A COMPETITIVE PROTEIN-BINDING METHOD FOR DETERMINATION OF URINARY ANDROSTANEDIOL

by

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THESIS

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ABSTRACT

A new method using sex-steroid binding globulin in a competitive protein-binding assay for the determination of urinary 5α-androstane-3α,17β-diol has been studied in this paper, and compared to that by gas-liquid chromatography previously described. Results obtained by the two methods are statistically the same. With the increased sensitivity of the competitive protein-binding method, determination of the steroid using very small amounts of urine can be conveniently performed for screening purposes in assessing androgenic states.

INTRODUCTION

It had been demonstrated in vivo and in vitro that testosterone was efficiently converted to dihydrotestosterone (17β-hydroxy-5α-androstan-3-one) by androgen target tissue,[1] including the human skin, accessory sex organs, and hypothalmic tissue.[2-5] Ito and Horton had shown that in males, 70% of plasma dihydrotestosterone arise from testosterone conversion, while in females, androstenedione conversion gives rise to over two-thirds of plasma dihydrotestosterone.[6] Furthermore, they suggested that these events occur in peripheral androgen target tissues. Mauvais-Jarvis et al. had shown that 5α-androstanediol (5α-androstane-3α,17β-diol) is the main metabolite of dihydrotestosterone through 3α-hydrogenation, at least in normal individuals,[7] with 50 to 70% of the urinary androstanediol* in normal males arising from extra-hepatic tissue, the rest being formed from hepatic catabolism of testosterone and dihydrotestosterone.[8]

Mauvais-Jarvis et al. had used the difference between urinary testosterone and androstanediol as an index of extrahepatic testosterone catabolism to androstanediol via dihydrotestosterone,[9] since testosterone glucuronide seems to be formed in the splanchnic compartment only.[10]

In hirsute females, although mean urinary excretion of testosterone was in the normal range for females,[11] and plasma testosterone may sometimes be normal,[12] Mauvais-Jarvis

*When not specified, androstanediol refers to 5α-androstane-3α,17β-diol

et al. had shown that the mean excretion of androstanediol was elevated to levels found in normal men. This reflects androstenedione and other precursors being reduced to testosterone in target cells, giving rise to dihydrotestosterone in the plasma, and finally to androstanediol in the urine.

Therefore androstanediol, being a common product in several pathways of androgen metabolism, may be a very useful index in assessing androgenic activity, since it reflects extra-hepatic metabolism of testosterone as well as hepatic catabolism of testosterone and dihydrotestosterone.

A method for the determination of urinary androstanediol using gas-liquid chromatography had been reported,[9] involving β-glucuronidase and sulfatase hydrolysis, separation of testosterone and androstanediol by Girard's T reagent, purification by thin-layer chromatography, and final determination by gas-liquid chromatography. Measurement of the steroid by competitive protein-binding instead of gas-liquid chromatography at the final step has been studied in this paper.

Many methods have been developed to measure testosterone using sex-steroid binding globulin in plasma, the concentration of which is elevated in late pregnancy and in women taking oral contraceptives. In addition to testosterone, the globulin also binds strongly with a number of other steroids having the 17β-hydroxy configuration, including dihydrotestosterone, androstanediol, androstenediol, and 17β-estradiol.[13] With the increased sensitivity of competitive protein-binding

assays, nanogram amounts of the steroids can be measured. This potentially provides a simpler and less time-consuming procedure for the measurement of androstanediol using very small amounts of urine. Clinically, this can be employed as a useful screening test for excess androgenicity, where many samples can be set up at the same time.

MATERIALS & EQUIPMENT

I. GENERAL (COMMON TO BOTH METHODS)

Helix pomatia β-glucuronidase (140,000 Fishman units/ml.) - aryl sulfatase (160,000 Whitehead units/ml.) from Calbiochem

^{3}H androstanediol (5α-androstan-3α,17β-diol [1,2-^{3}H]), specific activity 40-60 Ci/mmole, from New England Nuclear Corp., used after repurification by paper chromatography

5α-androstane-3α,17β-diol : from Sigma Chemicals, stored refrigerated at concentrations of 1 mg./ml., 5 ug./ml., 20 ng./ml.

Testosterone, androstenedione : from Sigma Chemicals

Organic solvents : methylene dichloride, used after re-distillation
ethyl ether, analytical grade
methanol, used after re-distillation
benzene, reagent grade
ethyl acetate, reagent grade

Flash evaporator : Buchler Universal

Extraction apparatus : Hershberg-Wolfe

Girard's T reagent : from Matheson, Coleman and Bell

Thin-layer plates : Bakerflex Silica Gel IB2-F from J.T.Baker, scraped into 1 inch strips, washed with

methanol, and stored in a dessicated container

Whatman filter paper No. 2 : washed with methanol in a chromatography cabinet for at least 24 hours, then in a Soxhlet with 50% ethanol in benzene for at least another 24 hours before use

II. COMPETITIVE PROTEIN-BINDING :

Plasma containing the steroid-binding protein, obtained from women taking oral contraceptives. The plasma was divided into 0.5 ml. aliquots, lyophilized and stored at -20°* until use; reconstituted with distilled water to the appropiate dilution

Ammonium sulfate : from J.T.Baker Chemical Co., 50% solution in distilled water

Liquid scintillation fluid : 42 ml. Liquifluor, from New England Nuclear, added to 1000 ml. toluene containing 2% ethanol, giving a final concentration of 4 gm. PPO (2,5-diphenyl oxazol) and 40 mg. POPOP (p-bis(2-(5-phenyl oxazol) benzene) per liter of solvent

Liquid scintillation counter : Nuclear Chicago Unilux, counting efficiency for ^{3}H about 25%

III. GAS-LIQUID CHROMATOGRAPHY :

Reagent for trimethylsilylation : SIL-PREP from Applied

*Temperatures are given in degrees Centigrade

Science Laboratories, Inc; pyridine:hexamethyl-disilazane:trimethylchlorosilane (9:3:1)

Carbon disulfide

Gas Chromatograph : F & M Model 400 (Hewlett-Packard), with flame ionization detector. Records obtained on a Honeywell Electronik 15 recorder operated at 0.25 in./min.

Hamilton syringes : 10 ul., 25 ul.

Planimeter : from Gelman Instrument Co.

METHOD

Hydrolysis : 24-hour urine specimens were collected in boric acid and stored at -20° until processed. A 1/20 aliquot of male urine and 1/10 aliquot of female urine were used; the pH was adjusted to 5 with 1/10 volume 2N acetic acid/2M sodium acetate buffer. Approximately 10,000 cpm ^{3}H androstanediol and 1500 units of Helix pomatia per ml. urine were added. The mixture was incubated for 48 hours at 37°.

Extraction : The urine sample was diluted up to 1100 ml. with distilled water, and placed into a continuous extraction apparatus. 500 ml. of methylene dichloride, volatilized at 40° was allowed to pass through the specimen continuously for 24 hours. The methylene dichloride extract was washed twice with 1/10 volume of 10% sodium carbonate and twice with distilled water. It was then dried over anhydrous sodium sulfate, and the methylene dichloride was removed in a rotary evaporator.

Girard's T reaction for separation of ketonic and non-ketonic steroids : The dried extract was transferred to an Erlenmeyer flask with 25 ml. methanol. 2.5 ml. acetic acid and 300 mg. Girard's T reagent was added, and the mixture incubated overnight at 37°. The reaction was then stopped by adding 50 ml. of ice-cold distilled water; the pH was adjusted to 6.5 with 10N sodium hydroxide. The hydroxy-steroids were extracted twice with equal volume of anhydrous ethyl ether.

The combined ether extract was washed once with 10% sodium carbonate, once with distilled water, dried over anhydrous sodium sulfate and evaporated to dryness in a rotary evaporator. The residue was transferred to a conical tube with 10 ml. methylene dichloride/methanol (1:1), and evaporated under reduced pressure in a 45° water bath.

Thin-layer chromatography : The dried extract was transferred with methylene dichloride/methanol onto a thin-layer plate with a 10 ul. capillary pipet, 2 cm. from the base of the plate. The plate was developed at room temperature using ascending migration in a benzene:ethyl acetate (1:1) solvent system. Testosterone, with a R_f value similar to androstanediol as shown by spraying with alcohol:sulfuric acid (1:1) and heating for 5 min. at 100°, was used as the reference. The area corresponding to the testosterone standard as located by ultraviolet absorption was cut off and eluted with 7 ml. of methanol into a conical tube, and then evaporated to dryness.

Paper chromatography : The extract was spotted with methylene dichloride/methanol on Whatman filter paper No. 2, 2.5 cm.X 40 cm. A testosterone:epitestosterone:androstenedione mixture in equal amounts was used as the reference. The paper strips were placed in a Bush tank containing cyclohexane:p-dioxane: methanol:water (100:25:100:10), equilibrated and run for 15-17 hours at 22° by descending chromatography.

Relative mobilities of relevant steroids from origin with respect to testosterone :

testosterone	100
β-androstanediol	100
α-androstanediol	118
epitestosterone	124
androstenedione	>145

Position of androstanediol was located between testosterone and epitestosterone, both of which were identified by ultra-violet absorption. The area containing androstanediol was cut off and eluted with 5-7 ml. methanol into a conical tube, and the methanol was evaporated to dryness.
5 ml. methanol was added to the dried extract, and aliquots were taken for competitive protein-binding (0.2 ml. for dilution), gas-liquid chromatography (3 ml.), and recovery (0.5 ml.).

Competitive protein-binding : A 1:5 dilution of the extract was made. 5 ul. and 20 ul. samples of this were pipetted into 0.5 ml. of methanol, evaporated to dryness, and carried through the competitive protein-binding procedure. Standards containing 0, 0.1, 0.2, 0.4, 0.6, 0.8 ng. of androstanediol in methanol were also evaporated. All samples and standards determinations were done in duplicates in 12X75 mm. test tubes.
Preparation of the ^{3}H androstanediol binding plasma was done by using appropiate amounts of ^{3}H androstanediol (evaporated

to dryness) and reconstituted lyophilized plasma (1:70 dilution with distilled water) to give a final concentration of approximately 20,000 cpm per ml. of solution. 0.5 ml. of the plasma solution (containing approximately 10,000 cpm) was added to each tube, and duplicate aliquots were also pipetted into counting vials for determination of the total number of counts added to each tube. The tubes were gently mixed on a Vortex mixer, and kept at room temperature for 20 min. The protein-bound androstanediol was precipitated by adding 1.5 ml. of 50% ammonium sulfate solution and mixing gently by inversion. The tubes were allowed to stand for 10 min., and then centrifuged at 3000 rpm for 20 min. A 0.5 ml. aliquot of the supernatant was pipetted into a counting vial, and 10 ml. of scintillation fluid was added. The counting vials were shaken thoroughly and allowed to stand 30 min. in the dark before counting. The amount of unbound ^{3}H androstanediol was calculated as number of counts X 4, and was expressed as a percentage of the total number of counts added, A standard curve of % unbound ^{3}H androstanediol vs. amount of standard was plotted, and the corresponding unknowns were read off the graph. The total amount of androstanediol present in the extract was calculated, taking into account dilution and recovery. The 24-hour androstanediol excretion was then determined.

Gas-liquid chromatography : A 3 ml. aliquot of the redissolved extract was evaporated to dryness in a 5-ml. conical tube.

50 ul. of SIL-PREP reagent was added, vortexed, and allowed to react for 30 min. at room temperature. Standards of 0.25, 0.5, 1.0, 2.0 ug. androstanediol were treated in a similar way. The excess reagent was evaporated under reduced pressure, and 25 ul. of carbon disulfide was added just before injection to reduce loss from evaporation. 5 ul. of the sample was injected with a 10-ul. Hamilton syringe into the gas-liquid chromatograph having a 6 ft. X 0.34 cm. I.D. glass column packed with Gas-Chrom Q coated with 3% XE-60. Helium was used as the carrier gas, with a flow-rate of 60 ml./min. Temperatures were : 260° for flash heater, 200° for column, and 240° for detector.

The androstanediol peaks were quantitated by triangulation using a planimeter, and a graph of area vs. amount of androstanediol standard was plotted. The unknowns were read off the graph, and the total amount of androstanediol present in the extract was calculated, after correcting for recovery.

Relative retention times of relevant steroids with respect to 5α-androstanediol trimethylsilyl derivative obtained by gas-liquid chromatography (3% XE-60, 200°, 60 ml./min. carrier gas flow) :

5α-androstanediol	1.00
5β-androstanediol	1.21
androstenediol	1.42
5β-pregnanediol	2.50
testosterone	5.47

Recovery : A 0.5 ml. aliquot from the paper chromatography extract was dried in a counting vial. 10 ml. liquid scintillation fluid was added, shaken, and counted. The recovery was calculated from the number of counts recovered as a percentage of the number of counts added at the beginning, and was used to correct for losses in the procedure.

Proposed micro-determination : 0.5 ml. of urine, buffered at pH 5, and containing 2000 cpm ^{3}H androstanediol, was hydrolyzed in a 15-ml. conical tube. 10 ml. of methylene dichloride was used for extraction by shaking. After washing, the extract was dried, and 0.5 ml. methanol, 0.05 ml. acetic acid and 6 mg. Girard's T reagent were added. After overnight incubation, 1 ml. ice-cold water was added, and the mixture pH'd to 6.5 by 2 drops of 10N sodium hydroxide. The hydroxysteroids were extracted twice with 10 ml. ethyl ether; the extract was washed and dried. It was then carried through thin-layer and paper chromatography as described above. The final extract was dissolved in 2 ml. methanol; 0.2 ml. was taken for recovery, 100-200 ul. (for females) and 50-100 ul. (for males) for competitive protein-binding assay.

RESULTS

Thin-layer chromatography and paper chromatography

After hydrolysis, extraction, reaction with Girard's T reagent, and purification by thin-layer chromatography, trimethylsilyl derivatives of the extract gave a gas chromatogram very similar to that shown previously by other authors. The main peaks were 5α-androstanediol, 5β-androstanediol, androstenediol and pregnanediol (Fig.1). However, when the extract was carried through an additional paper chromatography step, the various steroids could be separated so that the gas-chromatogram showed only 5α-androstanediol as the dominant peak (Fig.2).

Furthermore, when the thin-layer chromatography extract was diluted and carried through the competitive protein-binding procedure, results obtained were 60-80% higher than that obtained from quantitation of the gas chromatogram peaks :

androstanediol (ug./24 hrs.)

CPB after TLC	GLC
100	66
235	141

It was shown that equal amounts of 5β-androstanediol and androstenediol had 10% and 70% binding affinity respectively compared to that of 5α-androstanediol. Therefore the paper chromatography step was necessary to remove interfering steroids.

Since position of 5α-androstanediol on the paper chromatogram was located with reference to testosterone and

GAS CHROMATOGRAM OF A URINARY EXTRACT AFTER THIN-LAYER CHROMATOGRAPHY

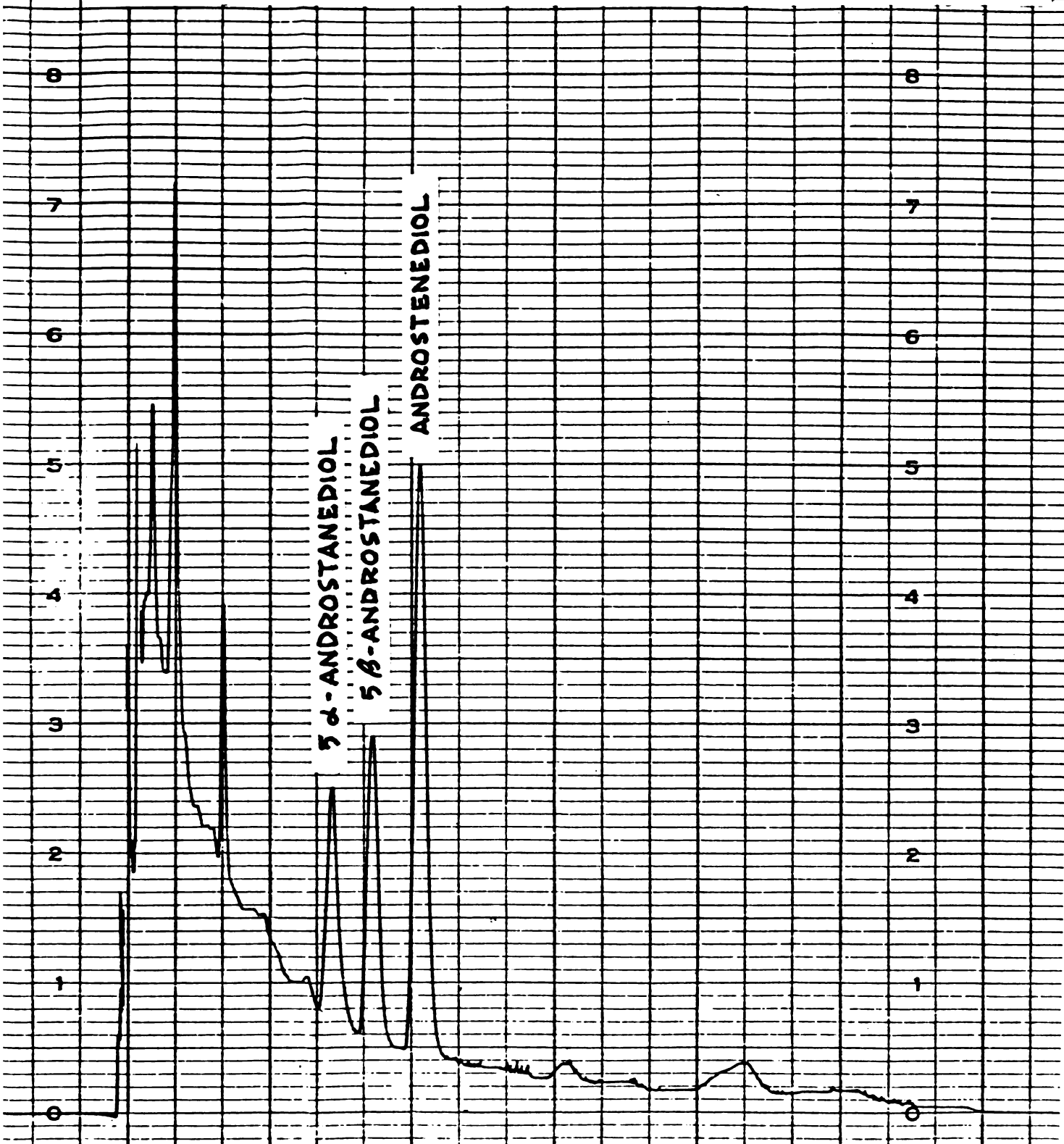

Figure 1

GAS CHROMATOGRAM OF A URINARY EXTRACT AFTER THIN-LAYER AND PAPER CHROMATOGRAPHY

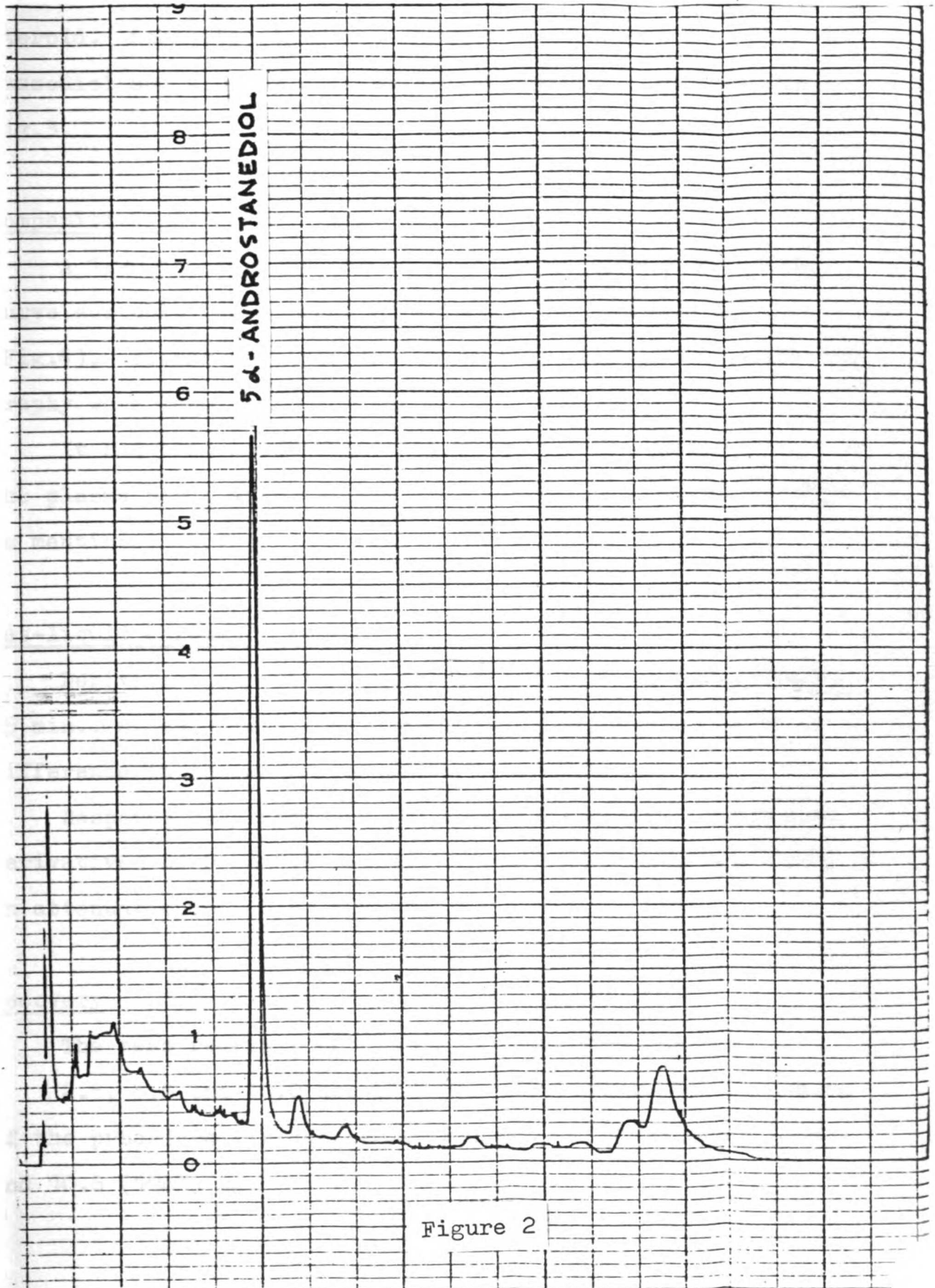

Figure 2

epitestosterone rather than authentic androstanediol, it was important to cut the paper accurately to prevent loss of the steroid. Mobility of androstanediol as obtained by ^{3}H androstanediol and also by androstanediol standard was shown in Fig.3.

Competitive protein-binding

A 1:70 dilution of the binding plasma gave a standard curve suitable for a range of 0 to 0.8 ng. androstanediol (Fig.4). Blanks obtained from thin-layer and paper chromatography were less than 0.05 ng.

It had been demonstrated that the binding globulin in the plasma binds with various affinities for various steroids as mentioned above.

Gas-liquid chromatography

Derivative formation of the steroid was complete after 15 min., since 15 min. and overnight incubation produced no difference in the amount of derivative formed.

Response obtained from the chromatogram was linear when derivatives of 0 to 0.4 ug. of standard were injected, using an attenuation of 10X8 (Fig.5).

Recovery

The mean recovery of the whole procedure was 41% .

2.5, 5.0 and 10.0 ug. of standard added at the beginning of the procedure yielded recoveries of 2.6 (104%), 4.3 (86%), and 10.8 (108%) ug.

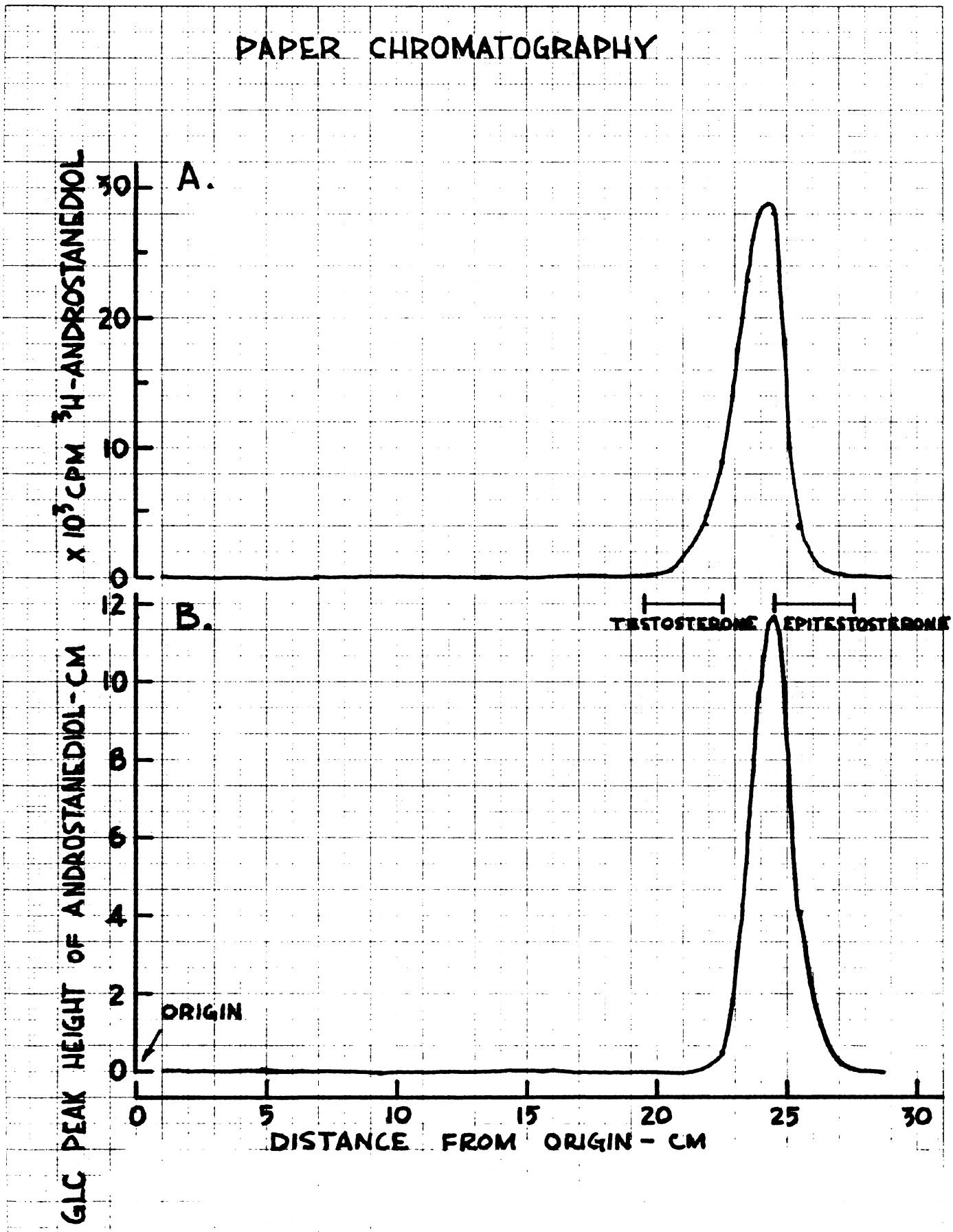
PAPER CHROMATOGRAPHY
A.
B.
^{3}H-ANDROSTANEDIOL x 10^3 CPM
GLC PEAK HEIGHT OF ANDROSTANEDIOL - CM
TESTOSTERONE
EPITESTOSTERONE
ORIGIN
DISTANCE FROM ORIGIN - CM
0
5
10
15
20
25
30
2
4
6
8
12

Figure 3

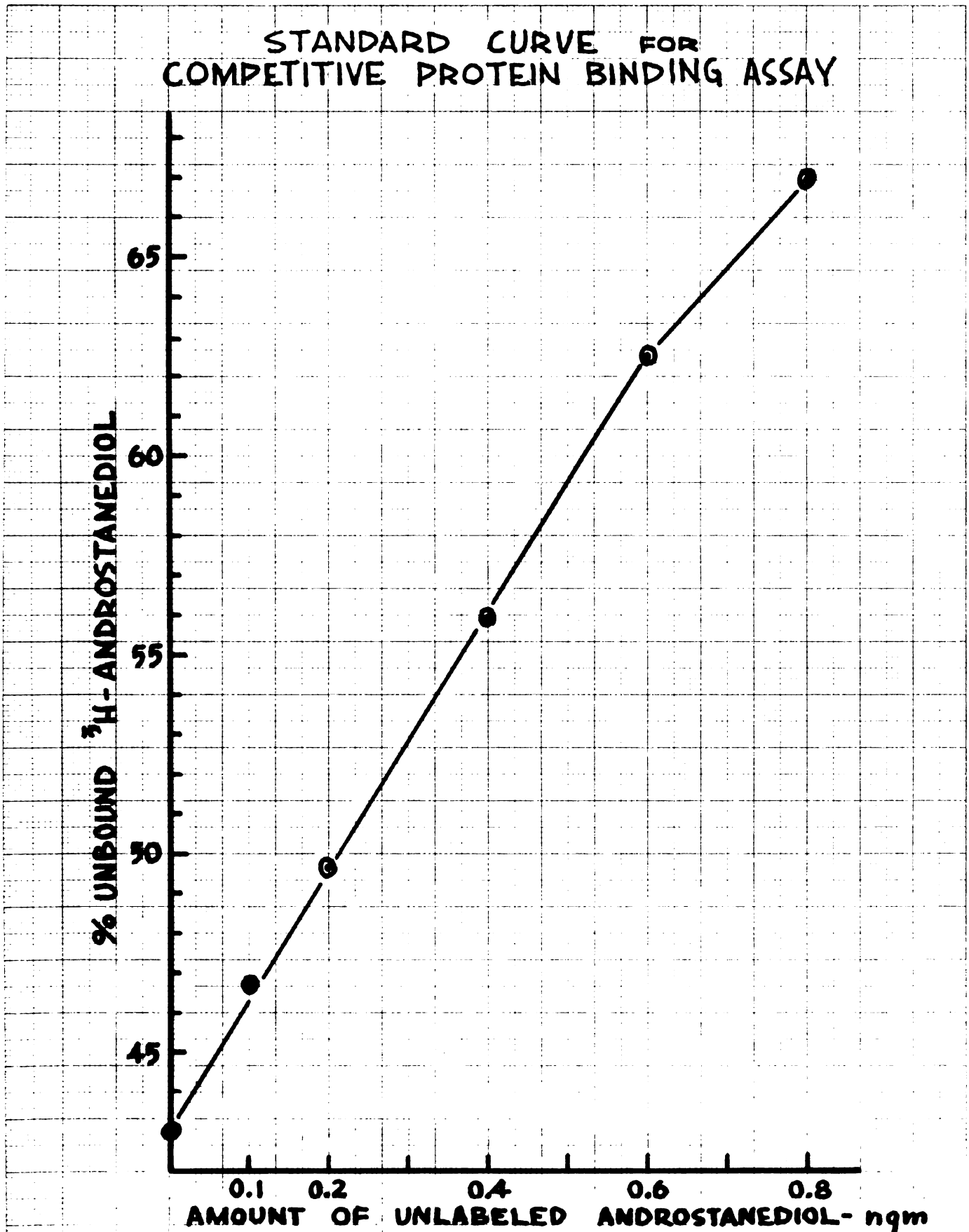
STANDARD CURVE FOR
COMPETITIVE PROTEIN BINDING ASSAY
% UNBOUND ³H-ANDROSTANEDIOL
65
60
55
50
45
0.1
0.2
0.4
0.6
0.8
AMOUNT OF UNLABELED ANDROSTANEDIOL- ngm

Figure 4

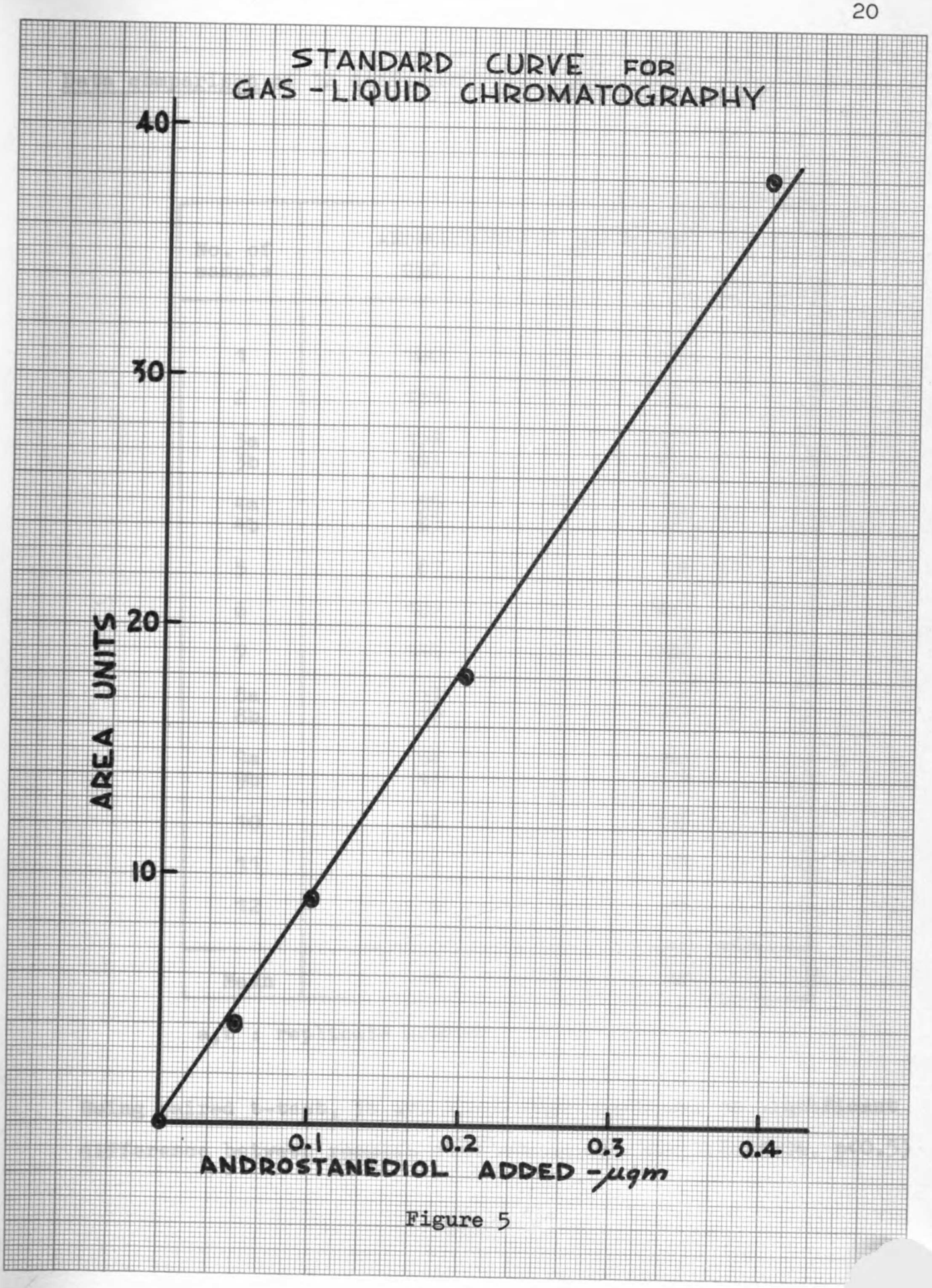
STANDARD CURVE FOR
GAS - LIQUID CHROMATOGRAPHY
40
30
20
10
AREA UNITS
0.1
0.2
0.3
0.4
ANDROSTANEDIOL ADDED - µgm

Figure 5

Data comparison of 12 different urine specimens

No. of sample	ANDROSTANEDIOL ug./24 hrs. GLC	CPB
1	100	111
2	159	133
3a	178	194
3b	183	164
4a	59	64
4b	67	65
5	67	59
6	187	150
7	144	132
8a	17	17
8b	17	13
9a	68	62
9b	48	58
10	138	156
11	19	19
12	6	8
Mean	91	88

a,b : replicate assays

Using paired t-test, it was shown that there was no significant difference between results obtained by GLC and CPB ($N=16$, $p<0.5$)

'Micro' determination of androstanediol :

ANDROSTANEDIOL ug./24 hrs.		
GLC	CPB	MICRO
100	111	112
159	133	170
181	179	171
63	65	69
144	132	125
19	19	20
$\bar{X}$ = 111	107	111

As shown above, results obtained were in good agreement to each other.

CLINICAL CASES

	17-KETO STEROIDS mg.DEA/24°	17-HYDROXY STEROIDS mg.F/24°	PREGNANE-TRIOL mg./24°	ANDRO-STANEDIOL ug./24°
NORMAL RANGE	5-15	4-10	up to 3	8-28*
ADRENOGENITAL SYNDROME:				
untreated	37.3	12.6	23.1	147
suppression by dexamethasone	0.7	no recovery	0.7	<2
IDIOPATHIC HIRSUTISM:	10.6	5.3	2.1	59
NORMAL:	6.1	5.4	2.9	19

*Range taken from data described previously by other authors

Abbreviations : DEA=Dehydroepiandrosterone
F=Cortisol

DISCUSSION

In the method previously described by Mauvais-Jarvis et al.[9] and Berthou et al.[14] for the determination of urinary andrsotanediol, it was essential to use gas-liquid chromatography for accurate quantitation. Using an additional paper chromatography step to remove interfering steroids, determination by competitive protein-binding could be undertaken. The protein-binding assay is faster and more convenient than measurement by gas-liquid chromatography since it does not require a gas chromatograph and more samples can be processed simultaneously. The same procedure can be modified for 1/2 ml. urine specimens, where hydrolysis and extraction can be carried through in the same test tube, which also eliminates the time required for continuous extraction. Furthermore, since only such a small amount of urine is used, the thin-layer chromatography purification can probably be dispensed with as the paper chromatography may be adequate in removing interfering substances. In this way, the determination of urinary androstanediol can be used as a screening test in assessing the androgenic status.

ACKNOWLEDGMENT

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