single HPLC separation with on-line UV detection. The 11β-DIONE corrects for extraction and injection losses and provides the reference mass for compound mass determination simultaneously (see ref. 1 for detail of the 11β-DIONE internal standard technique). Therefore, samples are easily prepared and determinations made. For example, an investigator with a freshly collected or thawed sample is capable of preparing and making a single determination in less than 1 h. None of the techniques discussed above offers this ease of operation, especially the GLC methods which all require prior chromatography (TLC, paper, Celite, etc.), derivative formation and isotopic tracer measurement for recovery before the GLC

separation and mass determination.

The HPLC system offers additional flexibility since either of the solvent systems that form the basis of the simultaneous assay may be used in the preparative mode because the column and detector do not damage or destroy the steroids.

The tetrahydrofuran–methanol–water solvent system might also be exploited for the study of 17-PREG and PREG. Both of these compounds were found to be resolved (data not shown) from the Δ^4-3-ketosteroids measured by the technique presented in this paper. They could potentially be measured by their absorbance at 210 nm, although at decreased sensitivity compared to that of the Δ^4-3-ketosteroids at 240 nm.

We have described a rapid, reliable method for the measurement of nanogram amounts of T, ADIONE, 17-PROG and PROG, which should be readily applicable to the study of testicular Δ^4 pathway steroidogenesis. The HPLC techniques that form the basis of this method may have broader applications in either preparative separation or measurement of other testicular steroids. Finally, the method is easier to perform than any other currently available method for the measurement of these important steroids.

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REFERENCES

1 R. C. Cochran, K. J. Darney, Jr. and L. L. Ewing, *J. Chromatogr.*, 173 (1979) 349.
2 L. L. Ewing, B. R. Zirkin, R. C. Cochran, N. Kromann, C. Peters and N. Ruiz-Bravo, *Endocrinology*, 105 (1979) 1135.
3 L. L. Ewing, R. C. Cochran, B. Zirkin and C. E. Chubb, in A. Steinberger and E. Steinberger (Editors), *Testicular Development, Structure, and Function*, Raven Press, New York, 1980, p. 117.
4 B. R. Zirkin, L. L. Ewing, N. Kromann and R. C. Cochran, *Endocrinology*, 107 (1980) 1867.
5 D. D. Dykman, R. Cochran, P. M. Wise, C. A. Barraclough, N. H. Dubin and L. L. Ewing, *Biol. Reprod.*, 25 (1981) 235.
6 G. J.-L. Lee, R. M. K. Carlson and S. Kushinsky, *J. Chromatogr.*, 212 (1981) 108.
7 J. L. Glajch, J. J. Kirkland, K. M. Squire and J. M. Minor, *J. Chromatogr.*, 199 (1980) 57.
8 R. Lehrer, *Amer. Lab.*, 13 (1981) 113.
9 L. L. Ewing, B. Brown, D. C. Irby and I. Jardine, *Endocrinology*, 96 (1975) 610.
10 L. L. Ewing, C. E. Chubb and B. Robaire, *Nature (London)*, 264 (1976) 84.
11 C. Chubb and L. L. Ewing, *Amer. J. Physiol.*, 6 (1979) E231.
12 L. R. Snyder and J. J. Kirkland, *Introduction to Modern Liquid Chromatography*, Wiley, New York, 2nd ed., 1979, p. 41.
13 D. G. Walters, P. M. D. Foster and R. C. Cottrell, *J. Chromatogr.*, 219 (1981) 152.
14 M. J. Kessler, *Steroids*, 39 (1982) 21.
15 A. Vermeulen, *Clin. Chim. Acta*, 34 (1971) 223.
16 M. Novotny, M. P. Maskarinec, A. T. G. Steverink and R. Farlow, *Anal. Chem.*, 48 (1976) 468.
17 M. Axelson, *J. Steroid Biochem.*, 8 (1977) 693.
18 D. C. Anderson, B. R. Hopper, B. L. Lasley and S. S. C. Yen, *Steroids*, 28 (1976) 179.