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RADIOIMMUNOASSAY OF PLASMA ANDROSTENEDIONE, TESTOSTERONE AND 11β-HYDROXYANDROSTENEDIONE AFTER CHROMATOGRAPHY ON LIPIDEX-5000 (HYDROXYALKOXYPROPYL SEPHADEX)

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SUMMARY

11β-Hydroxyandrostenedione is a C_{19}-steroid almost exclusively of endogenous adrenal origin. It represents a major pathway of biological inactivation of adrenal androstenedione, an important androgen precursor. If the proportion of biosynthesized adrenal androstenedione that becomes 11β-hydroxylated were constant, measurement of this product could serve as an indicator of the contribution of the adrenal to overall androstenedione production. For these reasons a procedure for the isolation of testosterone, androstenedione, and 11β-hydroxyandrostenedione by chromatography on Lipidex-5000, followed by radioimmunoassay measurement, has been developed and the analytical characteristics of the procedure evaluated.

INTRODUCTION

The problem of selectively measuring adrenal or gonadal sources of androgens remains extremely difficult. Subjects who have undergone castration or adrenalectomy are not readily available for clinical research, and, in any event, are not necessarily "normal" in all other respects. "Selective" suppression tests using dexamethasone or contraceptive steroids do not yield unambiguous answers since corticosteroids affect gonadotropin secretion and estrogens affect adrenal function. The production of androgens from circulating "prehormones" may also be affected. Venous catheterization yields important information, although the procedure is frequently unsuccessful and the total venous effluent cannot be measured accurately [1]. However, this method is not without hazards and must be ethically justified. It is therefore important to develop some unequivocal and convenient marker of ovarian or adrenal androgen production. Since 11-hydroxylation of C_{19}-steroids is not found in the nontumorous ovary [2], one can assume that 11-hydroxylated C_{19}-steroids originate in the adrenal. It has been shown that most of the 11β-hydroxyandrostenedione (OHA) produced by the adrenal arises from 11β-hydroxylation of androstenedione rather than from sidechain cleavage of 11-oxygenated corticosteroids [3, 4]. If production of OHA were a constant fraction of the total production of androstenedione, as is the case with 11β-hydroxylation of adrenal C_{21}-steroids [5, 6], it might serve as a marker for adrenal androstenedione secretion, and discriminate it from androstenedione derived from other sources.

The availability of chromatography and radioimmunoassays for androstenedione and 11β-hydroxyandrostenedione is a prerequisite for testing this hypothesis.

EXPERIMENTAL

Glassware. Disposable glassware was rinsed with methanol; other glassware was acid-washed and rinsed with methanol. Glass chromatographic columns were Kontes Chromaflex standard sections, 5 mm i.d., of three-component construction: a straight 500 mm column section, an upper 250 mm column section with an integral 50 ml reservoir, and a terminal stopcock adapter with a removable sintered glass disc. Viton O-ring fitted balljoint connections were secured with screw-type Thomas clamps. A glass wool plug placed under the sintered disc was used to trap column packing particles.

Chromatographic materials. Fifty % (by weight) C_{14}-alkylated hydroxyalkoxypropyl Sephadex was obtained as "Lipidex-5000", from Packard Instrument Company (Downers Grove, IL).

Chromatographic solvents and reagents. Diethyl ether (Mallinckrodt analytical reagent) from freshly opened containers, previously stored at 4°C, was used. Chloroform, cyclohexane, hexane, and dichloromethane were Mallinckrodt nanograde quality reagents, used as supplied. Absolute ethanol (USI) and tert-butanol (Mallinckrodt) were reagent grade. Azobenzene was obtained from Eastman Chemicals.

Column preparation. Columns were prepared in advance to permit determination of flow rates. Two extra columns were prepared to permit selection of

the most uniform flow characteristics within a set of columns. "Lipidex-5000" supplied in methanol suspension was collected by filtration and washed in a filter funnel with about 200 ml of mobile phase solvent, water-saturated cyclohexane–chloroform–*tert*-butanol (85:15:2, by vol.). The washed gel was resuspended in enough of the solvent to yield a pourable consistency and allowed to swell for at least 1 hr. The column and the lower 10–15 mm of the reservoir were filled with solvent and the slurry poured into the reservoir. After the Lipidex had settled to constant height under gravity, its top was adjusted to be 25 mm below the reservoir. The 5 × 725 mm bed was washed with another 100 ml of mobile phase solvent.

Plasma extractions. Into each 15 ml conical glass centrifuge tube, 0.1 ml (approx. 2000 d.p.m.) each of androstenedione (11 pg), testosterone (6 pg), and OHA (8 pg) standards in phosphate buffer was introduced. Following the addition of 1.0 ml of plasma, the contents of the tube were thoroughly mixed in a vortex mixer. After 15 min of equilibration, 2 ml of diethyl ether from a freshly opened can were added and the mixture vortexed. The aqueous phase was frozen by placing the tube into a dry ice–ethanol bath. After decanting the ether phase, the aqueous layer was thawed and the procedure repeated three more times. The combined ether extract was evaporated with nitrogen. For storage (usually overnight, but not to exceed 2 days), the residue was dissolved in 1 to 2 ml of the chromatographic solvent and kept at 4°C; this solvent was removed by evaporation immediately before chromatography.

The dried plasma extract was transferred to the column with five 0.1 ml portions of mobile phase, with the first 0.1 ml portion containing azobenzene (4 mg/ml). The steroids of interest were eluted in three consecutive 8 ml fractions with cuts made as follows: androstenedione, 7–15 ml, testosterone, 15–23 ml; and OHA, 23–31 ml. The azobenzene was used to monitor column performance. The eluted steroid fractions were divided into two equal portions, one for radioimmunoassay (RIA) and the other for radioactivity recovery determinations. Both portions were evaporated to dryness under nitrogen. The portion for RIA was dissolved in 0.5 ml methanol and that for recovery determination in 15 ml of the appropriate scintillation counting fluid.

Radioimmunoassay reagents. Androstenedione-6-BSA antiserum (AN6-22) was obtained from Endocrine Sciences (Tarzana, CA), 11α-hydroxytestosterone-11-succinyl-BSA antiserum (5224) from Organon-Oss. The 11β-hydroxyandrostenedione antiserum was prepared in our laboratories [7]. After Rivanol treatment, it was stored in BSA-phosphate buffer at a 1:5 dilution. Borate buffer (pH 8, 0.05 M) for androstenedione RIA [8], phosphate buffer (pH 7.4, 0.02 M) for testosterone RIA [9] and phosphate buffer (pH 7.0, 0.1 M) for OHA RIA [7] were stored at 4°C. The diluting medium for each antiserum was 0.1% BSA in its buffer, while the assay buffer contained 200 mg human gamma-globulin (Fraction II, Grade B) in 100 ml of phosphate buffer. Dextran charcoal suspension for testosterone and OHA assay contained Norit A charcoal (1.0 g) and Dextran T-70 (0.1 g) in 200 ml deionized water. The androstenedione assay used saturated ammonium sulfate solution for precipitation [8].

Miscellaneous solvents for radioimmunoassays included methanol (reagent grade, redistilled), diethyl ether redistilled, and ethanol (absolute, reagent grade). Sources of nonradioactive steroids were: 4-androstene-3,-17-dione, Steraloids Inc., Pawling, NY; testosterone, Nutritional Biochemicals Corp., Cleveland, OH; 11β-hydroxy-4-androstene-3,17-dione, Upjohn Co., Kalamazoo, MI. These were purified by recrystallization before use. All nonradioactive steroid standard solutions were prepared with absolute ethanol. [1,2,6,7-^{3}H]-Testosterone (S.A. 85 Ci/mmol) and [1,2-^{3}H]-4-androstene-3,17-dione (S.A. 46 Ci/mmol) were obtained from New England Nuclear Corp. Dr. P. N. Rao prepared the [1,2,6,7-^{3}H]-11β-hydroxy-4-androstene-3,17-dione (S.A. 69 Ci/mmol). Stock solutions of the radioactive steroids dissolved in benzene–ethanol (9:1, v/v) were stored at 4°C and repurified at 3-month intervals. Androstenedione was purified on a 30 × 500 mm Sephadex LH-20 column by elution with benzene–methanol (9:1, v/v); testosterone and OHA on 15 × 450 mm Sephadex LH-20 columns with a mobile phase of water-saturated dichloromethane–hexane–ethanol (50:50:0.5, by vol).

Scintillation counting fluids. For androstenedione measurements, a scintillation fluid consisting of 42 ml Liquifluor (New England Nuclear) per liter of toluene was used. For testosterone and OHA "Scintisol complete" (Isolab, Akron, OH 44321) was used as the counting medium.

Sample treatment for RIA. Dried column eluates containing the equivalent of 0.5 ml plasma were dissolved in 0.5 ml nanograde methanol. Aliquots used for assays in duplicate were: androstenedione, 25 μl; testosterone, 200 μl for low-value samples, 100 μl for high-value samples, OHA, 100 μl.

Androstenedione. Dilute antiserum was prepared fresh for each assay by adding, in order: 10 ml borate buffer, 100×10^3 d.p.m. [^{3}H]androstenedione (480 pg, 30 μl), 0.15 ml 10% solution of BSA, 0.20 ml 2.5% bovine gammaglobulin solution, and 100 μl of well but gently mixed antiserum stock solution. The 1:10,000 antiserum dilution was thoroughly mixed with gentle agitation, then centrifuged at 2,000 rev./min for 5 min before use.

Duplicates of the column eluate (25 μl per assay tube) and androstenedione standards were prepared in 12 × 75 mm disposable tubes. The standards contained 0, 25, 50, 75, 100, and 150 pg of androstenedione. To each assay tube, 0.20 ml of dilute antiserum mixture containing 10 pg of labeled androstenedione was added. Incubation was carried out at room temperature for 2 h. After incubation, 0.20 ml of saturated ammonium sulfate was added to each tube, which

was then vortexed. The tubes were centrifuged for 10 min at 3,000 rev./min at room temperature. The entire supernatant was decanted into counting vials.

Testosterone. The freeze-dried antiserum, as supplied, was reconstituted to its original volume with phosphate buffer. This solution was dispensed in 10 µl aliquots into serum vials and quick-frozen for storage. The working solution was freshly prepared by adding 70 ml of buffer to 10 µl of stock antiserum with a resulting 1:7000 dilution.

Testosterone assay standards contained 0, 50, 100, 250, 500, 750, and 1,000 pg per tube were prepared in duplicate from a working standard solution containing 10 ng/ml testosterone in absolute ethanol. Column fraction samples were also prepared in duplicate, using 200 µl per assay tube for low testosterone subjects (e.g. women) and 100 µl per assay tube for high-value samples.

11β-Hydroxyandrostenedione. The 1:5 stock solution of Rivanol-treated antiserum was further diluted to a 1:1500 working solution by adding 25 µl of stock solution to 7.5 ml of phosphate buffer (pH 7.0, 0.1 M) containing 0.1% BSA. This assay was the only one of the three affected by the column solvent; therefore a blank column (i.e., no plasma extract applied) was run just with mobile phase and the eluate corresponding to the OHA fraction collected. One hundred microliters of this material was added to each assay standard tube after the addition of the appropriate volume of the steroid standard to provide 0, 50, 100, 250, 500, and 1,000 pg per assay tube. The working standard solution was prepared by diluting the stock solution (10 µg/ml) 1,000-fold with absolute ethanol. Solvent was evaporated under N_2. Sample assays were performed on 100 µl of column eluate, using dextran-charcoal in the standard manner.

All counting was performed in Packard liquid scintillation spectrometers to a precision of ±2%.

RESULTS

Column performance

Figure 1, a composite derived from seven separate chromatograms, shows the separation of androstenedione, testosterone, and OHA. For each chromatogram, the total radioactivity under each peak, corrected for mean background, was determined, and the counts in each 1 ml fraction recalculated as a percent of the total. The seven chromatograms were obtained with seven different columns over a span of 6 weeks; the mean values for the peaks are shown in the figure. For comparison, we have included the resolution characteristics of an earlier system using LH-20 instead of Lipidex-5000; the improved resolution with the latter preparation is evident. Recoveries of the three compounds were: androstenedione—94.0 ± 3.6% ($n = 32$), testosterone—96.2 ± 3.4%, and OHA—93.1 ± 4.6%.

Of even greater significance for routine operations involving many samples is reproducibility of chromatographic behavior with different columns and for the same column in repeated use. Individual column performance was monitored by including azobenzene as a marker which emerges with the androstenedione front and does not interfere with either the scintillation counting for recovery measurement or the RIA. No departure in the emergence of this dye in a 1–2 ml fraction, at from 9–11 ml effluent volume, was experienced with repeated use of 12 different Lipidex-5000 columns over a 6-month period. Column operations were always performed in a temperature-controlled room. Initially, variations in column performance were encountered due to varying moisture content of the mobile phase solvents; this was readily overcome by water-saturation of the solvent mixture.

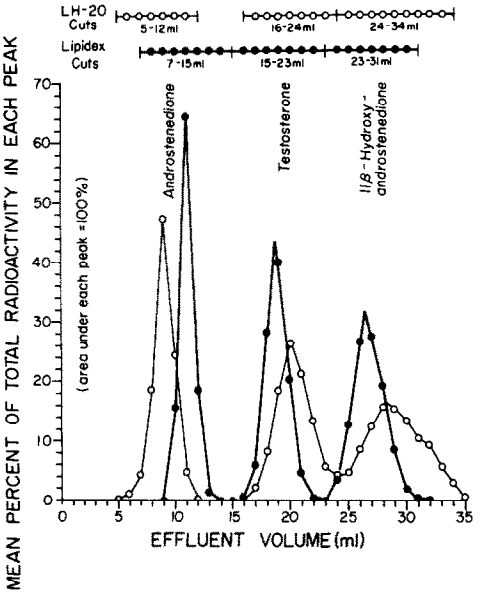

Fig. 1.

Statistical measures for evaluation of precision and sensitivity of the *overall* procedure were as follows: precision $s - [\Sigma d^2/2N]^{1/2}$ where d = difference between duplicate values and N = number of duplicate sets; sensitivity = $ts/N^{1/2}$ where $t = 2.576$ ($P = 0.01$). For testosterone values in the range of 0–100 ng/dl plasma, d for 13 duplicate sets was 5.3 ng/dl and sensitivity was 3.5 ng/dl based on 100 µl samples. For androstenedione (10 sets in the 0–200 ng/dl range) d = 19.2 and sensitivity = 15 ng/dl on 25 µl samples. For OHA (13 sets in the 0–200 ng/dl range) d = 11.8 and sensitivity = 15 ng/dl for 100 µl samples.

For the RIA curves, a computer program [10] performed the statistical analysis for assay sensitivity (least amount of unlabelled steroid that can be distinguished from zero). This value is approximately two standard deviations from zero with a level of significance of $P = 0.025$. The program also determines the coefficient of variation (*c.v.*). Analyzing seven assays gave the data shown in Table 1.

Inter- and intra-assay variation ranged between 8 and 10% for all three RIA procedures.

Table 1. Sensitivity and precision of standard curves

Steroid	Limit of sensitivity (ng/dl)	Range of precision, ng/dl; (c.v. in %)
Testosterone	12	50–125; (15.9–9.5)
Androstenedione	10	50–250; (8.5–11.2)
11β-Hydroxyandrostenedione	21	125–500; (9.3–6.8)

The only plasma steroid known to have clinically significant cross-reactivity with any of the three antisera used in this study, and not eliminated by chromatographic purification, is dihydrotestosterone (DHT). As a consequence of a 20–30% cross-reaction with our testosterone antiserum, the testosterone RIA values include a DHT contribution. The 54% C_{11}–C_{14} alkylated Sephadex LH-20 system employed by Jänne *et al.* [11] separates DHT from testosterone completely, but it requires elution of a 2.5 g column with more than 70 ml of hexane–chloroform (95:5, v/v), whereas our total elution volume for all three steroids of interest on a 3.5 g Lipidex-5000 column was only 31–32 ml. DHT and testosterone can be separated on a 2.5 g Lipidex-5000 column using cyclohexane–chloroform (95:5, v/v) with a total elution volume of less than 45 ml. The development of antibodies which can discriminate these two compounds completely [12] affords a solution to this problem in circumstances where this separation is of importance. A low level of cross-reactivity is found in the other two antisera with respect to the 5-reduced metabolites of the measured steroid.

This procedure is being applied to several clinical problems in the interrelation of androgens from adrenal and gonadal sources. Values of some random plasma samples are shown in Table 2. The "normal" subjects received this classification after extensive endocrine evaluation; the castrate females, all of middle age, were on replacement therapy with conjugated estrogens with one exception; the males and females with adrenal hyperplasia were treated with dexamethasone (DXM) so that the cortisol level of the particular plasma sample was less than 4 μg/dl.

DISCUSSION

A preliminary series of investigations with Sephadex LH-20 showed the feasibility of isolating the androgens of interest in a form compatible with subsequent radioimmunoassay. Hydroxyalkoxypropyl Sephadex, Lipidex-5000, was introduced as an effective support for separations of labile compounds in the picogram range [13]. It was successfully employed as the means of purification because of negligible blanks, high recovery, and repeatable use of columns over long periods of time. Moreover, the separations achieved and the sharpness of the elution peaks were improved with Lipidex, resulting in better reproducibility both within and between assays. The procedure still requires meticulous attention to detail, and is not suitable for the large throughput associated with most RIA methods. Clinical investigations would be benefited by the development of simpler but equally precise methods for separation of these steroids.

Both adrenal and gonadal steroid secretion show a pulsatile pattern, which may account for the difficulty of identifying elevated plasma androgen levels consistently in subjects who show unequivocal clinical evidence of androgen excess. An approximation of the mean level of hormones which pulsate can be made by multiple sampling, and studies of the most efficient and practical schedule of sampling have been published [14].

Table 2. Plasma steroid values in various groups of human subjects

Subjects	Plasma steroid, mean (range) in ng/dl		
	Androstenedione	Testosterone	11β-Hydroxy-androstenedione
8 Normal males	227 (108–410)	516 (195–1015)	138 (60–238)
4 Normal females	243 (70–350)	44 (<10–76)	157 (64–244)
22 Castrate females on estrogen	175 (60–397)	32 (13–102)	172 (77–319)
2 Castrate males	77, 112	<10, <10	75, 76
7 Adrenal hyperplasias on DXM (male and female)	136 (66–252)		33 (<20–48)

Aside from being a potential marker for adrenal androstenedione production, 11β-hydroxyandrostenedione is interesting, of itself, with regard to the mechanism of adrenal androgen production. The proximal steps in the biosynthetic pathway consist of the cleavage of progesterone to androstenedione, and the secretion of this substance or its protonated product, testosterone. However, a substantial part of the synthesized androstenedione is 11-hydroxylated, rendering it biologically inactive. In this sense, both desmolase and C_{19}-11β-hydroxylase are critical enzyme activities. (Production of androgens by the conversion from dehydroepiandrosterone is a minor pathway, which may be neglected for the moment.) Whether or not changes in the activity of the C_{19}-11β-hydroxylase can regulate adrenal androgenic activity remains to be investigated.

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