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AN IMPROVED METHOD FOR EXTRACTION AND DETERMINATION OF PROSTATE CONCENTRATIONS OF ENDOGENOUS ANDROGENS

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SUMMARY

Our published method for determination of prostate concentrations of endogenous androgens has been modified to increase the precision, sensitivity and reliability. A more efficient tissue homogenizer (Tekmar) was used instead of the VirTis-driven blades. Silica gel adsorption of androgens in the solvent extract and subsequent separation from nonpolar lipids by selective solvent extraction in a batch process in tubes was substituted for column elution. Time, solvent and blanks were decreased. Cyproterone acetate, as a more reliable, internal relative R_F marker on thin-layer chromatography (t.l.c.), replaced medrogestone. With increased sensitivity, better precision and ability to homogenize smaller tissue masses, our method was also suitable for scaling down from 1-g to biopsy-size (50 mg) specimens. The method appears to be quantitatively adequate for dihydrotestosterone (DHT), the most concentrated and biologically significant androgen in prostate, but present measurements of androstanediol (1/3 as concentrated as DHT in benign prostatic hypertrophy) are at least semi-quantitative.

INTRODUCTION

Measurement of intracellular levels of androgen metabolites in androgen target tissues is important in elucidating mechanisms of androgen action and in understanding pathogenesis of disease states in such tissues. Using our original method for tissue androgens [1], we have already uncovered some useful information in this regard [2]. In order to extend such measurements to biopsy-size whole tissue samples and to various intracellular compartments, optimization of our method in terms of precision, sensitivity and reliability was found to be essential.

EXPERIMENTAL

The following represent changes in our previously described method:

Preparation of materials. Hot oven-cleaned silica gel spread in a pyrex dish or large porcelain evaporating dish, was poured into a beaker and allowed to cool in a closed chamber of air saturated with distilled deionized water, so that the activated silica gel adsorbed the water instead of air pollutants and the dry gel's static electricity was reduced.

Solvents were nanograde or distilled in glass and were tested for contamination by radioimmunoassay (RIA).

Radioactive androgens were purified by Celite column chromatography when purity fell below 90% as tested by t.l.c. or when antiserum binding decreased below quality control limits [1] to indicate significant interference by contaminants.

Homogenization. Approximately 2 g of tissue was minced to about 1 mm diam., and after transferring to a pre-weighed, 40 ml round-bottom, heavy-wall centrifuge tube with a pour spout, the minced tissue was weighed. Radioactive androgens, about 200 c.p.m. each of [^{3}H]-DHT, [^{14}C]-T and [^{3}H]-Diol (25 μl benzene solutions of each)/tissue replicate, was added along with 1 μg of butylated hydroxytoluene, as an anti-oxidant in 100 μl of ethyl acetate. The tissue was homogenized with a Tekmar Super Dispax Tissumizer 100 N using a 182 EN shaft and generator at maximum speed (23,000 rev./min) for 30 s, 6 times, with 30-s cooling intervals in ice water to maintain the temperature under 10°C.

After centrifuging the homogenate at 1000 *g* for 10–20 min, the supernatant was poured into a clean 18 × 150 mm tube and the tissue debris was homogenized again using the same procedure and solvent volume as described above. Three such supernatants were combined. A solvent blank was prepared as described above prior to homogenizing the tissue.

Silica gel adsorption and extraction. Silica gel, equivalent to a volume of 1–2 ml, was added to each sample and blank replicate, followed by vortex mixing. Solvent was gradually evaporated by an ex-

Steroid nomenclature (name or trivial name acceptable by IUPAC-IUB; trivial name used here, if different, abbreviated): 5α-Androstane-3α or 3β, 17α or 17β-diol; androstanediol; Diol. 4-Androstene-3, 17-dione; androstenedione; Ad. 17β-Hydroxy-5α-androstan-3-one; dihydrotestosterone; DHT. 6,17-Dimethyl-4, 6-pregnadiene-3, 20-dione; medrogestone; M. 1,2α-Methylene-6-chloro-17-acetoxy-4, 6-pregnadiene-3, 20-dione; cyproterone acetate; Cyp A. Testosterone; T.

tremely fine stream of dry nitrogen in a 40° water bath. After drying, the silica gel was washed 2 or 3 times by vortexing with 5 ml of hexane each time, and each nonpolar lipid extract was discarded by decanting the centrifuged slurry. Steroids were extracted from the silica gel with five 2-ml aliquots of ethyl acetate, vortexing each time and combining the five extracts in a 15 ml centrifuge tube by decanting each centrifuged slurry.

Procedural losses. To minimize losses of androgens in the extraction procedure (monitored by sampling aliquots and counting added radioactive androgens), it was found necessary to carefully follow these steps, in addition to those reported previously [1]: 1. The silica gel used to adsorb androgens from the evaporated Delsal's solvent extract must be completely dried before washing with hexane. As much as 6% of radioactive DHT was lost in hexane washes when Delsal's solvent was not completely dried from the silica gel-adsorbed sample. 2. Excess silica gel must be avoided. Recovery of [^{3}H]-DHT decreases as the amount of silica gel increases. After evaporation of 1 ml solutions of [^{3}H]-DHT in Delsal's solvent on 1, 2, and 3 times the recommended amount of silica gel, followed by extraction with 1 ml of ethyl acetate, the following recoveries (means of duplicates) were obtained: 72% using 0.3 ml of silica gel recommended for a 50-mg tissue extract, 67% using 0.6 ml of silica gel, and 53% using 0.9 ml. In the presence of tissue extract a more pronounced effect is observed on the delay or diminution of [^{3}H]-DHT recovery from silica gel with additional aliquots of ethyl acetate. 3. The silica gel-adsorbed sample must be thoroughly vortex mixed with each of the 5 ethyl acetate aliquots to obtain efficient extraction of androgens. With these precautions, overall recovery of [^{3}H]-DHT added to tissue prior to homogenization averaged 54% in 22 studies: range 41 to 81%. Overall recoveries of [^{3}H]-DHT ranged between 90 and 100% after homogenization and 60 to 90% after silica gel adsorption and ethyl acetate extraction. Recoveries of [^{3}H]-Diol tended to be 5–20% higher.

Thin-layer chromatography. Solvent was evaporated, 10 μg of cyproterone acetate (Schering)/25 μl of ethyl acetate was added to each sample of blank replicate, and each vortexed solution was transferred as a 2-cm streak in a 4-cm lane on a silica gel plate. Three ethyl acetate rinses of each tube were also transferred to the corresponding, initially applied streak. Other t.l.c. techniques and solvent development were previously described [1]. Androgens were located on the basis of predetermined R_F values (DHT = 0.76, 0.63; T = 0.52, 0.40; Diol = 0.35, 0.24) of the leading and trailing edges of the androgens relative to the leading and trailing edges of Cyp A, respectively.

t.l.c. Separation of radioactive androgens. The reliability of t.l.c. separation was improved when Cyp A replaced medrogestone as an internal R_F marker, the temperature of solvent development was controlled at 2–5°C, and moisture content of the applied sample on the silica gel plate was minimized. Cyp A was more stable than medrogestone over long-term storage in ethyl acetate solution at 4°C. Cyp A co-chromatographs with androstenedione in the androgen zone ahead of DHT (relative R_F's for DHT: Cyp A are thus higher than for DHT:M). Microgram amounts needed for visualization of Cyp A on t.l.c. did not significantly affect blanks eluted from DHT, T, or Diol zones. (Other internal markers which may be substituted for Cyp A with equally good results include: flutamide or 4'-nitro-3'-trifluoromethylisobutyranilide and MK-316 or 2',3'α-tetrahydrofuran-2'-spiro-17-(6,7α-difluoromethylene-1,2α-methylene-4-androsten-3-one, both from Merck, Sharp & Dohme, and megestrol acetate or 6-methyl-17-acetoxy-4,6-pregnadiene-3,20-dione from Mead Johnson.)

Recoveries and separation of adjacent radioactive androgens on t.l.c. were measured as described [1], except that 1.0 ml (or $\frac{1}{2}$) of each t.l.c. eluate (evaporated to dryness and redissolved in 2.0 ml of ethyl acetate) was evaporated in a vial before counting in 8 ml of PPO scintillant.

A flow diagram for the modified extraction of tissue androgens is displayed in Fig. 1.

Extraction of biopsy-size specimens. For biopsy-size specimens (50–60 mg/replicate) in 16 × 100 mm Siliclad-treated tubes, homogenization was carried out with a 100 N Tekmar shaft in 1 ml of Delsal's solvent, plus 0.1 μg of butylated hydroxytoluene/replicate. Other conditions and techniques were the same as described above for 1-g tissue replicates. Supernatant

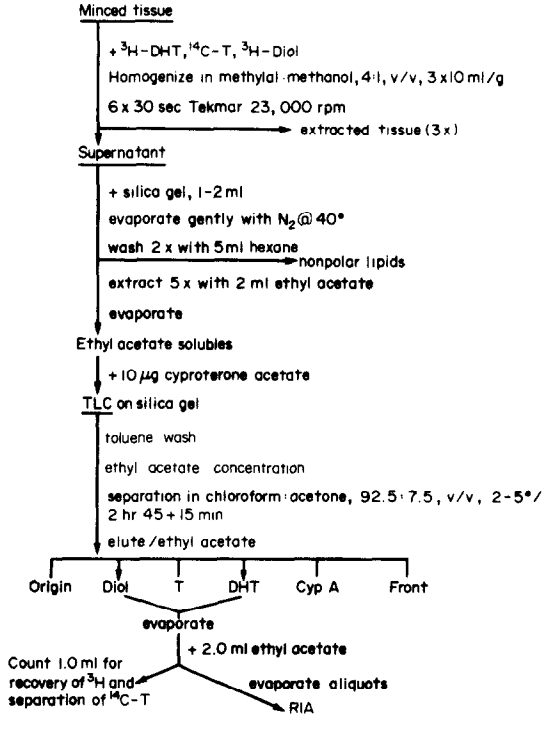

Fig. 1. Extraction of prostate androgens.

extracts were combined in a 13 × 100 mm tube, and 0.3 ml of silica gel was added. After drying, the silica gel was washed twice with 1 ml of hexane, then extracted 5 times with 1-ml aliquots of ethyl acetate. It was convenient to vortex each solvent aliquot with the silica gel-adsorbed sample, centrifuge at 3000 rev./min for 5 min and decant the solvent supernatants. Ethyl acetate extracts were poured into a 13 × 100 mm Siliclad-treated tube and evaporated to dryness. After elution from t.l.c., the dried residue was redissolved in 0.4 ml of ethyl acetate, 0.2 ml was taken for counting recoveries, and the remainder was pipetted into appropriate aliquots in tubes for RIA.

Radioimmunoassays were described [1]. Androgen standards: prepared as 1 ng/ml of ethyl acetate and stored in glass tubes stoppered with Teflon-lined screw caps at −15°C, were pipetted in triplicate (10, 25, 35, 50, 75, 100 μl) into 13 × 100 mm glass tubes and evaporated in a vacuum oven at 40°C to obtain 10, 25, 35, 50, 75, 100 pg.

Internal RIA controls were pipetted in duplicate from a pool of BPH (benign prostatic hypertrophic) extracts in ethyl acetate (stored at −15°C) so that 3 portions of the standard curve could be monitored for interassay variation.

Radioimmunoassay precision. Interassay variation of internal controls pipetted in duplicate from a pool of BPH extracts was determined at 3 ranges of the standard curve in 10 assays ($\bar{x} \pm 2$ S.D.): low (9–19 pg) = 23.8% C.V.; medium (31–51 pg) = 10.1% C.V.; high (57–95 pg) = 8.5% C.V. Intra-assay variation for the same controls, 10 of each in one assay: low = 14.2%, medium = 4.1%.

RESULTS

Sensitivity limits

Interassay blank means +2 S.D. ranged from 0.4–0.6 ng androgen/total blank extract used for 1 g of tissue and 0.1–0.3 ng/total blank extract used for 50 mg of tissue. Sensitivity limits reported previously were 1.0–1.1 ng androgen/1-g blank extract [1].

Methodological precision

The inter- and intra-assay precision of the entire method as modified for DHT and Diol were determined using the same minced pool of BPH tissue (stored frozen in Parafilm). The coefficients of variation (C.V.'s) ranged from 3.6 to 18% for 1 g of tissue and from 10 to 14% for 50 mg of tissue, for DHT levels indicated in Table 1. C.V.'s ranged from 7.5 to 16% for Diol in 1 g of tissue and 18 to 45% in 50 mg of tissue for Diol levels that were less than 1/3 that of DHT.

Accuracy

Adequate accuracy of the original method in recovering 1 to 5 ng amounts of added DHT and Diol was reported [1] for 1-g replicates. Accuracy of the method for 50-mg replicates was established by comparing ng androgen/g BPH to concentrations obtained with 1-g replicates (Table 1). Comparing the interassay mean concentrations, 4.4 vs. 5.7 ng DHT/g BPH (both corrected for procedural losses), the method has a 77% accuracy or recovery for DHT in 50-mg samples (10% C.V.), and 70% for Diol (45% C.V.), 1.2 vs. 1.7 ng Diol/g BPH.

Specificity

t.l.c. Separation and antiserum specificity were reported [1]. The 11% cross-reactivity of androsterone reported for our DHT antiserum was based on a crude estimate at a low % bound [Table 3 in ref. 1]. A more carefully measured cross-reactivity at the 50% bound level yielded a value of 0.6%.

DISCUSSION

Our method for determination of prostate concentrations of endogenous androgens [1] has been modified to improve the precision, sensitivity and reliability. Precision has increased by at least a factor of 2, down to less than 10% for a coefficient of variation (mean of 3 trials) for DHT and to less than 12% (mean of 2 trials) for Diol. Sensitivity limits have similarly been reduced by a factor of 2 (down to 0.4–0.6 ng androgen/total blank extract used for 1 g of tissue). These improvements were noted after several modifications were introduced, the main ones being.:

(1) Separation of nonpolar lipids from silica gel-adsorbed steroids, followed by extraction of the latter, using selective solvents in a batch process in tubes

Table 1. Precision of tissue extraction—RIA method

		Run	N	ng DHT/g BPH	C.V.	N	ng Diol/g BPH	C.V.
A.	Intra-assay							
	1-g Replicates	1	4	6.3	18%	—	—	—
		2	4	5.6	3.6%	4	2.3	7.5%
		3	4	5.2	8.1%	4	1.1	16%
	Original method [1]:				21%			24%
	50-mg Replicates	1	2	4.9	—	2	0.6	—
		2	4	4.0	14%	3	1.5	27%
		3	2	4.3	—	4	1.5	18%
B.	Inter-assay							
	1-g Replicates		3	5.7	9.9%	2	1.7	12%
	Original method [1]:				19%			22%
	50-mg Replicates		3	4.4	10%	3	1.2	45%

instead of column elution. Volume of solvents, blank values and time were significantly reduced.

(2) Use of a more efficient tissue homogenizer (Tekmar) instead of the VirTis-driven blades. With the Tekmar, tissue and solvent are confined to a smaller volume during homogenization; no special homogenizing flask is needed; the same tube is used for homogenizing and centrifuging which minimizes losses in transfer of the homogenate. The Tekmar is also easier and more convenient to operate.

(3) Use of cyproterone acetate, a more reliable, internal relative R_F marker on t.l.c. instead of medrogestone.

With increased sensitivity, better precision and ability to homogenize smaller tissue masses, our modified method was also suitable for scaling down from 1-g to biopsy-size (50 mg) specimens. The interassay means (3 trials) were 77% of that obtained with 1-g replicates for DHT (10% C.V.) and 70% for Diol (45% C.V.). This high interassay C.V. for a level of 1–2 ng Diol/g BPH is based on only 2 runs, so at present the method is at least semi-quantitatively reliable for Diol in biopsy-size specimens of prostate.

Precision is acceptable above 20 pg on our standard androgen curve. For this reason, assays of samples do not have reproducible precision on aliquots containing less than 20 pg. Within the above described limitations, our modified method appears to be quantitatively adequate for DHT and at least semi-quantitatively reliable for Diol in prostate biopsies. However, the method should be quantitatively adequate for Diol in normal prostate having Diol concentrations about 5 times that in BPH [2].

A technician inexperienced with this extraction method may obtain low recoveries of [^{3}H]-DHT (<30%) added to tissue. Use of the minimal amounts of silica gel, thorough vortex mixing to maximally adsorb androgens in the Delsal's extract to the silica gel, complete removal of Delsal's solvent with a nitrogen steam and vigorous vortex mixing in each of the 5 ethyl acetate extractions of the silica gel-adsorbed DHT has resulted in acceptable recoveries (>40%).

Our original method has already provided a reliable means of obtaining useful data on concentrations of DHT and Diol in (a) normal human prostate compared to benign hypertrophy with an indication of possible endocrine pathogenesis of this common disease [2], (b) anti-androgen treated benign prostatic hypertrophy, as part of our investigation on the mechanism of anti-androgen effect on biochemical steps mediating androgen action [3, 4], and (c) treated and untreated prostate cancer compared to other androgen target tissues and non-androgen target tissues in the human [5].

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