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MEASUREMENT OF TESTOSTERONE WITH A HIGH-PERFORMANCE LIQUID CHROMATOGRAPH EQUIPPED WITH A FLOW-THROUGH ULTRAVIOLET SPECTROPHOTOMETER

ROGER C. COCHRAN, KIRWIN J. DARNEY, Jr. and LARRY L. EWING

Division of Reproductive Biology, Department of Population Dynamics, The Johns Hopkins University School of Hygiene and Public Health, 615 N. Wolfe Street, Baltimore, Md. 21205 (U.S.A.)

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SUMMARY

A technique is described for measuring nanogram amounts of testosterone using high-performance liquid chromatography with detection by a flow-through spectrophotometer. The addition of a non-radioisotopic internal standard (4-androsten-11β-ol-3,17-dione) to the biological specimen automatically corrects for testosterone losses due to extraction and non-quantitative sample injection into the high-performance liquid chromatograph. This new method, which can be performed by inexperienced personnel, is shown to be rapid, precise, accurate and specific for testosterone.

INTRODUCTION

Testosterone is the major androgen synthesized and secreted by the mammalian testis[1]. Techniques developed to measure the concentration of steroids in biological fluids generally fall into three categories: (a) radioligand assays, which include both competitive protein binding assays[2–4] and radioimmunoassays[5–7]; (b) physicochemical methods, which include double isotope dilution[8–10] or gas–liquid chromatography (GLC) with flame-ionization, electron-capture or mass spectrometer detectors[11–13] and (c) enzymatic measurement of steroids[14–16].

The measurement of testosterone by double isotope dilution and gas–liquid chromatography is tedious, time consuming and requires considerable technical skill. Radioligand assays of testosterone, while sensitive and less tedious, are subject to non-specific interference from unknown sources. The enzymatic measurement of any steroid requires access to highly purified, stable and steroid specific enzymes. Unfortunately, such an enzyme is not available for testosterone.

Recent advances in the separation of steroids by high-performance liquid chromatography (HPLC) followed by the measurement of light absorbance at 240 nm with a flow-through spectrophotometer led to the development of a new method to measure dexamethasone in urine[17]. Briefly, this method involved the addition to urine samples of a non-radioisotopic internal standard which chromatographed

differently than dexamethasone in an HPLC system; extraction of the sample with an organic solvent; and finally, injection of an aliquot of the solvent residue into the HPLC system.

We adopted the principle of this method to develop a similar, simple and specific method for determining the concentration of testosterone in spermatic venous effluent from the rabit testis perfused *in vitro*.

MATERIALS AND METHODS

Materials

A Waters Assoc. (Milford, Mass., U.S.A.) Model 6000A chromatography pump coupled with a U6K injector and a μBondapak C_{18} column were used for HPLC. The mass of the Δ^4-3-keto steroids was measured by monitoring the absorbance of light at 240 nm with a spectroflow Model 770 UV analyser (Schoeffel, Westwood, N.J., U.S.A.) interfaced with an Autolab System IV integrator computer (Spectra-Physics, Stirling, N.J., U.S.A.). A Packard Model 420 gas–liquid chromatograph with a 3% OV-210 on 80–100 mesh Gas-Chrom Q column, flame-ionization detector, and interfaced with the Autolab System IV integrator–computer was used for estimation of testosterone mass by a previously published gas–liquid chromatographic method[18].

Testosterone (4-androsten-17β-ol-3-one), testosterone acetate (4-androsten-17β-ol-3-one-acetate) and 4-androsten-11β-ol-3,17-dione (11β-DIONE) were obtained from Steraloids (Wilton, N.H., U.S.A.) and recrystallized to constant melting point before use. [^{3}H]Testosterone was purchased from New England Nuclear (Boston, Mass., U.S.A.) and purified by thin-layer chromatography (TLC) on pre-coated plates (250 μm, Analtech, Newark, Del., U.S.A.) prior to use. Solvents were acetic anhydride, pyridine, carbon disulfide, ethyl ether (reagent grade; Mallinckrodt, St. Louis, Mo., U.S.A.), benzene, ethylacetate (Nanograde; Mallinckrodt), Photrex reagent (specially denatured ethanol; J. T. Baker, Phillipsburg, N.J., U.S.A.) and spectrophotometric-grade acetonitrile (Burdick & Jackson Labs., Muskegon, Mich., U.S.A.). Water was double-distilled in glass.

New Zealand white rabbits (12 months of age), purchased from Bunnyville (Altoona, Pa., U.S.A.), were housed in an air-conditioned (20°) and light-controlled (14 h light: 10 h dark) room and supplied with 120 g Rabbit Checkers (Ralston Purina, St. Louis, Mo., U.S.A.) per day and water *ad libitum*. Testes were perfused *in vitro* with a medium constituted of Krebs-Ringers bicarbonate solution with 3% (w/v) fraction V bovine serum albumin (KRB–BSA), and 25% bovine red blood cells as described previously[18,19].

HPLC testosterone assay

A 400-ng amount of 11β-DIONE in benzene was pipetted into the bottom of 12-ml disposable, glass, screw-cap tubes using a micromedic automatic pipette (Micromedic Systems, Huntsville, Ala., U.S.A.). For the standard curve, different amounts of testosterone in benzene were next added to the appropriate test tubes. The benzene was evaporated under nitrogen. Two drops of ethanol followed by 1 ml of KRB–BSA, or testicular venous effluent were added to each tube. A 10-ml volume of ice-cold diethyl ether from a freshly opened can was added next. The mixture was shaken

vigorously for 1 min, centrifuged at 1500 *g* for 10 min. The aqueous layer was snap-frozen in a dry-ice–acetone bath and the ether decanted into another disposable glass test tube. The ether extract was evaporated under nitrogen and the residue dissolved in 50 μl of Photrex reagent. Approximately one-half of this solution was placed in the U6K injector of the chromatograph. The internal standard (11β-DIONE) and testosterone were eluted differentially from the μBondapak C_{18} column with acetonitrile–water (40:60, v/v) at 2 ml/min. The areas of the resultant peaks of UV absorbance were integrated and the quantity of testosterone present calculated automatically by the Spectra-Physics integrator computer according to the formula

$$\text{Testosterone quantity} = \frac{\text{Area of T peak}}{\text{Area of 11}\beta\text{-DIONE peak}} \times KF \times 100\,\text{ng 11}\beta\text{-DIONE}$$

where *KF* is the ratio of the absorbance peak area for 100 ng of 11β-DIONE to that of 100 ng testosterone. Estimates of *KF* were performed with each series of testosterone assays.

GLC testosterone assay

Testosterone measurement by the GLC technique was described in detail and validated in an earlier report[18].

RESULTS

General

Testosterone was readily separated from the 11β-DIONE internal standard on the μBondapak C_{18} column with acetonitrile–water (40:60, v/v) at a flow-rate of 2 ml/min. Testosterone and 11β-DIONE eluted at 480 and 270 sec, respectively.

The partition coefficient for testosterone and 11β-DIONE between diethyl ether and the aqueous sample were similar. This was tested in the following experiment. One hundred ng each of testosterone and 11β-DIONE internal standard were pipetted into ten test tubes. These test tubes were divided into two groups of five tubes each. An aliquot from each of the first five tubes was injected directly into the chromatograph. The absorbance of light at 240 nm by the T and 11β-DIONE peaks was measured and the 11β-DIONE/T ratio computed. The second group of five samples were extracted with 10 volumes of diethyl ether, aqueous phase snap-frozen in dry ice and acetone, ether extract decanted, evaporated to dryness and an aliquot injected into the HPLC system. The absorbance of light at 240 nm by the T and 11β-DIONE peaks was measured and the 11β-DIONE/T ratio computed. Analysis of variance showed that there was no difference in the ratio (11β-DIONE/T) of light absorbed at 240 nm for the unextracted and extracted samples (0.93 ± 0.01; $\bar{x} \pm$ S.E.M. and 0.91 ± 0.03, respectively).

Sensitivity

The smallest amount of testosterone contained in a biological sample which could be measured consistently by the HPLC assay was 10 ng. Biological samples containing 5 ng of testosterone failed to generate a detectable testosterone peak at 240 nm.

Accuracy

Recrystallized testosterone, from 10 to 2000 ng, was added to 1-ml portions of KRB–BSA. Five replicates at each of seven concentrations were measured by the HPLC method (Fig. 1). Regression analysis showed the estimated amounts of testosterone to be linearly correlated with the mass added. The highly significant correlation coefficient was 0.99. The slope of the regression line and the y intercept were 1.0 and 1.5, respectively. The measured testosterone values were identical to results obtained when the same amounts of standard testosterone were injected directly into the HPLC system without prior extraction.

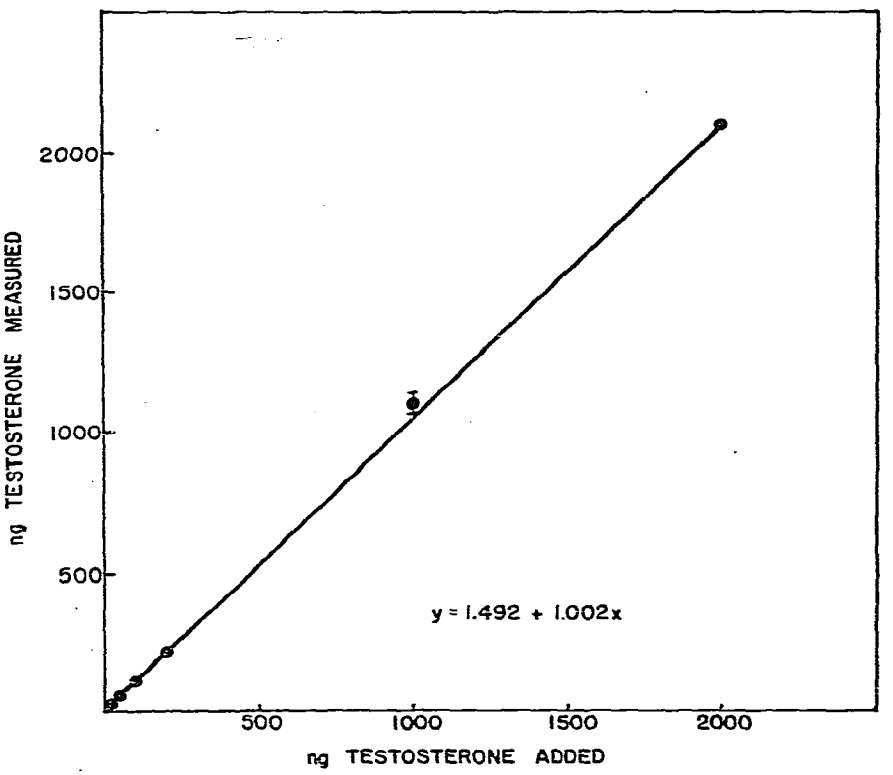

Fig. 1. Accuracy of HPLC for measuring testosterone using regression analysis.

Precision

The precision of the HPLC method was examined using a two-way analysis of variance to measure the intra- and inter-assay variability. The data required for this analysis was collected by measuring the testosterone concentration in five 1-ml aliquots taken from each of 3 samples, which contained 75, 150, or 300 ng T/ml, on 3 different days. The results in Table I show that the estimates of testosterone in each pool were not significantly different ($P > 0.25$) on any given day (intra-assay variance) or from day to day (inter-assay variance). The intra-assay coefficient of variation (standard deviation/mean $\times$ 100%) was 3.2% and the inter-assay coefficient of variation was 3.6%.

TABLE I

RESULTS OF HPLC TESTOSTERONE ASSAY FOLLOWING EXTRACTION OF ADDED STANDARD FROM KRB–BSA

Five samples were taken from each of 3 different pools on 3 different days.

Testosterone added	*Testosterone estimated (mean ± standard error of mean, ng)*		
	Day 1	*Day 2*	*Day 3*
75	75.6 ± 1.6	73.6 ± 4.5	72.4 ± 3.6
150	141 ± 4.3	147 ± 5.4	145 ± 2.4
300	284 ± 3.4	291 ± 9.9	295 ± 8.1

Specificity

The specificity of testosterone measurement by the HPLC method described above was due to three physico-chemical properties of testosterone. First, the differential extraction of the biological sample with ethyl ether assured the separation of testosterone and other lipophilic molecules from polar hydrophilic molecules. Secondly, the large extinction coefficient for light absorbance at 240 nm by testosterone is unique to Δ^4-3-ketosteroids. Thirdly, the retention time of testosterone, eluted from a μBondapak C_{18} column with acetonitrile–water (40:60), is unlike that of any other testicular Δ^4-3-ketosteroid.

Experimental verification of specificity was obtained by comparing the testosterone secretion rate of rabbit testes perfused *in vitro* as described previously[19]. The venous effluent was collected for 1 h. Triplicate testosterone determinations were made on aliquots of spermatic venous effluent from each of the five perfusions by both HPLC and GLC. The mean ± the standard error of the mean for testosterone secretion (μg T/h) was 7.3 ± 0.7 and 7.8 ± 0.9 when measured by HPLC and GLC, respectively. A two-way analysis of variance revealed no significant difference ($P > 0.25$) between testosterone secretion of perfused rabbit testes measured by the HPLC and GLC methods. In contrast, there was a highly significant difference ($P > 0.001$) between the amount of testosterone secreted by the individual rabbit testes.

DISCUSSION

Progress in studying the synthesis and secretion of testosterone by mammalian testes has been hampered by the lack of a simple, rapid, yet specific method for measuring testosterone in biological samples containing complex mixtures of steroids.

Discovery of an internal standard (11β-DIONE) which partitions similarly to testosterone between ethyl ether and an aqueous phase but which elutes differently than testosterone from a μBondapak C_{18} column allowed us to develop a HPLC method to measure testosterone in biological samples. The diagram in Fig. 2 shows that the HPLC method for testosterone measurement requires only an extraction followed by HPLC. In contrast, the GLC technique used routinely in our laboratory requires extraction, derivative formation, TLC, liquid scintillation spectrometry and GLC. The sensitivity, accuracy, precision and specificity of this new HPLC testosterone measurement method compares favorably with the GLC technique used

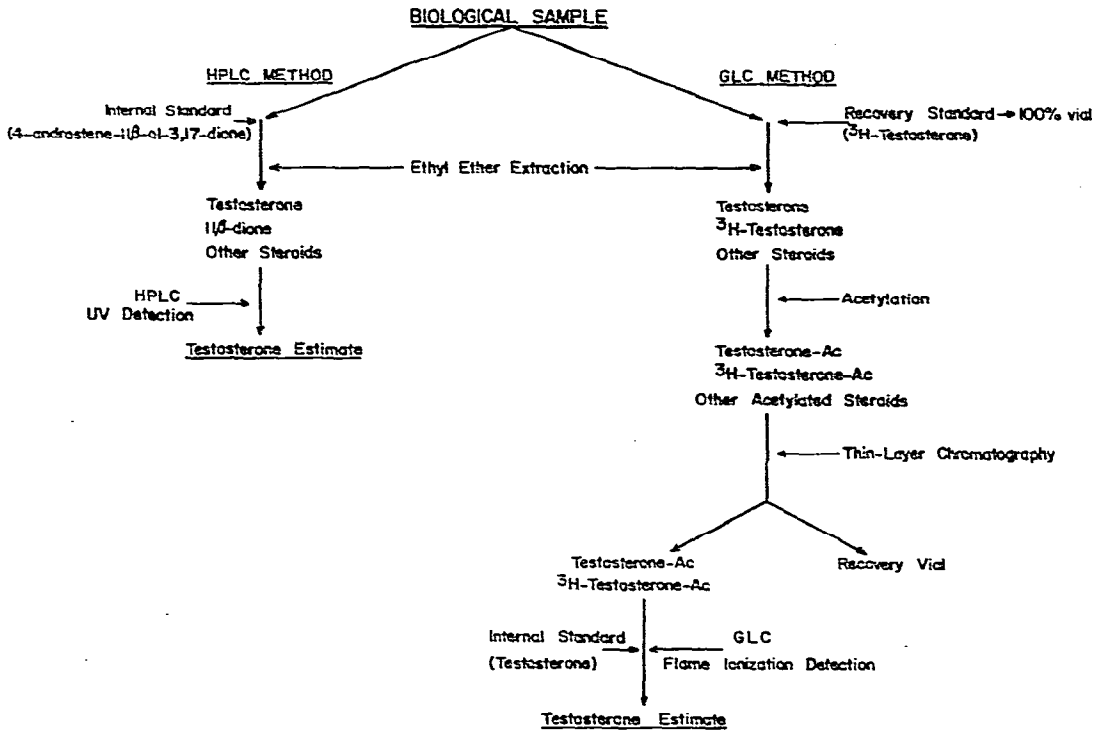

Fig. 2. Comparison of the new HPLC technique for measuring testosterone with an established GLC method[18] routinely used in our laboratory.

routinely in our laboratory[18]. Thus, for measuring testosterone, the HPLC method can be operated reliably by inexperienced personnel to make determinations rapidly on large numbers of samples.

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